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High-level antimicrobial resistance or hypervirulence in emerging and re-emerging "super-bug" foodborne pathogens: Detection, mechanism, and dissemination from omics insights

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High-level antimicrobial resistance or hypervirulence in emerging and re-emerging "super-bug" foodborne pathogens: Detection, mechanism, and dissemination from omics insights

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Dr. Scott Van Nguyen works for ATCC. All other topic editors declare no conflict of interest.

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Editorial: High-level antimicrobial resistance or hypervirulence in emerging and re-emerging "super-bug" foodborne pathogens: detection, mechanism, and dissemination from omics insights

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Editorial on the Research Topic
High-level antimicrobial resistance or hypervirulence in emerging and re-emerging "super-bug" foodborne pathogens: detection, mechanism, and dissemination from omics insights

With the development of the global livestock and poultry industry, food processing industry and international trade, the spread of foodborne pathogenic microorganisms is accelerating and becoming more complex. This has resulted in a variety of foodborne diseases and increasingly serious antimicrobial resistance (AMR), which have brought an incremental disease burden to the health of people around the world. This is already one of the most challenging public health issues internationally and still causes a substantial economic and social burden worldwide (Pires et al., 2021).

The most common pathogens responsible for spreading foodborne diseases in humans include but are not limited to *Salmonella*, *Campylobacter*, *Clostridium*, *Cronobacter*, pathogenic *Escherichia coli* (*E. coli*), *Listeria monocytogene* (*L. monocytogene*), *Staphylococcus aureus* (*S. cureus*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Bacillus cereus* (*B. cereus*), *Yersinia enterocolitica* (*Y. enterocolitica*), among others. These pathogens contaminate various types of foods throughout the food chain including cereal, vegetables, fruits, meat, dairy, and aquatic products in entire proceedings from farmland to fork and disseminate AMR and virulence.

In addition to the virulence-related phenotypes expressed, different metabolites, including various toxins that can be produced, several pathogenic bacteria of the so called "ESKAPEE" group, that often express an AMR phenotype, can cause serious

human illnesses, resulting in a substantial disease burden. According to estimates published in 2019, 1.27 million deaths were directly attributed to drug-resistant infections globally, and 4.95 million deaths were associated with bacterial AMR in total (Murray et al., 2022). Some estimates also indicate that by 2050 there could be up to 10 million deaths globally per year—on par with the 2020 worldwide death toll from cancer (O'Neill, 2016; United Nations Environment Programme, 2023).

The term "ESKAPE" is composed of seven different pathogens with continuously expanding multidrug resistance (MDR) and virulence phenotypes, responsible for majority of nosocomial infections and are capable of "escaping" the biocidal action of antimicrobial agents (Mulani et al., 2019) the World Health Organization (WHO) published a list of pathogens for which new antimicrobial compound development was urgently needed in an effort to focus and guide research and development related to new antibiotics (Tacconelli et al., 2018; De Oliveira et al., 2020). Within this broad list, ESKAPEE pathogens were designated "priority status", e.g., Carbapenem resistant *A. baumannii* and *P. aeruginosa* (CRAB and CRPA) along with extended spectrum β-lactamase (ESBL) or carbapenem resistant *K. pneumoniae* (CRKP) and *Enterobacter* spp. are listed in the critical priority list of pathogens; whereas, vancomycin resistant *E. faecium* (VRE) and methicillin and vancomycin resistant *S. aureus* (MRSA and VRSA) are in the list of high priority group (Mulani et al., 2019). Building on the 2017 edition, WHO released a Bacterial Priority Pathogens List (BPPL) in May 2024, updating and refining the prioritization of 15 families of antimicrobial resistant bacterial pathogens and grouping them into critical, high and medium categories for prioritization, to address the evolving challenges of AMR (WHO, 2024).

In this context, these clinically important antimicrobial-resistant foodborne pathogens with high-level AMR or hypervirulence have spread so quickly that they could be found emerging in clinical hospitals, agricultural farmlands, foods, food animals, environments and also humans/animals guts, by virtue of the fact that they have several key biological characteristics, including adaptations for survival in the modern health-care setting, diverse methods for acquiring resistance determinants and the dissemination of successful high-risk clones around the world (Miller and Arias, 2024). However, it is still unclear and understudied as to how prevalent these pathogens are, where they originated from, mechanisms for their extensive resistance and/or hypervirulence, their dissemination, evolution, and potential impacts, and so on. We need more intensive and compelling evidence, explanation, and interpretation. Multi-omics approaches, including genomics, transcriptomics, proteomics and metabolomics etc, are important tools to untangle the genetic, immunologic, (post)transcriptional, (post)translational, and metabolic mechanisms underlying progression from contamination to dissemination, from AMR to pathogenesis, from infection to clearance of the mechanisms of foodborne pathogens (Khan et al., 2019).

This Research Topic aims to provide a platform for recent discoveries and the latest progress in detection, mechanism, and dissemination from omics insights with regards to the emerging or re-emerging foodborne pathogens with high-level resistance or hypervirulence, to increase our understanding of these superbugs, to track their sources, to discover the mechanisms that make them behave thus, and to uncover the dissemination along the animal-food-human chain using big data analysis, to assess human health risks.

Salmonella is one of the most common foodborne pathogens causing sporadic cases or outbreaks of gastroenteritis around the world. Salmonellosis, including gastroenteritis and typhoid fever, is the disease mainly caused by consuming food contaminated with *Salmonella*, has a high health and economic burden globally. Poultry meat production supply chain has frequently been associated with human salmonellosis cases and is an important cause of *Salmonella* transmission between poultry farms and humans (Rincón-Gamboa et al., 2021). *Salmonella* is also a significant repository of AMR genes (ARGs), posing substantial challenges to public health and security (Jajere, 2019). Several papers included in this Research Topic reported on AMR and virulence factors: Wang et al. investigated the prevalence, AMR, and genomic characteristics of *Salmonella* isolated from chilled chicken carcasses and humans with diarrhea in Qingdao, China. The most common serotypes (Enteritidis and Typhimurium) and the top four detected resistance phenotypes (to nalidixic acid, ampicillin, tetracycline and chloramphenicol) in *Salmonella* were reported from both chicken and human sources. High prevalence of plasmid replicons and prophages were observed among the isolates and a total of 79 ARGs were found, including ESBL genes, bla_{NDM-1}, *mcr-1.1*, and *mcr-9.1*, mediating resistance to these critically important compounds for the clinical treatment of infections caused by ESKAPEE pathogens. Zhou L. et al. uncovered a marked increasing trend in MDR *Salmonella* isolated from retail foods in Guizhou province, China, (2016–2021), with strains from meat products showing significantly higher drug resistance than those from other sources. Their data also showed that *S.* Typhimurium and *S.* Enteritidis were the most prevalent serovars, with a notable presence of MDR strains and key virulence genes. Mora et al. investigated the antimicrobial profiles, virulence, and susceptibility of 105 *S. enterica* isolates from swine and chicken samples obtained from slaughterhouses and public wet markets in Metropolitan Manila using whole genome sequencing (WGS) analysis. This study provides proof of principle that WGS approaches can untangle the complex AMR and virulence patterns and shows that sequencing should be implemented by meat inspection authorities to augment the existing presence–absence detection tests as acceptability, safety, and quality criteria. In addition, WGS can also give insights into strain clustering and evidence of infection cross-contamination. Ghoshal et al. analyzed the phenotypic and transcriptomic alterations in the acid-evolved lineages (EL) of *Salmonella enterica* serovar Enteritidis after acid stress exposure to delve into the molecular mechanisms driving adaptive laboratory evolution (ALE) of *Salmonella* in acid stress. They found the elevated antibiotic minimum inhibitory concentration (MIC) observed after exposure to acetic acid for 70 days was lost when acid stress was removed and the MIC swiftly elevated again after stress reintroduction. This phenomenon was observed against several human antimicrobials such as meropenem, ciprofloxacin, gentamicin, and streptomycin. Transcriptomic

analysis demonstrated the upregulation of drug resistance, virulence, iron metabolism, and stress adaptation genes during continuous acid stress. The reversible nature of antimicrobial resistance highlights the adaptability of bacterial populations and the potential consequences of stress-induced adaptations. This research provides important insights into the process of adaptive evolution in bacterial populations and underscores the intricate relationship between stress adaptation, antibiotic resistance, and bacterial fitness. This study also emphasizes the importance of the need for a comprehensive and holistic approach, e.g., a combination of genomics and transcriptomics, to understand bacterial responses to stress and their impact on public health and food safety.

Carbapenem-resistant Enterobacterales (CRE), especially *E. coli*, carrying New Delhi Metallo-β-lactamase (NDM) have increased rapidly over the last decades and have become an urgent public health threat (El-Gamal et al., 2017), leading to the increased clinical use of colistin, which has been considered as the last therapeutic option for treating infections caused by MDR microorganisms. However, the efficacy of colistin has been challenged by the emergence of plasmid-mediated mobile colistin resistance (MCR), which was also found in Enterobacteriaceae in 2015 (Liu et al., 2016). Liu et al. reported the first case of *E. coli* carrying $bla_{\mathrm{NDM-5}}$ of retail eggs in Guangdong province of China. The IncI1-plasmid-carrying $bla_{\mathrm{NDM-5}}$ displayed high homology with a clinical plasmid pEC6363-NDM5, while the IncHI2 plasmid harboring $bla_{\mathrm{NDM-5}}$ shared highly similar structures with plasmids of animal origin. Considering the clinical importance of carbapenem together with the fact that the consumption of eggs is substantial in human diet, the carbapenem resistance in eggs has the risk to spread to humans throughout the food chain. Feng et al. identified a colistin resistant *E. coli* ST744 isolate from a fecal sample collected in Shanghai, carrying a conjugable IncI2 plasmid with a stable transferable *mcr-1.1* gene and exhibiting extensive AMR profiles and additional AMR genes, indicating a high risk to disseminate the extensively-drug-resistance phenotype among Enterobacteriaceae.

S. aureus exhibits vital adaptability across diverse habitats including food products, human skin, wastewater, treatment plants, public spaces, and households, etc. (Fang et al., 2024). It is also a major cause of infections in both inpatient and outpatient settings. The emergence of MRSA, particularly in communities in the 1990s, raised alarms and resulted in both greater surveillance of MRSA and implementation of strategies to limit MRSA transmission in clinical settings (Carrel et al., 2024). Zhang et al. investigated the prevalence, AMR and genomic characterization of MRSA isolated from ground pork, retail whole chicken, and patient samples in Hanzhong, China. Their data showed that 83.9% MRSA isolates expressed an MDR phenotype and three dominant livestock-associated MRSA (LA-MRSA) sequence types were identified: ST59-t437, ST9-t899, and ST398, among which, the previous major human MRSA ST59 had become the predominant interspecies MRSA sequence type among humans and retail livestock products with high adaption and transmission capacity, warranting special attention and active surveillance in China. In view of the fact that *S. aureus* has developed resistance against glycopeptides which were considered the last drug of choice against MRSA, and the emergence of vancomycin-resistant, and teicoplanin-resistant strains is globally reported, Habib et al. explored the role of the *tcaRAB* operon in *S. aureus* persister cells formation using WGS and RNA sequencing technology by studying a clinical MRSA isolate from a COVID-19 patient which showed a high level of resistance to teicoplanin, vancomycin, and methicillin. Their data revealed that Δ*tcaA* mutants resulted in a significant increase in persister cell formation in comparison to the wild type and *tcaA* might be one of the key genes that increase persister cells and glycopeptide resistance and could be a potential therapeutic target in *S. aureus*, and *tcaA* inactivation would give rise to persisters that tolerated a high concentration of glycopeptides and resuscitated the bacterial population. Their findings might begin to explain in part the mechanism of persister cell formation during host infection of MRSA.

K. pneumoniae has an exceptional ability to acquire exogenous resistance-encoding and hypervirulence-encoding genetic elements and the emergence of carbapenem-resistant *K. pneumoniae* (CRKP) has led to the extremely limited antibiotic therapy options (Ernst et al., 2020). At first, concurrent hypervirulence and MDR phenotypes have not been reported in *K. pneumoniae*. However, hypervirulent CRKP (hv-CRKP) isolates have been increasingly reported in recent years. The epidemic of hv-CRKP strains have emerged as a worldwide public health concern as they may cause untreatable, severe infections (Yang et al., 2021). Wu et al. reported the uncommon co-existence of $bla_{\mathrm{OXA-232}}$, *rmtF*, and a mobile pLVPK-like virulence plasmid in a ST15 CRKP isolate, after the screening of 207 CRKP isolates from patients at 12 tertiary China teaching hospitals in eight Chinese provinces from January 2015 to May 2021. It not only exhibited resistance to carbapenems and high-level resistance to aminoglycosides, but also possessed typical pathogenic characteristic, including hypermucoviscosity and hypervirulence phenotypes. Notably, they found the co-existence of resistance and virulence plasmids not only generated the high-risk hypervirulent multidrug-resistant phenotype, but also increased the transmission of non-conjugative virulence plasmid. Jiang et al. attempted to characterize the differences in molecular characteristics and expression of virulence genes between 150 clinical isolates of CRKP and 213 isolates of carbapenem-sensitive *K. pneumoniae* (CSKP) from the local area in Ningbo, China. Their data showed that CSKP could carry a significantly greater number of virulence genes and higher virulence gene expression efficiency and virulence phenotype than CRKP. Compared to CSKP, CRKP strains had noticeable homogeneity with ST11 being the predominant sequence type, whereas CSKP strains exhibited relative diversity, but there is still some evidence of clonal dissemination (ST23).

B. cereus is one of the leading etiological agents of toxin-induced foodborne diseases, and its omnipresence in different environments, its capacity for spore formation, and its ability to adapt to varying conditions and produce harmful toxins make this pathogen a health hazard that should not be underestimated; food poisoning attributed to *B. cereus* can manifest itself as an emetic or diarrheal syndrome which were caused by the potent peptide toxin cereulide and proteinaceous enterotoxins, respectively (Jovanovic et al., 2021). As a foodborne pathogen, as well as a causative agent of non-gastrointestinal infections

and even nosocomial complications, *B. cereus* has inspired vast volumes of multidisciplinary research in food and clinical domains. However, we only collected one manuscript in our Research Topic, Zhou Q. et al. reported nine strains of *B. cereus* from four foodborne outbreaks in Guizhou Province in southwest China collected from June to September 2021. WGS, comparative genomic and secondary metabolite analysis were performed to give a thorough exploration in terms of the mechanism of toxin production. They observed a contraction of gene families among the isolates, which were mainly associated with prophages and which contributed to the species diversity of *B. cereus*. The Hsp20 gene family underwent a rapid evolution in these strains, which facilitated the adaptation to adverse environmental conditions. They also found a higher copy number in the non-ribosomal polypeptide synthetase (NRPS) genes and found isolates carried the complete cereulide synthetase (ces) gene cluster sequences, which is a classical regulatory mechanism for emetic toxin synthesis. These findings are important for further investigation into the evolutionary relationship between *B. cereus* and their related species, as well as the underlying mechanisms governing the synthesis and secretion of bacterial toxins.

It was thought that housemaids operating inside a kitchen could be the source of infection and may transmit disease-inflicting pathogens through contaminated hands. This is an interesting hypothesis that historically was considered to be linked to cases such as Typhoid Mary in the United States. To assess the prevalence and antimicrobial susceptibility profile of bacteria isolated from the hands of housemaids in Jimma City, Ethiopia, a laboratory-based cross-sectional study including 234 housemaids were carried out by Ango et al.. The proportion of housemaids' hands containing one or more positive bacterial isolates was 72%, and the dominant bacterial isolates included *S. aureus* (31.6%), *E. coli* (21.3%), *Klebsiella* species (23.1%), *Proteus* species (14.7%), which accounts for the proportion of 90%, following by *Shigella* species (6.7%) and *Salmonella* species (1.3%). Fingernail status and the removal of a watch, ring, and bracelet during hand washing were significantly associated with the prevalence of bacterial isolation. Most of the bacterial isolates were susceptible to chloramphenicol and gentamycin, while the majority of them were resistant to tetracycline, vancomycin, and ceftazidime.

Due to the long-term and inappropriate use of antibiotics for the prevention and control of bacterial diseases in aquaculture, ARGs have become a new source of pollution in aquatic products. Factors such as the spread of drug-resistant strains and the horizontal transfer of drug-resistant genes have led to multi-drug resistance in fish pathogens, which seriously affects the quality and safety of aquatic products. Gao et al. characterized the phenotypic characteristics of the bacteria to common drugs used in aquaculture such as sulfonamides, amide alcohols, quinolones, aminoglycosides and tetracyclines, and screened for AMR genes in horse mackerel and puffer fish products sold in aquatic product markets and seafood supermarkets in Dalian, China. Their statistical analyses demonstrated that the drug resistance phenotypes and genotypes of bacteria detected in marine aquaculture fish samples in the Bohai Sea area of Dalian were complex, and the multi-drug resistance rate reached 80%, which might be closely related to the practice of intensive aquaculture. The detection rate of the AMR genes *tetA*, *sul1*, *sul2*, *qnrA*, *qnrS*, and *floR* exceeded 70% and all samples carried more than three drug resistance genes. Their data also indicated that the aminoglycosides gentamicin and tobramycin could still be considered effective in controlling bacterial infection in marine fish in the study area.

In this Research Topic, we also included a study on the analysis of clinical drug resistance mechanisms of the fungus *Aspergillus fumigatus*, and a new method of multi-detection and identification of food-borne pathogens in agricultural wastewater using next-generation sequencing technology. Zeng et al. investigated the biological characteristics of five strains of *A. fumigatus* in terms of sporulation, biofilm formation, evasion of phagocytosis, virulence, and drug sensitivity. A series of comprehensive experimental techniques, including Sanger sequencing, three-dimensional (3D) protein construction, high-performance liquid chromatography (HPLC), real-time quantitative polymerase chain reaction (RT-qPCR), biochemical analyses and transcriptomics, were employed to explore and clarify the mechanisms underlying drug resistance of the five strains. They found notable differences in the biological characteristics and pathogenic ability among the five test strains. The *cyp51A* mutations combined with the *hmg1* mutation S541G were associated with the mechanism of ITR resistance; the VRC resistance mechanism was associated with *cyp51A* mutations, changes in energy production, and increased expression of genes drug efflux pumps. Park et al. developed and optimized a novel NGS panel method that achieves the rapid and accurate detection and identification of 18 specific virulence factor genes from six target food-borne pathogens (*Bacillus cereus*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*) in contaminated samples with efficiency, sensitivity, and accuracy. They introduced the term "NGS panel" to the readers, which refers to an NGS-based assay that allows for the simultaneous analysis of multiple genes, genetic variants, microbial genomes, or other genomic features, with which hundreds to thousands of target gene sequences can be screened at once and many samples can be simultaneously analyzed to rapidly and efficiently detect and identify various foodborne pathogens. It also avoids the issue of producing an overabundance of sequence information which occur in 16S rRNA sequencing based on Sanger sequencing, 16S rRNA-based metagenome and random genome sequencing-based shotgun metagenomics approaches prior to the use of NGS panels. Therefore, this technology could be useful for ensuring food safety through the prevention of foodborne disease outbreaks via the rapid and accurate detection and identification of foodborne pathogens.

In conclusion, this Research Topic provides a platform for recent discoveries and the latest progress in detection, mechanisms, and dissemination from omics insights with regards to the emerging or re-emerging foodborne pathogens with high-level AMR (Multi-drug resistant/Extensively-drug resistant/Pan-drug resistant, MDR/XDR/PDR) or hypervirulence, expending our current understanding of these superbugs, and enabling us to track their sources, to discover the mechanisms and dissemination paths along the

animal-food-human chain. This Research Topic presented 15 papers that showed important information and new directions for related research, contributing to a better understanding of AMR and pathogenic mechanisms. Simultaneously, the concept of an integrated multi-disciplinary "One Health" approach has also been mentioned for widespread and sustained surveillance of foodborne pathogens, based on a multi-sectoral collaboration framework, to mitigate and prevent the threats of pathogens of animal-, human-, environment-, and food-origins (Erkyihun and Alemayehu, 2022). Moreover, this Research Topic emphasises the necessity for continued multi-disciplinary research and surveillance to mitigate the threats posed by these formidable pathogens.

Whereas, humans and pathogenic bacteria have coexisted in this earth and been intertwined through all of humanity's history. Despite recent progresses in this struggle, mankind has not really defeated these microbes, and emerging and re-emerging infectious diseases have long been a major threat to human health, since they are always on a path of mutation, development, and evolution, however, we still know very little about these aspects. For this reason, further research is needed to understand the terms of pathogenicity, the evolutionary arms race, and prevention in our food supplies. We are planning a sequel series of this Research Topic, which will aim to focus more on the virulence phenotype detection, pathogenic mechanism, fitness cost, toxicological evaluation techniques and risk assessment to human health of food-borne pathogenic microorganisms. We expect the next Research Topic would provide the research community with some of the most recent updates in the area.

Author contributions

YH: Funding acquisition, Investigation, Resources, Writing – original draft. WW: Investigation, Resources, Writing – original draft, Writing – review & editing. SN: Investigation, Resources, Writing – original draft, Writing – review & editing. GM: Investigation, Resources, Writing – original draft, Writing – review & editing. FL: Methodology, Resources, Supervision, Writing – review & editing. SF: Methodology, Resources, Supervision, Writing – review & editing.

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Conflict of interest

SN is employed by ATCC.

The remaining authors declare that the research was conducted in the absence of any commercial financial relationships that could be construed as a potential conflict of interest.

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References

Carrel, M., Smith, M., Shi, Q., Hasegawa, S., Clore, G. S., Perencevich, E. N., et al. (2024). Antimicrobial resistance patterns of outpatient *Staphylococcus aureus* isolates. *JAMA Netw. Open* 7:e2417199. doi: 10.1001/jamanetworkopen.2024.17199

De Oliveira, D. M. P., Forde, B. M., Kidd, T. J., Harris, P. N. A., Schembri, M. A., Beatson, S. A., et al. (2020). Antimicrobial resistance in ESKAPE pathogens. *Clin. Microbiol. Rev.* 33, e00181–e00119. doi: 10.1128/CMR.00181-19

El-Gamal, M. I., Brahim, I., Hisham, N., Aladdin, R., Mohammed, H., Bahaaeldin, A., et al. (2017). Recent updates of carbapenem antibiotics. *Eur. J. Med. Chem.* 131, 185–195. doi: 10.1016/j.ejmech.2017.03.022

Erkyihun, G. A., and Alemayehu, M. B. (2022). One Health approach for the control of zoonotic diseases. *Zoonoses* 2:37. doi: 10.15212/ZOONOSES-2022-0037

Ernst, C. M., Braxton, J. R., Rodriguez-Osorio, C. A., Zagieboylo, A. P., Li, L., Pironti, A., et al. (2020). Adaptive evolution of virulence and persistence in carbapenem-resistant *Klebsiella pneumoniae*. *Nat. Med.* 26, 705–711. doi: 10.1038/s41591-020-0825-4

Fang, G. Y., Wu, F. H., Mu, X. J., Jiang, Y. J., and Liu, X. Q. (2024). Monitoring longitudinal antimicrobial resistance trends of *Staphylococcus aureus* strains worldwide over the past 100 years to decipher its evolution and transmission. *J. Hazard. Mater.* 465:133136. doi: 10.1016/j.jhazmat.2023.133136

Jajere, S. M. (2019). A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Vet. World* 12, 504–521. doi: 10.14202/vetworld.2019.504-521

Jovanovic, J., Ornelis, V. F. M., Madder, A., and Rajkovic, A. (2021). *Bacillus cereus* food intoxication and toxicoinfection. *Compr. Rev. Food Sci. Food Saf.* 20, 3719–3761. doi: 10.1111/1541-4337.12785

Khan, M. M., Ernst, O., Manes, N. P., Oyler, B. L., Fraser, I. D. C., Goodlett, D. R., et al. (2019). Multi-omics strategies uncover host-pathogen interactions. *ACS Infect. Dis.* 5, 493–505. doi: 10.1021/acsinfecdis.9b00080

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., et al. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* 16, 161–168. doi: 10.1016/S1473-3099(15)00424-7

Miller, W. R., and Arias, C. A. (2024). ESKAPE pathogens: antimicrobial resistance, epidemiology, clinical impact and therapeutics. *Nat. Rev. Microbiol.* doi: 10.1038/s41579-024-01054-w. [Epub ahead of print].

Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., and Pardesi, K. R. (2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: a review. *Front. Microbiol.* 10:539. doi: 10.3389/fmicb.2019.00539

Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Naghavi, M., et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* 399, 629–655. doi: 10.1016/S0140-6736(21)02724-0

O'Neill, J. (2016). *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. Review on Antimicrobial Resistance*. London: Wellcome Trust. Available online at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf (accessed July 28, 2024).

Pires, S. M., Desta, B. N., Mughini-Gras, L., Mmbaga, B. T., Fayemi, O. E., Salvador, E. M., et al. (2021). Burden of foodborne diseases: think global, act local. *Curr. Opin. Food Sci.* 39, 152–159. doi: 10.1016/j.cofs.2021.01.006

Rincón-Gamboa, S. M., Poutou-Piñales, R. A., and Carrascal-Camacho, A. K. (2021). Analysis of the assessment of antimicrobial susceptibility. Non-typhoid *Salmonella* in meat and meat products as model (systematic review). *BMC Microbiol.* 21:223. doi: 10.1186/s12866-021-02268-1

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., et al. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* 18, 318–327. doi: 10.1016/S1473-3099(17)30753-3

United Nations Environment Programme (2023). *Bracing for Superbugs: Strengthening Environmental Action in the One Health Response to Antimicrobial Resistance*. Geneva

WHO (2024). *WHO Bacterial Priority Pathogens List, 2024: Bacterial Pathogens of Public Health Importance to Guide Research, Development and Strategies to Prevent and Control Antimicrobial Resistance*. Geneva: World Health Organization.

Yang, X., Dong, N., Chan, E. W., Zhang, R., and Chen, S. (2021). Carbapenem resistance-encoding and virulence-encoding conjugative plasmids in *Klebsiella pneumoniae*. *Trends Microbiol.* 29, 65–83. doi: 10.1016/j.tim.2020.04.012

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Co-occurrence of OXA-232, RmtF-encoding plasmids, and pLVPK-like virulence plasmid contributed to the generation of ST15-KL112 hypervirulent multidrug-resistant *Klebsiella pneumoniae*

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The emergence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains and restricted therapeutic options pose a global threat to public health. Aminoglycosides are a wise choice, which can effectively reduce the mortality rate when combined with β-lactam drugs. However, in this study, we identified a ST15-KL112 CRKP FK3006 which not only exhibited resistance to carbapenems, but also exhibited high level resistance to aminoglycosides. In addition to the multidrug resistant phenotype, FK3006 also owned typical pathogenic characteristic, including hypermucoviscosity and hypervirulence phenotypes. According to the whole-genome sequencing, one pLVPK-like virulence plasmid, and three key resistant plasmids ($bla_{OXA-232}$, $bla_{CTX-M-15}$, and *rmtF*) were observed in FK3006. Compared to other typical ST15 CRKP, the presence of pLVPK-like virulence plasmid (p3006-2) endowed the FK3006 with high virulence features. High siderophore production, more cell invasive and more resistant to serum killing was observed in FK3006. The *Galleria mellonella* infection model also further confirmed the hypervirulent phenotype of FK3006 *in vivo*. Moreover, according to the conjugation assay, p3006-2 virulence plasmid also could be induced transfer with the help of conjugative $IncFII_K$ p3006-11 plasmid ($bla_{CTX-M-15}$). In addition to the transmissible plasmid, several insertion sequences and transposons were found around $bla_{CTX-M-15}$, and *rmtF* to generate the mobile antimicrobial resistance island (ARI), which also make a significant contribution to the dissemination of resistant determinants. Overall, we reported the uncommon co-existence of $bla_{OXA-232}$, *rmtF*-encoding plasmids, and pLVPK-like virulence plasmid in ST15-KL112 *K. pneumoniae*. The dissemination threatens of these high-risk elements in *K. pneumoniae* indicated that future studies are necessary to evaluate the prevalence of such isolates.

KEYWORDS

***bla*$_{OXA-232}$, *rmtF*, *Klebsiella pneumoniae*, carbapenemase, mobile element, plasmid**

Introduction

The emergence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has become a major challenge facing clinical management and global public health, because of the extremely limited antibiotic therapy options (Ernst et al., 2020). Aminoglycosides are important options for treating infections caused by CRKP and are generally administered in combination with β-lactam agents and tigecycline (Daikos et al., 2014; Karaiskos et al., 2019). However, increasing rates of aminoglycoside resistance in CRKP have been reported in recent years, posing a new challenge for treatment (Galani et al., 2019). Hence, verifying the related mechanism and demonstrating the potential of the spread of these resistant phenotypes in clinical isolate are vital clues to solving antibiotic resistance.

It has been highlighted *Klebsiella pneumoniae* carbapenamase (KPC) is the most prevalent in China, it is noteworthy that OXA-48-like carbapenemases are common carbapenemases in *Enterobacterales* in certain regions of the world (Pitout et al., 2019). To date, several variants that differ from OXA-48 by only a few amino acids and display similar enzymatic profiles with OXA-48 have been identified. OXA-232 differs from OXA-48 by five amino acid substitutions, exhibiting a lower ability to hydrolyze carbapenems but greater hydrolytic activities against penicillin than OXA-48 (Potron et al., 2013; Miltgen et al., 2020). Since the first report of an OXA-232-producing *K. pneumoniae* strain in China in 2016, such isolates has became epidemic in China, and usually associated with a clonal dissemination of ST15 *K. pneumoniae* (Yin et al., 2017; Wang et al., 2022). Most aminoglycoside resistance mechanisms were associated with the aminoglycoside-modifying enzymes, among which only 16S rRNA methyltransferase (16S-RMTase)-encoding genes could mediate high-level resistance to aminoglycosides (Ramirez and Tolmasky, 2010). Among these genes, *rmtB* and *armA* present the most widespread 16S rRNA methylase genes, with *rmtF* is rarely reported (Nagasawa et al., 2014). Notably, these antibiotic resistances could be carried by various mobile genetic elements (MGEs), once these resistant elements co-existence in one host, the therapeutic options would be very limited.

Klebsiella pneumoniae has an exceptional ability to acquire exogenous resistance-encoding and hypervirulence-encoding genetic elements. For a long period, *K. pneumoniae* did not simultaneously encode the phenotypes of multidrug-resistance (MDR) and hypervirulence. However, carbapenem-resistant hypervirulent *K. pneumoniae* (hv-CRKP) isolates have been increasingly reported in recent years. The epidemic of hv-CRKP strains has emerged as a worldwide public health concern as they may cause untreatable, severe infections (Yang et al., 2021).

Although the hv-CRKP has increased rapidly, most were associated with the KPC carbapenemases and ST11 *K. pneumoniae*, the ST15-OXA-232 hv-CRKP was uncommon. In this study, we identified a hypermucovious multidrug-resistant ST15 *K. pneumoniae* (FK3006), exhibiting resistance to both aminoglycosides and ertapenem. Then, we applied whole-genome-sequencing (WGS) to explore the potential molecular mechanisms and observed four key plasmids. We also applied the conjugation assay to further determine the dissemination of these high-risk determinants and verified the relevant virulence phenotype of FK3006. In addition to the plasmids, we described other related MEGs through genetic comparisons as well. Overall, our goal was to report and describe a clinical hypervirulent carbapenem-resistant *K. pneumoniae* clearly, and emphasize the possible risk of these strains.

Materials and methods

Bacterial isolates

First, the 207 CRKP isolates from patients at 12 tertiary China teaching hospitals in eight Chinese provinces were collected From January 2015 to May 2021. And then the detached samples were cultured on blood agar plate and identified by MALDI-TOF MS. FK3006 was isolated from the sputum sample, for it is the only isolate harboring the co-existence of $bla_{\text{OXA-232}}$, $bla_{\text{CTX-M-15}}$, *rmtF*, and virulence plasmid analyzed by WGS. *K. pneumoniae* strain 3036 (FK3036) was used as control strain for the virulence-negative resistant strain (ST15, none Virulence factors) and NUTH-K2044(ST23) was used as a virulence-positive control (Supplementary Table S1). Plasmid conjugation was performed with *Escherichia coli* 600(EC600, rifampicin-resistant), which was used as the recipient strain.

Antimicrobial susceptibility test

The MICs of the FK3006, EC600, and transconjugants (FK3006JH-1, FK3006JH-2) were determined by standard broth microdilution method following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2020), except for colistin and tigecycline, for which the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used (EUCAST, 2020). Each AST was independently repeated three times in our study. *Escherichia coli* ATCC 25922 was used as the quality control organism in MIC determination.

Conjugation assay

The horizontal transferability of $bla_{\text{OXA-232}}$, $bla_{\text{CTX-M-15}}$, *rmtF*, and *iucA* were examined using the conjugation assay with *E. coli* 600. The FK3006 was used as donor strain, and *E. coli* 600 was used as the recipient strain. The recipients and donors were cultured in Luria-Bertani broth (37°C) until logarithmic phase (OD600 = 0.4–0.6), mixed in a ratio of 2:1 (200 μl,100uL) in 4 ml LB broth for 24 h. And then the serial dilutions were plated on selective media with appropriate antibiotics (gentamicin, 8 μg/ml[*rmtF*]; ampicillin, 100 μg/ml[$bla_{\text{CTX-M-15}}$]; meropenem, 1 μg/ml[$bla_{\text{OXA-232}}$]; dipotassium tellurite, 8 μg/ml[*iucA*]; rifampicin, 600 μg/ml). The frequency of plasmid transfer was calculated as the number of transconjugants per recipient. The transconjugants acquiring gene were confirmed by PCR and primers are listed in Supplementary Table S2.

Growth assays to assess in fitness

After the plasmid was confirmed to be obtained, we applied growth curve assays to investigate fitness. Transconjugants (FK3006JH-1 and FK3006JH-2) and EC600 were cultured in LB

medium overnight, then diluted to an OD600 of 0.01 and grown at 37°C for 24 h. Culture densities were determined by measuring the OD600 every 1 h for the first 12 h and then 24 h (Liu et al., 2016).

Whole genome sequencing and bioinformatics analysis

Bacterial genomic DNA of FK3006 was isolated using the Qiagen DNA extraction Kit (Qiagen, Germany) and the genome sequencing was then performed by the PacBio Sequel platform and the Illumina NovaSeq 6,000 platform. CANU (version 1.7.1) software was applied to assemble the data acquired by PacBio platform sequencing. The ORF prediction was measured in SnapGene (version 4.2.4). Resistant plasmid replicons were predicted using the PlasmidFinder tool[1]. To verify whether the plasmid was also a conjugative plasmid, VRprofile[2] and OriT Finder website[3] were performed to analysis of the four conjugal modules in the plasmid, including the relaxase gene, the origin of transfer site (oriT), the type IV secretion system gene cluster (T4SS), and the type IV coupling protein (T4CP) gene. The transposons (Tns) and insertion sequences (ISs) were also annotated and determined through the VRprofile and ISFinder[4]. We used BLAST Ring Image Generator (BRIG) to determine similar plasmids by comparing their identities and coverages. The circular representation was performed by Proksee[5]. The gene environments surrounding the antibiotic resistance gene was analyzed by Easyfig software. VFDB[6] was performed to annotate the virulence factors.

Quantitative siderophore production assay

Briefly, bacterial clones were diluted with saline to a concentration of approximately 10^8 CFU/ml, and then 1 μl bacterial clones were dropped on CAS and King's B (2:1) plates. After incubation for 24 h at 37°C, siderophore production was determined by the presence of an orange halo around the bacterial colonies (Zhou et al., 2022).

Galleria mellonella killing studies

Caterpillars of *Galleria mellonella* were stored at 4°C before use for ensuring their health and fitness. Caterpillars were selected weighing 200–250 mg each for the study. For the FK3006, EC600, FK3006JH-1 and FK3006JH-2 groups, the caterpillars were injected with 10 μl (~1×10^6 CFU) bacterial suspension at the left proleg, while the control groups were injected with PBS or empty syringe. A minimum of 10 caterpillars was used in each treatment group; they then were kept in culture at 37°C and inspected for 144 h. The survival rates of each group were recorded for each day (Li et al., 2020).

Serum killing assay

Briefly, serum was separated from blood samples and stored at-80°C. An inoculum of 25 μl (~1×10^6 CFU) bacteria prepared from the mid-log phase was incubated with 75 μl pooled human sera. The mixture was taken at 0, 1, 2, and 3 h, and then incubated on the MHA plate. The numbers of viable bacteria were determined at 24 h (Liu et al., 2019).

Infection of human cells and *Klebsiella pneumaniae*-mediated cytotoxicity

Approximately 1×10^5 A549 human lung epithelial cells (ATCC CCL-185) were grown in each well of 24-well plates in DMEM medium containing 10% fetal calf serum at 37°C with 5% CO_2 for 12 h and then incubated for 21 h at 37°C with 2×10^7 CFU bacteria (Gottig et al., 2016). After centrifugation (3,000 rpm, 5 min, 4°C), the LDH in the supernatant was measured using the LDH Cytotoxicity Assay kit according to the instructions (Solarbio BC0685).

S1 pulsed-field gel electrophoresis

The S1-Nuclease Pulsed Field gel electrophoresis (S1-PFGE) was used to determine the existence of plasmids profiles in donor strains, and recipient strains. In short, the isolates were embedded in 1% Seakem Gold agarose and digested with S1 nuclease (Takara, Dalian, China). PFGE analysis was carried out using a CHEF-Mapper system (Bio-Rad) for 19 h with a switch time 4.0–40 s (parameters: 14°C, voltage 6 V/cm, and electric field angle 120°). The *Xba*I digested DNA of Salmonella serotype Braenderup H9812 was considered as a molecular size marker (Ai et al., 2021).

Statistical analysis

Data analyses were executed with the software GraphPad Prism 8.0.2. Results are shown as a two-tailed non-parametric Student's test. For *in vivo* and vitro experiments survival data were analyzed by the Log Rank test (Mantel-Cox). $P < 0.05$ was considered to be statistically significant.

Nucleotide accession number

The complete nucleotide sequences of the chromosome and p3006-2, p3006-3, p3006-7, and p3006-11 plasmid were deposited as GenBank accession numbers JANCTJ010000000 consists of sequences JANCTJ010000001-JANCTJ010000014.

Results

FK3006 was a multidrug-resistant strain

To characterize the antibiotic-resistant phenotype of FK3006, the 18 antibiotics susceptibility was tested in this strain. Results showed that FK3006 was a multi-drug resistant strain, which not only exhibited

1 https://cge.cbs.dtu.dk/services/PlasmidFinder/
2 https://tool2-mml.sjtu.edu.cn/VRprofile/
3 https://tool-mml.sjtu.edu.cn/oriTfinder/oriTfinder.html
4 https://www-is.biotoul.fr/
5 https://proksee.ca/projects/10a9d9fa-c055-4dfe-b615-a55049475a52
6 http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi

high-level resistance to both aminoglycoside antibiotics and a series of β-lactam antibiotics, but also was resistant to ciprofloxacin, sulfamethoxazole, minocycline, and piperacillin-tazobactam (Table 1). Although it was sensitive to imipenem, it still exhibited low-level resistance to other carbapenems including meropenem and ertapenem.

FK3006 co-harboring multiple resistance and virulence determinants

According to the subsequent WGS-based analysis, we further found that FK3006 belonged to ST15-KL112 isolates, a typical MDR clone. Moreover, 13 resistant elements, three resistant plasmids, and one virulence plasmid were detected in this isolate (Table 2). In addition, three key resistance genes played an important role in the acquisition of resistance to aminoglycoside antibiotics (*rmtF*), β-lactam antibiotics ($bla_{CTX\text{-}M\text{-}15}$), and carbapenems ($bla_{OXA\text{-}232}$). Moreover, rmpA2 as a critical factor activating the expression of capsular polysaccharides (CPS) genes which is responsible for CPS biosynthesis was detected in the IncFIB(K)/HI1B plasmid of FK3006. The *iucABCD-iutA* operon which encoding proteins necessary for aerobactin siderophore biosynthesis was also found in p3006-2.

The non-conjugative pLVPK-like virulence plasmid could be transferred with the help of conjugative $IncFII_K$ p3006-11 plasmid

We have identified three key resistant plasmids, and one virulence plasmid in FK3006. As plasmids are often transmissible between bacteria, we made a detailed analysis of these plasmids, aiming to further clarify potential resistance and virulence dissemination threats of FK3006. In FK3006, we observed three resistant plasmids: p3006-3, p3006-7, and p3006-11. p3006-11 ($bla_{CTX\text{-}M\text{-}15}$) was a typical $IncFII_K$-type MDR plasmid and shared high identity with the mobilizable plasmid pKP7450-3(identity 99.95%, $bla_{CTX\text{-}M\text{-}15}$, IncFII(K), CP090471.1), as well as the mobilizable plasmid pMS3802-CTXM-vir (identity 99.59%, $bla_{CTX\text{-}M\text{-}15}$, IncHI1B(pNDM-MAR), CP068016.1; Figure 1A). p3006-11 harbored four conjugation modules, holding the potential to self-transfer. Hence, we applied conjugation assay to imitate and evaluate the dissemination ability of p3006-11 plasmid. We found the p3006-11 was successfully transferred from FK3006 to *E. coli* EC600 (1.1×10^{-6}-9.7×10^{-5}). Moreover, the S1-PFGE pattern (Figure 2A) and MICs of FK3006JH-1(EC600 harboring $bla_{CTX\text{-}M\text{-}15}$ plasmid) also confirmed that the resistance phenotype dissemination of FK3006 (Table 1). Notably, the obtain of p3006-11 did not affect the growth of *E. coli* 600, which ensuring the stable existence of the resistance plasmid (Supplementary Figure S1).

p3006-2 was a typical IncFIB(K)/HI1B type virulence plasmid and shared ~99% identity with pK2044 (CP026012.1) and pLVPK (AY378100) plasmids (Figure 1B). Notably, p3006-2 also harbored the core *oriT* site and T4CP, which were identical to pK2044 and pLVPK. Previous studies have confirmed the pK2044 and pLVPK virulence plasmid could transferred from hypervirulent *K. pneumoniae* (hvKP) to ST11 CRKP and *E. coli* strains with the help of a self-transferable $IncFII_K$ plasmid. In this study, we also observed that the p3006-2 virulence plasmid could be induced transfer with the help of p3006-11 plasmid (8.3×10^{-8}-3.2×10^{-7}; Figure 2B).

TABLE 1 Antimicrobial drug susceptibility profiles.

Drug class	Antibiotics	MIC (mg/L)/antimicrobial susceptibility							
		FK3006	S/I/R	EC600	S/I/R	FK3006JH-1	S/I/R	FK3006JH-2	S/I/R
Carbapenems	Ipm	<=0.5	S	<=0.5	S	<=0.5	S	<=0.5	S
	Etp	1	I	<=0.5	S	<=0.5	S	<=0.5	S
	Mer	2	I	<=0.5	S	<=0.5	S	<=0.5	S
β-Lactam/β-lactamase	P/T	>128/4	R	<=4/4	S	<=4/4	S	<=4/4	S
Inhibitor complexes	Caz/Avi	<=0.25/4	S	<=0.25/4	S	<=0.25/4	S	<=0.25/4	S
Monocyclic β-lactam	Azt	>32	R	<=1	S	>32	R	>32	R
Cephalosporin	Fox	32	R	4	S	4	S	2	S
	Ctx	>64	R	<=1	S	>64	R	>64	R
	Cpe	>16	R	<=0.5	S	>16	R	>16	R
	Caz	>32	R	<=1	S	>32	R	>32	R
Fluoroquinolones	Cip	>4	R	<=0.25	S	0.5	I	0.5	I
Folate metabolic pathway	CoSMZ	>2/38	R	<=0.5/9.5	S	>2/38	R	>2/38	R
Inhibitors	Te	8	I	<=1	S	<=1	S	<=1	S
Tetracyclines	Min	>8	R	<=2	S	<=2	S	<=2	S
	TGC	1	S	<=0.25	S	<=0.25	S	<=0.25	S
Polymyxin B	PB	1	I	1	S	1	S	1	S
Aminoglycosides	Gen	16	R	<=0.5	S	<=0.5	S	<=0.5	S
	AMK	>64	R	<=2	S	<=2	S	<=2	S

MIC, minimum inhibitory concentration; S, susceptible; I, intermediate; R, resistant; Ipm, imipenem; Etp, Ertapenem; Mer, meropenem; P/T, piperacillin–tazobactam; Caz/Avi, ceftazidime–avibactam; Azt, aztreonam; Min, minocycline; Cpe, cefepime; Ctx, cefotaxime; Caz, ceftazidime; Fox, cefoxitin; Cip, ciprofloxacin; Te, tetracycline; CoSMZ, sulfamethoxazole; TGC, tigecycline; PB, polymyxin B; Gen, gentamicin; AMK, amikacin.

TABLE 2 Several features of the FK3006 genome.

Parameter	p3006-2	p3006-3	p3006-7	p3006-11
Accension number	JANCTJ010000002	JANCTJ010000003	JANCTJ010000007	JANCTJ0100000011
Length(bp)	177,803	128,305	10,397	139,495
No. of ORF[a]	387	296	22	318
Incompatibility group	IncFIB(K)/HI1B	IncFIB(pKPHS1)	ColKP3	IncFII(K)
Conjugal ability	T4CP	NO	NO	T4CP
	oriT			T4SS
				oriT
				Relaxase
Resistance gene(s)	NO	*rmtF*	$bla_{OXA-232}$	*sul2*
		ARR-3		*aph(3″)-Ib*
				aph(6)-Id
				bla_{TEM-1B}
				$bla_{CTX-M-15}$
				dfrA14
				qnrB1
Virulence factors	*rmpA2*	NO	NO	NO
	iucABCD			
	iutA			

[a]ORF, open reading frame.

Genetical features of other resistance plasmids

p3006-3 plasmid harbored *rmtF* gene and belonged to IncFIB type plasmid (Figure 1C), which was nearly identical (99.98%) to the human *K. pneumoniae* plasmid pKP3295-3 (*rmtF*, IncFIB(pKPHS1) CP079728.1) and pWSD411-4 (*rmtF*, IncFIB(pKPHS1), CP045677.1) previously reported in Hangzhou, China. Meanwhile, p3006-3 plasmid was similar to the pPMK1-B(coverage 86%, identity 99.96%, IncFIB(pKPHS1), CP008931.1) which also could be self-transferred to the recipient strain except for a multidrug resistance region of p3006-3 (Shi et al., 2020).

p3006-7 plasmid was a 10,397 bp circular molecule, harboring $bla_{OXA-232}$ resistance element, and contained no necessary elements for transmission by bioinformatic analysis. The conjugation assay suggested that p3006-7 was not self-transferable. According to the genomic comparison, we found p3006-7 was almost identical to pKNICU5 plasmid (identity 99.93%, ColKP3, KY454616.1), isolated from the first OXA-232-producing *K. pneumoniae* strain in China (Figure 1D; Yin et al., 2017). As the self-transmissible modules were usually absent in such ColKP3 type plasmid, the $bla_{OXA-232}$ genes may be mainly clonal dissemination.

FK3006 was a typical hypervirulent strain and the virulence phenotype could be transferred to the recipients

The existence of pLVPK-like plasmid p3006-2 and the hypermucoviscosity indicated that the FK3006 may be a hypervirulent strain, here we applied several experiments to confirm the virulent phenotype. We found that FK3006 strain have a survival of about 74% after 60 min of incubation with the serum, which was significantly higher than that of the hypervirulence-negative resistant strain FK3036 (ST15 CRKP; Figure 3A). Moreover, there was a significant increase of siderophore production in FK3006 ($d = 25$ mm), compared with FK3036 ($d = 10$ mm), which did not have the *iuc* operon (Figure 3B). A549 human lung epithelial cells were infected with FK3006 (LDH, 1.70 μmol/l) led to cell LDH release of 76.9% compared with the positive control strain NUTH-K2044 group (LDH, 2.21 μmol/l). And, the negative control FK3036 group only release 0.85 μmol/l LDH. We also applied *G. mellonella* infection model to analyze the virulence of FK3006 *in vivo*. Testing showed that the survival rate of FK3006 group was 20% at 72 h, which was almost identical to that of a virulent strain of NUTH-K2044 (Figure 3C). A whole genome BLAST search was performed against VFDB to validate virulence factors harbored by the FK3006 (Supplementary Table S3). These results were in accordance with the fact that FK3006 populations were typical hypervirulent strains.

Notably, according to the conjugation assay, we observed the virulence plasmid p3006-2 could successfully transferred to the recipient strain EC600, and obtained the transconjugant FK3006JH-2. We found that FK3006JH-2(*iucA*) strains have a survival of about 53% after 60 min of incubation with the serum, which was significantly higher than that of the recipient strain EC600 (Figure 3D). When the EC600 obtained the p3006-2 plasmid, siderophore production also increased with the diameter of the halo increased ~2-fold (Figure 3E). *G. mellonella* infection model showed that the survival rate of FK3006JH-2 group was significantly lower than that of EC600 group (Figure 3F). All these results indicated that the virulence features of

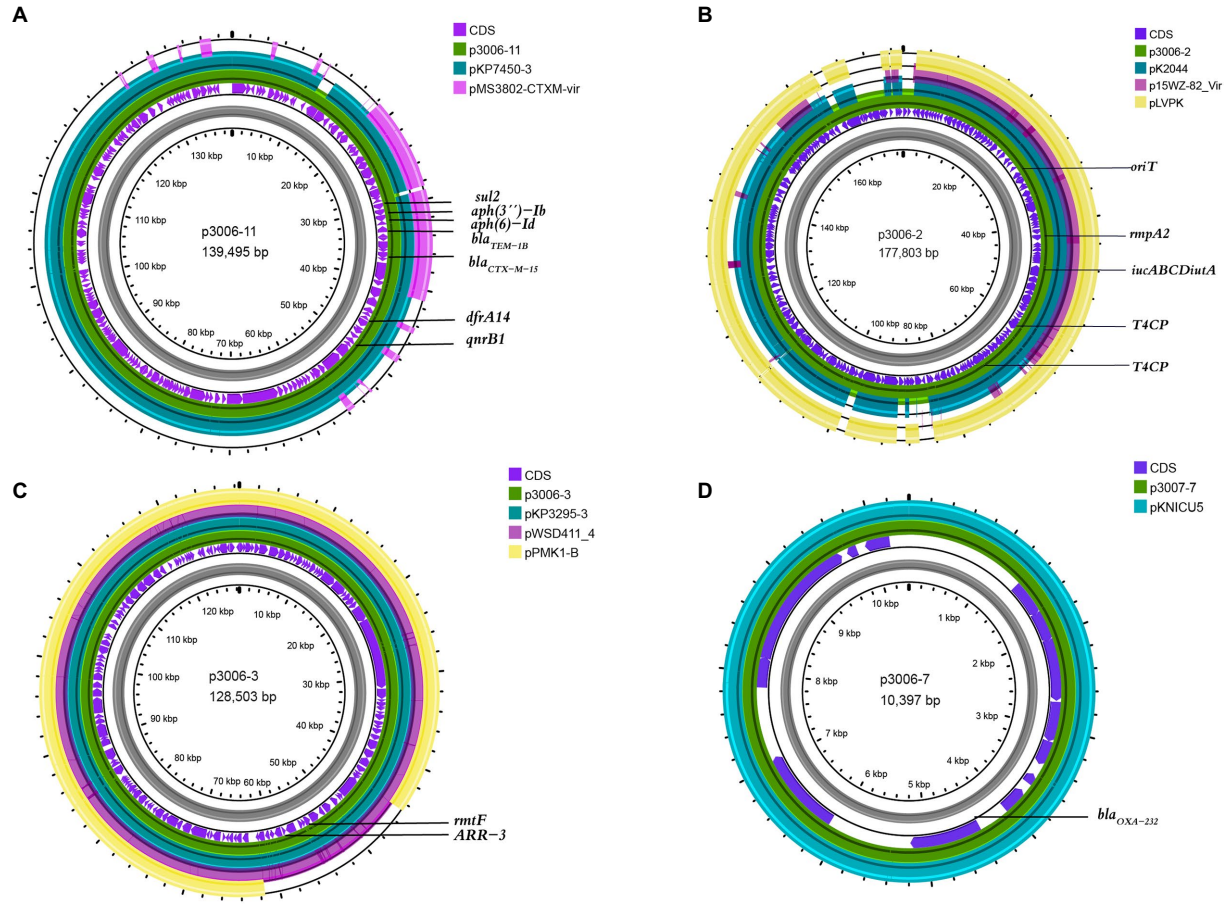

FIGURE 1
Comparative analysis of p3006-2, p3006-3, and p3006-7 plasmids with other reference plasmids. **(A)** Genome alignment was performed with p3006-11(JANCTJ010000011), pKP7450-3(99.95%, CP090471.1), and pMS3802-CTXM-vir(99.59%, CP068016.1). **(B)** Genome alignment was performed with p3006-2(JANCTJ010000002), virulent plasmid pK2044 (99.46%, CP026012.1), mobilisable virulence plasmid p15WZ-82-Vir(99.53%, CP032356.1), and pLVPK(99.51%, AY378100). **(C)** Genome alignment was performed with p3006-3(JANCTJ010000003), pKP3295-3(99.98%, CP079728.1), pWSD411_4(99.98%, CP045677.1), and mobilisable plasmid pPMK1-B(99.96%, CP008931.1). **(D)** Genome alignment was performed with p3006-7(JANCTJ010000007), and pKNICU5(KY454616.1).

the FK3006 could be transferred to the recipients through the key virulence plasmid p3006-2.

Mobile genetic elements associated with key resistance elements

The plasmid not only contains genes that promote the survival of the host but also carries other MGEs, such as Tn and IS, which also make a significant contribution to the dissemination of resistance genes. To thoroughly analyze the dissemination potential of *rmtF* and $bla_{\text{CTX-M-15}}$ in FK3006, we also analyze each of the MGEs surrounding them. In p3006-11, the $bla_{\text{CTX-M-15}}$, together with other antibiotic resistance genes (*sul2-aph(3")-Ib-aph(6)-Id-blaTEM*$_{\text{-1B-dfrA14-}}$ *qnrB1*), was part of a large AMR of 37,526 bp that was bracketed by two opposite orientation copies of the IS*26* (Figure 4A) and it was closely associated with the presence of class 1 integrons. IS*Ecp1*, a member of the IS*1380* family frequently upstream of the $bla_{\text{CTX-M-15}}$, seems to be a crucial element in the mobilization and dissemination of $bla_{\text{CTX-M-15}}$, as it not only located surrounding $bla_{\text{CTX-M-15}}$ in the p3006-11 plasmid, but also surrounding the $bla_{\text{CTX-M-15}}$ in the pE16K0288-1(CP052263.1, IncFIB(K), IncFII(K), Korea), pDA33141-217(CP029588.1, IncFIB(K), IncFII(K), Sweden) and pMS3802-CTX-M-Vir(CP068016.1, IncHI1B(pNDM-MAR), repB, Spain) (Upadhyay et al., 2015; Fu et al., 2020; Hernandez et al., 2021). Similar to pCRKP-1,215_1 (CP024839.1, IncFII(pKPX1), Korea) and pARLG-3,135-1(CP033947.1, IncFIB(pQil), IncFII(K), United States), the typical IS*6100-rmtF-ARR-3* ARI was identified in p3006-3, containing class 1 integrons (Figure 4B). IS*6100*, like IS*26*, is a member of the IS*6* family, and has previously been described as the most common IS element adjacent to *rmtF* (Mataseje et al., 2014). However, most of IS*6100* appears in the vicinity of drug resistance genes alone unlike IS*26* (Partridge et al., 2018).

Discussion

Globally, CRE including CRKP pose a major public health threat (Logan and Weinstein, 2017; Ernst et al., 2020). The notorious nosocomial pathogen hvKP exhibits enhanced virulence

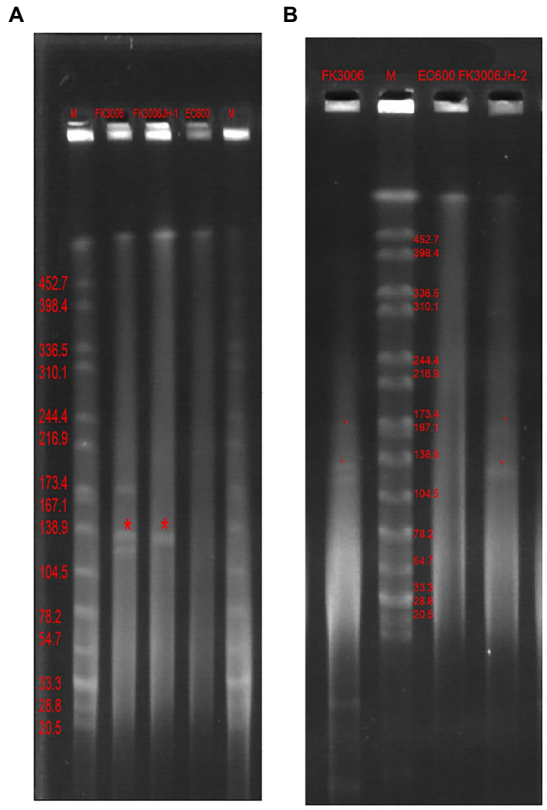

FIGURE 2
S1-PFGE profiles of FK3006, its transconjugants and *E.coli* 600 Lane marker was *Xba*I digested genomic DNA from *Salmonella* Braenderup H9812. **(A)** The transconjugant(FK3006JH-1, the serial dilutions were plated on selective media with ampicillin, 100μg/ml) only had one plasmid, with the size of ~139kb(p3006-11, $bla_{CTX\text{-}M\text{-}15}$). **(B)** The transconjugant (FK3006JH-2, the serial dilutions were plated on selective media with dipotassium tellurite, 8μg/ml) had two plasmid, with the size of ~139kb(p3006-11, $bla_{CTX\text{-}M\text{-}15}$) and~178kb(p3006-2, *iucA*).

features and causes metastatic, and invasive infections (Russo and Marr, 2019). The phenotypes of MDR and hypervirulence in *K. pneumoniae* did not overlap for a long time as MDR phenotypes are often exhibited by classical *K. pneumoniae* (cKP) strains while the carriage of MDR genes in hvKP isolates was rare(Lee et al., 2017). However, more and more isolates with MDR and hypervirulence have been detected in the face of antibiotic selection pressure, this poses a wide array of problems for the treatment (Hennequin and Robin, 2016; Tang et al., 2020). According to epidemiological researches, most hv-CRKP were associated with $bla_{KPC\text{-}2}$ and ST11 *K. pneumoniae*. However, in this study, we report an un-common co-existence of $bla_{OXA\text{-}232}$, *rmtF*, and pLVPK-like virulence plasmid in a ST15 *K. pneumoniae*.

FK3006, which owned the typical hypermucoviscosity feature, was isolated from the sputum sample of a young patient admitted to the ICU direct postoperatively. In FK3006, we got three resistant plasmids: p3006-3 (*rmtF*, *ARR-3*), p3006-7($bla_{OXA\text{-}232}$) and p3006-11 ($bla_{CTX\text{-}M\text{-}15}$), as well as a pLVPK-like virulence plasmid. The p3006-11 plasmid was conjugative and could be successfully self-transferred to EC600. Except for p3006-11 plasmid, other resistant plasmids were typed as non-mobile plasmid, as the core *oriT* site was absent in p3006-3 and p3006-7. Notably, although the p3006-2 virulence plasmid was typed as non-conjugative plasmid like pK2044 and pLVPK plasmid, we observed it could be induced mobilized with the help of p3006-11 plasmid as previously studied (Xu et al., 2021; Tian et al., 2022). The pLVPK-like virulence plasmids of *K. pneumoniae* are generally regarded as nonconjugative, these results indicated that the co-existence of $IncFII_K$ resistant plasmid and pLVPK-like virulence plasmid would increase the risk for the virulence dissemination. Although previous studies have reported the co-existence of $bla_{OXA\text{-}232}$ and virulence-like plasmid in a ST15 *K. pneumoniae*, such *isolate* did not exhibit the hypervirulent phenotype (Shu et al., 2019). However, in our study, the existence of pLVPK-like virulence plasmid endowed the typical hypervirulent characteristics to the FK3006, and such difference may be attributed to the expression variance or other unrecognized virulence determinants in FK3006.

The dissemination of resistance genes is not only *via* plasmids but also *via* other mobile structures. IS*Ecp1* was located in upstream of the $bla_{CTX\text{-}M\text{-}15}$ gene, which was common in other reported $bla_{CTX\text{-}M\text{-}15}$ elements. In addition, the promoter sequence-35(TTGAAA) and-10(TACAAT) regions in IS*Ecp1* provide a potential promoter for $bla_{CTX\text{-}M\text{-}15}$ gene, inducing high expression of it (Ben Slama et al., 2011; Kieslich et al., 2016). Further, IS*26* surrounds $bla_{CTX\text{-}M\text{-}15}$ gene and plays a key role in the dissemination (Seo and Lee, 2021). IS*26* can be located at the upstream of the CTX-M-15 gene, either alone or in combination with IS*Ecp1*. It was reported that IS*Ecp1* was often truncated when it co-existed with IS26, and the truncation position was not fixed. Notably, the promoter sequence of IS*Ecp1* was preserved (Diestra et al., 2009). In our study, IS*Ecp1* was not truncated by IS26. All in all, the surrounding environment of $bla_{CTX\text{-}M\text{-}15}$ contains a variety of transposons and integrons. Meanwhile, coexisting with other drug-resistant genes in the same plasmid makes it easier for them to survive in the environment.

Enterobacterales isolates producing *rmtF* used to be extremely rare in China, but in recent years relevant reports have emerged and are always accompanied by coproduction of OXA-232. Plasmids with *rmtF* gene often acquired through multiple mobile elements. In this study, the *rmtF* plasmid p3006-3 could not self-transferred to other isolates, but the MGEs surrounding the *rmtF* generated a mobile ARI. The genetic background of the *rmtF* is associated with insertion sequence IS*6100*. Previous studies showed Tn*6229* was related to the Tn*3* family and carried a class 1 integron harboring the *rmtF* and IS*6100* (Mataseje et al., 2014). This ARI, together with several MGEs (IS*6100* and Tn*3*), can form a highly active transmission among the strains, which is extremely harmful.

In this study, we report the uncommon co-existence of $bla_{OXA\text{-}232}$, *rmtF*, and a movable pLVPK-like virulence plasmid a ST15 *K. pneumoniae*. The association of antibiotic resistance genes with mobile genetic elements in FK3006 could promote rapid emergence of hv-CRKP strains. Notably, we found the co-exsistence of resistance and virulence plasmid not only generated the high-risk hypervirulent multidrug-resistant phenotype, but also increased the transmission threaten of non-conjugative virulence plasmid. In the future, when plasmid analysis becomes a routine detection method for such high-risk bacteria in medical institutions, necessary interventions could be carried out as early as possible, and the mortality rate might be reduced.

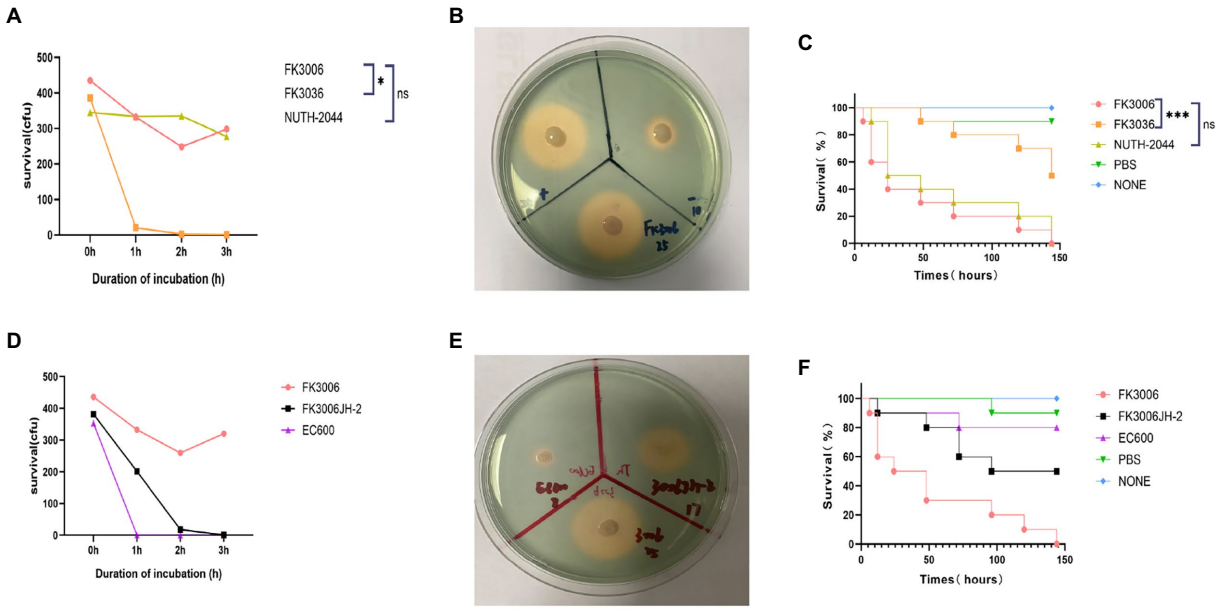

FIGURE 3
The virulence analysis of FK3006. **(A)** Serum killing assay of FK3006, FK3036 and NUTH-K2044 strains. Survival of each strain was evaluated by enumerating viable counts on MHA agar plate for 0, 1, 2, and 3h of incubation in the pooled human serum at 37°C. There was a difference (p<0.05) in the growth of the strain FK3006 and FK3036 *p<0.05. **(B)** Siderophore production of FK3006, FK3036 and NTUH-K2044. FK3036 and NUTH-K2044 were used as negative and positive control, respectively. An orange halos zone indicated Siderophore production. **(C)** Survival rates of *G. mellonella* infected with FK3006, FK3036, NUTH-K2044, PBS, and none. Log-rank Mantel-638 Cox test was performed for analysis of the indicated curves. A difference (p<0.001) was observed between FK3006 and FK3036 *** p<0.001. **(D)** Serum killing assay of FK3006, FK3036JH-2 and EC600 strains. **(E)** Siderophore production of FK3006(25mm), FK3006JH-2(17mm) and EC600(8mm). An orange halos zone indicated Siderophore production. **(F)** Survival rates of G. mellonella infected with FK3006, FK3006JH-2, EC600, PBS, and none.

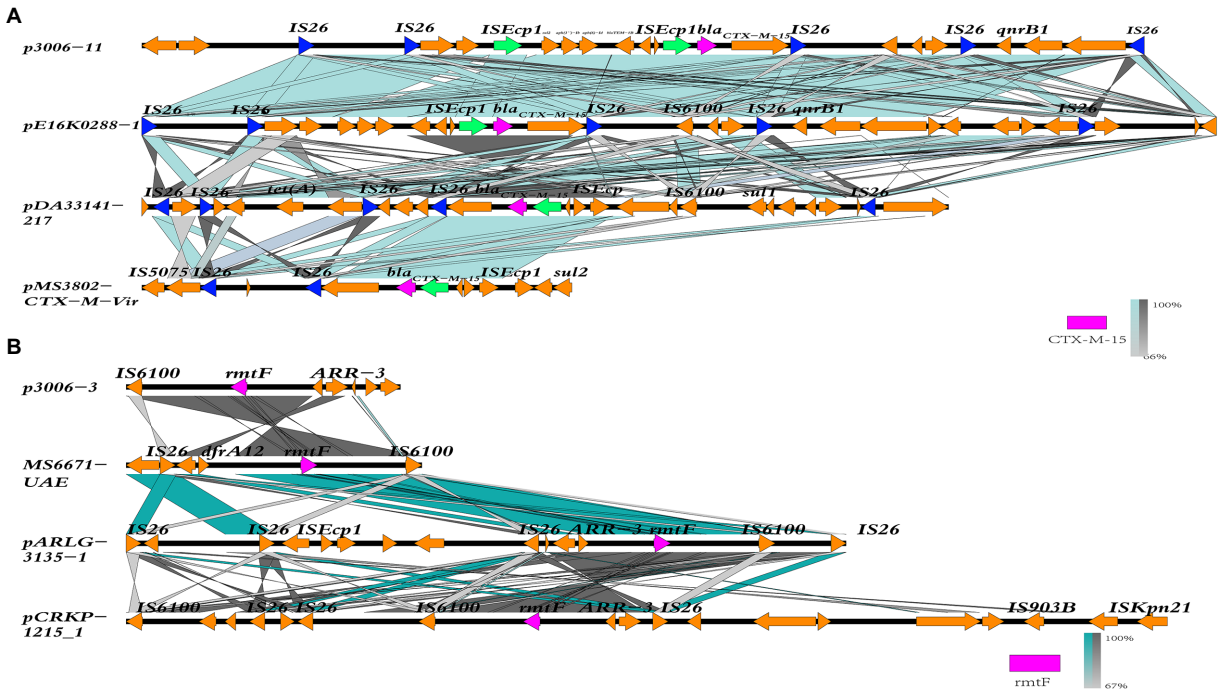

FIGURE 4
Linear comparison of the $bla_{CTX\text{-}M\text{-}15}$ and *rmtF* region. **(A)** The $bla_{CTX\text{-}M\text{-}15}$ region was compared with the regions extracted from pE16K0288-1(CP052263.1), pDA33141-217(CP029588.1), and pMS3802-CTX-M-Vir(NZ_CP068016.1). **(B)** The *rmtF* region was compared with the regions extracted from MS6671-UAE(LN824138.1), pARLG-3,135-1(CP033947.1), and pCRKP-1,215-1(NZ_CP024839.1).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

CW: conceptualization, data curation, formal analysis, methodology, and writing–original draft. YZ: data curation, methodology, and writing–original draft. WA and HZ: methodology. YG, BW, and XnW: software. XaW: formal analysis. LR and JZ: writing–original draft. FY: conceptualization, project administration, and writing–review and editing. LW: conceptualization and writing–review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1133590/full#supplementary-material

References

Ai, W., Zhou, Y., Wang, B., Zhan, Q., Hu, L., Xu, Y., et al. (2021). First report of coexistence of blaSFO–1 and blaNDM–1 β-lactamase genes as well as colistin resistance gene mcr-9 in a transferrable plasmid of a clinical isolate of *Enterobacter hormaechei*. *Front. Microbiol.* 12:676113. doi: 10.3389/fmicb.2021.676113

Ben Slama, K., Ben Sallem, R., Jouini, A., Rachid, S., Moussa, L., Saenz, Y., et al. (2011). Diversity of genetic lineages among CTX-M-15 and CTX-M-14 producing Escherichia coli strains in a Tunisian hospital. *Curr. Microbiol.* 62, 1794–1801. doi: 10.1007/s00284-011-9930-4

CLSI. (2020). *Performance Standards for Antimicrobial Susceptibility Testing. CLSI Supplement M100. 30th*. Wayne: Clinical and Laboratory Standards Institute.

Daikos, G. L., Tsaousi, S., Tzouvelekis, L. S., Anyfantis, I., Psichogiou, M., Argyropoulou, A., et al. (2014). Carbapenemase-producing *Klebsiella pneumoniae* bloodstream infections: lowering mortality by antibiotic combination schemes and the role of carbapenems. *Antimicrob. Agents Chemother.* 58, 2322–2328. doi: 10.1128/AAC.02166-13

Diestra, K., Juan, C., Curiao, T., Moya, B., Miro, E., Oteo, J., et al. (2009). Characterization of plasmids encoding blaESBL and surrounding genes in Spanish clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* 63, 60–66. doi: 10.1093/jac/dkn453

Ernst, C. M., Braxton, J. R., Rodriguez-Osorio, C. A., Zagieboylo, A. P., Li, L., Pironti, A., et al. (2020). Adaptive evolution of virulence and persistence in carbapenem-resistant *Klebsiella pneumoniae*. *Nat. Med.* 26, 705–711. doi: 10.1038/s41591-020-0825-4

EUCAST. (2020). *Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 10.0*. Available at: http://www.eucast.org (Accessed January 20, 2020).

Fu, Y., Xu, X., Zhang, L., Xiong, Z., Ma, Y., Wei, Y., et al. (2020). Fourth generation cephalosporin resistance among *Salmonella enterica* Serovar Enteritidis isolates in Shanghai, China conferred by Bla CTX-M-55 harboring plasmids. *Front. Microbiol.* 11:910. doi: 10.3389/fmicb.2020.00910

Galani, I., Nafplioti, K., Adamou, P., Karaiskos, I., Giamarellou, H., Souli, M., et al. (2019). Nationwide epidemiology of carbapenem resistant *Klebsiella pneumoniae* isolates from Greek hospitals, with regards to plazomicin and aminoglycoside resistance. *BMC Infect. Dis.* 19:167:167. doi: 10.1186/s12879-019-3801-1

Gottig, S., Riedel-Christ, S., Saleh, A., Kempf, V. A., and Hamprecht, A. (2016). Impact of blaNDM-1 on fitness and pathogenicity of Escherichia coli and *Klebsiella pneumoniae*. *Int. J. Antimicrob. Agents* 47, 430–435. doi: 10.1016/j.ijantimicag.2016.02.019

Hennequin, C., and Robin, F. (2016). Correlation between antimicrobial resistance and virulence in *Klebsiella pneumoniae*. *Eur. J. Clin. Microbiol. Infect. Dis.* 35, 333–341. doi: 10.1007/s10096-015-2559-7

Hernandez, M., Lopez-Urrutia, L., Abad, D., De Frutos Serna, M., Ocampo-Sosa, A. A., and Eiros, J. M. (2021). First report of an extensively drug-resistant ST23 *Klebsiella pneumoniae* of capsular serotype K1 co-producing CTX-M-15, OXA-48 and ArmA in Spain. *Antibiotics (Basel)* 10:157. doi: 10.3390/antibiotics10020157

Karaiskos, I., Lagou, S., Pontikis, K., Rapti, V., and Poulakou, G. (2019). The "old" and the "new" antibiotics for MDR gram-negative pathogens: for whom, when, and how. *Front. Public Health* 7:151. doi: 10.3389/fpubh.2019.00151

Kieslich, K., Littlejohns, P., and Weale, A. (2016). Drug appraisal issues must be resolved at policy level. *BMJ* 354:i4519. doi: 10.1136/bmj.i4519

Lee, C. R., Lee, J. H., Park, K. S., Jeon, J. H., Kim, Y. B., Cha, C. J., et al. (2017). Antimicrobial resistance of Hypervirulent *Klebsiella pneumoniae*: epidemiology, Hypervirulence-associated determinants, and resistance mechanisms. *Front. Cell. Infect. Microbiol.* 7:483. doi: 10.3389/fcimb.2017.00483

Li, G., Shi, J., Zhao, Y., Xie, Y., Tang, Y., Jiang, X., et al. (2020). Identification of hypervirulent *Klebsiella pneumoniae* isolates using the string test in combination with *Galleria mellonella* infectivity. *Eur. J. Clin. Microbiol. Infect. Dis.* 39, 1673–1679. doi: 10.1007/s10096-020-03890-z

Liu, D., Liu, Z. S., Hu, P., Cai, L., Fu, B. Q., Li, Y. S., et al. (2016). Characterization of surface antigen protein 1 (SurA1) from *Acinetobacter baumannii* and its role in virulence and fitness. *Vet. Microbiol.* 186, 126–138. doi: 10.1016/j.vetmic.2016.02.018

Liu, Y., Long, D., Xiang, T. X., Du, F. L., Wei, D. D., Wan, L. G., et al. (2019). Whole genome assembly and functional portrait of hypervirulent extensively drug-resistant NDM-1 and KPC-2 co-producing *Klebsiella pneumoniae* of capsular serotype K2 and ST86. *J. Antimicrob. Chemother.* 74, 1233–1240. doi: 10.1093/jac/dkz023

Logan, L. K., and Weinstein, R. A. (2017). The epidemiology of Carbapenem-resistant *Enterobacteriaceae*: the impact and evolution of a global menace. *J. Infect. Dis.* 215, S28–S36. doi: 10.1093/infdis/jiw282

Mataseje, L. F., Boyd, D. A., Lefebvre, B., Bryce, E., Embree, J., Gravel, D., et al. (2014). Complete sequences of a novel blaNDM-1-harbouring plasmid from *Providencia rettgeri* and an FII-type plasmid from *Klebsiella pneumoniae* identified in Canada. *J. Antimicrob. Chemother.* 69, 637–642. doi: 10.1093/jac/dkt445

Miltgen, G., Cholley, P., Martak, D., Thouverez, M., Seraphin, P., Leclaire, A., et al. (2020). Carbapenemase-producing *Enterobacteriaceae* circulating in the Reunion Island, a French territory in the Southwest Indian Ocean. *Antimicrob. Resist. Infect. Control* 9:36. doi: 10.1186/s13756-020-0703-3

Nagasawa, M., Kaku, M., Kamachi, K., Shibayama, K., Arakawa, Y., Yamaguchi, K., et al. (2014). Loop-mediated isothermal amplification assay for 16S rRNA methylase genes in gram-negative bacteria. *J. Infect. Chemother.* 20, 635–638. doi: 10.1016/j.jiac.2014.08.013

Partridge, S. R., Kwong, S. M., Firth, N., and Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.* 31:e00088. doi: 10.1128/CMR.00088-17

Pitout, J. D. D., Peirano, G., Kock, M. M., Strydom, K. A., and Matsumura, Y. (2019). The global ascendency of OXA-48-type carbapenemases. *Clin. Microbiol. Rev.* 33:e00102. doi: 10.1128/CMR.00102-19

Potron, A., Rondinaud, E., Poirel, L., Belmonte, O., Boyer, S., Camiade, S., et al. (2013). Genetic and biochemical characterisation of OXA-232, a carbapenem-hydrolysing class D beta-lactamase from *Enterobacteriaceae*. *Int. J. Antimicrob. Agents* 41, 325–329. doi: 10.1016/j.ijantimicag.2012.11.007

Ramirez, M. S., and Tolmasky, M. E. (2010). Aminoglycoside modifying enzymes. *Drug Resist. Updat.* 13, 151–171. doi: 10.1016/j.drup.2010.08.003

Russo, T. A., and Marr, C. M. (2019). Hypervirulent *Klebsiella pneumoniae*. *Clin. Microbiol. Rev.* 32:e00001. doi: 10.1128/CMR.00001-19

Seo, K. W., and Lee, Y. J. (2021). The occurrence of CTX-M-producing *E. coli* in the broiler parent stock in Korea. *Poult. Sci.* 100, 1008–1015. doi: 10.1016/j.psj.2020.09.005

Shi, Q., Han, R., Guo, Y., Zheng, Y., Yang, Y., Yin, D., et al. (2020). Emergence of ST15 *Klebsiella pneumoniae* clinical isolates producing plasmids-mediated RmtF and OXA-232 in China. *Infect. Drug Resist.* 13, 3125–3129. doi: 10.2147/IDR.S257298

Shu, L., Dong, N., Lu, J., Zheng, Z., Hu, J., Zeng, W., et al. (2019). Emergence of OXA-232 carbapenemase-producing *Klebsiella pneumoniae* that carries a pLVPK-like virulence plasmid among elderly patients in China. *Antimicrob. Agents Chemother.* 63:e02246. doi: 10.1128/AAC.02246-18

Tang, M., Kong, X., Hao, J., and Liu, J. (2020). Epidemiological characteristics and formation mechanisms of multidrug-resistant Hypervirulent *Klebsiella pneumoniae*. *Front. Microbiol.* 11:581543. doi: 10.3389/fmicb.2020.581543

Tian, D., Liu, X., Chen, W., Zhou, Y., Hu, D., Wang, W., et al. (2022). Prevalence of hypervirulent and carbapenem-resistant *Klebsiella pneumoniae* under divergent evolutionary patterns. *Emerg Microbes Infect* 11, 1936–1949. doi: 10.1080/22221751.2022.2103454

Upadhyay, S., Hussain, A., Mishra, S., Maurya, A. P., Bhattacharjee, A., and Joshi, S. R. (2015). Genetic environment of plasmid mediated CTX-M-15 extended Spectrum Beta-lactamases from clinical and food borne bacteria in north-eastern India. *PLoS One* 10:e0138056. doi: 10.1371/journal.pone.0138056

Wang, M., Guo, H., He, F., and Xu, J. (2022). Genomic and phylogenetic analysis of a multidrug-resistant *Klebsiella pneumoniae* ST15 strain co-carrying blaOXA-232 and blaCTX-M-15 recovered from a gallbladder infection in China. *J. Glob. Antimicrob. Resist.* 30, 228–230. doi: 10.1016/j.jgar.2022.06.023

Xu, Y., Zhang, J., Wang, M., Liu, M., Liu, G., Qu, H., et al. (2021). Mobilization of the nonconjugative virulence plasmid from hypervirulent *Klebsiella pneumoniae*. *Genome Med.* 13:119. doi: 10.1186/s13073-021-00936-5

Yang, X., Dong, N., Chan, E. W., Zhang, R., and Chen, S. (2021). Carbapenem resistance-encoding and virulence-encoding conjugative plasmids in *Klebsiella pneumoniae*. *Trends Microbiol.* 29, 65–83. doi: 10.1016/j.tim.2020.04.012

Yin, D., Dong, D., Li, K., Zhang, L., Liang, J., Yang, Y., et al. (2017). Clonal dissemination of OXA-232 Carbapenemase-producing *Klebsiella pneumoniae* in neonates. *Antimicrob. Agents Chemother.* 61:e00385. doi: 10.1128/AAC.00385-17

Zhou, Y., Ai, W., Guo, Y., Wu, X., Wang, B., Xu, Y., et al. (2022). Co-occurrence of rare ArmA-, RmtB-, and KPC-2-encoding multidrug-resistant plasmids and Hypervirulence iuc operon in ST11-KL47 *Klebsiella pneumoniae*. *Microbiol Spectr* 10:e0237121. doi: 10.1128/spectrum.02371-21

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Investigation of antibiotic resistance genotypic and phenotypic characteristics of marine aquaculture fish carried in the Dalian area of China

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Due to the long-term and irrational use of antibiotics for the prevention and control of bacterial diseases in aquaculture, antibiotic resistance genes have become a new source of pollution in aquatic products. Factors such as the spread of drug-resistant strains and the horizontal transfer of drug-resistant genes have led to multi-drug resistance in fish-infecting bacteria, which seriously affects the quality and safety of aquatic products. In this study, 50 samples of horse mackerel and puffer fish sold in Dalian aquatic products market and seafood supermarket were collected, and the phenotypic characteristics of the bacteria carried by the fish for drugs such as sulfonamides, amide alcohols, quinolones, aminoglycosides and tetracyclines were tested and analyzed, and the resistance genes carried by fish samples were detected by SYBG qPCR. Our statistical analyses demonstrated that the drug resistance phenotypes and genotypes of bacteria carried by mariculture horse mackerel and puffer fish in the Dalian area of China were complex, and the multi-drug resistance rate reached 80%. Among the examined antibiotics, the resistance rates to cotrimoxazole, tetracycline, chloramphenicol, ciprofloxacin, norfloxacin, levofloxacin, kanamycin, and florfenicol exceeded 50%, whereas the resistance rates to gentamicin and tobramycin were 26 and 16%, respectively. The detection rate of the drug resistance genes tetA, sul1, sul2, qnrA, qnrS, and floR exceeded 70% and all samples carried more than three drug resistance genes. The correlation analysis of drug resistance genes and drug resistance phenotypes showed that the detection of the drug resistance genes sul1, sul2, floR, and qnrD was correlated with the detection of drug resistance phenotypes ($p<0.01$). However, the correlation between the resistance genes cmlA, cfr, tetA, qnrA, qnrS, and aac(6')-Ib-cr and the corresponding resistance phenotype was not significant ($p>0.05$). In general, our findings indicated that the multi-drug resistance of bacteria carried by marine horse mackerel and puffer fish in the Dalian area was serious. From the perspective of drug resistance rate and drug resistance gene detection rate, the aminoglycosides gentamicin and tobramycin are still considered effective in controlling bacterial infection in marine fish in the study area. Collectively, our findings provide a scientific basis for the management of drug use in mariculture, which can prevent the transmission of drug resistance through the food chain and minimize the associated human health risks.

KEYWORDS

Dalian, marine aquaculture fish, drug resistance, drug resistance genes, SYBG qPCR

Introduction

Antibiotics are widely used to prevent and control bacterial infections in the medical, animal husbandry, and aquaculture fields, and can also be used as growth promoters in aquaculture, thus playing an important role in ensuring human health and the healthy development of aquaculture (Shi and Wang, 2018; Cheng et al., 2021). With the development of intensive farming patterns, large quantities of antibiotics are currently being used in aquaculture. Previous studies have estimated that global antibiotic consumption increased by approximately 69% between 2000 and 2015 and continues to grow at an annual rate of 4%. By frequency of use, the most commonly used antimicrobial classes were quinolones (27%), tetracyclines (20%), mycin (18%), and sulfonamides (14%) (Schar et al., 2020). In China, the world's largest antibiotic producer and consumer, the total antibiotic use in 2015 alone reached 97,000 tons (Shi and Wang, 2018; Al Salah et al., 2019; Chen et al., 2020). Overuse and misuse of antibiotics have led to the rapid development of bacterial resistance, leading to increased healthcare costs, bacterial infections, and mortality (Du et al., 2022). In fact, more than 700,000 deaths worldwide each year are attributed to microbial drug resistance, and this figure is expected to increase to 10 million deaths by 2050 (Zhao et al., 2019; Sultan et al., 2020; Davtyan et al., 2021; Lai et al., 2021). The overuse and misuse of antibiotics in human disease control, as well as in the veterinary, agricultural, and aquaculture industries, can also lead to new sources of antibiotic resistance genes (ARGs), which may spread at higher rates between species through horizontal gene transfer (HGT), thereby exacerbating the problem of antibiotic resistance (Ye, 2020; Okeke et al., 2022). The ingestion of drug resistance genes carried by fish can lead to an imbalance of the normal flora in the human body, increase the resistance of pathogenic bacteria and conditionally pathogenic bacteria, and pose a serious threat to human health and disease prevention and control.

Antimicrobial susceptibility testing (AST) is a commonly used method for the detection of bacterial resistance, including paper diffusion, agar dilution, broth dilution, concentration gradients, and other classical methods (Jiang et al., 2022). For the detection of drug resistance genes, fluorescent probes, nucleic acid amplification, and high-throughput sequencing technologies can be used in conjunction with polymerase chain reaction (PCR) and quantitative reverse transcription PCR (RT-qPCR) to detect drug resistance genes (Nnadozie and Odume, 2019; Jiang et al., 2022). Among these approaches, paper diffusion is a simple and reproducible approach that does not require expensive equipment. However, it only provides phenotypic information about bacterial resistance and cannot detect resistance genes (Yalew, 2020; Jiang et al., 2022). The detection of drug resistance genes based on fluorescent probe technology has several advantages, including strong specificity. However, synthesizing fluorescent-labeled probes can be prohibitively costly. Moreover, high-throughput sequencing technology can be time-consuming, costly, and highly complex, in addition to lacking standardized and automated analysis processes, and is currently limited to pathogen diagnosis and screening of complex clinical diseases. In contrast, SYBG based qPCR methods are simple, time-saving, and cost-effective for rapid diagnosis and screening of clinical bacterial resistance genes (Jiang et al., 2022). Hossain et al. (2018) studied the resistance patterns of 43 strains of *Aeromonas* isolated from 46 zebrafish and the results showed that each isolate was resistant to at least four antibiotics, with multiple antibiotic resistance index values ranging from 0.22 to 0.50. Li et al. (2022) reported that nine species of freshwater fish and marine fish in a city in northern China contained four antibiotics, including doxycycline, tetracycline, sulfamethoxazole, and roxithromycin, in addition to 10 ARGs including strA. Hemamalini et al. (2022) reported that bacterial isolates in freshwater ornamental carp goldfish and tiger hooks had high resistance to bacitracin, rifampicin, trimethoprim, cephalexin, ampicillin, amoxicillin, nalidixic acid, and nitrofurantoin, and most bacterial isolates exhibited a multidrug resistance index of >0.2. Fauzi et al. (2021) used PCR and paper diffusion to detect drug resistance genes and their sensibility to 14 antibiotics in *Aeromonas* sp. isolated from 221 fish samples from Malaysia, and the results showed that the multiple antibiotic resistance index of the isolates ranged from 0.07 to 0.64, and resistance genes such as sul1, strA-strB, aadA, tetA-tetE, and tetM were detected. Khairy et al. (2019) evaluated vancomycin resistance using agar dilution and RT-qPCR and identified the VanB phenotype/vanA genotype in 33.3% vancomycin-resistant enterococcal isolates. Previous studies suggest that the detection of drug-resistant phenotypes and genotypes can be used to monitor drug-resistant bacterial infections and epidemics. However, few studies have investigated the correlation between bacterial resistance phenotypes and drug resistance genes. Liang Shaoshan et al. (Liao et al., 2022) investigated the drug resistance of *E. coli* in an aquaculture environment *via* the paper diffusion method and PCR method. The authors demonstrated that bacteria exhibited varying levels of drug resistance genes and drug resistance rates, but no specific analysis of the correlation between the two was conducted. Shen et al. (2022) tested antibiotic susceptibility and whole-genome sequencing in *Listeria monocytogenes* isolated from 1797 imported food samples collected between 2018 and 2020, and the analysis results demonstrated that the tetracycline- and chloramphenicol-resistant phenotypes were closely related to the genotype, whereas no clear relationship was identified between the remaining phenotypes and genotypes.

Dalian is an important city in the Bohai Rim region of China, whose primary aquaculture species are horse mackerel and pufferfish. This study analyzed samples of horse mackerel and puffer fish sold in Dalian aquatic products market and seafood supermarket were used as the research object, and the resistance of the samples to 10 antibiotics was determined through the antimicrobial-sensitive paper sheet method. SYBG qPCR was used to detect relevant resistance genes. Additionally, the correlation between the two methods was statistically analyzed and the consistency of drug resistance genes and drug resistance phenotypes was discussed. Finally, we assessed the applicability of drug resistance genes in drug resistance screening. The expression of drug resistance genes is regulated by a variety of substances and the causes of drug resistance are also affected by many factors. Therefore, it is important to study the correlation between bacterial drug resistance phenotypes and the detection of drug resistance genes. Here, we assessed the distribution and prevalence of antibiotic resistance and resistance genes in marine fish in Dalian, thus providing data support for the development of drug use strategies for aquaculture in Dalian. In turn, these measures could prevent the transmission of antibiotic resistance through the food chain and minimize their associated human health risks.

Materials and methods

Collection, storage and preparation of fish samples

Fifty fresh or frozen samples of horse mackerel and puffer fish were collected from Dalian aquatic products market and seafood supermarket, and the experimental fish samples collected were all healthy and disease-free, and each fish sample was individually packaged in a clean polyethylene bag, and transferred to an ice box and transported to the laboratory for storage at −20°C for later use.

Sample pretreatment and DNA extraction

Under sterile conditions, after wiping the surface of the fish body with 70% alcohol for disinfection, 0.1–0.2 g homogenization was taken from the gill tissue sample, and placed in nutritional broth (Difco 240230 LB Broth, Lennox, BD Company, USA), and cultured at 36°C for 24 h for DNA extraction.

The Chelex 100 method was used to extract bacterial genomic DNA using the MightyPrep reagent for DNA kit (Code No. 9182; Baori Medical Biotechnology (Beijing) Co., Ltd) according to the manufacturer's instructions. Next, 20 μL of bacterial solution was collected in 1.5 mL microcentrifuge tubes and 100 μL of the MightyPrep reagent for DNA reagent was added. The sample was then thoroughly mixed in a vortex shaker. Next, the samples were heated in a water bath at 95°C for 10 min, cooled to room temperature, and centrifuged at 12000 rpm in a Mini-15 K high-speed centrifuge (Hangzhou, Aosheng Instrument Co., Ltd.) for 2 min. The supernatant containing the DNA was then stored at −20°C for later use as a template for the PCR reactions.

Drug resistance gene detection primer synthesis

This study used PCR primers for the analysis of 10 resistance genes for 5 classes of antibiotics, including the tetracycline resistance gene tetA; the sulfonamide resistance genes sul1 and sul2; the quinolone resistance genes qnrA, qnrD, and qnrS; the amide alcohol resistance genes cmlA, floR, and cfr; and the aminoglycoside resistance gene aac(6′)-Ib-cr. The primers were synthesized by Bao Bioengineering (Dalian) Co., Ltd., and the specific sequences are shown in Table 1.

qPCR detection of drug resistance gene

SYBG qPCR analysis was performed on a Flex QuantStudio TM 7 real-time fluorescence thermocycler (Thermo Fisher Scientific, USA). The amplifications were conducted in 25 μL reaction volumes containing 12.5 μL of TB Green Premix Ex Taq II (Tli RnaseH Plus) 2x, 1 μL of PCR Forward Primer (10 μM), 1 μL of PCR Reverse Primer (10 μM), 0.5 μL of ROX Reference Dye II (50×), 2 μL of DNA template, and 8 μL of DEPC treated water. The reaction consisted of a 30 s pre-denaturation step at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s, and finally 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The presence of resistance genes was confirmed based on the fluorescence curve.

Drug resistance phenotype detection

Drug susceptibility and resistance phenotyping were evaluated using the paper diffusion method (K-B) recommended by the American Committee for Clinical Laboratory Standardization (NC-CLS) (Ministry of Health of the People's Republic of China, 2000). First, 1 mL of nutrient broth culture was evenly coated on three plates, after which 3–4 susceptibility paper sheets were adhered at equal intervals in the center of the plate. The plate with the antimicrobial susceptibility paper was then placed in a 36°C incubator for 16–18 h, after which the diameter of the antibacterial circle of the drug-sensitive paper was observed and recorded. *Escherichia coli* ATCC 35218 was used for testing and quality control. The antibiotic susceptibility paper tablets were purchased from Hangzhou Binhe Microbial Reagent Co., Ltd., China. A total of 10 antibiotics were tested in this study, including kanamycin (KAN, 30 μg/tablet), gentamicin (GEN, 10 μg ± 2.5 μg/tablet), tobramycin (TOB, 10 μg/tablet), ciprofloxacin (CIP, 5 μg/tablet), norfloxacin (NOR, 10 μg/tablet), levofloxacin (LEV, 5 μg/tablet), tetracycline (TET, 30 μg/tablet), florfenicol (FFC, 30 μg/tablet), chloramphenicol (CHL, 30 μg/tablet), and trimoxazole (SMZ, 25 μg/tablet). All statistical analyses for the drug resistance phenotypic data were conducted using the SPSS Statistics 17.0 software.

Drug resistance spectrum analysis

Resistance spectra were analyzed against the Clinical and Laboratory Standards Institute (CLSI) antibiotic susceptibility testing standards (Clinical and Laboratory Standards Institute, 2011). According to the diameter of the antibiotic circle, the resistance of each antibiotic was classified as "S" (Sensitive), "I" (Intermediate), or "R" (Resistant), and each sample carried bacteria to form a unique resistance spectrum to the 10 antibiotics evaluated herein. The resistance spectrum was statistically classified, the drug resistance rate, multidrug resistance index, and drug resistance spectrum richness were calculated, and the resistance spectrum carried by the samples was plotted. The antibiotic resistance rate (ARR) refers to the ratio of the number of samples resistant to an antibiotic to the total number of samples tested. The multi-antibiotic resistance index (MARI) is the ratio of the number of antibiotics that a bacterial sample resisted from the 10 antibiotics tested in total. Resistance spectrum richness refers to the ratio of the number of resistance spectra of a sample to the total number of samples (Deng et al., 2020).

Statistical analysis

For the fish samples collected in this study, the paper sheet method and the drug resistance gene method were used for drug resistance screening, and the diagnostic performance of the two methods in clinical samples was then discussed. The kappa values were calculated using the SPSS Statistics 17.0 software to assess method consistency. The kappa calculation results range from −1 to 1, and kappa values between 0 and 1 can be generally divided into five groups to represent different levels of consistency: 0.0–0.20, very low consistency (slight); 0.21–0.40, fair consistency (fair); 0.41–0.60, medium consistency (moderate); 0.61–0.80, high consistency (substantial); 0.81–1, almost completely consistent (nearly perfect).

TABLE 1 Primers for SYBG qPCR detection of drug resistance genes.

Types of antibiotics	Genes	Primer pair	Sequences (5′→3′)	Annealing temp. (°C)	Amplicon size (bp)	Reference
Sulfonamides	sul 1	FW	CGC ACC GGA AAC ATC GCT GCA C	62	163	Pei et al. (2006)
		RV	TGA AGT TCC GCC GCA AGG CTC G			
	sul 2	FW	TCC GGT GGA GGC CGG TAT CTG G	62	191	Pei et al. (2006)
		RV	CGG GAA TGC CAT CTG CCT TGA G			
Amide alcohols	cmlA	FW	GCC AGC AGT GCC GTT TAT	55	158	Li et al. (2013)
		RV	GGC CAC CTC CCA GTA GAA			
	floR	FW	CGG TCG GTA TTG TCT TCA CG	56	171	Li et al. (2013)
		RV	TCA CGG GCC ACG CTG TAT			
	cfr	FW	TGT GCT ACA GGC AAC ATT GGA T	55	148	Li et al. (2013)
		RV	CAA ATA CTT GAC GGT TGG CTA GA			
Quinolones	qnrA	FW	AGG ATT TCT CAC GCC AGG ATT	57	124	Vien le et al. (2012)
		RV	CCG CTT TCA ATG AAA CTG CA			
	qnrD	FW	AGT GAG TGT TTA GCT CAA GGA G	56.8	175	Vien le et al. (2012)
		RV	CAG TGC CAT TCC AGC GAT T			
	qnrS	FW	GTA TAG AGT TCC GTG CGT GTG A	54.6	189	Vien le et al. (2012)
		RV	GGT TCG TTC CTA TCC AGC GAT T			
Tetracyclines	tetA	FW	GCT ACA TCC TGC TTG CCT TC	62	210	Huang et al. (2015)
		RV	CAT AGA TCG CCG TGA AGA GG			
Aminoglycosides	aac(6′)-Ib-cr	FW	TGC GAT GCT CTA TGA GTG GCT A	55	482	Park et al. (2006)
		RV	CTC GAA TGC CTG GCG TGT TT			

FW, upstream primer; RV, downstream primers.

Results and analysis

Resistance phenotype results

Fish samples were statistically analyzed for drug resistance to 10 antibiotics, and our findings revealed that the bacteria in the fish samples exhibited strong resistance to SMZ, TET, CHL, CIP, NOR, LEV, KAN, and FFC (drug resistance >50%), moderate resistance to GEN (25–50%), and low resistance to TOB (10–25%). Among the tested antibiotics, the bacteria exhibited a general resistance to sulfonamides and tetracyclines, with resistance rates of 94 and 80%, respectively. The resistance rates to the quinolones CIP and LEV were 66 and 64%, respectively, which were comparable to those of the banned NOR (66%). CHL is an amide alcohol drug whose application has been banned in aquaculture. However, its resistance rate was still 76%. FFC is an approved amide alcohol drug with an intermediate resistance of 32% and a resistance rate of 52%. The test samples were generally sensitive to aminoglycosides, except for KAN (54% resistance), GEN (26% resistance and 60% sensitivity), and TOB (16% resistance and 76% sensitivity). Table 2 and Figure 1A illustrate the patterns of resistance to 10 antibiotics in fish samples.

Resistance gene results

The statistical analysis of our drug resistance gene detection results (Table 3; Figure 1B) indicated that the detection rate of drug resistance genes exhibited the following descending order: sul2 > sul1 > floR > tetA > qnrS > qnrA > cmlA > qnrD > cfr > aac(6′)-Ib-cr. Particularly, the frequency of detection of the tetA, sul1, sul2, qnrA, qnrS, and floR genes exceeded 70%. The detection rate of sulfonamide resistance genes sul1 and sul2 was also very high, reaching up to 90%, which was consistent with the 94% resistance rate of sulfonamides. The detection rate of the tetracycline tetA resistance gene was 86%, which was consistent with the detection rate of 80% of tetracycline resistance phenotypes. The floR gene is a florfenicol-specific resistance gene, and the detection rate for this gene was as high as 88%. This study focused on investigating the prevalence of quinolone qnr gene families, for which the detection rate exceeded 50%. Although only low-level quinolone resistance is mediated by the qnr genes, these genes can be horizontally transferred, thus accelerating the spread of quinolone-resistant strains and promoting the generation of resistant mutant strains. The detection rate of the aminoglycoside resistance gene aac(6′)-Ib-cr was 22%, which was consistent with the low detection rate of the aminoglycoside resistance phenotype.

Drug resistance analysis of different species of fish samples

Our study analyzed the resistance of pufferfish and horse mackerel bacterial samples to 10 antibiotics (Figure 2). The horse mackerel samples showed strong resistance to SMZ, TET, FFC, and CHL, with resistance rates of 87.5, 62.5, 62.5, and 54.17%, respectively. The

TABLE 2 Statistics of phenotype screening results of fish samples for drug resistance.

Types of antibiotics	Drug name (abbreviation)	Sensitivity (%)	Intermediary rate (%)	Drug resistance rate (%)
Sulfonamides	SMZ	6%	0%	94%
Amide alcohols	FFC	16%	32%	52%
	CHL	10%	14%	76%
Quinolones	CIP	26%	8%	66%
	NOR	30%	4%	66%
	LEV	28%	8%	64%
Aminoglycosides	KAN	28%	18%	54%
	GEN	60%	14%	26%
	TOB	76%	8%	16%
Tetracyclines	TET	6%	14%	80%

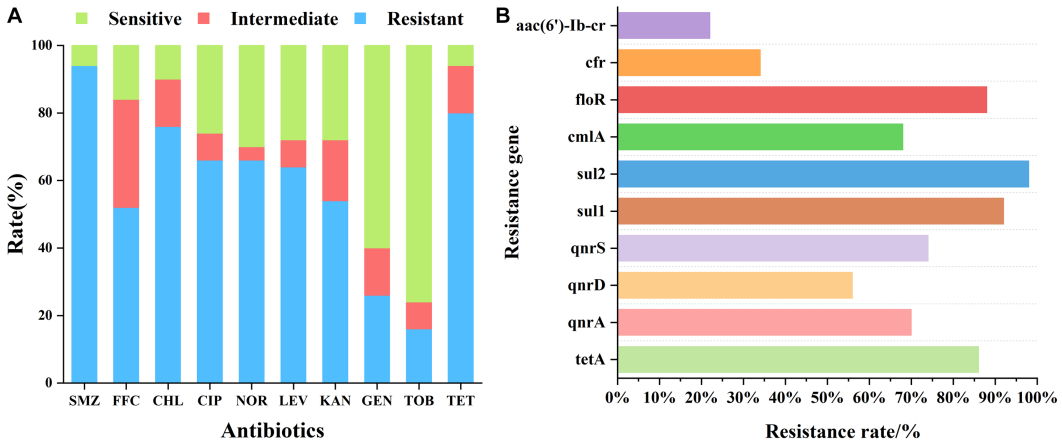

FIGURE 1
(A) Phenotypic screening of drug resistance in fish samples. The x-axis indicates the tested antibiotics and the y-axis indicates the resistance ratios of the fish samples to 10 antibiotics. **(B)** Detection of drug resistance genes in fish samples: the x-axis represents the detection rate of the corresponding resistance genes in all samples and the y-axis represents the resistance genes.

samples also exhibited moderate resistance to LEV, CIP, NOR, and KAN, with resistance rates of 45.83, 41.67, 41.67, and 37.5%, respectively. Finally, we also detected low resistance rates to GEN and TOB of 20.83 and 8.33%, respectively. The pufferfish samples showed strong resistance to SMZ TET, CHL, CIP, NOR, LEV, and KAN, with resistance rates of 100, 96.15, 96.15, 88.46, 88.46, 80.77, and 69.23%, respectively; moderate resistance to FFC and GEN, with resistance rates of 42.31 and 30.77%, respectively; and a low TOB resistance rate of 23.08%. Both fish were sensitive to GEN and TOB, and the resistance and resistance rates were relatively consistent.

Drug resistance spectrum analysis

Our findings demonstrated that the examined fish samples carried complex drug-resistant phenotypes and genotypes, with 26 drug-resistant phenotypes and 33 drug-resistant genotypes. The multidrug resistance index (MARI) ranged from 0 to 1 and the richness of the resistance spectrum was 0.52. Figure 3A illustrated the distribution of the multidrug resistance index. All samples carried three or more resistance genes, with some carrying up to ten, of which eight were the main resistance genes, accounting for 42% (21/50). Two of the 50 samples were not resistant to any antibiotic and the remaining 48 samples were resistant to two or more antibiotics, some of which were resistant to all ten antibiotics. These 48 samples were predominantly resistant to six antibiotics and seven antibiotics, both with the same number of resistant samples, accounting for 16% of the total (8/50) (Figure 3B).

Statistical analysis of the carrying resistance phenotypes indicated that there were 26 resistance phenotypic spectra (Figure 4A), of which 6 samples were resistant to TET, CIP, NOR, LEV, KAN, GEN, TOB, SMZ, FFC, and CHL; 5 samples were resistant to TET, CIP, NOR, LEV, KAN, SMZ, CHL, TET, CIP, NOR, LEV, SMZ, and CHL; 4 samples were resistant to TET, CIP, NOR, LEV, KAN. SMZ, FFC, and CHL had 4 samples; 3 samples were resistant to SMZ and TET, SMZ, FFC, and CHL; 2 samples were resistant to TET, CIP, NOR, LEV, KAN, GEN, TOB, SMZ, FFC, and CHL; and the other 19 spectra had 1 sample each. Samples 17 and 19 were not resistant to any of the 10 antibiotics.

Statistical analysis of drug-resistant genotypes was conducted and a total of 33 drug-resistant genotypes were obtained (Figure 4B), of

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which 6 samples carried tetA, qnrA, qnrD, qnrS, sul1, sul2, cmlA, and floR; 5 samples carried tetA, qnrA, sul1, sul2, cmlA, floR; 4 samples carried tetA, qnrA, qnrD, sul1, sul2, cmlA, floR, and cfr; 3 samples carried tetA, qnrA, qnrS, sul1, sul2, cmlA, loR, and cfr; 2 samples carried tetA, qnrA, qnrD, qnrS, aac(6′)-Ib-cr, sul1, sul2, cmlA, floR; and 2 more samples carried tetA, qnrA, qnrD, qnrS, sul1, sul2, loR, and cfr and tetA, qnrA, sul1, sul2, and floR, respectively. The other 26 resistance genotypes had 1 sample each.

TABLE 3 Detection of drug resistance genes.

Types of antibiotics	Drug resistance gene	Number of drug resistance gene detected samples	Drug resistance gene carrying rate (positive/total number of samples)
Sulfonamides	sul 1	46	92%(46/50)
	sul 2	49	98%(49/50)
Amide alcohols	cmlA	34	68%(34/50)
	floR	44	88%(44/50)
	cfr	17	34%(17/50)
Quinolones	qnrA	35	70%(35/50)
	qnrD	28	56%(28/50)
	qnrS	37	74%(37/50)
Aminoglycosides	aac(6′)-Ib-cr	11	22%(11/50)
Tetracyclines	tetA	43	86%(43/50)

Consistency analysis of drug resistance phenotype and drug resistance gene

The correlation between drug-resistant phenotypes and drug-resistant genes has recently garnered increasing attention among scholars. In this study, the kappa value was used to statistically analyze the consistency of the drug resistance phenotype and drug resistance gene detection results, as well as to explore the applicability of drug resistance genes in actual sample detection.

Statistical analysis of the kappa values between the drug-resistant genes and the susceptibility paper sheet method (Figure 5A) indicated that the kappa value of the alignment between the sul1 gene and the SMZ assay was 0.728, which was highly consistent between the two methods, and the kappa value of the alignment between the sul2 gene and the SMZ assay was 0.380, which was fairly consistent between the two methods, indicating that the sul1 gene could better reflect the resistance of sulfonamides. The consistency between the cmlA, floR,

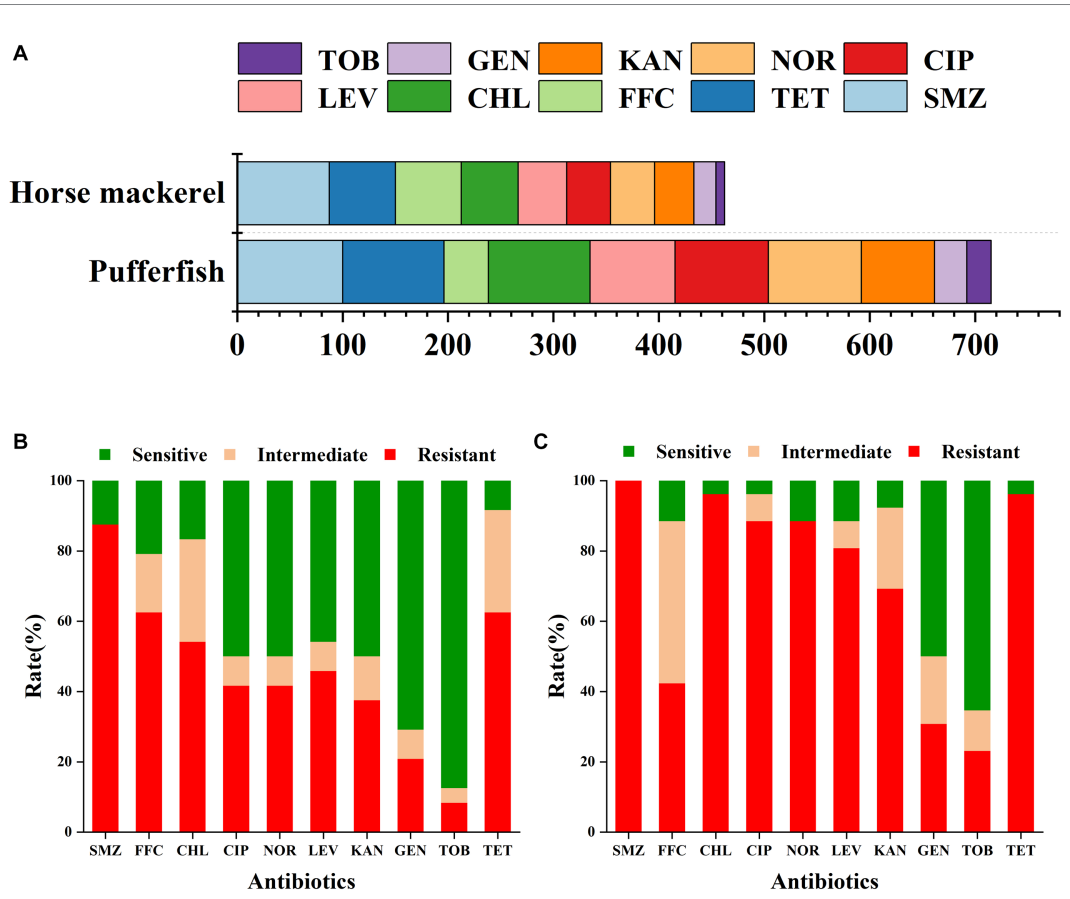

FIGURE 2
(A) Cumulative resistance rate of the ten examined antibiotics in the horse mackerel and pufferfish samples. Drug resistance of **(B)** horse mackerel and **(C)** pufferfish samples: the x-axis represents antibiotics whereas the y-axis represents the ratios of the resistance, mediation, and sensitivity of the two fish samples to 10 antibiotics.

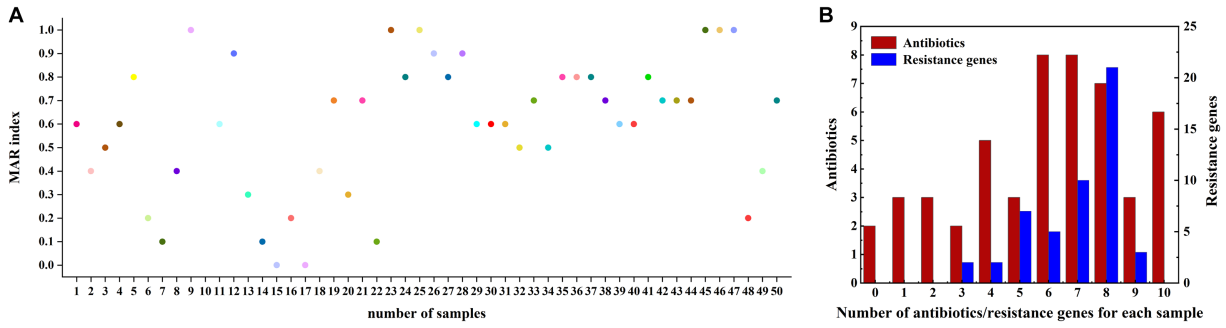

FIGURE 3
(A) Multidrug resistance MAR index plot of fish samples for 10 different antibiotics. **(B)** Multidrug resistance in fish samples; the x-axis represents the types of antibiotics tolerated or the number of resistance genes detected for each sample (including the detection of a minimum of 0 antibiotics or containing 0 resistance genes, and detecting a maximum of 10 antibiotics or containing 10 resistance genes). The left vertical axis represents the number of samples with a resistance phenotype detected (red histogram) and the right vertical axis represents the number of samples with resistance genes detected (blue histogram).

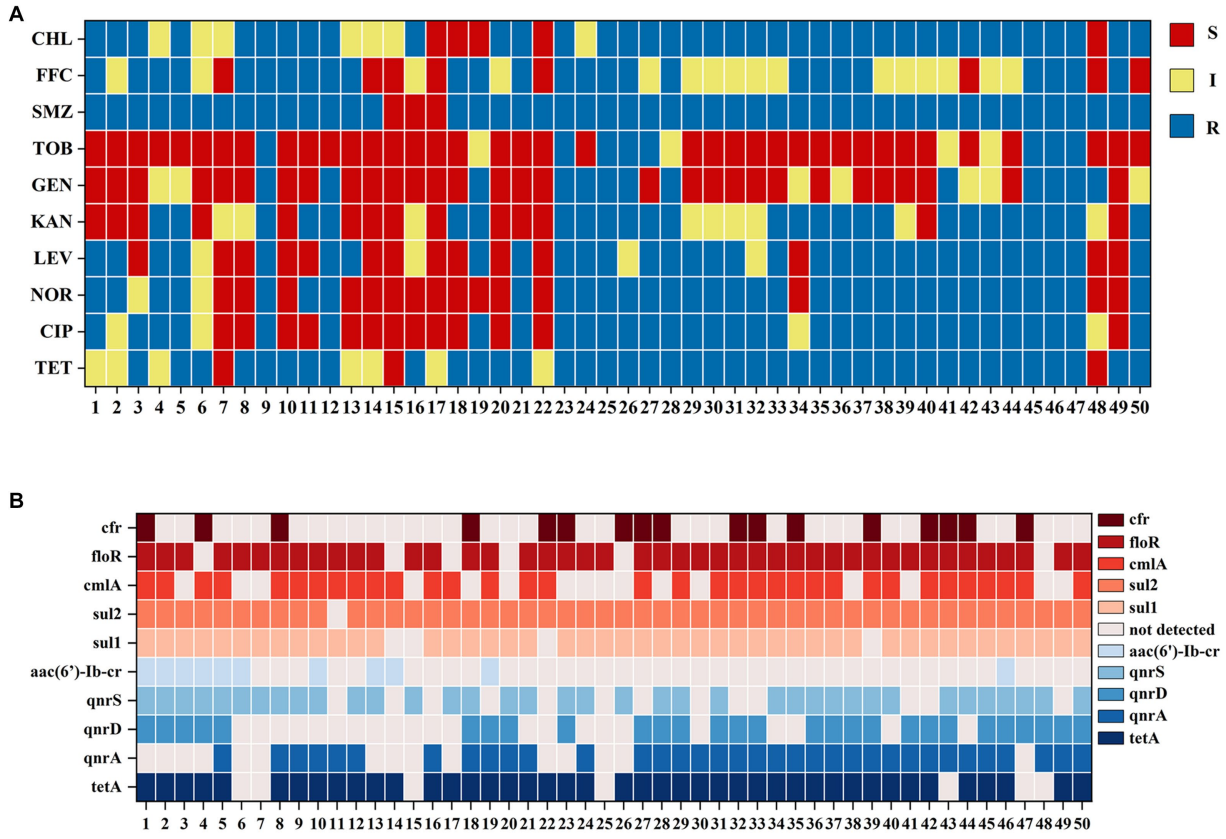

FIGURE 4
(A) Fish samples carrying bacterial resistance to drug-sensitive paper pieces. A total of 26 resistance phenotypes spectra were detected. **(B)** Fish samples carrying drug resistance genes were profiled. A total of 33 drug resistance genotypes were detected.

and cfr genes of amide alcohols and FFC was very low. The cmlA and floR genes were generally consistent with CHL, and the consistency of the cfr gene was very low with CHL. The tetracycline tetA gene was generally consistent with the TET phenotype. The quinolone qnrA and qnrD genes were generally consistent with CIP, NOR, and LEV, whereas the consistency of the qnrS genes was very low. The aminoglycoside aac(6′)-Ib-cr gene exhibited slightly less consistency with KAN, GEN, and TOB, which may be related to the complexity of the mechanism of aminoglycoside resistance.

Next, we conducted a correlation analysis of the drug resistance genes and the drug resistance phenotypic detection results (Figure 5B). The detection of drug resistance genes sul1, sul2, floR, and qnrD was correlated with the drug resistance phenotype of the sample ($p < 0.05$), whereas the correlation between the cmlA, cfr, tetA, qnrA, qnrS,

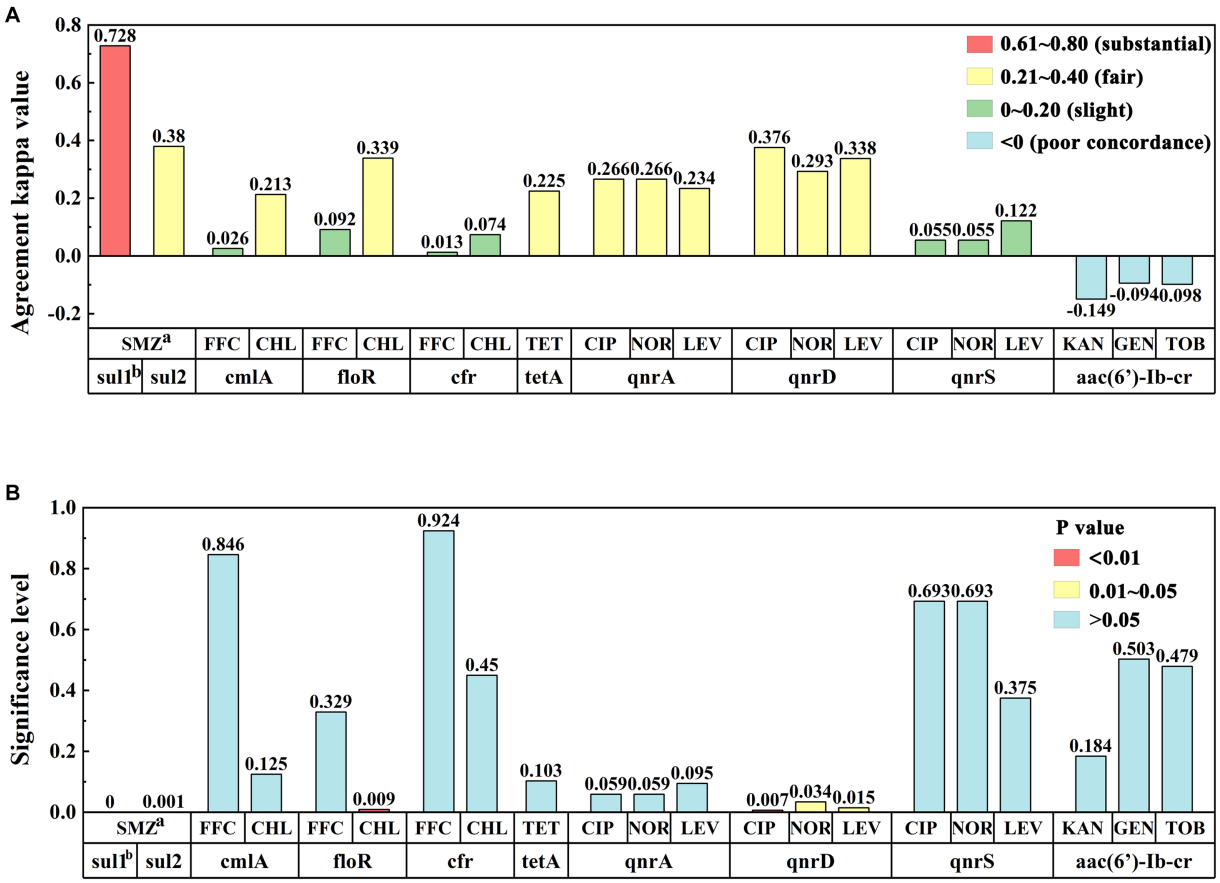

FIGURE 5
(A) Consistency analysis of kappa values between drug resistance phenotypes and drug resistance gene results. Row "a" represents antibiotics, row "b" represents resistance genes, and the y-axis represents the degree of consistency between the detection results of the two methods. **(B)** Significance analysis of drug resistance phenotype and resistance gene results: Row "a" represents antibiotics, row "b" represents resistance genes, and the y-axis represents significance levels between the test results of the two methods.

aac(6′)-Ib-cr genes and their respective drug resistance phenotype was not obvious ($p > 0.05$). Among them, the sulfonamide resistance genes sul1 and sul2 were significantly correlated with the SMZ resistance phenotype ($p < 0.01$). There was a significant correlation between the floR gene and the CHL resistance phenotype ($p < 0.01$). For quinolones, the qnrD gene was associated with CIP, NOR, and LEV resistance phenotypes ($p < 0.05$). For tetracyclines and aminoglycosides, the tetA and aac(6′)-Ib-cr resistance genes carried by the samples were not associated with any resistance phenotypes.

Discussion

Our findings demonstrated that drug resistance was serious and complex in marine aquaculture fish in Dalian, China. The test samples exhibited strong resistance to SMZ, TET, CHL, CIP, NOR, LEV, KAN, and FFC. Resistance to sulfonamides and tetracyclines was extremely high and therefore these antibiotics are not recommended in the study region. Although the use of CHL and NOR has been banned in aquaculture, these drugs still exhibited a relatively high resistance rate. This was presumably due to the high concentration of residues in the breeding environment caused by the indiscriminate use of these drugs, resulting in bacterial resistance. FFC is a broad-spectrum amide alcohol antibiotic that is currently used in mariculture to replace the banned drug CHL due to its low toxicity. This study examined whether CHL developed cross-resistance with FFC after banning, and our findings indicated that 21 of the 38 samples resistant to CHL also showed resistance to FFC, indicating that CHL-resistant bacteria also developed resistance to FFC. Additionally, our findings demonstrated that drug resistance to FFC was generally moderate (intermediary resistance rate of 32%). These findings suggest that the resistance to this drug gradually increased with increased administration, meaning that these drugs should be used with caution in mariculture. These findings were consistent with previous reports on the resistance of farmed fish to FFC in South China (Yin, 2022). The antibiotics with the lowest resistance rates among the test samples were the aminoglycosides GEN and TOB, and aminoglycoside resistance genes also had the lowest detection rates, indicating that the farmed fish in the study region were more sensitive to these drugs.

The correlation analysis of drug resistance phenotypes and drug resistance genes demonstrated that only the sulfonamide resistance genes sul1 and sul2, the chloramphenicol resistance gene floR, and the quinolone resistance gene qnrD were associated with the corresponding resistance phenotypes ($p < 0.01$), showing a good diagnostic consistency

for resistance detection in clinical samples. Furthermore, our findings confirmed that the emergence of drug resistance is closely related to the occurrence of drug resistance genes. However, this relationship is not completely consistent. For example, the resistance phenotypes of multiple types of microbes did not show obvious correspondence with the resistance genes that they carry. These findings may be related to variations in the resistance mechanisms of each drug, as well as selection pressures in the environment. Several factors can inhibit the expression of resistance genes. However, ARG-carrying bacteria may have other potential mechanisms of drug resistance, thus highlighting the complexity of drug resistance in bacteria. Based on the drug resistance phenotypes and the detection rate of drug resistance genes elucidated in this study, marine aquaculture fish in the Bohai Sea area of Dalian exhibit strong antibiotic resistance and carry multiple drug resistance genes, which is closely related to the practice of intensive aquaculture. Therefore, other green prevention and control measures can be combined to focus on the use of aminoglycosides to control aquaculture diseases in the study region and reduce the use of other types of antibiotics. In turn, these measures could substantially decrease the expression of drug resistance genes and the horizontal spread of drug resistance to promote the healthy development of the aquaculture industry.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Author contributions

ZG conducted methodology, validation, investigation, writing-original draft, writing review and editing and the acquisition, analysis, and interpretation of data. YP and CY performed investigation and validation. BH performed sample detection. XZ performed actual sample detection. QZ performed conceptualization, supervision, funding acquisition, project administration, and substantively revised it. JC performed project administration and substantively revised it. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Al Salah, D. M. M., Laffite, A., and Poté, J. (2019). Occurrence of bacterial markers and antibiotic resistance genes in Sub-Saharan rivers receiving animal farm wastewaters. *Sci. Rep.* 9:14847. doi: 10.1038/s41598-019-51421-4

Chen, Y., Shen, W., Wang, B., Zhao, X., Su, L., Kong, M., et al. (2020). Occurrence and fate of antibiotics, antimicrobial resistance determinants and potential human pathogens in a wastewater treatment plant and their effects on receiving waters in Nanjing, China. *Ecotoxicol. Environ. Saf.* 206:111371. doi: 10.1016/j.ecoenv.2020.111371

Cheng, X., Lu, Y., Song, Y., Zhang, R., Shang Guan, X., Xu, H., et al. (2021). Analysis of antibiotic resistance genes, environmental factors, and microbial community from aquaculture farms in five provinces, China. *Front. Microbiol.* 12:679805. doi: 10.3389/fmicb.2021.679805

Clinical and Laboratory Standards Institute. (2011). *Performance standards for antimicrobial susceptibility testing. Twenty-First Informational Supplement M100-S21*. Wayne, PA: Clinical and Laboratory Standards Institute.

Davtyan, H., Grigoryan, R., Niazyan, L., Davidyants, M., Ghalechyan, T., and Davtyan, K. (2021). Antimicrobial resistance in a tertiary Care Hospital in Armenia: 2016–2019. *Trop. Med. Infect. Dis.* 6:31. doi: 10.3390/tropicalmed6010031

Deng, Y. Q., Xu, L. W., Zhang, Y. Q., Guo, Z. X., Feng, J. (2020). Analysis of virulence genes and antibiotic resistance of *Photobacterium damselae* isolated from marine fishes in coastal South China. *Acta Microbiol Sin.* 60, 2606–2619. doi: 10.13343/j.cnki.wsxb.20200129

Du, Y., Hu, X. M., Miao, L., and Chen, J. (2022). Current status and development prospects of aquatic vaccines. *Front. Immunol.* 13:1040336. doi: 10.3389/fimmu.2022.1040336

Fauzi, N. N. F. N. M., Hamdan, R. H., Mohamed, M., Ismail, A., Mat Zin, A. A., and Mohamad, N. F. A. (2021). Prevalence, antibiotic susceptibility, and presence of drug resistance genes in *Aeromonas spp.* isolated from freshwater fish in Kelantan and Terengganu states, Malaysia. *Vet. World* 14, 2064–2072. doi: 10.14202/vetworld.2021.2064-2072

Hemamalini, N., Shanmugam, S. A., Kathirvelpandian, A., Deepak, A., Kaliyamurthi, V., Suresh, E., et al. (2022). Prevalence, antimicrobial susceptibility and resistance gene detection in bacteria isolated from goldfish and tiger barb from ornamental fish farms of Tamil Nadu. *Indian J. Microbiol.* 62, 441–446. doi: 10.1007/s12088-022-01023-y

Hossain, S., De Silva, B. C. J., Dahanayake, P. S., and Heo, G. J. (2018). Characterization of virulence properties and multi-drug resistance profiles in motile *Aeromonas spp.* isolated from zebrafish (*Danio rerio*). *Lett. Appl. Microbiol.* 67, 598–605. doi: 10.1111/lam.13075

Huang, M., Qi, F., Wang, J., Xu, Q., and Lin, L. (2015). Changes of bacterial diversity and tetracycline resistance in sludge from AAO systems upon exposure to tetracycline pressure. *J. Hazard. Mater.* 298, 303–309. doi: 10.1016/j.jhazmat.2015.05.039

Jiang, G. L., Yu, S. Y., Yu, W. Q., Zhou, Y. X., Zhou, Z. M., and Li, H. (2022). Research progress in the detection techniques and methods of bacterial drug resistance and drug-resistant genes. *Chin. J. New Clin. Med.* 15, 907–913. doi: 10.3969/j.issn.1674-3806.2022.10.04

Khairy, R. M., Mahmoud, M. S., Esmail, M. A. M., and Gamil, A. N. (2019). First detection of vanB phenotype-vanA genotype vancomycin-resistant enterococci in Egypt. *J. Infect. Dev. Ctries.* 13, 837–842. doi: 10.3855/jidc.10472

Lai, F. Y., Muziasari, W., Virta, M., Wiberg, K., and Ahrens, L. (2021). Profiles of environmental antibiotic resistomes in the urban aquatic recipients of Sweden using

high-throughput quantitative PCR analysis. *Environ. Pollut.* 287:117651. doi: 10.1016/j.envpol.2021.117651

Li, W., Niu, Z., Zhang, X., Zhang, K., and Luo, S. (2022). Antibiotics and resistant genes in the gut of Chinese nine kinds of freshwater or marine fish. *J. Environ. Sci. Health B* 57, 316–324. doi: 10.1080/03601234.2022.2051401

Li, J., Shao, B., Shen, J., Wang, S., and Wu, Y. (2013). Occurrence of chloramphenicol-resistance genes as environmental pollutants from swine feedlots. *Environ. Sci. Technol.* 47, 2892–2897. doi: 10.1021/es304616c

Liao, C. Y., Balasubramanian, B., Peng, J. J., Tao, S. R., Liu, W. C., and Ma, Y. (2022). Analysis of antimicrobial resistance and resistance genes of *Escherichia coli* from aquaculture farm in Zhanjiang. *Front. Vet. Sci.* 41, 73–78. doi: 10.13823/j.cnki.jtcvm.2022.02.015

Ministry of Health of the People's Republic of China. (2000). *WS/T 125–1999 standard of antidbiotics susceptibility test (Kirby-bauer method)*. Beijing: Standards Press of China.

Nnadozie, C. F., and Odume, O. N. (2019). Freshwater environments as reservoirs of antibiotic resistant bacteria and their role in the dissemination of antibiotic resistance genes. *Environ. Pollut.* 254:113067. doi: 10.1016/j.envpol.2019.113067

Okeke, E. S., Chukwudozie, K. I., Nyaruaba, R., Ita, R. E., Oladipo, A., Ejeromedoghene, O., et al. (2022). Antibiotic resistance in aquaculture and aquatic organisms: a review of current nanotechnology applications for sustainable management. *Environ. Sci. Pollut. Res. Int.* 29, 69241–69274. doi: 10.1007/s11356-022-22319-y

Park, C. H., Robicsek, A., Jacoby, G. A., Sahm, D., and Hooper, D. C. (2006). Prevalence in the United States of aac(6′)-Ib-cr encoding a ciprofloxacin-modifying enzyme. *Antimicrob. Agents Chemother.* 50, 3953–3955. doi: 10.1128/AAC.00915-06

Pei, R., Kim, S. C., Carlson, K. H., and Pruden, A. (2006). Effect of river landscape on the sedient concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* 40, 2427–2435. doi: 10.1016/j.watres.2006.04.017

Schar, D., Klein, E. Y., Laxminarayan, R., Gilbert, M., and Van Boeckel, T. P. (2020). Global trends in antimicrobial use in aquaculture. *Sci. Rep.* 10:21878. doi: 10.1038/s41598-020-78849-3

Shen, J., Zhang, G., Yang, J., Zhao, L., Jiang, Y., Guo, D., et al. (2022). Prevalence, antibiotic resistance, and molecular epidemiology of *Listeria monocytogenes* isolated from imported foods in China during 2018 to 2020. *Int. J. Food Microbiol.* 382:109916. doi: 10.1016/j.ijfoodmicro.2022.109916

Shi, X. M., and Wang, S. L. (2018). Antibiotic resistance in environment of animal farms. *Chin. J. Biotechnol.* 34, 1234–1245. doi: 10.13345/j.cjb.180177

Sultan, I., Ali, A., Gogry, F. A., Rather, I. A., Sabir, J. S. M., and Haq, Q. M. R. (2020). Bacterial isolates harboring antibiotics and heavy-metal resistance genes co-existing with mobile genetic elements in natural aquatic water bodies. *Saudi. J. Biol. Sci.* 27, 2660–2668. doi: 10.1016/j.sjbs.2020.06.002

Vien le, T. M., Minh, N. N., Thuong, T. C., Khuong, H. D., Nga, T. V., Thompson, C., et al. (2012). The co-selection of fluoroquinolone resistance genes in the gut flora of Vietnamese children. *PLoS One* 7:e42919. doi: 10.1371/journal.pone.0042919

Yalew, S. T. (2020). Review on antibiotic resistance: resistance mechanisms, methods of detection and its controlling strategies. *Biomed. J. Sci. Tech. Res.* 24, 18651–18657. doi: 10.26717/BJSTR.2020.24.004121

Ye, F. (2020). *A study of contamination and detection methods of antibiotic resistance genes from aquatic products*. dissertation/master's thesis. Hangzhou: Zhejiang Gongshang University.

Yin, M.H. (2022). *Analysis of antibiotic resistance phenotype and genotype of Aeromonas from freshwater aquaculture in Jiangsu*. dissertation/master's thesis. Shanghai: Shanghai Ocean University.

Zhao, H., Yan, B., Mo, X., Li, P., Li, B., Li, Q., et al. (2019). Prevalence and proliferation of antibiotic resistance genes in the subtropical mangrove wetland ecosystem of South China Sea. *Microbiology* 8:e871. doi: 10.1002/mbo3.871

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Novel next generation sequencing panel method for the multiple detection and identification of foodborne pathogens in agricultural wastewater

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Detecting and identifying the origins of foodborne pathogen outbreaks is a challenging. The Next-Generation Sequencing (NGS) panel method offers a potential solution by enabling efficient screening and identification of various bacteria in one reaction. In this study, new NGS panel primer sets that target 18 specific virulence factor genes from six target pathogens (*Bacillus cereus*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*) were developed and optimized. The primer sets were validated for specificity and selectivity through singleplex PCR, confirming the expected amplicon size. Crosscheck and multiplex PCR showed no interference in the primer set or pathogenic DNA mixture. The NGS panel analysis of spiked water samples detected all 18 target genes in a single reaction, with pathogen concentrations ranging from 10^8 to 10^5 colony-forming units (CFUs) per target pathogen. Notably, the total sequence read counts from the virulence factor genes showed a positive association with the CFUs per target pathogen. However, the method exhibited relatively low sensitivity and occasional false positive results at low pathogen concentrations of 10^5 CFUs. To validate the detection and identification results, two sets of quantitative real-time PCR (qPCR) analyses were independently performed on the same spiked water samples, yielding almost the same efficiency and specificity compared to the NGS panel analysis. Comparative statistical analysis and Spearman correlation analysis further supported the similarity of the results by showing a negative association between the NGS panel sequence read counts and qPCR cycle threshold (Ct) values. To enhance NGS panel analysis for better detection, optimization of primer sets and real-time NGS sequencing technology are essential. Nonetheless, this study provides valuable insights into applying NGS panel analysis for multiple foodborne pathogen detection, emphasizing its potential in ensuring food safety.

KEYWORDS

next-generation sequencing, NGS panel, foodborne pathogen, multiple detection, real-time PCR

Introduction

Foodborne pathogens, including *Escherichia coli* O157:H7, *Salmonella, Bacillus cereus, Yersinia enterocolitica, Staphylococcus aureus,* and *Vibrio*, are widespread and frequently cause foodborne diseases. In the USA from 2009 to 2020, 9,720 foodborne pathogen associated disease outbreaks occurred, causing 168,656 illness, 10,983 hospitalizations, and 268 deaths (Lee and Yoon, 2021). In South Korea from 2010 to 2018, there were 2,815 outbreaks of foodborne and waterborne diseases, which posed health risks to the population (Lee et al., 2021). To prevent or reduce such serious foodborne disease outbreaks, it is necessary to rapidly detect foodborne pathogens; thus, the development of efficient foodborne pathogen detection methods is essential (Lee et al., 2001).

Foodborne pathogen detection methods can be divided into four types: (1) culture-based detection, (2) immunological detection, (3) biosensor-based detection, and (4) DNA-based detection. Culture-based detection is the traditional foodborne pathogen identification method; thus, it has a long history and is considered the gold standard (Bhunia, 2014). Using this method, viable colony forming units (CFUs) of foodborne pathogens are detected in genus-specific selective media cultures, and live CFUs and cell numbers can be confirmed in contaminated samples cost-effective and well-established manner (Bolton, 1998). However, at least 2–3 days are required to obtain the results of culture-based foodborne pathogen detection tests, and these are followed by biochemical tests, molecular tests, and/or mass spectrometry (Zhao et al., 2014). Therefore, alternative rapid foodborne pathogen detection methods have been developed. Immunological detection involves the use of an antibody–antigen reaction to detect foodborne pathogens; the methods used include enzyme-linked immunosorbent assays (Fusco et al., 2011), lateral flow immunoassays (Shi et al., 2015), and immunomagnetic separation assays (Shim et al., 2008). Monoclonal or polyclonal antibodies are used for different specificities to detect specific antigens, offering rapid, portable, and economic detection in the commercial ELISA-based detection kit via their massive production (Umesha and Manukumar, 2018). However, the influence of environmental stress on the antibody leads to low accuracy in immunological detection (Hahn et al., 2008). Biosensor-based detection was developed to overcome the disadvantages of immunological detection. Specifically, optical piezoelectric biosensors have been developed that provide a wide working range, rapid results, portability, and enhanced detection accuracy and limit of detection (Velusamy et al., 2010). Therefore, biosensors for bacterial quantification are generally rapid, specific, sensitive and very reliable. However, the development and commercialization cost of biosensors are relatively high, including the production of inexpensive sensors, storage and stabilization of biosensors, calibration methods, and achieving complete integration of the sensor system. Once developed and optimized, the cost of biosensors for bacterial quantification can be lowered (Tokarskyy and Marshall, 2008; Nnachi et al., 2022). DNA-based detection via specific gene-based polymerase chain reaction (PCR) is generally used in foodborne pathogen diagnostics in laboratories (Priyanka et al., 2016). Because of DNA amplification, conventional PCR in which specific gene-targeting primers are used exhibits high sensitivity up to the femtogram level (Palka-Santini et al., 2009). However, this method still requires a time-consuming electrophoresis step for the detection and confirmation of specific genes, and only one gene can be detected in each analysis (Joensen et al., 2014). To overcome the limitations of conventional PCR, real-time PCR or multiplex PCR methods were developed and optimized. Real-time PCR using specific gene-targeting primers and a probe does not require the electrophoresis step, and specific genes can be detected via the fluorescence signal from the probe (Yang et al., 2015). Determining fluorescence intensity also enables the quantification of DNA concentrations (Liu et al., 2019). Multiplex PCR can detect a few targeted genes at the same time because a mixture of primer sets is used (Chen et al., 2012). Combining these advantages, multiplex real-time PCR was developed. Many PCR-based detection kits developed in recent years use multiplex real-time PCR, which achieves rapid and multiple detection with high specificity and sensitivity (Park et al., 2020). Next-generation sequencing (NGS) has enabled the generation of large quantities of DNA sequences in an economical and time-efficient manner (Gupta and Verma, 2019). The most frequently used NGS sequencers are those from Illumina, which provide the prevailing high-throughput technology with the highest fidelity (Yohe and Thyagarajan, 2017). Although NGS produces massive amounts of DNA sequences in one run, the technology was highly expensive at the early stage (De Magalhães et al., 2010). However, NGS services have been popularized and subject to reduced costs given the continuous development of new technologies such as nanopore (Oxford NanoPore Technologies, United Kingdom) sequencing (Vega et al., 2016). Given the reduced costs, this NGS sequencing service is now available for use in molecular studies of foodborne pathogens to achieve rapid detection and identification and facilitate microbial genomics, metagenomics, and even shotgun metagenomics analyses (Chung et al., 2021). The term "NGS panel" refers to an NGS-based assay that allows for the simultaneous analysis of multiple genes, genetic variants, microbial genomes, or other genomic features. In particular, NGS panels are promising analysis methods with which hundreds to thousands of target gene sequences can be screened at once and many samples can be simultaneously analyzed to rapidly and efficiently detect and identify foodborne pathogens (Ferrario et al., 2017).

The NGS panel method was initially evaluated and used in clinical cancer diagnoses and genetically modified organism (GMO) determination. In a previous study, an NGS panel with 13 endometrial cancer gene target primers was developed and evaluated, and 20 randomly chosen cases of patients with endometrial cancer were successfully classified, highlighting the rapid and accurate diagnosis ability of NGS panels (López-Reig et al., 2019). In another study, a NGS panel with four GMO-related target gene sequences was developed and evaluated using real-time PCR as the control; the NGS panel and real-time PCR provided a 92% GMO detection rate, indicating the reliability of screening performed via this method (Arulandhu et al., 2018). Given the advantages of NGS panels, they have also been evaluated and tested for the multiple detection and determination of various foodborne pathogens. Prior to the use of NGS panels, the detection and identification of foodborne pathogens was conducted using 16S rRNA sequencing based on Sanger sequencing, 16S rRNA-based metagenome and random genome sequencing-based shotgun metagenomics approaches (Bridier, 2019); however, these detection methods produce an overabundance of sequence information (Zakotnik et al., 2022). To overcome this problem, NGS panels were developed and evaluated using specific primer sets, generally targeting the virulence factors and antibiotic resistance genes of foodborne pathogens. However, only one NGS

panel study has involved the detection and identification of multiple foodborne pathogens; in this study, a species-specific multiplex PCR amplicon was sequenced using an Illumina MiSeq sequencer to a sensitivity of 10^1 CFUs/g (Ferrario et al., 2017). This study demonstrates that, compared with metagenome and shotgun metagenomics sequencing, the NGS panel approach achieves rapid and accurate species-specific identification via the one-time compact NGS sequencing of virulence factors and antibiotic resistance genes. Only one primer set per pathogen was used in this study, and the specificity and sensitivity of the primer sets were not fully evaluated; however, the importance of NGS panel primer set quality and the requirement of multiple primer sets per pathogen should be considered. Indeed, the NGS panel method should be optimized with reliable multiple primer sets.

In the present study, we aimed to optimize the NGS panel method for the detection and identification of six major foodborne pathogens in South Korea: *Bacillus cereus*, *Yersinia enterocolitica*, *Staphylococcus aureus, Vibrio cholerae, Vibrio parahaemolyticus,* and *Vibrio vulnificus*. In addition, 2–5 species-specific primer sets per pathogen were designed and evaluated. With these new primer sets, the NGS panel method was tested and evaluated using the six selected foodborne pathogens. To verify the sensitivity and accuracy of the NGS panel, multiplex real-time PCR was performed as a control and compared with the NGS panel results. This study provides a novel optimized NGS panel method that achieves the rapid and accurate detection and identification of selected foodborne pathogens in contaminated samples with efficiency, sensitivity, and accuracy. Therefore, this technology could be useful for ensuring food safety through the prevention of foodborne disease outbreaks via the rapid and accurate detection and identification of foodborne pathogens.

Materials and methods

Bacterial strains, selective/culture media, and growth conditions

The bacterial strains and selective/culture media used in this study are listed in Table 1. All bacterial strains were aerobically incubated at 37°C for 18 h. All culture media were purchased from Oxoid (United Kingdom), and the agar medium was prepared with 1.8% BACTO Agar (BD, United States).

Isolation of foodborne pathogens

For the isolation of foodborne pathogens, five seafood samples were collected from Garak Fisheries Wholesale Market (Seoul, Korea) and Noryangjin Seafood Wholesale Market (Table 1). After sample collection, 25 g of the collected samples were transferred to a 3 M sterilized bag (USA) and suspended with 225 mL of sterilized phosphate-buffered saline buffer. Suspended samples were homogenized using a BagMixer 400 (Interscience, France) with a speed of 4 m/s for 30 s. After homogenization, the samples were serially diluted to 10^{-6}, plated on selective agar plates specific for each pathogen (Table 1), and incubated as described previously. From each selective agar plate, multiple colonies were picked and each colony was separately inoculated into fresh broth culture media. After broth culture incubation, the selected bacterium was identified using 16S rRNA gene sequencing technology (Table 1). The selected bacterium was identified using 16S rRNA gene sequencing technology, and the identified bacterium was stored at −80°C in 10% (w/v) sterilized skim milk solution.

DNA extraction

Bacterial genomic DNA was extracted and purified using a Genelix™ Bacterial Extraction Kit (Sanigen, South Korea) according to the manufacturer's instructions. In preparation for NGS panel analysis, total bacterial DNA was extracted from prepared agricultural water samples spiked with the six selected foodborne pathogens or agricultural water free of these pathogens using a QIAamp DNA Stool Mini Kit (Qiagen, United States) according to manufacturer's standard protocol.

16S rRNA gene sequencing

All PCRs were performed using a C1000 Touch Thermal Cycler (Bio-Rad, United States). In addition, 16S rRNA gene sequencing was performed for bacterial identification under the following conditions. The PCR mixture (final volume: 25 μL) contained 1 μL of template DNA (40 ng/μl), 0.5 μL of forward primer (20 μM; 27F, 5′-AGAGTTTGATCCTGGCTCAG-3′) and 0.5 μL of reverse primer (20 μM; 1492R, 5′-GGTTACCTTGTTACGACTT-3′; Montagner et al., 2010), 12.5 μL of BioFACT™ 2X Taq PCR Master Mix (BioFact, South Korea), and 10.5 μL of molecular water. The PCR conditions were as follows: 1 cycle of 95°C for 3 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 5 min. Following PCR, 16S rRNA amplicons were purified using a NICSROprep™ PCR Clean-up S and V Kit (Bionics, South Korea) and sequenced using a 3730xl DNA Analyzer (Thermo Fisher, United States) at Bionics in South Korea according to manufacturer's standard protocols.

Genome sequencing and analysis

For sequencing library preparation with the bacterial genomic DNA, a TruSeq Nano DNA LT Kit (Illumina, United States) was used to add sequencing barcodes to NGS sequencing templates. The sequencing library was then sequenced using an Illumina MiSeq system according to the Illumina MiSeq 2 × 150 bp paired-end run protocol. The qualified sequence reads were assembled using the Unicycler program (Wick et al., 2017) and the assembled contigs of each foodborne pathogen were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016).

NGS panel primer design and optimization

The publicly available complete genome sequences of target pathogens were collected from the GenBank database in the NCBI.[1]

1 https://www.ncbi.nlm.nih.gov/genbank

TABLE 1 Bacterial strains, culture medium, samples, and sampling locations.

Bacterium	Strain	Selective media[a]	Culture media[b]	Reference[c]	Sample	Sampling location
Selected foodborne pathogens						
Bacillus cereus	SG_003	BBC	LB	This study	Seaweed fulvescens	Garak Agricultural and Fisheries Wholesale Market, Seoul
Yersinia enterocolitica	SG_002	CIN	LB	This study	Pollack roe	Garak Agricultural and Fisheries Wholesale Market, Seoul
Staphylococcus aureus	ATCC 23235	-	LB	ATCC	-	-
	Newman	-	LB	ATCC	-	-
	CCARM 3089	-	LB	CCARM	-	-
	SG_001	MSA	LB	This study	Crab	Garak Agricultural and Fisheries Wholesale Market, Seoul
Vibrio cholerae (non-O1-type)	SG_017	TCBS	LB	This study	Octopus	Noryangjin Seafood Wholesale Market, Seoul
Vibrio vulnificus	SG_012	TCBS	LB	This study	Mussel	Noryangjin Seafood Wholesale Market, Seoul
Vibrio parahaemolyticus	SG_014	TCBS	LB	This study	Sea urchin	Noryangjin Seafood Wholesale Market, Seoul

[a]MSA, mannitol salt medium; CIN, cefsulodin–irgasan–novobiocin medium; BBC, Brillance *Bacillus cereus* medium; TCBS, thiosulfate–citrate–bile salts–sucrose medium.
[b]LB, Luria–Bertani medium.
[c]ATCC, American Type Culture Collection; CCARM, Culture Collection of Antimicrobial Resistance Microbes.

Comparative pan-genome analysis with the complete genome sequences of other pathogens was performed using the panX program (Ding et al., 2018) to identify target pathogen-specific genes. Among the detected pathogen-specific genes, virulence factors and antibiotic resistance genes were primarily considered for selection. New primer sets for the NGS panel were then designed using the sequences of the selected genes and the Primer3 program (Untergasser et al., 2012) with the following parameters: size: 100–300 bp; GC content: 40%–60%; T_m value: 53°C–60°C; self-compatibility: ≥4. After primer set design, the stability of the primers, e.g., self-binding and dimer formation, and specificity of the primer set to the target pathogen genome sequence were confirmed using Primer3. For NGS panel sequencing analysis, 2–5 genes per target pathogen were selected for primer design. Therefore, a single pathogen had 2–5 specific primer sets, and each primer set was optimized as previously explained. The selected pathogen-specific genes and their targeting primer sets are listed in Supplementary Table S1.

Singleplex PCR and crosscheck PCR

To validate the primer specificity to the target pathogen genome sequence, singleplex and crosscheck PCRs were performed. For singleplex PCR, the PCR mixture (final volume: 25 μL) contained 1 μL of template DNA (4 ng/μL), 0.5 μL of forward and reverse primers (20 μM), and 12.5 μL of KAPA HiFi HotStart ReadyMix (Roche, Germany), and the final volume was adjusted with molecular water. Crosscheck PCR was used to evaluate the selected primer set, and two approaches were taken: (1) a single primer set with the genomic DNA of 9 target strains and (2) multiple primer sets (2–5 primer sets per reaction) with the genomic DNA of a single target strain. The PCR mixture of the first crosscheck PCR test was prepared with the same composition as that used in singleplex PCR, except for the genomic DNA templates. The test genomic DNA template mixture for the first crosscheck PCR was prepared with the genomic DNA of a target pathogen and other nontarget pathogens, and the negative control genomic DNA mixture was prepared with the genomic DNA of only the nontarget pathogens. These template DNA mixtures contained 4 ng/μL of DNA per pathogen. The PCR mixture of the second crosscheck PCR test had the same composition as that used in the singleplex PCR, except for the multiple primer sets, which themselves contained 2–5 primer sets (20 μM of each) per reaction with a single target strain. The PCR conditions for both the singleplex and crosscheck PCRs were as follows: 1 cycle of 95°C for 3 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 5 min. To verify the PCR results, agarose gel electrophoresis was performed with 2.5% agarose gel containing ethidium bromide (0.2 μg/mL), and the size of each PCR amplicon was confirmed in the gel using 100 bp DNA ladder (Bioneer, South Korea) after the gel was run at 135 V for 20 min.

Multiplex PCR

In addition to singleplex and crosscheck PCRs, multiplex PCR was performed to confirm the specificity of the primer sets in the multi-detection of target pathogens. The multiplex PCR mixture (final volume: 25 μL) contained 1 μL of template DNA (4 ng/μL per pathogen; 9 pathogens in total), 0.5 μL of forward and reverse primer sets (2–5 primer sets; 20 μM of each), and 12.5 μL of KAPA HiFi HotStart ReadyMix (Roche, Germany), and the final volume was adjusted with molecular water. The test and negative control genomic

DNA mixtures were prepared with the same composition as that used in the first crosscheck PCR. The PCR results were verified following the same procedure used in singleplex and crosscheck PCRs.

Collection of agricultural water, and the preparation of simulated agricultural water samples with selected pathogens

Six agricultural water samples were collected from a vegetable farm in Hadong-gun, Gyeongsangnam-do, South Korea. Four samples, namely B4GNG1-1 (chive), B4GNG4-2 (chive), B1GNG8-1 (cabbage), and B1GNG8-2 (cabbage), were collected from ground water, whereas two samples, B1GNS10-1 (cabbage) and B1GNS10-2 (chive), were collected from stream water. One liter of each water sample was collected and transferred into a 2-L sterilized water pack (Worldmedi, South Korea). For the preparation of the spiked water samples, nine pathogenic strains were selected as follows: *V. vulnificus* SG_012; *V. parahaemolyticus* SG_014; non-O1-type *V. cholerae* SG_017; *Y. enterocolitica* SG_002; *S. aureus* strains SG_001, ATCC 23235, Newman, and CCARM 3089; and *B. cereus* SG_003 (Table 1). Six selected foodborne pathogenic strains and three reference strains were separately inoculated into fresh Luria–Bertani (LB) media and incubated up to 1.0 optical density at a wavelength of 600 nm. Subsequently, the CFUs of each culture was adjusted to 1.0×10^8 CFUs/mL using sterilized LB broth medium. To prepare a single *S. aureus* culture containing four different strains, 25% of each *S. aureus* strain culture was mixed. The negative control was prepared with an agricultural water sample without inoculation of target pathogens. Each CFU-adjusted culture of a selected pathogen was mixed, and the culture mixture containing six selected pathogenic species was centrifuged at 13,000 rpm for 10 min to harvest the bacterial cell mixture (1.0×10^8 CFUs per pathogen). This mixed cell pellet (6.0×10^8 CFUs) was resuspended using 250 mL of each collected agricultural water sample. The resuspended bacterial mixture was then serially 10-fold diluted to 6.0×10^5 CFUs per sample (1.0×10^5 CFUs per target pathogen in the sample). These serially diluted agricultural water samples (10^8, 10^7, 10^6, and 10^5 CFUs per target pathogen) were used for total bacterial DNA extractions before further NGS panel analysis. This experiment was performed in triplicate.

NGS panel analysis

To prepare the NGS panel sequencing template DNA via PCR, two sets of template DNA were prepared: (1) total DNA for test samples from one of six agricultural water samples containing target pathogens and (2) total DNA for negative controls from one of six agricultural water samples without the addition of target pathogens. The PCR mixture (final volume: 25 μL) contained 1 μL of template DNA (the total DNA template for test samples or total DNA template for negative controls), 0.1 μL of forward and reverse primers per primer set (18 primer sets; 100 μM of each), and 12.5 μL of KAPA HiFi HotStart ReadyMix (Roche), and the final volume was adjusted with molecular water. The PCR conditions used were the same as those used in singleplex PCR. Following PCR, target PCR amplicons were gel-extracted and purified using a NICSROprep™ DNA Gel Extraction S & V Kit (Bionics) according to the manufacturer's standard protocol. To prepare the sequencing library, a TruSeq Nano DNA LT Kit (Illumina, United States) was used to add sequencing barcodes to NGS sequencing templates. Subsequently, the sequencing library was sequenced using an Illumina MiniSeq system according to the Illumina MiniSeq 2 × 150 bp paired-end run protocol. After NGS sequencing, the following steps were taken: (1) a filtering step in which the raw reads were filtered using Trimmomatic (Bolger et al., 2014) to obtain a Phred quality score of >20; (2) a merging step in which the filtered reads were merged using Pandaseq (Masella et al., 2012) with its default parameters; and (3) a mapping step in which the merged reads were mapped to the six selected pathogen-specific gene sequences using BLASTN with a >95% nucleotide identity (Camacho et al., 2009). Finally, the number of mapped reads was counted. The detection criteria for false positive (<6 read counts) was determined using NGS panel analysis with negative controls, the agricultural water samples without inoculation of target pathogens. The false positive was determined using this detection criteria after counting the number of read counts from the NGS panel analysis result.

Quantitative real-time PCR

To evaluate the NGS panel analysis, quantitative real-time PCR (qPCR) was performed, and the results were compared with the NGS panel analysis results. The qPCR was performed using a CFX96 deep-well plate reader (Bio-Rad). The two sets of NGS panel sequencing template DNA previously described were used as the template DNA for qPCR. A Genelix™ Multiplex Real-Time PCR Kit (#G102, Sanigen) was used to detect *V. vulnificus, V. parahaemolyticus*, and *V. cholera*, whereas a Genelix™ Multiplex Real-Time PCR Kit (#G104, Sanigen) was used to detect *B. cereus, Y. enterocolitica*, and *S. aureus*. The qPCR was performed according to the manufacturer's standard protocols, and the Ct was determined automatically using CFX Manager Software version 3.1 (Bio-Rad). All tests were performed in triplicate.

Statistical analysis

GraphPad version 7.0 (Prism, United States; http://www.graphpad.com) and R version 4.1.2 (R Core Team, 2013) were used to perform all correlations and visualizations.

Results

Isolation and identification of foodborne pathogens

In total, 54 pathogenic bacteria were isolated from 6 seafood samples (crab, pollack roe, seaweed fulvescens, octopus, mussel, and sea urchin). These pathogens were identified as *V. vulnificus* (1 strain), *V. parahaemolyticus* (1 strain), non-O1-type *V. cholerae* (1 strain), *Y. enterocolitica* (1 strain), *B. cereus* (1 strain), *S. aureus* (1 strain), *Pseudomonas aeruginosa* (16 strains), *E. coli* (8 strains), *Klebsiella pneumonia* (2 strains), *Enterococcus hirae* (9 strains), *Enterococcus faecalis* (3 strains), *Listeria innocua* (4 strains), and *Serratia liquefaciens* (6 strains) at the molecular level using 16S rRNA gene sequencing. Among these pathogens, six strains of *B. cereus*, *Y. enterocolitica*, *S. aureus, V. cholera, V. parahaemolyticus,* and *V. vulnificus* were

selected as target pathogens, and three *S. aureus* type strains were also selected (Table 1) as these bacterial species have been associated previously with agricultural water contamination related to potential foodborne disease outbreaks (Pianetti et al., 2004; Silva et al., 2020; Elshikh et al., 2022; Roulová et al., 2022).

General genome features of selected foodborne pathogens, and the design of primer sets

The genome sequence information of selected target pathogens is required to design specific primer sets and confirm their binding sites in the genomes. Therefore, NGS genome sequencing was performed, and draft genome sequences were obtained for *B. cereus*, *Y. enterocolitica*, *V. cholera, V. parahaemolyticus,* and *V. vulnificus* as well as two *S. aureus* strains (SG_001 and CCARM 3089). In addition, the previously reported genome sequences of two *S. aureus* strains (ATCC 23235 and Newman) were obtained from the NCBI GenBank database. The general genome features of these foodborne pathogens are summarized in Table 2. Based on the genome sequences, primer sets targeting specific toxin genes and virulence factors were designed to meet the criteria of primer design given in Materials and Methods. The sequence information of the designed primer sets is shown in Table 3, and the primer target genes and primer binding locations are listed in Supplementary Table S1.

Singleplex PCR for NGS panel primer validation

To evaluate the specificity of primer sets to the target pathogens, singleplex PCR was performed with a single target pathogen and an associated single primer set. For the six target pathogens, the selected specific genes with their encoded functions, designed specific primer sets, and expected PCR amplicon sizes are listed in Table 3. Following singleplex PCR, agarose gel electrophoresis analysis revealed that all PCR amplicons were of the expected size according to the single PCR bands, confirming the specificity of all the PCR primer sets to the associated target pathogens (Figure 1). Thus, the designed primer sets qualified for crosscheck PCR evaluation in the next stage.

Crosscheck PCR for NGS panel primer validation

To confirm the specificity of the primer sets to the target and nontarget pathogens, two different crosscheck PCRs were conducted: (1) an evaluation of the target pathogen and eight different nontarget pathogens with a single primer set and (2) an evaluation of a single pathogen-targeting primer set mixture (2–5 primer sets) with an associated target pathogen.

For the first crosscheck PCR, two genomic DNA template sets (test DNA template mixture and negative control DNA template mixture without target pathogenic DNA) were prepared to confirm the nonspecific binding of a selected single primer set to nontarget pathogenic DNA. In this crosscheck PCR, the PCR amplicon bands specific to the selected gene were found in the target pathogen but not the nontarget pathogens (Figure 2). In addition, the sizes of the PCR amplicon bands matched those expected, indicating that the primer sets were highly specific to the target pathogenic DNA, even though the template DNA mixture contained all other nontarget pathogenic DNA. Thus, the PCR primer sets were specific to the associated target gene as well as the target pathogen.

TABLE 2 General genome features of foodborne pathogens.

Bacterium	Strain	Genome size (bp)	Assembly	Contig	GC (%)	CDS	tRNA	rRNA	Reference[d]
Selected foodborne pathogens									
Bacillus cereus	SG_003	5,908,983	Draft	83	34.81	5,920	62	3	This study
Yersinia enterocolitica	SG_002	4,357,829	Draft	123	46.92	3,932	66	3	This study
Staphylococcus aureus	ATCC 23235[a]	2,789,574	Draft	2	32.68	2,705	59	19	ATCC
	Newman[b]	2,878,897	Complete	1	32.89	2,851	59	16	ATCC
	CCARM 3089[c]	2,865,317	Draft	54	32.72	2,822	56	2	CCARM
	SG_001	2,944,975	Draft	111	32.77	2,781	59	4	This study
Vibrio cholerae	SG_017	4,005,842	Draft	91	47.52	3,592	69	4	This study
Vibrio parahaemolyticus	SG_014	6,040,036	Draft	81	44.01	5,740	135	5	This study
Vibrio vulnificus	SG_012	5,012,927	Draft	114	46.66	4,401	83	4	This study

[a]NCBI GenBank BioProject accession number, PRJNA224116.
[b]NCBI GenBank BioProject accession number, PRJDA18801.
[c]NCBI GenBank BioProject accession number, PRJNA870224.
[d]ATCC, American Type Culture Collection; CCARM, Culture Collection of Antimicrobial Resistance Microbes.

TABLE 3 Selected pathogen species-specific genes, their functions, and the associated designed primer sets.

Bacterium	Gene	Function	Primer	Sequence (5′ to 3′)	Size (bp)	Reference
Bacillus cereus	*entFM1*	Enterotoxin	ent_F	GAACTGCTGGTACAACACCTG	229	This study
			ent_R	TCTGCACTAATGAACTGACCG		
	tpi	Triose phosphate isomerase	tpi_F	GCGCTCTTCTAAAGTCTCAC	175	This study
			tpi_R	CGAAATTAGCCCAGTAGCAC		
Yersinia enterocolitica	*ail*	Attachment invasion locus protein	ail_F	TGGGGCCATCTTTCCGCATTA	235	This study
			ail_R	TACCCTGCACCAAGCATCCAA		
	gspE	Type II secretion system ATPase	gspE_F	AACGGGGCATCTGGTTCTCTC	190	This study
			gspE_R	TGGTGGTGTCAGGAAAGGGAC		
Staphylococcus aureus	*femA*	Methicillin resistance factor	femA_F	GCAGCTTGCTTACTTACTGCT	214	This study
			femA_R	TACCTGTAATCTCGCCATCAT		
	sea1	Exotoxin A	sea_F	ATTCATTGCCCTAACGTGGAC	191	This study
			sea_R	GCTGTAAAAATTGATCGTGACTCTC		
	seb1	Exotoxin B	seb_F	GTATGGTGGTGTAACTGAGC	212	This study
			seb_R	CCGTTTCATAAGGCGAGTTG		
	sec1	Exotoxin C	sec_F	CTGCTATTTTTCATCCAAAGA	180	This study
			sec_R	TTCTTATCAGTTTGCACTTCA		
	sed1	Exotoxin D	sed_F	TGTCACTCCACACGAAGGTA	162	This study
			sed_R	TGCAAATAGCGCCTTGCTTG		
Vibrio cholerae	*ctxA*	Enterotoxin	ctxA_F	GCCAAGAGGACAGAGTGAGTA	253	This study
			ctxA_R	ATGAGGACTGTATGCCCCTA		
	hlyA	Cytolysin and hemolysin	hlyA_F	GTTTGTATGTGCGAGCGGGTG	175	This study
			hlyA_R	GTGAATGTCAGCGCCACCAAC		
	toxS	Transmembrane regulator	toxS_F	TAAGACCAACAGCAACCGCCC	209	This study
			toxS_R	ACTCGACTGGCGTAACCAAAAGG		
Vibrio parahaemolyticus	*plsX*	Phosphate acyltransferase	plsX_F	GCACTGTCTCATTTCCCAGAG	219	This study
			plsX_R	CGCTTCTTGGTCAGAAACCAG		
	tdh	Thermostable direct hemolysin	tdh_F	TCCATCTGTCCCTTTTCCTGCC	187	This study
			tdh_R	CAGCCATTTAGTACCTGACGTTGTG		
	tlh	Thermolabile hemolysin precursor	tlh_F	GCGAGCGATCCTTGTTTGGAC	144	This study
			tlh_R	GCGGTGAGTTGCTGTTGTTGG		
	toxR	Transcriptional activator	toxR_F	ACCTGTGGCTTCTGCTGTG	178	This study
			toxR_R	CCAGTTGTTGATTTGCGGGTG		
Vibrio vulnificus	*glnA*	Glutamate ammonia ligase	glnA_F	AGCACATCTCTATTCCTTCTC	170	This study
			glnA_R	TAGCGTTGCTTCTTCAGTAA		
	vvh	Hemolysin	vvh_F	CTCTGCCTAGATGTTTATGG	199	This study
			vvh_R	CAATACCATTTCTGTGCTAAG		

The second crosscheck PCR was conducted to determine whether one PCR reaction can multidetect the target genes in a single pathogen with a single pathogen-targeting primer set mixture that combines primer sets targeting 2–5 selected genes in a single pathogen (Table 3). In this crosscheck PCR, the PCR amplicons of all target genes in each pathogen were confirmed in the gel electrophoresis (Figure 3), and their amplicon sizes matched those expected. Therefore, PCR with a mixture of primer sets detected target genes in one reaction without any primer interference.

Multiplex PCR for NGS panel primer validation

For multiplex PCR, template DNA was prepared with the same sets used in the first crosscheck PCR, and the mixture of primer sets was the same as that used in the second crosscheck PCR. Multiplex PCR results showed that the PCR amplicons of all target genes in each pathogen were detected in gel electrophoresis, and their band sizes were the same as those expected (Figure 4). Therefore, multiplex PCR

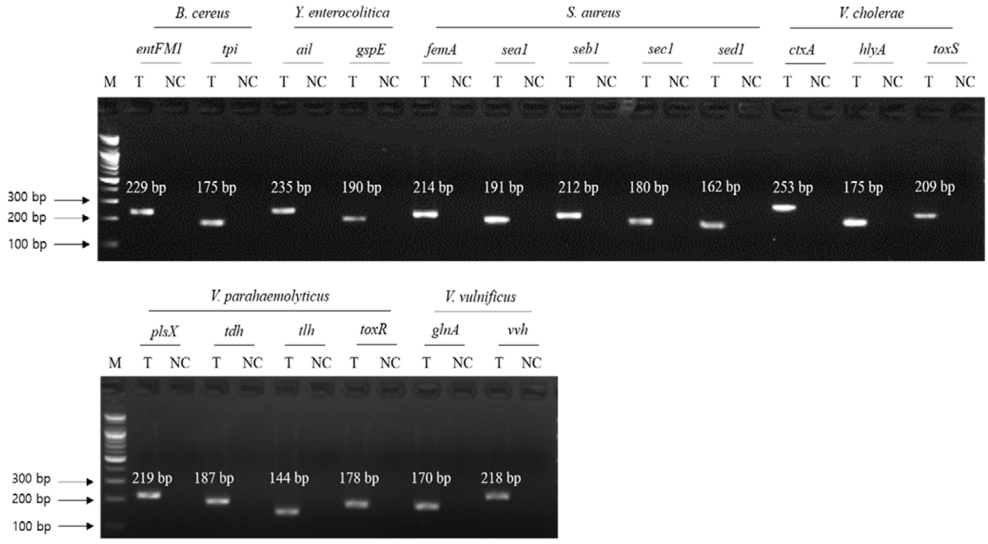

FIGURE 1
Gel electrophoresis results of singleplex PCR. Target pathogens and their specific genes are shown above the gel electrophoresis results. PCR mixture of the test (T) lane contained the associated target pathogen genomic DNA and specific gene primer set. PCR mixture of the negative control (NC) lane contained molecular water and the target pathogen-specific gene primer set. M: 100 bp DNA ladder.

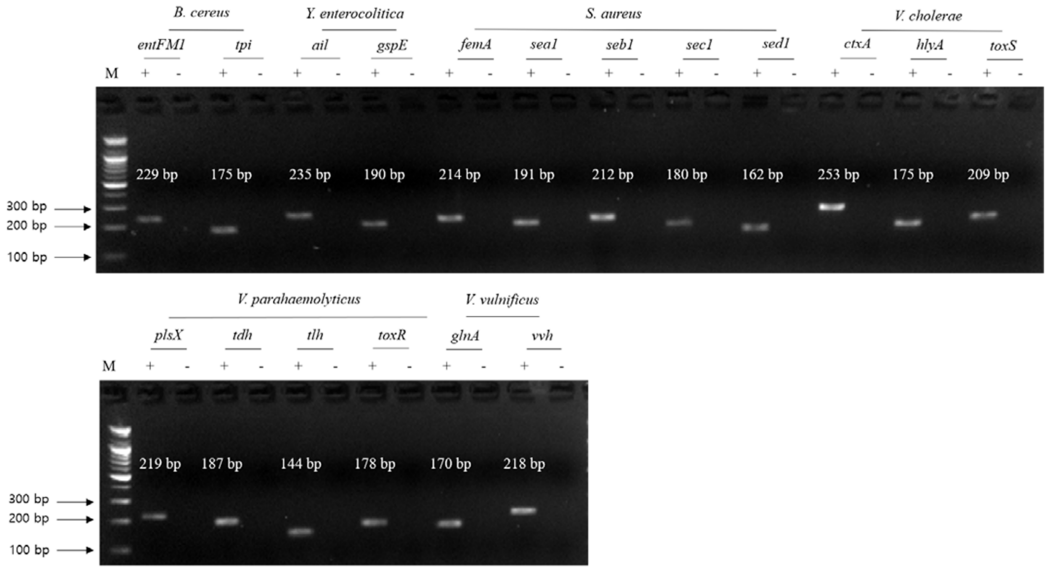

FIGURE 2
Gel electrophoresis results of the first crosscheck PCR. Target pathogens and their specific genes are shown above the gel electrophoresis results. PCR mixture of the test (+) lane contained a genomic DNA mixture including the associated target pathogen and target pathogen-specific gene primer set. PCR mixture of the negative test (–) lane contained a genomic DNA mixture lacking the associated target pathogen and target pathogens-specific gene primer set. M: 100 bp DNA ladder.

confirmed that a mixture of primer sets can be used to detect target genes in one reaction without any template DNA or primer interference.

NGS panel analysis

NGS panel analysis was performed with six different agricultural water samples spiked with a mixture of target pathogens. Following NGS panel sequencing, the qualified sequence reads were collected and mapped to the target pathogen-specific gene sequences. The average number of sequence reads mapped to target pathogen-specific genes was 228,915 (93.263% of total qualified sequence reads), 125,902 (61.501%), 35,360 (23.125%), and 3,218 (1.879%) at dilutions of 10^8, 10^7, 10^6, and 10^5 CFUs per target pathogen, respectively. Interestingly, the averages number of sequence reads mapped to target pathogen-specific genes and the CFU number per target pathogen were positively associated ($y = 78011x - 95630$; $R^2 = 0.9532$; Figure 5A). The prepared

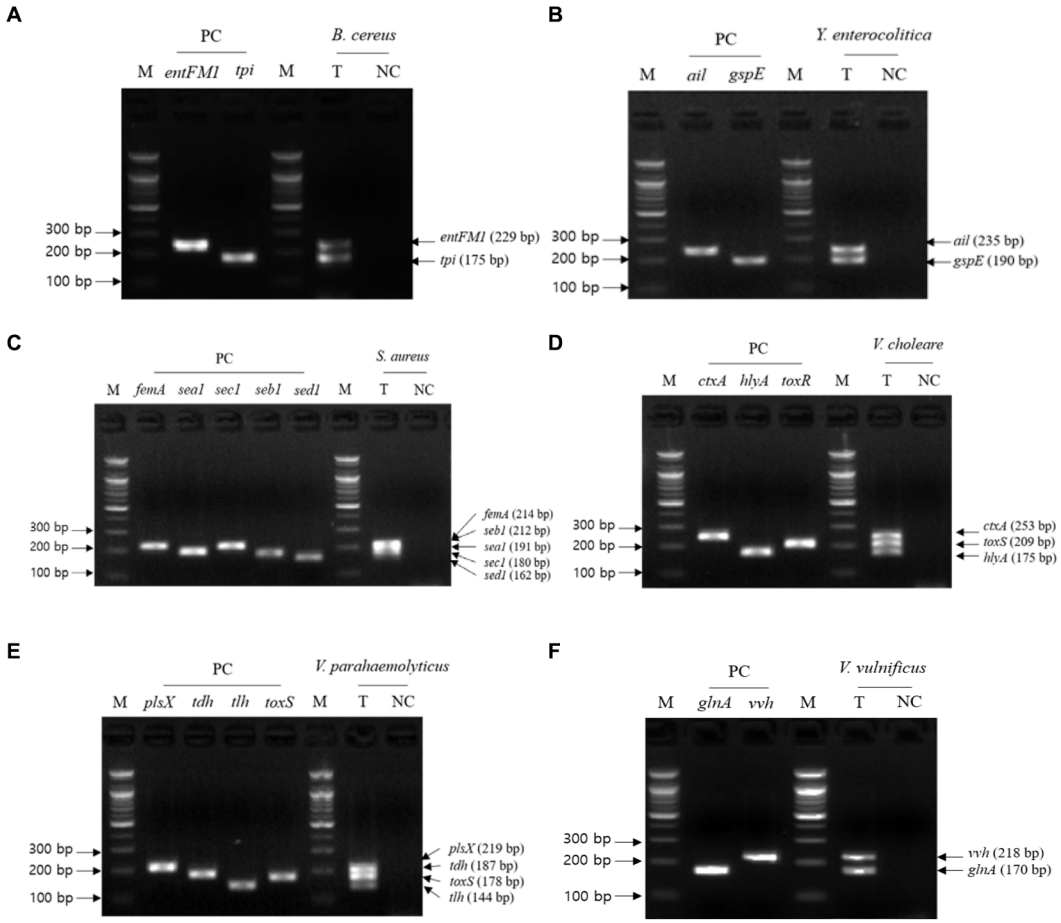

FIGURE 3
Gel electrophoresis results of the second crosscheck PCR. Target pathogens and their specific genes are shown above the gel electrophoresis results. The target pathogen-specific genes and their corresponding pathogens are as follows: **(A)** *B. cereus*, **(B)** *Y. enterocolitica*, **(C)** *S. aureus*, **(D)** *V. cholerae*, **(E)** *V. parahaemolyticus*, and **(F)** *V.vulnificus*. Lanes contain each target pathogen-specific gene with singleplex PCR amplicons as positive controls (PC). The PCR mixture of the test (T) lane contained the associated target pathogen genomic DNA and 2–5 target pathogen-specific gene primer sets. The PCR mixture of the negative control (NC) lane contained molecular water and 2–5 target pathogenspecific gene primer sets. M: 100 bp DNA ladder.

negative control samples, which were not intentionally spiked with specific pathogen, exhibited 1–6 sequence reads mapped to target pathogen-specific genes, suggesting that a small number of pathogens were present in the original agricultural water samples and produced false positive results (Supplementary Figure S1A). Thus, ≤6 reads were determined as the false positive rate for further NGS panel analysis.

After mapping to 18 different target genes of six target pathogens, all qualified NGS panel sequence reads were collected from the six different agricultural water samples. The collected read counts for each dilution factor (10^8, 10^7, 10^6, and 10^5 CFUs per target pathogen) were compared in terms of the detection and identification of specific target pathogens (Figure 6A). For the dilution factors 10^6 to 10^8, all 18 target genes were multi-detected, and the dilutions were enough to identify the six target pathogens in one NGS panel analysis without false positives (Supplementary Figures S1B–D). This result was confirmed in triplicate tests of all agricultural water samples. As expected, the serial dilution of target pathogens was proportionally associated with the read count, i.e., the highest and lowest numbers of read counts were associated with the dilution factors 10^8 and 10^6, respectively, supporting the results shown in Figure 5A. However, when the dilution factor was 10^5, many false positive results were detected (Supplementary Figure S1E). In particular, *tlh* of *V. parahaemolyticus* and *seb1* of *S. aureus* were poorly detected by NGS panel analysis. The number of read counts for each target gene was compared among dilution factors, and the numbers of *tlh* and *seb1* were always lower than those of the other 16 target genes, supporting the results shown in Supplementary Figure S2. Therefore, *tlh* and *seb1* could be removed as target genes to increase the limit of detection and improve the identification of specific target pathogens in NGS panel analysis. Although false positive results were found at 10^5, using the average of triplicate tests in NGS panel analysis removed most of the false positive results at this dilution, thereby enhancing the detection and identification of all target pathogens (Supplementary Figure S1E). Nevertheless, the results of NGS panel analysis suggest that the limit of detection and identification of target pathogens may be at a dilution of 10^5 CFUs.

qPCR analysis

The qPCR template DNA used was the same as that used in the NGS panel analysis. According to the qPCR results, the average Ct values of the target pathogens were 18.84 (10^8 CFUs per target

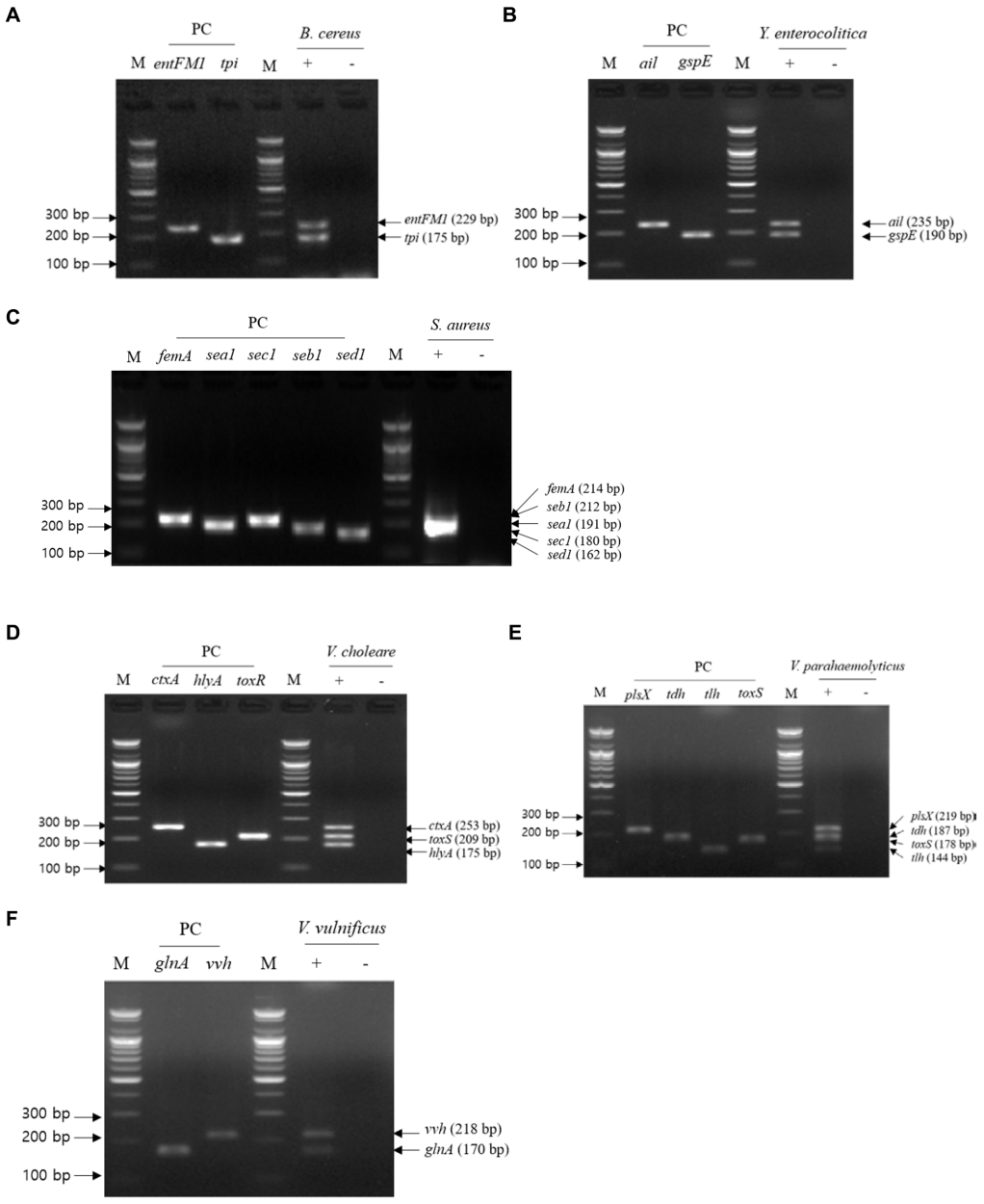

FIGURE 4
Gel electrophoresis results of multiplex PCR. Target pathogens and their specific genes are shown above the gel electrophoresis results. The target pathogen-specific genes and their corresponding pathogens are as follows: **(A)** *B. cereus*, **(B)** *Y. enterocolitica*, **(C)** *S. aureus*, **(D)** *V. cholerae*, **(E)** *V. parahaemolyticus*, and **(F)** *V.vulnificus*. Lanes contain each target pathogen-specific gene with singleplex PCR amplicons as positive controls. PCR mixture of the test (+) lane contained a genomic DNA mixture including the associated target pathogen and 2–5 target pathogen-specific gene primer sets. PCR mixture of the negative test (–) lane contained a genomic DNA mixture lacking the associated target pathogen and 2–5 target pathogens-specific gene primer sets. M: 100 bp DNA ladder.

pathogen), 22.33 (10^7), 24.97 (10^6), and 29.49 (10^5). Interestingly, a negative correlation existed between Ct and the number of cells of the target pathogen ($y = -3.4584x + 32.556$; $R^2 = 0.9895$), suggesting that target pathogens with a low Ct value or high number of cells could be rapidly detected and identified (Figure 5B). In contrast, the prepared negative control samples without the presence of specific pathogens showed no Ct values across all qPCR reactions (up to 40 cycles), suggesting that target pathogens were not present in these negative control samples (Supplementary Figure S3A).

Furthermore, the Ct values per target pathogen at four different dilution factors (10^8, 10^7, 10^6, and 10^5 CFUs per target pathogen) were compared to determine the sensitivity and detection limit of qPCR (Figure 6B). For all dilution factors, all target pathogens were detected in the qPCR reactions, and the six different target pathogens were identified without false positives (Supplementary Figures S3B–E). This result was confirmed in triplicate tests of all agricultural water samples. Overall, these results suggest that the sensitivity of qPCR may be higher than that of NGS panel analysis.

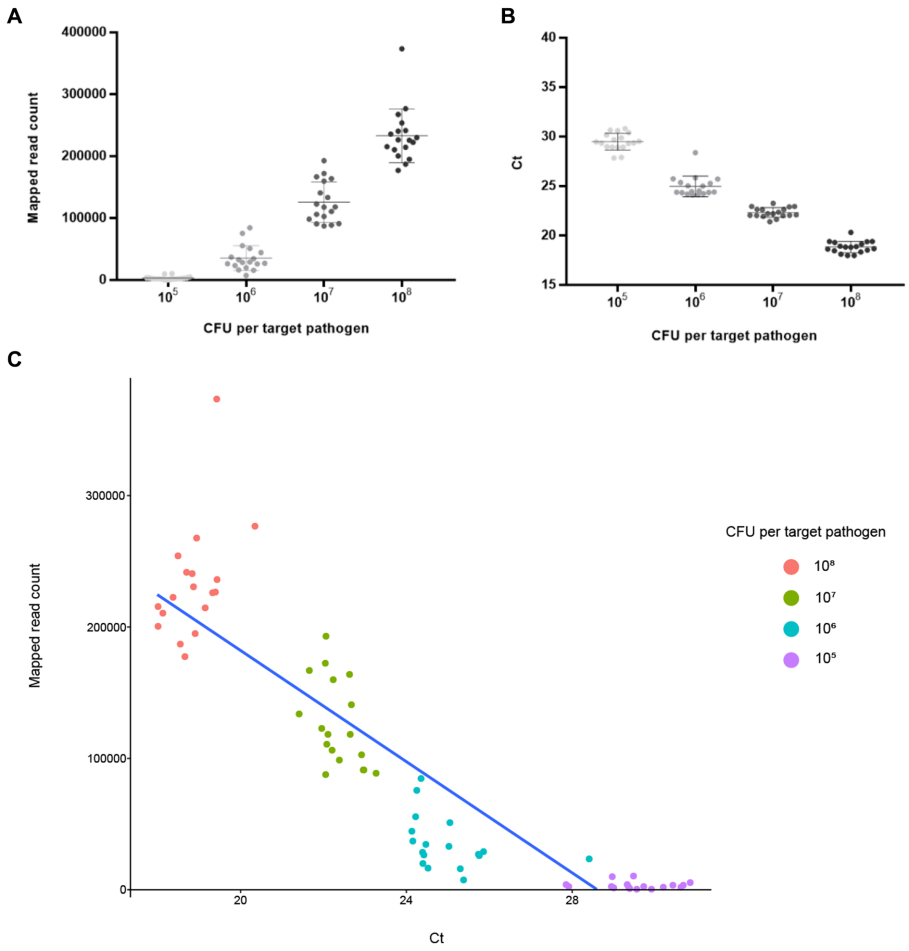

FIGURE 5
Analysis results of NGS panel and qPCR. Six different agricultural water samples with 10^8, 10^7, 10^6, and 10^5 CFUs per pathogen were analyzed using NGS panel analysis and qPCR. The analysis results are shown using linear regression with 10^8, 10^7, 10^6, and 10^5 CFUs per pathogen. Each point is the mean of six target pathogen-specific gene reads or Ct values in a single replicate. **(A)** Average reads mapped to the total target pathogen-specific genes (NGS panel). **(B)** Average total target pathogen Ct values (qPCR). **(C)** Correlation between the average reads mapped to the total target pathogen-specific genes and average total target pathogen Ct values.

Comparative evaluation of NGS panel analysis and qPCR

To further evaluate the detection and identification of specific target pathogens using NGS panel analysis, the results of NGS panel analysis and qPCR were compared. As the qualified read counts and Ct values were correlated with the number of cells of the target pathogens, additional correlation analyses between the qualified read counts and Ct values in each specific target pathogen were performed. Interestingly, the read counts and Ct values were negatively correlated, with comparative analysis revealing a negative relationship ($y = -21154x + 605174$; $R^2 = 0.7984$; Figure 5C), supporting the previous finding that a high number of target pathogen cells is associated with the quicker detection and identification of pathogens. To verify this correlation, Spearman correlation analysis was performed using the results of NGS panel analysis and qPCR for specific target genes, and negative correlations were found in all cases (Figure 7). The genes *entFM1* and *tpi* of *B. cereus* exhibited the highest correlations, whereas *seb1* of *S. aureus* exhibited the lowest correlation (Figure 7), which might have been due to the false negative results for this gene in NGS panel analysis (Supplementary Figure S1E).

The strong correlation between NGS panel analysis and qPCR in the specific target genes of pathogens suggests that the newly developed NGS panel analysis could serve as a supporting or alternative method to qPCR for the detection and identification of multiple target pathogens in given environments.

Discussion

Foodborne disease outbreaks are generally associated with foodborne pathogen contamination (Newell et al., 2010). However, food environments contain a plethora of food-related microbiota including a variety foodborne pathogens; (Lampel et al., 2000) therefore, it is often a challenge to detect and identify the specific foodborne pathogen that has caused an outbreak. To detect and identify outbreak-causing pathogens, various methods can be used such as culturing with specific selective media, immune detection

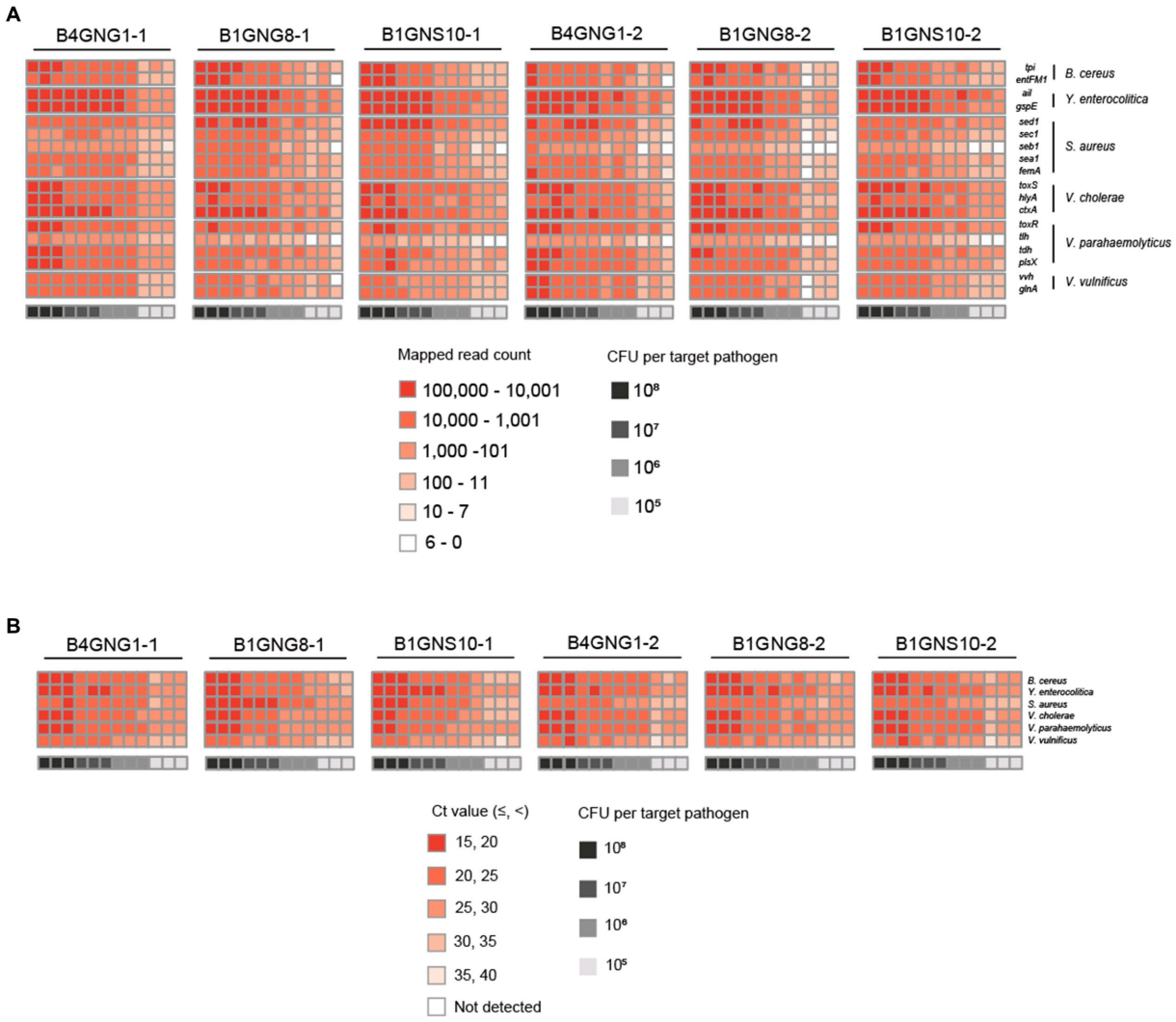

FIGURE 6
Heat maps of target pathogen detection results in six agricultural water samples with or without a mixture of target pathogens. **(A)** Target pathogen-specific gene reads of the NGS panel. **(B)** Target pathogen Ct values of qPCR. The color scale of the target pathogen-specific gene reads or target pathogen Ct values and the level of CFUs per target pathogen is indicated in the figure.

using specific antibodies, PCR using foodborne pathogen-targeting specific primer sets, and real-time PCR. However, these methods are not always appropriate for detecting and identifying specific pathogens in foodborne disease outbreaks owing to the complex bacterial ecosystem of food environments. Therefore, new screening methods must be developed and optimized. Accordingly, in the present study, a new method for detecting and identifying multiple pathogens in one reaction, an NGS panel method, was developed, optimized, and compared with a typical qPCR method.

Although the NGS panel method was originally developed for clinical diagnosis and GMO detection (Arulandhu et al., 2018; López-Reig et al., 2019) it has also been used for the multiple detection and identification of foodborne pathogens in food samples (Ferrario et al., 2017). In the current study, NGS panel primer sets targeting 18 pathogenic genes were developed and optimized. Using these primer sets, NGS panel analysis was conducted using six agricultural water samples spiked with pathogens. All pathogens were detected and identified, even with a sample dilution of 10^5 CFUs per pathogen, demonstrating the main advantage of the new method: multiple pathogen detection in one reaction. However, comparative analysis revealed that qPCR has a higher sensitivity than the NGS panel method, although all pathogens could not be detected in one reaction using qPCR. The NGS panel method also gave some false positive results when the number of target pathogen cells was low. Thus, the sensitivity of the NGS panel method when detecting and identifying pathogens must be increased through further optimization of the primer sets. Another major disadvantage of the NGS panel method is the time required to complete NGS sequencing, which could also be optimized through the use of new NGS sequencing technology. For example, nanopore sequencing technology can achieve real-time sequencing (Buytaers et al., 2021) and would therefore be a candidate sequencing method for minimizing the sequencing time in NGS panel analysis. In summary, although the potential and advantages of the developed NGS panel

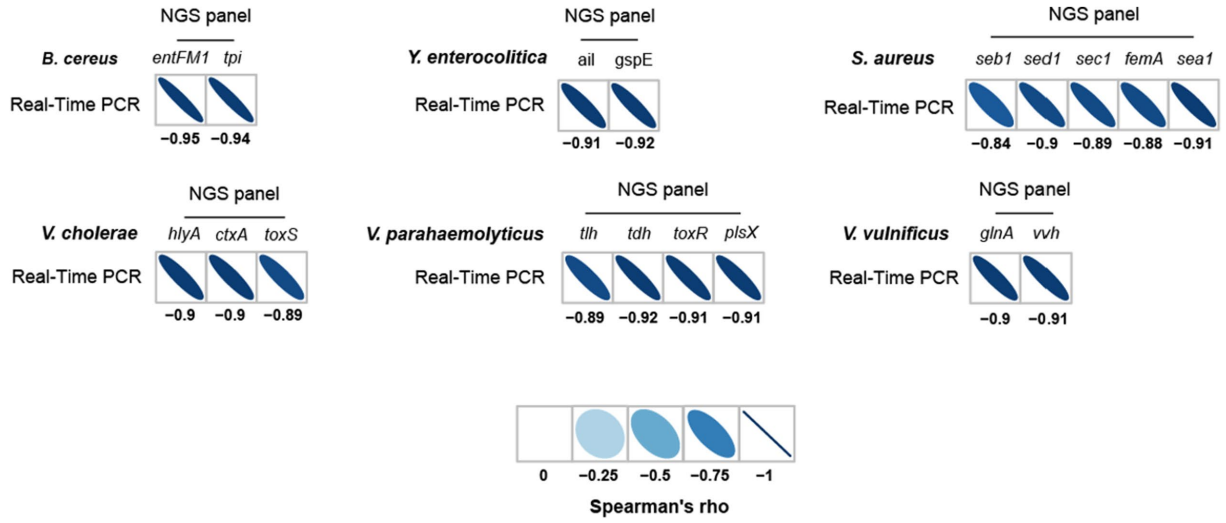

FIGURE 7
Spearman correlation between NGS panel analysis and multiplex qPCR results for six detected target pathogens.

analysis method were demonstrated in this study, further optimization of the NGS panel primer sets and the application of new real-time NGS sequencing technology will enhance the method's pathogen detection and identification capabilities and help popularize the technology for the improvement of food safety.

Conclusion

Because general methods cannot multi-detect and identify complex foodborne pathogens of a food sample in a reaction with high sensitivity, the NGS panel analysis method was optimized and its performance was evaluated with multiple detection of 18 virulence factor genes of 6 selected foodborne pathogens at the same time up to dilution factor of 10^5 in this study. Although this method overcomes the limitation of other detection methods in multiple detection and identification, its comparative analysis with qPCR showed that NGS panel analysis has lower sensitivity and longer detection/identification time than qPCR due to NGS sequencing. However, qPCR could detect and identify only a few pathogenic bacteria in a reaction. To improve the sensitivity and detection/identification time of NGS panel analysis, the primer sets need to be further optimized and a new real-time sequencing technology such as nanopore sequencing should be used in the next study. The optimized primer sets and the real-time nanopore sequencing technology would reduce the false-positive results as well as the sequencing time in NGS panel analysis. Although NGS panel analysis method still needs some improvements, this new technology enabled to multi-detect and identify numerous foodborne pathogens at the same time by a reaction, and even it would be able to determine the origin pathogen in foodborne outbreaks. Therefore, this study provides information on the usability and application of NGS panel analysis method. Furthermore, based on this, introduction of further optimized new NGS panel primer sets and nanopore sequencing technology in the next study will make possible the real-time detection and identification of pathogens during NGS sequencing as well as rapid determination of the origin pathogen from foodborne outbreaks.

Data availability statement

The datasets presented in this study can be found in online repositories. The genome sequences of six foodborne pathogenic bacteria listed in Table 2 were deposited in the GenBank database with the BioProject accession numbers PRJNA882507 (*S. aureus* SG_001) and PRJNA857825 (other pathogenic bacteria).

Author contributions

JP, SoK, and J-HL: conceptualization. D-GP, E-SH, and J-HL: methodology. D-GP, J-GK, E-SH, IC, JC, WL, SeK, SoK, and J-HL: validation. D-GP and E-SH: formal analysis. BK: software. D-GP, E-SH, BK, WL, SeK, and SoK: investigation. D-GP, E-SH, BK, and IC: data curation. WL, SeK, and SoK: resources. D-GP, J-GK, and J-HL: writing-original draft. J-GK and J-HL: writing-review and editing. D-GP: visualization. JC, JP, and J-HL: supervision. J-HL: project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

E-SH, BK, IC, JC, and JP were employed by Sanigen Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1179934/full#supplementary-material

References

Arulandhu, A. J., Van Dijk, J., Staats, M., Hagelaar, R., Voorhuijzen, M., Molenaar, B., et al. (2018). NGS-based amplicon sequencing approach; towards a new era in GMO screening and detection. *Food Control* 93, 201–210. doi: 10.1016/j.foodcont.2018.06.014

Bhunia, A. K. (2014). One day to one hour: how quickly can foodborne pathogens be detected? *Future Microbiol.* 9, 935–946. doi: 10.2217/fmb.14.61

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bolton, F. (1998). Strategies in the development of media for the detection of food-borne pathogens. *Int. J. Food Microbiol.* 45, 29–34. doi: 10.1016/S0168-1605(98)00144-5

Bridier, A. (ed.) (2019). "Exploring foodborne pathogen ecology and antimicrobial resistance in the light of shotgun metagenomics" in *Foodborne bacterial pathogens: Methods and protocols* (Springer), 229–245.

Buytaers, F. E., Saltykova, A., Denayer, S., Verhaegen, B., Vanneste, K., Roosens, N., et al. (2021). Towards real-time and affordable strain-level metagenomics-based food-borne outbreak investigations using Oxford Nanopore sequencing technologies. *Front. Microbiol.* 12:738284. doi: 10.3389/fmicb.2021.738284

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: architecture and applications. *BMC Bioinformatics* 10, 1–9. doi: 10.1186/1471-2105-10-421

Chen, J., Tang, J., Liu, J., Cai, Z., and Bai, X. (2012). Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. *J. Appl. Microbiol.* 112, 823–830. doi: 10.1111/j.1365-2672.2012.05240.x

Chung, H. Y., Kim, Y.-T., Kwon, J.-G., Im, H. H., Ko, D., Lee, J.-H., et al. (2021). Molecular interaction between methicillin-resistant *Staphylococcus aureus* (MRSA) and chicken breast reveals enhancement of pathogenesis and toxicity for food-borne outbreak. *Food Microbiol.* 93:103602. doi: 10.1016/j.fm.2020.103602

De Magalhães, J. P., Finch, C. E., and Janssens, G. (2010). Next-generation sequencing in aging research: emerging applications, problems, pitfalls and possible solutions. *Ageing Res. Rev.* 9, 315–323. doi: 10.1016/j.arr.2009.10.006

Ding, W., Baumdicker, F., and Neher, R. A. (2018). panX: pan-genome analysis and exploration. *Nucleic Acids Res.* 46:e5. doi: 10.1093/nar/gkx977

Elshikh, M. S., Alarjani, K. M., Huessien, D. S., Elnahas, H. A., and Esther, A. R. (2022). Enhanced biodegradation of Chlorpyrifos by *Bacillus cereus* CP6 and *Klebsiella pneumoniae* CP19 from municipal waste water. *Environ. Res.* 205:112438. doi: 10.1016/j.envres.2021.112438

Ferrario, C., Lugli, G. A., Ossiprandi, M. C., Turroni, F., Milani, C., Duranti, S., et al. (2017). Next generation sequencing-based multigene panel for high throughput detection of food-borne pathogens. *Int. J. Food Microbiol.* 256, 20–29. doi: 10.1016/j.ijfoodmicro.2017.05.001

Fusco, V., Quero, G. M., Morea, M., Blaiotta, G., and Visconti, A. (2011). Rapid and reliable identification of *Staphylococcus aureus* harbouring the enterotoxin gene cluster (egc) and quantitative detection in raw milk by real time PCR. *Int. J. Food Microbiol.* 144, 528–537. doi: 10.1016/j.ijfoodmicro.2010.11.016

Gupta, N., and Verma, V. K. (2019). Next-generation sequencing and its application: empowering in public health beyond reality. *Microbial Technol Welfare Soc* 17, 313–341. doi: 10.1007/978-981-13-8844-6_15

Hahn, M. A., Keng, P. C., and Krauss, T. D. (2008). Flow cytometric analysis to detect pathogens in bacterial cell mixtures using semiconductor quantum dots. *Anal. Chem.* 80, 864–872. doi: 10.1021/ac7018365

Joensen, K. G., Scheutz, F., Lund, O., Hasman, H., Kaas, R. S., Nielsen, E. M., et al. (2014). Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* 52, 1501–1510. doi: 10.1128/JCM.03617-13

Lampel, K. A., Orlandi, P. A., and Kornegay, L. (2000). Improved template preparation for PCR-based assays for detection of food-borne bacterial pathogens. *Appl. Environ. Microbiol.* 66, 4539–4542. doi: 10.1128/AEM.66.10.4539-4542.2000

Lee, W.-C., Lee, M.-J., Kim, J.-S., and Park, S.-Y. (2001). Foodborne illness outbreaks in Korea and Japan studied retrospectively. *J. Food Prot.* 64, 899–902. doi: 10.4315/0362-028X-64.6.899

Lee, H., and Yoon, Y. (2021). Etiological agents implicated in foodborne illness world wide. *Food Sci Anim Resourc* 41, 1–7. doi: 10.5851/kosfa.2020.e75

Lee, S. H., Yun, J.-W., Lee, J. H., Jung, Y. H., and Lee, D. H. (2021). Trends in recent waterborne and foodborne disease outbreaks in South Korea, 2015–2019. *Osong. Public Health Res. Perspect.* 12, 73–79. doi: 10.24171/j.phrp.2021.12.2.04

Liu, Y., Cao, Y., Wang, T., Dong, Q., Li, J., and Niu, C. (2019). Detection of 12 common food-borne bacterial pathogens by TaqMan real-time PCR using a single set of reaction conditions. *Front. Microbiol.* 10:222. doi: 10.3389/fmicb.2019.00222

López-Reig, R., Fernández-Serra, A., Romero, I., Zorrero, C., Illueca, C., García-Casado, Z., et al. (2019). Prognostic classification of endometrial cancer using a molecular approach based on a twelve-gene NGS panel. *Sci. Rep.* 9, 1–9. doi: 10.1038/s41598-019-54624-x

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., and Neufeld, J. D. (2012). PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* 13, 1–7. doi: 10.1186/1471-2105-13-31

Montagner, F., Jacinto, R. C., Signoretti, F. G., and Gomes, B. P. (2010). Treponema species detected in infected root canals and acute apical abscess exudates. *J. Endod.* 36, 1796–1799. doi: 10.1016/j.joen.2010.08.008

Newell, D. G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., et al. (2010). Food-borne diseases—the challenges of 20 years ago still persist while new ones continue to emerge. *Int. J. Food Microbiol.* 139, S3–S15. doi: 10.1016/j.ijfoodmicro.2010.01.021

Nnachi, R. C., Sui, N., Ke, B., Luo, Z., Bhalla, N., He, D., et al. (2022). Recent progress on biosensors for rapid detection of bacterial pathogens in water, food and environment. *Environ. Int.* 166:107357. doi: 10.1016/j.envint.2022.107357

Palka-Santini, M., Cleven, B. E., Eichinger, L., Krönke, M., and Krut, O. (2009). Large scale multiplex PCR improves pathogen detection by DNA microarrays. *BMC Microbiol.* 9, 1–14. doi: 10.1186/1471-2180-9-1

Park, M., Won, J., Choi, B. Y., and Lee, C. J. (2020). Optimization of primer sets and detection protocols for SARS-CoV-2 of coronavirus disease 2019 (COVID-19) using PCR and real-time PCR. *Exp. Mol. Med.* 52, 963–977. doi: 10.1038/s12276-020-0452-7

Pianetti, A., Sabatini, L., Bruscolini, F., Chiaverini, F., and Cecchetti, G. (2004). Faecal contamination indicators, Salmonella, Vibrio and Aeromonas in water used for the irrigation of agricultural products. *Epidemiol. Infect.* 132, 231–238. doi: 10.1017/S095026880300181X

Priyanka, B., Patil, R. K., and Dwarakanath, S. (2016). A review on detection methods used for foodborne pathogens. *Indian J. Med. Res.* 144, 327–338. doi: 10.4103/0971-5916.198677

R Core Team. (2013). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Available at: http://www.R-project.org

Roulová, N., Moťková, P., Brožková, I., Swiontek Brzezinska, M., and Pejchalová, M. (2022). Detection, characterization, and antimicrobial susceptibility of *Yersinia enterocolitica* in different types of wastewater in the Czech Republic. *J. Appl. Microbiol.* 133, 2255–2266. doi: 10.1111/jam.15786

Shi, L., Wu, F., Wen, Y., Zhao, F., Xiang, J., and Ma, L. (2015). A novel method to detect *Listeria monocytogenes* via superparamagnetic lateral flow immunoassay. *Anal. Bioanal. Chem.* 407, 529–535. doi: 10.1007/s00216-014-8276-8

Shim, W.-B., Choi, J.-G., Kim, J.-Y., Yang, Z.-Y., Lee, K.-H., Kim, M.-G., et al. (2008). Enhanced rapidity for qualitative detection of *Listeria monocytogenes* using an enzyme-linked immunosorbent assay and immunochromatography strip test combined with immunomagnetic bead separation. *J. Food Prot.* 71, 781–789. doi: 10.4315/0362-028X-71.4.781

Silva, V., Caniça, M., Capelo, J. L., Igrejas, G., and Poeta, P. (2020). Diversity and genetic lineages of environmental staphylococci: a surface water overview. *FEMS Microbiol. Ecol.* 96:fiaa191. doi: 10.1093/femsec/fiaa191

Tatusova, T., Dicuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., et al. (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res.* 44, 6614–6624. doi: 10.1093/nar/gkw569

Tokarskyy, O., and Marshall, D. L. (2008). Immunosensors for rapid detection of *Escherichia coli* O157: H7—perspectives for use in the meat processing industry. *Food Microbiol.* 25, 1–12. doi: 10.1016/j.fm.2007.07.005

Umesha, S., and Manukumar, H. (2018). Advanced molecular diagnostic techniques for detection of food-borne pathogens: current applications and future challenges. *Crit. Rev. Food Sci. Nutr.* 58, 84–104. doi: 10.1080/10408398.2015.1126701

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Res.* 40:e115. doi: 10.1093/nar/gks596

Vega, M., Lerner, B., Lasorsa, C. A., Pierpauli, K., and Perez, M. S. (2016). Automated and low cost method to manufacture addressable solid-state nanopores. *Microsyst. Technol.* 22, 109–117. doi: 10.1007/s00542-014-2399-x

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., and Adley, C. (2010). An overview of foodborne pathogen detection: in the perspective of biosensors. *Biotechnol. Adv.* 28, 232–254. doi: 10.1016/j.biotechadv.2009.12.004

Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* 13:e1005595. doi: 10.1371/journal.pcbi.1005595

Yang, Y.-W., Chen, M.-K., Yang, B.-Y., Huang, X.-J., Zhang, X.-R., He, L.-Q., et al. (2015). Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in mouse feces. *Appl. Environ. Microbiol.* 81, 6749–6756. doi: 10.1128/AEM.01906-15

Yohe, S., and Thyagarajan, B. (2017). Review of clinical next-generation sequencing. *Arch. Pathol. Lab. Med.* 141, 1544–1557. doi: 10.5858/arpa.2016-0501-RA

Zakotnik, S., Knap, N., Bogovič, P., Zorec, T. M., Poljak, M., Strle, F., et al. (2022). Complete genome sequencing of tick-borne encephalitis virus directly from clinical samples: comparison of shotgun metagenomic and targeted amplicon-based sequencing. *Viruses* 14:1267. doi: 10.3390/v14061267

Zhao, X., Lin, C.-W., Wang, J., and Oh, D. H. (2014). Advances in rapid detection methods for foodborne pathogens. *J. Microbiol. Biotechnol.* 24, 297–312. doi: 10.4014/jmb.1310.10013

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Stability and genetic insights of the co-existence of *bla*$_{CTX-M-65}$, *bla*$_{OXA-1}$, and *mcr-1.1* harboring conjugative IncI2 plasmid isolated from a clinical extensively-drug resistant *Escherichia coli* ST744 in Shanghai

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Background: Co-existence of colistin, β-lactam and carbapenem in multidrug-resistant *Enterobacteriaceae* isolates poses a serious threat to public health. In this study, we investigated and characterized the co-occurrence of *bla*$_{CTX-M-65}$, *bla*$_{OXA-1}$, and *mcr-1.1* strain isolated from a clinical extensively-drug-resistant *Escherichia coli* ST744 in Shanghai.

Methods: Antimicrobial susceptibility test was carried out by agar dilution methods. Whole genome sequencing was conducted, and resistance genes, and sequence types of colistin in *E. coli* isolates were analyzed. Plasmid stability and amino acid mutations were assessed in *E. coli* isolates.

Results: A colistin resistant *E. coli* ST744, named ECPX221, was identified out of 145 fecal samples collected. The strain carries a 60,168 IncI2 plasmid with the *mcr-1.1* gene. The strain also has *bla*$_{CTX-M-65}$, *bla*$_{OXA-1}$, *dfrA*14, *qnrS1*, *cmlA5*, *arr2*, *ampC*, *aph(4)-Ia*, *sul1*, and *aadA5* resistance genes. The plasmid pECPX221 was capable of conjugation with an efficiency of 2.6×10^{-2}. Notably, 45% of the transconjugants were determined as *mcr-1.1*-harboring in the colistin-free environment after 60 generation of passage. No mutations occurred in *pmrB*, *mgrB*, and *phoPQ* gene in the *mcr-1.1*-harboring transconjugants. Bioinformatic analysis indicated pECPX221 shared highly similar backbone with the previously reported *mcr-1.1*-harboring pAH62-1, pMFDS1339.1, pSCZE4, and p2018-10-2CC. Furthermore, sequencing and phylogenetic analyses revealed a similarity between other MCR-1-homolog proteins, indicating that ECPX221 was colistin resistant.

Conclusion: The stable transferable *mcr-1.1*-harboring plasmid found in the *E. coli* ST744 strain indicated the high risk to disseminate the extensively-drug-resistance phenotype among *Enterobacteriaceae*.

KEYWORDS

MCR-1, *Escherichia coli* STstrain-744 (ST744), colistin, stability, plasmid

Introduction

Multidrug resistance in *Escherichia coli* has become a concerning issue, displaying resistance to β-lactam antibiotics, particularly through the production of β-lactamases, including extended-spectrum β-lactamases (ESBLs) and carbapenemases (1). The increasing use of β-lactams and carbapenems over the past several decades has led to the increased use of colistin, which is now considered the last therapeutic option for treating infections caused by such organisms. However, the efficacy of colistin has been challenged by the emergence of plasmid-mediated mobile colistin resistance (*mcr-1*), which was found in *Enterobacteriaceae* in 2015 (2), and has since been disseminated in animals, meat products, humans (both fecal carriage and infections), and the environment in over 50 countries, covering six continents (3–5). In China, colistin has been adopted for treating carbapenem-resistant *Enterobacteriaceae* (CRE) infections since 2018 (6), thereby increasing the potential risk of the dissemination of *mcr-1* (7). To date, *mcr-1* positive plasmids have also been found in multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Enterobacteriaceae* isolates carrying plasmid-borne carbapenemase and ESBL genes (8), which could generate resistance to multiple drugs and contribute to the spread of MDR bacteria in human populations (9).

Previous studies have reported *mcr-1*-harboring plasmids found in different Inc. types, while IncI2, IncHI2, and IncX4 appear to be the most common carriers of *mcr-1* (3, 10–12). The mobility of *mcr-1* is difficult to control because *mcr-1*-harboring plasmids often carry other antimicrobial resistance (AMR) genes, including those encoding resistance to β-lactams, fluoroquinolones, and tetracyclines (8, 13, 14). For example, Wang et al. reported *E. coli* strain QE11-421, which was isolated from a sputum sample of a 90-year-old male patient receiving treatment in the ICU, was an *mcr-1*-positive colistin-resistant isolate that co-harbored the bla_{KPC-2} gene conferring carbapenem resistance (15). Zhang et al. (16) reported the coexistence of the bla_{NDM-5}, $bla_{CTX-M-65}$, bla_{OXA-10}, bla_{TEM-1} and *mcr-1.1* genes detected in *E. coli* 20IR1127 strain belonging to ST156 lineage isolated from children in China. Lu reported three *E. coli* ST6775 coharboring *tet*(X4), *mcr-1*, and bla_{NDM-5} isolates from pigeons (17). In this study, we investigated the prevalence of colistin-resistant *E. coli* isolates from fecal samples in Shanghai and characterized the plasmid stability and persistence that contribute to colistin resistance in *E. coli* isolates.

Materials and methods

Sample collection

Fresh fecal samples were collected from patients experiencing acute diarrhea within 3 days of symptom onset, and who had not received any antibiotics treatment from September 2021 to January 2022 from surveillance hospitals in Shanghai. The samples were collected using five sterile cotton swabs from multiple sites and placed into a 50 mL screw-cap-sealed centrifuge tube with C–B transport medium. For patients and infants who experienced difficulty in defecating, rectal swabs were used to collect the samples. The rectal cotton swab was soaked in normal saline and inserted 4–5 cm deep into the anus (2–3 cm for children), and gently rotated. At least two swabs were collected from the same patient and placed in C–B transport medium and 3 mL of preservation solution (containing 5% bovine serum cell maintenance solution). The samples were stored at 4°C and sent to the laboratory within 48 h after collection.

Bacterial strains, identification and *mcr-1* gene screening

Each fecal sample was placed in sterile plastic bags containing 225 mL of Mueller–Hinton broth and incubated overnight at 37°C. The samples were then seeded on Nutrient broth plates (COMAGAL Microbial Technology, Shanghai) with 2 μg/mL colistin and incubated for 24 h at 37°C. To identify the isolate, a positive colony was selected by amplifying the *mcr-1* gene via real-time PCR (RT-PCR). The strains were then identified by MALDI-TOF mass spectrometry using the VITEK MS system (BioMérieux Shanghai Co. Limited). Basic clinical data, including gender, age, and date of isolation, were collected for patients from whom the *mcr-1*-harboring strains were isolated. The *mcr-1* gene screening was performed as following: briefly, the genomic DNA from each of the strains was extracted by boiling and freeze-thawing processes, and the resulting supernatant was used as the template. The specific primer used in this study was: *mcr-1*-RT-F: 5′-CGCGATGCTACTGATCACCA-3′, *mcr-1*-RT-R: 5′-GGTCGTATCATAGACCGTGCC-3′, and the *mcr-1*-probe: VIC-5′-TTATCATCGTATCGCTATGTGCTA-3′-MGB.

Antimicrobial susceptibility testing

A total of 30 antimicrobial agents (Shanghai Fosun Biological Technology Co., Ltd.) were used for antimicrobial susceptibility testing via broth microdilution method, including ampicillin (AMP), ampicillin/sulbactam 2:1 ratio (AMS), tetracycline (TET), chloramphenicol (CHL), trimethoprim/sulfamethoxazole (SXT), cefazolin (CFZ), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (CFX), gentamicin (GEN), imipenem (IMP), nalidixic acid (NAL), azithromycin (AZI), tigecycline (TIG), ciprofloxacin (CIP), amoxicillin/clavulanic acid (AMC), cefotaxime/clavulanic acid (CTC), ceftazidime/clavulanic acid (CAC), colistin (CT), aztreonam (ATM), cefuroxime (CXM), amikacin (AMI), cefepime (CPM), meropenem (MEM), levofloxacin (LEV), ertapenem (ETP), ceftazidime/avibactam (CZA), streptomycin (STR), and norfloxacin (NOR). The double-disc synergy test and a modified carbapenem inactivation method were used to confirm the production of ESBL and carbapenemase, respectively, according to Clinical and Laboratory Standards Institute (CLSI) guidelines. *E. coli* ATCC®25922™ strain was used as quality control. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints defines colistin resistance as 2 μg/mL for *Enterobacteriaceae* (18).

Conjugation assay

To investigate whether the *mcr-1* gene was present on a transferable plasmid, we performed a filter mating assay using

rifamycin-resistant *E. coli* C600 as the recipient strain. Both the original isolates and recipient *E. coli* C600 were grown overnight in LB broth and adjusted to a 0.5 McFarland standard. The donor bacteria were mixed with recipient *E. coli* C600 at a ratio of 1:3 to 5 mL of fresh LB broth and then incubated at 37°C overnight. Transconjugants were selected on LB plates supplemented with rifampicin (40 μg/mL) and colistin (2 μg/mL). Putative transconjugants were confirmed using PCR and antimicrobial susceptibility tests. The mobilization efficiency were calculated by dividing the number of transconjugant colonies by the number of donor colonies (19).

Plasmid stability assay

The plasmid stability assay was conducted to determine the stability of the *mcr-1*-harboring plasmids in the absence of antibiotic selective pressure. The strains were grown in LB broth containing colistin (2 μg/mL) and then transferred to a fresh LB broth without antibiotics. The cultures were periodically passaged for 60 days, with serial passages performed every 24 h, resulting in approximately 600 generations of bacterial growth. Cultures from passages 2, 4, 6, 8, 10, 20, 30, 40, 50, and 60 were diluted and plated onto LB plates containing colistin and LB plates without antibiotics. The frequency of stable plasmids was calculated as the number of colonies grown on the LB plate containing colistin and the antibiotic-free LB plate, divided by the total number of colonies on both plates, multiplied by 100%. The distribution of all *mcr-1*-harboring plasmids from different antimicrobial environments and passages was monitored by RT-PCR and Sanger sequencing to identify any changes in the plasmid composition over time.

Genetic mutation in colistin-resistant isolates

To assess the genetic mutation in the colistin-resistant *E. coli* isolates, the colistin resistance genes including *mgrB*, *pmrAB*, *phoPQ* were amplified by real-time quantitative PCR as previously described (20, 21). Mutations that occurred in colistin-resistant *E. coli* isolates were determined by comparing to their corresponding parental reference genomes.

Whole genome sequencing

The genomic DNA was extracted and sequencing libraries were generated using the TruSeq DNA Sample Preparation Kit (Illumina, USA). The genome sequencing was then performed with standard protocol and were sequenced with 150-bp paired-end strategy by using the Illumina Novaseq 6,000 (Sangon Biotech Company, Shanghai, China). Data assembly was carried out after adapter contamination removal and data filtering by using AdapterRemoval and SOAPec. Scaffold and contig construction were performed using SPAdes (version 3.12.0) and A5-miseq, respectively, with integration of all assembled results to obtain a complete sequence.

Subsequently, the VFDB (Virulence Factors of Pathogenic Bacteria)[1] and CARD (The Comprehensive Antibiotic Resistance)[2] database were used to retrieve the pathogenicity genes and antibiotic resistance genes, respectively. The *mcr-1*-carrying contigs generated by Illumina sequencing were examined for Inc. types by PlasmidFinder version 2.1.[3] The set of close typing sequences was determined by PubMLST[4] and then compared with the typing sequences of *Escherichia* spp. The linear comparison of complete plasmid sequences was created by EasyFig version 2.2.2.[5] The plasmid construction map was generated by SnapGene 6.1.2 software (Insightful Science, United States). The genome-wide similarities was generated by FastANI version 1.33.[6] Sequences were deposited to NCBI website under the Bioproject PRJNA929103.

Phylogenetic analysis

The MCR-1 and MCR-1-like proteins' homologous sequences were extracted from NCBI through BLASTp search (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 20 June 2023), with MCR-1 protein of ECPX221 in this study obtained from the sequencing data. Aligned sequences of MCR-1 obtained from ClustalW version 2.0[7] were used to construct a phylogenetic tree through the Maximum Likelihood Method of MEGA X (Mega Limited, Auckland, New Zealand). To confirm the results, 1,000 bootstrap repetitions were used.

Ethical considerations

This study was reviewed and approved by the ethical committee of the Shanghai Municipal Centre for Disease Control and Prevention.

Results

Antimicrobial susceptibility profile of *mcr-1*-harboring *Escherichia coli* isolates

Out of 145 fecal samples collected between September 2021 and January 2022, only one *E. coli* isolate, named ECPX221, was found to harbor the *mcr-1.1* gene. Antimicrobial resistance testing was performed on this *mcr-1.1*-positive strain, and it was found to exhibit colistin resistance at 2 μg/mL (as shown in Table 1). Furthermore, ECPX221 was identified as an extended-spectrum β-lactamase producer, and was found to be resistant to CIP, AMP, AMS, CFZ, CTX, CXM, SXT, NAL, CHL, TET, ATM, LEV, and NOR. However, it was found to be susceptible to 15 other common antibiotics, including CFX, CPM, CZA, IMP, ETP, TIG, and others (as shown in Table 1).

1 http://www.mgc.ac.cn/VFs/main.htm
2 https://card.mcmaster.ca/
3 https://cge.cbs.dtu.dk/services/PlasmidFinder/
4 https://pubmlst.org/
5 https://mjsull.github.io/Easyfig/
6 https://github.com/ParBLiSS/FastANI
7 http://www.clustal.org/

TABLE 1 Information for the *mcr-1.1*-harboring *Escherichia coli* strain ECPX221 identified in this study and the transconjugant isolate ECPX221-T. The wild type EC C600 was used as control.

Antibiotic	ECPX221		ECPX221-T		EC C600	
	MIC	Result	MIC	Result	MIC	Result
CIP	4	R	8	R	≤0.015	S
AMP	>64	R	>64	R	4	S
AMS	32/16	R	16/8	I	4/2	S
CT	2	I	4	R	0.25	S
CFZ	>32	R	>32	R	2	S
CTX	16	R	16	R	≤0.25	S
CAZ/C	0.5/4	–	≤0.25/4	–	≤0.25/4	–
CTX/C	1/4	–	≤0.125/4	–	≤0.125/4	–
CFX	8	S	2	S	2	S
CPM	2	S	≤1	S	≤1	S
CXM	>32	R	>32	R	≤0.5	S
CZA	≤0.25/4	S	≤0.25/4	S	≤0.25/4	S
IMP	≤0.25	S	≤0.25	S	≤0.25	S
CAZ	1	S	2	S	0.5	S
AZI	8	–	4	–	≤2	–
ETP	≤0.25	S	≤0.25	S	≤0.25	S
SXT	>8/152	R	>8/152	R	>8/152	R
NAL	>64	R	>64	R	>64	R
CHL	>64	R	32	R	4	S
GEN	2	S	32	R	≤1	S
TET	>32	R	>32	R	≤1	S
TIG	0.5	S	≤0.25	S	≤0.25	S
AMK	4	S	4	S	≤2	S
ATM	16	R	16	R	≤2	S
LEV	>4	R	>4	R	≤0.125	S
MEM	≤0.125	S	≤0.125	S	≤0.125	S
STR	>32	–	>32	–	8	–
NOR	16	R	32	R	≤0.125	S

Ampicillin (AMP), ampicillin/sulbactam 2:1 ratio (AMS), tetracycline (TET), chloramphenicol (CHL), trimethoprim/sulfamethoxazole (SXT), cefazolin (CFZ), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (CFX), gentamicin (GEN), imipenem (IMP), nalidixic acid (NAL), azithromycin (AZI), tigecycline (TIG), ciprofloxacin (CIP), amoxicillin/clavulanic acid (AMC), cefotaxime/clavulanic acid (CTC), ceftazidime/clavulanic acid (CAC), colistin (CT), aztreonam (ATM), cefuroxime (CXM), amikacin (AMI), cefepime (CPM), meropenem (MEM), levofloxacin (LEV), ertapenem (ETP), ceftazidime/avibactam (CZA), streptomycin (STR), and norfloxacin (NOR).

Apart from *mcr-1.1*, ECPX221 was also found to carry resistance genes for $bla_{\text{CTX-M-65}}$, $bla_{\text{OXA-1}}$, *dfrA*14, *qnrS*1, *cmlA*5, *arr*2, *ampC*, *aph(4)-Ia*, *sul*1, and *aadA*5.

Transmissibility of *mcr-1.1* via conjugation

The result indicated that the *mcr-1.1*-harboring plasmid was capable of successful transfer from the donor strain to the recipient strain (*E. coli* C600). The conjugation of ECPX221 to *E. coli* C600 via horizontal transfer was achieved with an average efficiency of 2.6×10^{-2}. The transconjugant ECPX221-T, which was confirmed to harbor *mcr-1.1* gene, exhibited a MIC value of 4 μg/mL to colistin, which represented a significant increase when compared to the wild type *E. coli* C600 (0.25 μg/mL). Therefore, it was speculated that the transconjugants acquired the colistin resistance gene from the donor strain.

Plasmid stability and genetic mutations

In order to assess the plasmid stability, we analyzed the dynamics of the pECPX221 plasmid by passaging the ECPX221-T strain carrying the plasmid with colistin for 60 days. Next day, 97% of the transconjugants (29 positive colonies out of 30 colonies) were detected as *mcr-1.1* positive in an antibiotic-free environment, while all transconjugants were positive in the colistin environment. On day 30, only 60% of the transconjugants (15 positive colonies out of 25 colonies) were detected as *mcr-1.1*-carrying isolates in the antibiotic-free environment, and this result decreased further to 45% on day 60,

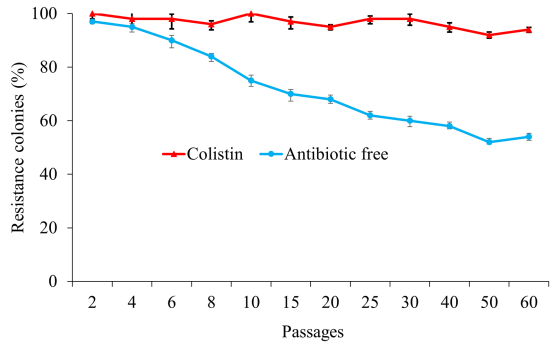

FIGURE 1
The resistance colonies that harboring *mcr-1.1* gene in the colistin (red) and antibiotic free environment (blue).

while the positive rate remained at 94% in the plate with colistin (Figure 1). To assess genetic mutations related to colistin resistance in the *mcr-1*-harboring plasmid in *E. coli*, we examined key genes such as *mgr*B, *pmr*AB, and *pho*PQ in the *mcr-1.1*-harboring and non-harboring transconjugants. However, our results showed that none of the aforementioned genes were mutated in the *mcr-1.1*-harboring transconjugants.

Molecular features of *mcr-1.1* harboring strain

We sequenced the genomes of the EXPX221 and the sequence data showed that it belonging to the replicon types IncI2. BLASTn analysis showed that the backbone of the plasmid pECPX221 (GenBank Accession No. GCA_028527545) was strikingly similar with (the query cover of 100% and the identities 99%) other previously sequenced *mcr-1.1*-harboring IncI2 plasmids, such as pAH62-1 from *E. coli* AH62 (GenBank Accession No. CP055260), pMFDS1339.1 from *E. coli* MFDS1339 (GenBank Accession No. MK852553), pSCZE4 from *E. coli* SCZE5 (GenBank Accession No. CP051226), and p2018-10-2CC from *E. coli* 2018–10-2CC (GenBank Accession No. LC511662). In all, the ANI heatmap showed that these IncI2 plasmids bearing *mcr-1.1* showed very high architectural conservation (Figure 2). Furthermore, the BLAST comparison of pECPX221, pAH62-1, pMFDS1339.1, pSCZE4 and p2018-10-2CC revealed that their *mcr-1.1* insertion sites differed (Figure 3). An approximately 2.5 kb *mcr-1.1-pap2* element was identified in the above-mentioned plasmids. In addition, an integrase core domain protein as IS481-like element ISEc19 family transposase (WP_010723086) was identified in pECPX221, but only found in pMFDS1339.1. The putative conjugal transfer components of pECPX221 were also detected by using oriTfinder. The *vir* gene family encoding VirB1 to VirB11 were identified as T4SS belonging to Type IV secretion system was predicted on pECPX221 (Figure 4). The relaxase in pECPX221 from 45,033 to 49,346 nt was found to be 99% identity with the relaxase (WP_124777228.1) that obtained from *E. coli*. This evidence confirms that pECPX221 is a conjugative plasmid.

Phylogeny analysis

A total of 54 MCR-1 proteins originated from *E. coli* (Supplementary File S1), and 26 proteins of MCR-1 gene obtained from bacteria other than *E. coli* (Supplementary File S2) were categorized for further analysis. In the case of protein acquisition from the NCBI database, above 50% of query coverage was set as the screening point. These two sets of proteins (Supplementary Files S1, S2), including ECPX221 strain harboring MCR-1 in this study, were used for phylogenetic analysis (Figure 5). The sequenced MCR-1 of ECPX221 in this study clearly showed its genomic confirmation as *mcr-1* genes by highly aligning with *mcr-1* genes of *E. coli* as well as other bacteria origins. In addition, the phylogenetic tree showed that ECPX221 strain in this study was mostly of Asian origin and that they were closely related.

Discussion

Colistin resistance has become a serious issue in food animals such as pigs and chickens, as it has been frequently used since the 1950s (22, 23). Livestock and poultry are known to be the main reservoir for colistin resistance, and the discovery of the stable plasmid-mediated *mcr-1* gene in *E. coli* has helped us understand the potential transmission of colistin resistance between animals and humans (2). In this study, we have identified the co-occurrence of ESBL genes ($bla_{\text{CTX-M-65}}$ and $bla_{\text{OXA-1}}$) and *mcr-1.1*-producing *E. coli* ST744 isolates from clinical fecal samples in Shanghai. The plasmid pECPX221 contains four typical conjugal modules: a T4CP gene, an *ori*T-like region for transfer origin, a relaxase gene, and a gene cluster for the bacterial T4SS apparatus. Additionally, pECPX221 contains the *mcr-1.1-pap2* cassette, which has been shown to be capable of horizontally transferring into various plasmid replicon types (24).

The prevalence of coexistence of *mcr-1,* ESBL genes carrying *E. coli* was found to be only 0.7% (1/145) in clinical fecal samples, which is similar as that one *E. coli* strain harboring *mcr-1* and $bla_{\text{NDM-5}}$ reported in companion animals that was collected from six different cities including Harbin, Yangzhou, Chongqing, Wuhan, Chengdu and Guangzhou (0.8%, 1/129) (25). Also, it was not that high when comparing with that has been reported in other regions in China such as in Shandong (3.5%) (26), Shanghai (3.9%) (27), Henan (7.7%) (28) and some other countries like in Pakistan (23.2%) (29), Lebanon (18%) (30), Japan (4.84%) (31) and Bolivia (38.3%) (32), but a little higher than that children patients with diarrhoea in Shanghai (0.28%) (33). This low prevalence could be attributed to the strict usage of colistin in Shanghai. According to the Shanghai Catalogue of Graded Management of Clinical Application of Antibacterial Drugs (2021 version) (34), colistin is only used for extensively resistant gram-negative infections such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacteriaceae*.

E. coli is the first and most prevalent species carrying the *mcr-1* gene in *Enterobacteriaceae* and can be isolated from raw meat and fecal samples of animals and humans in China (2, 35). The co-existence of ESBL genes such as $bla_{\text{CTX-M-65}}$ and *mcr-1*-harboring *E. coli* have been reported globally, particularly in animal source *E. coli* isolates in

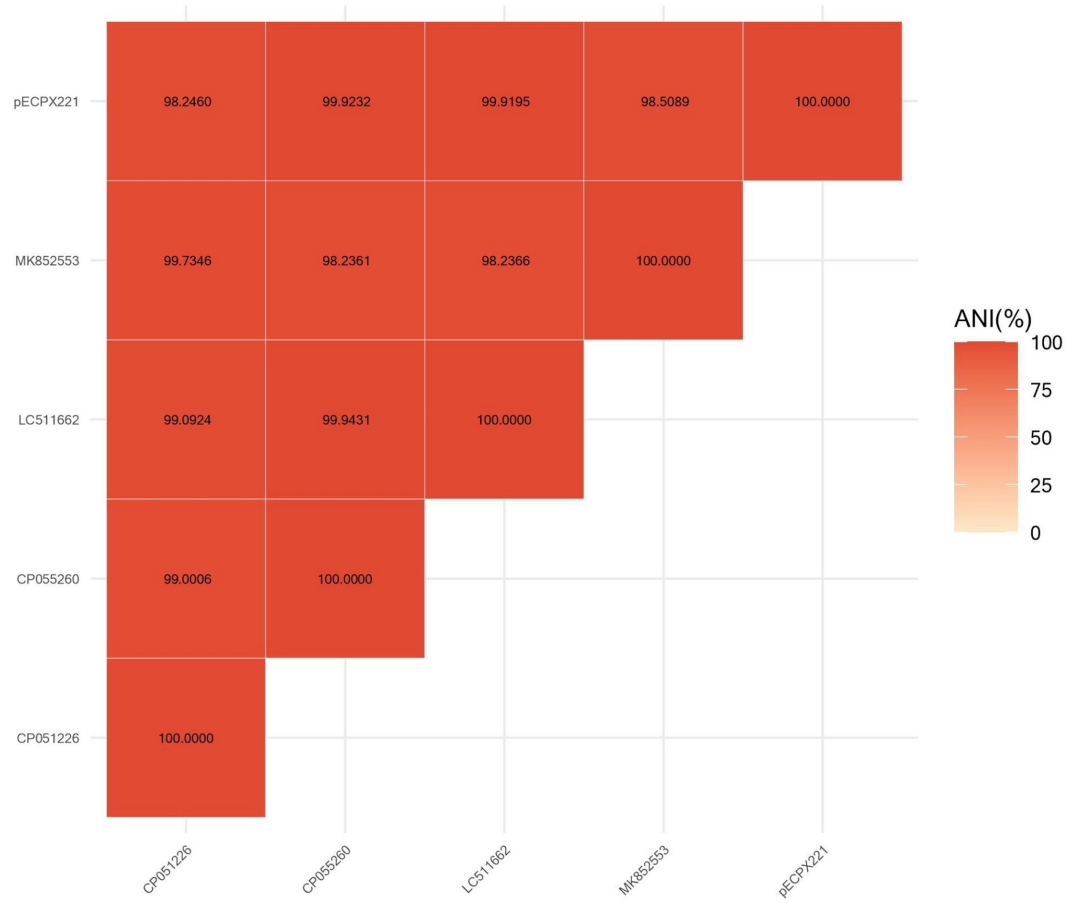

FIGURE 2
Comparison of pECPX221 and other *mcr-1*-harboring plasmids including pAH62-1, pMFDS1339.1, pSCZE4, and p2018-10-2CC by FastANI software.

northern China (36, 37). In eastern China, Zhejiang Province identified the co-occurrence of bla_{NDM-5}, $bla_{CTX-M-65}$, bla_{OXA-10}, bla_{TEM-1}, and *mcr-1.1* genes isolated from human *E. coli* ST156 in 2022 (16). Jiangsu Province reported an ESBL, carbapenemase- and *mcr-1*-producing *E. coli* ST648 strain isolated from a urine sample, which was found to have three transferable resistance plasmids (38). Apart from *E. coli*, one isolate named CFSA664 was found to co-harbor the *mcr-1* gene and $bla_{CTX-M-65}$ in *Salmonella enterica serotype* Indiana from retail chickens in Jiangsu (39). However, the co-existence of $bla_{CTX-M-65}$ and *mcr-1* was found in *E. coli* ST117 isolated from a veterinary hospital in Shanghai (40) and EC1CT136A isolated from broiler farms in Ecuador (41). Our study identified, for the first time, the co-existence of $bla_{CTX-M-65}$, bla_{OXA-1}, and *mcr-1.1* from a human fecal isolate in *E. coli* ST744 in Shanghai. This finding indicates a high risk of disseminating this extensively drug-resistant *E. coli*, which poses a threat to public health.

WGS data indicated the presence of various determinants of antibiotic resistance, suggesting that carbapenem and colistin co-resistant strains may be selected with the use of any antibiotics. Colistin resistance typically arises from selective pressure resulting from the use of polymyxins (2). In China, colistin was widely used as a growth promoter in animal production until 2017, when it was banned due to the identification of plasmid-mediated colistin-resistant isolates in the country (42, 43). Furthermore, colistin therapy in humans was introduced in February 2017 (7). As a result, it is essential to note that the prevalence of colistin-resistant isolates may be underestimated, as colistin susceptibility is not routinely tested in clinical samples from outpatients.

Plasmid horizontal transfer of *mcr-1* gene was widely reported in human, food, animals, and the environment in a lot of countries and regions worldwide (44). In this study, the plasmid replicon type of IncI2, the major type of plasmids spreading globally that promote *E. coli* resistance, was identified. The IS*Apl*1, which is consistently associated with the *mcr-1* gene and its related cassette that can be inserted into a variety of genetic loci in different plasmids, was missing in our study, this was similar to the previously reports on the absence of IS*Apl*1 in *E. coli* strain and other *Enterobacteriaceae* such as *E. fergusonii* (45). The diversity of plasmids carrying the *mcr-1* gene has been shown to increase with the use of colistin in clinical settings, suggesting that colistin administration could promote the dissemination of diverse resistance plasmids among *E. coli* isolates (46). The result of plasmid stability assay revealed that pECPX221 remained stable in the recipient bacterial strain, which was consistent with other *E. coli* strains (47, 48).

The phylogenetic analysis indicated that the sequenced *mcr-1* genes of *E. coli* are homologous to previously reported *mcr-1* genes

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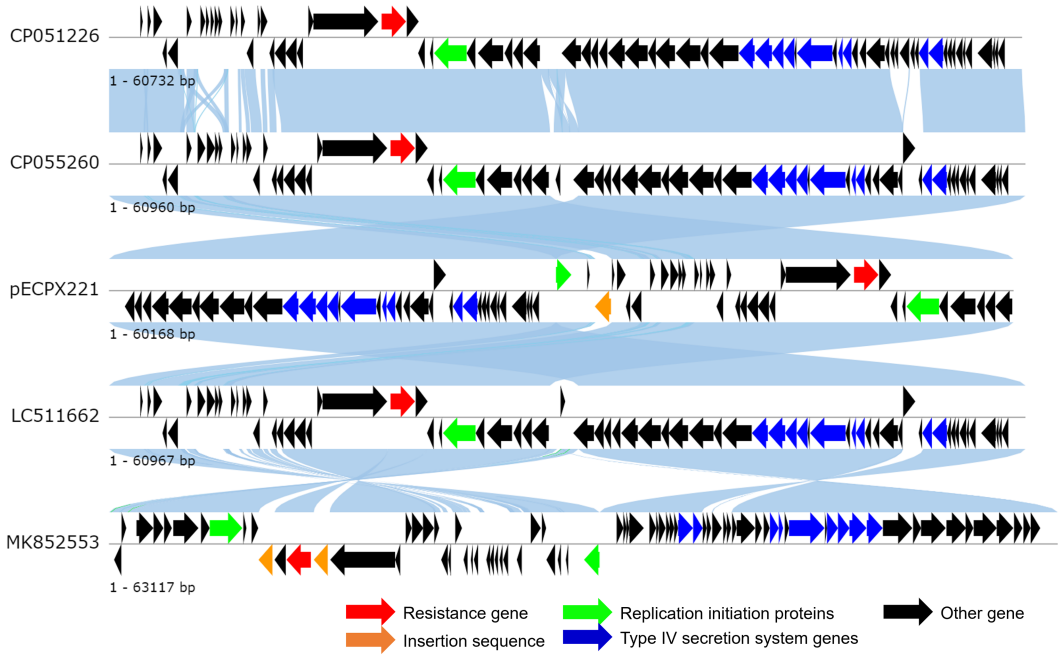

FIGURE 3
Linear comparison of complete plasmid sequences of plasmid *Escherichia coli* pECPX221 (this study), pAH62-1 from *E. coli* AH62 (GenBank Accession No. CP055260), pMFDS1339.1 from *E. coli* MFDS1339 (GenBank Accession No. MK852553), pSCZE4 from *E. coli* SCZE5 (GenBank Accession No. CP051226), and p2018-10-2CC from *E. coli* 2018–10-2CC (GenBank Accession No. LC511662).

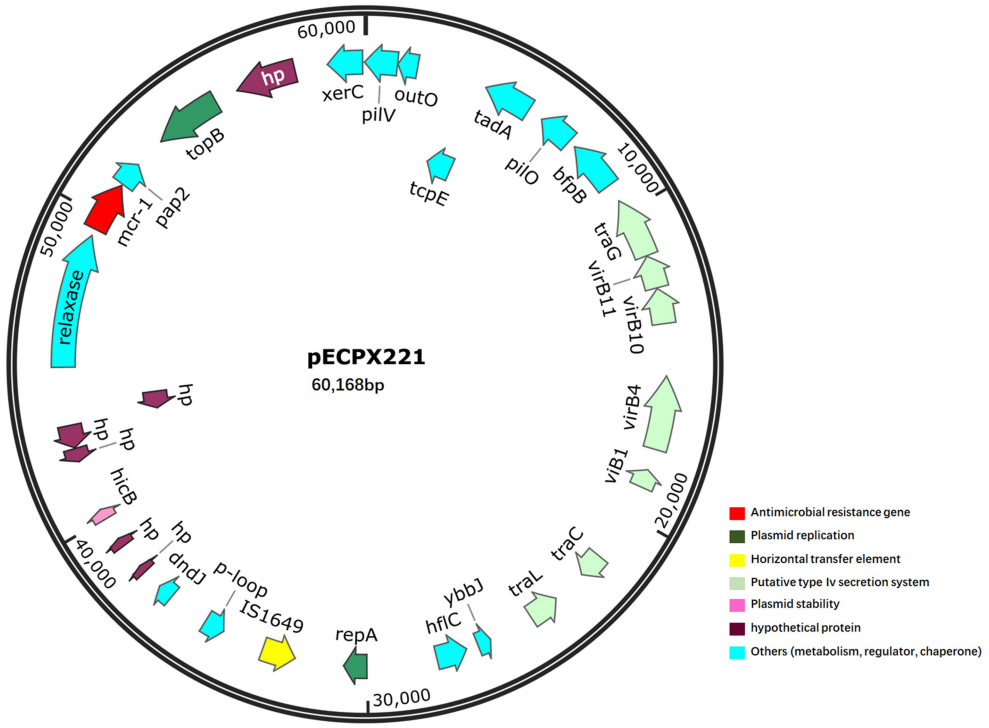

FIGURE 4
Map of *mcr-1*-harbouring plasmid pECPX221. The *mcr-1* gene is marked in red.

from *E. coli* and other bacteria origins. The sequence of the *mcr-1* identified in our study is identical to that of the *mcr-1* previously identified from *E. coli* (GenBank Accession No: A0A0R6L508). The phylogenetic tree demonstrates a close relation among some studied *mcr-1* strains, and an evolutionary relationship to other *mcr-1* genes like strain WP109545056, EF05030066, and

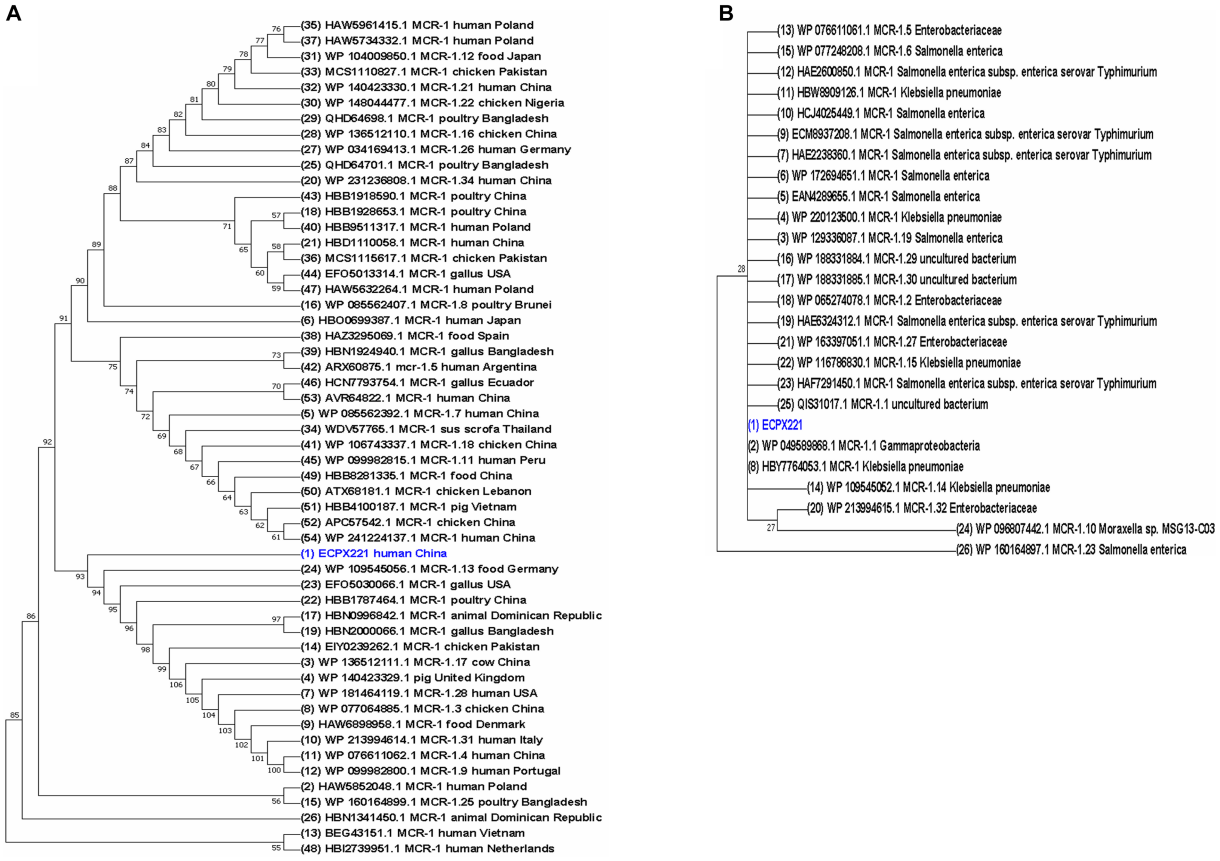

FIGURE 5
Phylogenetic study of MCR-1 and MCR-1-like proteins reveals ancestral origins and diversification. Using amino acid sequences from MCR-1 protein of ECPX221 in this study, the BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, Accessed on 20 June 2023) was used to retrieve related sequences of MCR-1 and MCR-1-like proteins from the NCBI database. MCR-1 and MCR-1-like proteins of *E. coli*, *Salmonella*, and strains containing LptA and others were among the sequences categorized. Using aligned MCR-1 sequences from CLUSTALW, the maximum likelihood method of MEGA X was used to create a phylogenetic tree. **(A)** Phylogeny of 54 MCR-1 proteins of *E. coli* origin retrieved from NCBI database including sequenced MCR-1 gene of ECPX221 from this study. **(B)** Phylogeny of 26 MCR-1 proteins retrieved from NCBI database except *E. coli* strains.

HBB1787464. Furthermore, the phylogenetic tree revealed that the *mcr-1*-positive ECPX221 in this study is related to many MCR-1 variants. For example, it is closely related to the *mcr-1.13* strain isolated from meat, and its spread could result in widespread resistance to colistin.

There are two limitations to this study: Firstly, the *E. coli* isolates were collected solely from fecal samples in Shanghai province, China. To obtain more accurate results, it would be beneficial to include isolates from additional regions with prolonged monitoring. Secondly, the fitness cost of ECPX221 needs to be assessed in order to evaluate the potential plasmid loss imposed by pECPX221.

Conclusion

The study found that the prevalence of *mcr-1.1*-harboring *E. coli* among clinical fecal isolates in Shanghai is low. However, the strain ECPX221, which carries the *mcr-1.1* gene, exhibited extensive antimicrobial resistance profiles and additional resistance genes. The results of the conjugation experiment confirmed the horizontal transfer of the *mcr-1.1* gene, and the *mcr-1.1*-harboring plasmid pECPX221 was found to be stable in the recipient strain. Phylogenetic analysis showed an evolutionary linkage between MCR-1 and MCR-1 homolog proteins. Given the crucial role of colistin as a last-line treatment option against infections caused by multidrug-resistant Gram-negative bacteria, continuous surveillance is urgently needed to monitor the spread of the coexistence of the *mcr-1.1* gene and other important resistance genes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA929103.

Ethics statement

The studies involving humans were approved by Shanghai Municipal Center for Disease Control and Prevention. The studies

were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

JF wrote the draft, revised the manuscript, designed this study, and responsible for the whole experiment. HYW, YZ, JYL, YC, YTW, JYF, and QS participated in the whole experiment process. ZAY and MC managed the experiment and provided suggestions and revisions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1216704/full#supplementary-material

References

1. van Duin D, Doi Y. The global epidemiology of carbapenemase-producing Enterobacteriaceae. *Virulence*. (2017) 8:460–9. doi: 10.1080/21505594.2016.1222343

2. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis*. (2016) 16:161–8. doi: 10.1016/S1473-3099(15)00424-7

3. Sun J, Zhang H, Liu YH, Feng Y. Towards understanding MCR-like colistin resistance. *Trends Microbiol*. (2018) 26:794–808. doi: 10.1016/j.tim.2018.02.006

4. Wang C, Feng Y, Liu L, Wei L, Kang M, Zong Z. Identification of novel mobile colistin resistance gene mcr-10. *Emerg Microbes Infect*. (2020a) 9:508–16. doi: 10.1080/22221751.2020.1732231

5. Wang R, van Dorp L, Shaw LP, Bradley P, Wang Q, Wang X, et al. The global distribution and spread of the mobilized colistin resistance gene mcr-1. *Nat Commun*. (2018) 9:1179. doi: 10.1038/s41467-018-03205-z

6. Liang Q, Huang M, Xu Z. Early use of polymyxin B reduces the mortality of carbapenem-resistant *Klebsiella pneumoniae* bloodstream infection. *Braz J Infect Dis*. (2019) 23:60–5. doi: 10.1016/j.bjid.2018.12.004

7. Shen Y, Zhou H, Xu J, Wang Y, Zhang Q, Walsh TR, et al. Anthropogenic and environmental factors associated with high incidence of mcr-1 carriage in humans across China. *Nat Microbiol*. (2018) 3:1054–62. doi: 10.1038/s41564-018-0205-8

8. Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev*. (2017) 30:557–96. doi: 10.1128/CMR.00064-16

9. Yang YQ, Zhang AY, Ma SZ, Kong LH, Li YX, Liu JX, et al. Co-occurrence of mcr-1 and ESBL on a single plasmid in *Salmonella enterica*. *J Antimicrob Chemother*. (2016) 71:2336–8. doi: 10.1093/jac/dkw243

10. Kong-Ngoen T, Santajit S, Tunyong W, Pumirat P, Sookrung N, Chaicumpa W, et al. Antimicrobial resistance and virulence of non-typhoidal *Salmonella* from retail foods marketed in Bangkok, Thailand. *Foods*. (2022) 11:661. doi: 10.3390/foods11050661

11. Matamoros S, van Hattem JM, Arcilla MS, Willemse N, Melles DC, Penders J, et al. Global phylogenetic analysis of Escherichia coli and plasmids carrying the mcr-1 gene indicates bacterial diversity but plasmid restriction. *Sci Rep*. (2017) 7:15364. doi: 10.1038/s41598-017-15539-7

12. Hu Y, Zhang C, Zhang J. Antimicrobial Resistance in Non-typhoidal Salmonella from Retail Foods Collected in 2020 in China. *Zoonoses* (2023) 3. doi: 10.15212/ZOONOSES-2023-0001

13. Guo L, Wang J, Wang S, Su J, Wang X, Zhu Y. Genome characterization of mcr-1-positive *Escherichia coli* isolated from pigs with postweaning Diarrhea in China. *Front Vet Sci*. (2020) 7:503. doi: 10.3389/fvets.2020.00503

14. Lu X, Xiao X, Liu Y, Li R, Wang Z. Emerging opportunity and Destiny of mcr-1- and tet(X4)-Coharboring plasmids in *Escherichia coli*. *Microbiol Spectr*. (2021) 9:e0152021. doi: 10.1128/Spectrum.01520-21

15. Wang Y, Liu H, Wang Q, Du X, Yu Y, Jiang Y. Coexistence of Bla(KPC-2)-IncN and mcr-1-IncX4 plasmids in a ST48 *Escherichia coli* strain in China. *J Glob Antimicrob Resist*. (2020b) 23:149–53. doi: 10.1016/j.jgar.2020.08.023

16. Zhang X, Fang C, Zhang J, Hua W, He R, Zhou M. Carbapenemase- and colistin resistant *Escherichia coli* strains from children in China: high genetic diversity and first report of Bla (NDM-5), Bla (CTX-M-65), Bla (OXA-10), Bla (TEM-1), and mcr-1.1 genes co-occurrence in *E. coli* ST156. *Infect Drug Resist*. (2022) 15:5315–20. doi: 10.2147/IDR.S378574

17. Lu X, Du Y, Peng K, Zhang W, Li J, Wang Z, et al. Coexistence of tet(X4), mcr-1, and Bla(NDM-5) in ST6775 *Escherichia coli* isolates of animal origin in China. *Microbiol Spectr*. (2022) 10:e0019622. doi: 10.1128/spectrum.00196-22

18. European Committee on Antimicrobial Susceptibility Testing. (2022). *Clinical breakpoints-bacteria (v 12.0)*.

19. Wang P, Xiong Y, Lan R, Ye C, Wang H, Ren J, et al. pO157_Sal, a novel conjugative plasmid detected in outbreak isolates of *Escherichia coli* O157:H7. *J Clin Microbiol*. (2011) 49:1594–7. doi: 10.1128/JCM.02530-10

20. Cannatelli A, Di Pilato V, Giani T, Arena F, Ambretti S, Gaibani P, et al. In vivo evolution to colistin resistance by PmrB sensor kinase mutation in KPC-producing *Klebsiella pneumoniae* is associated with low-dosage colistin treatment. *Antimicrob Agents Chemother*. (2014) 58:4399–403. doi: 10.1128/AAC.02555-14

21. Haeili M, Javani A, Moradi J, Jafari Z, Feizabadi MM, Babaei E. MgrB alterations mediate colistin resistance in *Klebsiella pneumoniae* isolates from Iran. *Front Microbiol*. (2017) 8:2470. doi: 10.3389/fmicb.2017.02470

22. Rhouma M, Beaudry F, Letellier A. Resistance to colistin: what is the fate for this antibiotic in pig production? *Int J Antimicrob Agents*. (2016) 48:119–26. doi: 10.1016/j.ijantimicag.2016.04.008

23. Feng J, Xu Z, Zhuang Y, Liu MX, Luo JY, Wu YT, et al. Research progress on detection technology of colistin resistance (in Chinese). *J Pathogen Biol*. (2023) 18:854–9. doi: 10.13350/j.cjpb.230722

24. Li A, Yang Y, Miao M, Chavda KD, Mediavilla JR, Xie X, et al. Complete sequences of mcr-1-harboring plasmids from extended-Spectrum-beta-lactamase- and Carbapenemase-producing Enterobacteriaceae. *Antimicrob Agents Chemother*. (2016) 60:4351–4. doi: 10.1128/AAC.00550-16

25. Kuang X, Yang R, Ye X, Sun J, Liao X, Liu Y, et al. NDM-5-producing *Escherichia coli* co-Harboring mcr-1 gene in companion animals in China. *Animals (Basel)*. (2022) 12:1310. doi: 10.3390/ani12101310

26. Bi Z, Berglund B, Sun Q, Nilsson M, Chen B, Tarnberg M, et al. Prevalence of the mcr-1 colistin resistance gene in extended-spectrum beta-lactamase-producing *Escherichia coli* from human faecal samples collected in 2012 in rural villages in Shandong Province, China. *Int J Antimicrob Agents*. (2017) 49:493–7. doi: 10.1016/j.ijantimicag.2016.12.018

27. Xie J, Liang B, Xu X, Yang L, Li H, Li P, et al. Identification of mcr-1-positive multidrug-resistant *Escherichia coli* isolates from clinical samples in Shanghai, China. *J Glob Antimicrob Resist*. (2022) 29:88–96. doi: 10.1016/j.jgar.2022.02.008

28. Lu X, Zhang P, Du P, Zhang X, Wang J, Yang Y, et al. Prevalence and genomic characteristics of mcr-positive *Escherichia coli* strains isolated from humans, pigs, and foods in China. *Microbiol Spectr*. (2023) 11:e0456922. doi: 10.1128/spectrum.04569-22

29. Hameed F, Khan MA, Bilal H, Muhammad H, Tayyab Ur R. Detection of MCR-1 gene in multiple drug resistant Escherichia coli and *Klebsiella pneumoniae* in human clinical samples from Peshawar, Pakistan. *Comb Chem High Throughput Screen*. (2021) 24:737–42. doi: 10.2174/1386207323666200914100119

30. Hassan J, Mann D, Li S, Deng X, Kassem II. Emergence of the Mobile colistin resistance gene, mcr-1, in multidrug-Resistant *E. coli* isolated from the Fecal matter of toddlers in a community. *Antimicrob Agents Chemother*. (2021) 65:e00243-21. doi: 10.1128/AAC.00243-21

31. Nakano A, Nakano R, Nishisouzu R, Suzuki Y, Horiuchi S, Kikuchi-Ueda T, et al. Prevalence and relatedness of mcr-1-mediated colistin-resistant *Escherichia coli* isolated from livestock and farmers in Japan. *Front Microbiol*. (2021) 12:664931. doi: 10.3389/fmicb.2021.664931

32. Giani T, Sennati S, Antonelli A, Di Pilato V, di Maggio T, Mantella A, et al. High prevalence of carriage of mcr-1-positive enteric bacteria among healthy children from rural communities in the Chaco region, Bolivia, September to October 2016. *Euro Surveill*. (2018) 23:1800115. doi: 10.2807/1560-7917.ES.2018.23.45.1800115

33. Feng J, Zhuang Y, Luo J, Xiao Q, Wu T, Chen Y, et al. Prevalence of colistin-resistant *mcr-1*-positive *Escherichia coli* isolated from children patients with diarrhoea in Shanghai, 2016-2021. *J Glob Antimicrob Resist*. (2023) 34:166–175. doi: 10.1016/j.jgar.2023.06.006

34. Shanghai Municipal Health Commission (2021). "Shanghai catalogue of graded management of clinical application of antibacterial drugs (2021 version)", Shanghai Municipal Health Commission Shanghai.

35. Zhang S, Huang Y, Yang G, Lei T, Chen M, Ye Q, et al. High prevalence of multidrug-resistant *Escherichia coli* and first detection of IncHI2/IncX4-plasmid carrying mcr-1 *E. coli* in retail ready-to-eat foods in China. *Int J Food Microbiol*. (2021) 355:109349. doi: 10.1016/j.ijfoodmicro.2021.109349

36. Chen Y, Liu Z, Zhang Y, Zhang Z, Lei L, Xia Z. Increasing prevalence of ESBL-producing multidrug resistance *Escherichia coli* from diseased pets in Beijing, China from 2012 to 2017. *Front Microbiol*. (2019) 10:2852. doi: 10.3389/fmicb.2019.02852

37. Wu C, Wang Y, Shi X, Wang S, Ren H, Shen Z, et al. Rapid rise of the ESBL and mcr-1 genes in *Escherichia coli* of chicken origin in China, 2008-2014. *Emerg Microbes Infect*. (2018) 7:30. doi: 10.1038/s41426-018-0033-1

38. Lu Y, Kang H, Fan J. A novel Bla (CTX-M-65)-Harboring IncHI2 plasmid pE648CTX-M-65 isolated from a clinical extensively-drug-resistant *Escherichia coli* ST648. *Infect Drug Resist*. (2020) 13:3383–91. doi: 10.2147/IDR.S269766

39. Hu Y, He Y, Nguyen SV, Liu C, Liu C, Gan X, et al. Antimicrobial resistance of Salmonella Indiana from retail chickens in China and emergence of an mcr-1-harboring isolate with concurrent resistance to ciprofloxacin, cefotaxime, and colistin. *Front Microbiol*. (2022) 13:955827. doi: 10.3389/fmicb.2022.955827

40. Lin H, Chen W, Zhou R, Yang J, Wu Y, Zheng J, et al. Characteristics of the plasmid-mediated colistin-resistance gene mcr-1 in *Escherichia coli* isolated from a veterinary hospital in Shanghai. *Front Microbiol*. (2022a) 13:1002827. doi: 10.3389/fmicb.2022.1002827

41. Vinueza-Burgos C, Ortega-Paredes D, Narvaez C, De Zutter L, Zurita J. Characterization of cefotaxime resistant *Escherichia coli* isolated from broiler farms in Ecuador. *PLoS One*. (2019) 14:e0207567. doi: 10.1371/journal.pone.0207567

42. Walsh TR, Wu Y. China bans colistin as a feed additive for animals. *Lancet Infect Dis*. (2016) 16:1102–3. doi: 10.1016/S1473-3099(16)30329-2

43. Wang Y, Xu C, Zhang R, Chen Y, Shen Y, Hu F, et al. Changes in colistin resistance and mcr-1 abundance in *Escherichia coli* of animal and human origins following the ban of colistin-positive additives in China: an epidemiological comparative study. *Lancet Infect Dis*. (2020c) 20:1161–71. doi: 10.1016/S1473-3099(20)30149-3

44. Touati A, Mairi A. Plasmid-determined colistin resistance in the north African countries: a systematic review. *Microb Drug Resist*. (2021) 27:121–33. doi: 10.1089/mdr.2019.0471

45. Lin J, Tang B, Zheng X, Chang J, Ma J, He Y, et al. Emergence of Incl2 plasmid-mediated colistin resistance in avian *Escherichia fergusonii*. *FEMS Microbiol Lett*. (2022b) 369:fnac016. doi: 10.1093/femsle/fnac016

46. Huang H, Dong N, Shu L, Lu J, Sun Q, Chan EW, et al. Colistin-resistance gene mcr in clinical carbapenem-resistant Enterobacteriaceae strains in China, 2014-2019. *Emerg Microbes Infect*. (2020) 9:237–45. doi: 10.1080/22221751.2020.1717380

47. Cheng P, Yang Y, Cao S, Liu H, Li X, Sun J, et al. Prevalence and characteristic of swine-origin mcr-1-positive *Escherichia coli* in Northeastern China. *Front Microbiol*. (2021) 12:712707. doi: 10.3389/fmicb.2021.712707

48. He T, Wei R, Zhang L, Sun L, Pang M, Wang R, et al. Characterization of NDM-5-positive extensively resistant *Escherichia coli* isolates from dairy cows. *Vet Microbiol*. (2017) 207:153–8. doi: 10.1016/j.vetmic.2017.06.010

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Teicoplanin associated gene *tcaA* inactivation increases persister cell formation in *Staphylococcus aureus*

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Staphylococcus aureus is part of normal human flora and is widely associated with hospital-acquired bacteremia. *S. aureus* has shown a diverse array of resistance to environmental stresses and antibiotics. Methicillin-resistant *S. aureus* (MRSA) is on the high priority list of new antibiotics discovery and glycopeptides are considered the last drug of choice against MRSA. *S. aureus* has developed resistance against glycopeptides and the emergence of vancomycin-intermediate-resistant, vancomycin-resistant, and teicoplanin-resistant strains is globally reported. Teicoplanin-associated genes tcaR-tcaA-tcaB (tcaRAB) is known as the *S. aureus* glycopeptide resistance operon that is associated with glycopeptide resistance. Here, for the first time, the role of tcaRAB in *S. aureus* persister cells formation, and ΔtcaA dependent persisters' ability to resuscitate the bacterial population was explored. We recovered a clinical strain of MRSA from a COVID-19 patient which showed a high level of resistance to teicoplanin, vancomycin, and methicillin. Whole genome RNA sequencing revealed that the tcaRAB operon expression was altered followed by high expression of *glyS* and *sgtB*. The RNA-seq data revealed a significant decrease in *tcaA* ($p = 0.008$) and *tcaB* ($p = 0.04$) expression while *tcaR* was not significantly altered. We knocked down *tcaA*, *tcaB*, and *tcaR* using CRISPR-dCas9 and the results showed that when *tcaA* was suppressed by dCas9, a significant increase was witnessed in persister cells while *tcaB* suppression did not induce persistence. The results were further evaluated by creating a *tcaA* mutant that showed ΔtcaA formed a significant increase in persisters in comparison to the wild type. Based on our findings, we concluded that *tcaA* is the gene that increases persister cells and glycopeptide resistance and could be a potential therapeutic target in *S. aureus*.

KEYWORDS

MRSA, *tcaA*, *tcaB*, persister cell, glycopeptides resistance, *glyS*

1. Introduction

Staphylococcus aureus has two major types of resistant strains, namely, methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA). MRSA is considered the major cause of hospital and community-acquired infections (Aqib and Rodriguez-Morales, 2021) and is

largely treated with glycopeptides. However, the emergence of teicoplanin resistance, vancomycin-intermediate *S. aureus* (VISA), and VRSA has made the clinical treatment unsuccessful and increased death rates across the world (Howden et al., 2010). *S. aureus* resistant to glycopeptides are a serious threat to public health and teicoplanin-resistant (Elsaghier et al., 2002; Szymanek-Majchrzak et al., 2018), VISA, and VRSA strains are globally reported (Appelbaum, 2006; Shariati et al., 2020). A meta-analysis of 155 articles from 2010 to 2019 reported a global prevalence of VRSA of 2.4 and 4.3% for VISA (Shariati et al., 2020), whereas a similar study reported a 7% frequency of VRSA from 2015 to 2020, with a prevalence of 16% in Africa, 5% in Asia, and 4% in America (Wu et al., 2021). Different genes were reported for glycopeptide resistance in *S. aureus* such as the *vanA*, *yycF*, *yycG*, *tcaA*, and *ccpA* (Renzoni et al., 2009). Other include mutations in accessory gene regulator, *vraSR*, and *graSR* two-component regulatory systems (Howden et al., 2008; Hu et al., 2016). Likewise, mutations in *sigB* and *trfAB* genes also contributed to glycopeptide resistance (Renzoni et al., 2009; Schulthess et al., 2009). According to the Clinical and Laboratory Standards Institute (CLSI) guidelines, *S. aureus* strains with a minimum inhibitory concentration (MIC) of ≤2 μg/mL would be considered susceptible (Clinical and Laboratory Standards Institute, 2018) and MRSA isolates with a glycopeptide MIC of greater than 2 μg/mL reflect poor clinical outcomes (Chen et al., 2013; Song et al., 2017). Several studies documented that teicoplanin resistance developed earlier than vancomycin resistance (Brunet et al., 1990; Hiramatsu, 2001) and was related to teicoplanin resistance operon tcaRAB (Brandenberger et al., 2000; Maki et al., 2004). A study revealed that teicoplanin resistance resulted in a slight increase in vancomycin resistance (Hiramatsu, 2001). Furthermore, penicillin binding protein-2 (pbp2) ectopic expression was associated with an increase in vancomycin MIC from 1 to 2 μg/mL and teicoplanin MIC from 2 to 8 μg/mL (Sieradzki et al., 1998). Interestingly, both Gram-negative and Gram-positive bacteria use different strategies to evade antibiotic actions. One of the mechanisms bacteria have adopted is the formation of persister cells. Persisters are nongrowing or metabolically less active cells that can survive high antibiotic concentrations without being resistant (Lewis, 2010; Personnic et al., 2023). Upon favorable conditions, persisters become metabolically active, regain their virulence potential, and resuscitate the whole population that remains fully susceptible to antibiotics (Eisenreich et al., 2021). Persistence should not be confused with tolerance and resistance because tolerance is the ability of a bacterial population to survive a transient exposure to antibiotics usually higher than the MIC (Handwerger and Tomasz, 1985), whereas resistance is an inherited trait that develops due to genetic changes in bacteria (Blair et al., 2015). Resistance to antibiotics is quantified by MIC testing and substantially higher than the MIC for a susceptible strain of bacteria, whereas the MIC for a tolerant strain is similar to the susceptible strain. Similarly, a persistent strain has a similar MIC and minimum duration for killing (MDK99) to a susceptible strain, whereas the MDK99 for a tolerant strain is substantially higher than the MDK99 for a susceptible strain. However, the MDK for 99.99% (MDK99.99) of bacterial cells in the population is substantially higher for a persistent strain than the MDK99.99 for a susceptible strain (Brauner et al., 2016). Several factors have been reported in different bacteria that contributed to persister cell formation, including nutrient starvation, acidic pH, accumulation of insoluble proteins, ATP depletion, and antibiotics exposure (Levin et al., 2017; Mohiuddin et al., 2021). Particularly, ATP depletion has induced persister cell formation in *S. aureus*, *E. coli*, and *P. aeruginosa* (Cameron et al., 2018). *S. aureus purB* and *purM* mutants showed defective persistence in low pH, heat stress, and through rifampicin treatment (Lin et al., 2020). The work of Wang et al. (2015) revealed the results of the transposon mutant library of a clinical MRSA strain where gene mutations in the tricarboxylic acid cycle, oxidative phosphorylation, and ABC transporters showed a lower number of persisters. For instance, the mutant of succinate dehydrogenase was defective in persister cells formation against levofloxacin (Wang et al., 2015). Shang and coworkers inactivated *phoU* which decreased vancomycin and levofloxacin persisters (Shang et al., 2020). Tricarboxylic acid cycle genes increased persister cell formation due to reduced ATP level and membrane potential (Wang et al., 2018). Likewise, *S. aureus* grown in polymicrobial cultures displayed increase antibiotic tolerance, accompanied by low intracellular ATP and membrane potential (Jia et al., 2013). Of note, persister cells can facilitate the evolution of drug resistance because tolerance precedes resistance and can boost the chances for resistance mutations in bacterial populations (Levin-Reisman et al., 2017). Due to the COVID-19 pandemic, numerous bacterial resistant strains were reported worldwide and were associated with antibiotic resistance and treatment failure (Ghanizadeh et al., 2021; Habib et al., 2022, 2023; Tariq et al., 2023). In this study, we assessed teicoplanin- and vancomycin-intermediate-resistant *S. aureus* recovered from a COVID-19 patient and explored the involvement of tcaABR operon in glycopeptide resistance and persistence. The role of *tcaA* in glycopeptide resistance has been widely studied but *tcaA* involvement in persister cell formation has not been discovered. Using CRISPR-dCas-9, the genetic basis was explored that revealed the teicoplanin resistance gene *tcaA* was associated with an increase in persister cell formation. The inactivation of *tcaA* has shown persistence and resistance to glycopeptides whereas *tcaB* and *tcaR* have no significant effects.

2. Materials and methodology

2.1. Bacterial strains, media, vectors, and growth parameters

The *S. aureus* teicoplanin- and vancomycin-intermediate-resistant clinical strain, hereafter referred to as wild-type (WT), was obtained from Lady Reading Hospital Peshawar. Tryptic soy broth (TSB), Luria Bertani broth (LB), and cation-adjusted Mueller Hinton broth (MHB) were used for *S. aureus*, *E. coli*, and MIC testing, respectively. Bacterial cultures were refreshed from −80°C and were grown in TSB (*S. aureus*) and LB (*E. coli*) at 37°C with shaking at 220 rpm. Bacterial strains were grown in round bottom tubes (14 mL tube with 3 mL culture media) and persister assays were performed in a conical flask (100 mL flask with 30 mL culture media). Cells were washed with ddH_2O. Temperature-sensitive (at 30°C) vector pBTs were used for mutant construction in *S. aureus*, pRMC-2 as an inducible vector for gene expression, and pALC with green fluorescent protein (GFP) gene was used for fluorescence assay. For plasmid maintenance, media were supplemented with appropriate antibiotic concentration, i.e., ampicillin 150 μg/mL and chloramphenicol 20 μg/mL. The pSD1 is a pRMC2 derivative vector that contains dCas9 and sgRNA expression cassettes. *S. aureus* RN4220 and *E. coli* DH5α were used for transformation. The primers, plasmids, recombinant vectors, and bacterial strains used in this study are listed in Table 1.

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TABLE 1 The list of primers, plasmids, and bacterial strains used in this study.

Strain	Relevant genotype	Source
S. aureus strains		
MRSA MW2	Methicillin-resistant *S. aureus* strain MW2	NARSA
S. aureus WT	Clinical isolate, glycopeptide intermediate resistant strain	Hospital source
RN4220	*S. aureus* restriction modification deficient strain	NARSA
ΔtcaA	*S. aureus* WT *tcaA* mutant strain	This study
E. coli DH5α	Transformation	TransGen
Plasmids		
pRMC2	ATC inducible plasmid, Amp ^{r} Chl ^{r}	Corrigan and Foster (2009)
pBT2	Temperature sensitive (30°C) plasmid for knockout	Brückner (1997)
pBTs	Modified pBT2 plasmid for knockout, Amp ^{r} Chl ^{r}	Hu et al. (2015)
pBTs-tcaA	*TcaA* mutant vector with 1,400 bp fragment	This study
pALC	pALC1484 derivative, harboring ORF of GFP and the promoter of the S10 ribosomal gene, Amp ^{r} Chl ^{r}	Bao et al. (2013)
WTpRMC-tcaA	pRMC2 derivative, with ORF of tcaA, Amp ^{r} Chl ^{r}	This study
pSD1	pRMC2 derivative with dCas9 and sgRNA expression cassette	Zhao et al. (2017)
dCas9-tcaA	pSD1 with sgRNA1 targeting tcaA	This study
dCas9-tcaB	pSD1 with sgRNA2 targeting tcaB	This study
dCas9-tcaR	pSD1 with sgRNA3 targeting tcaR	This study
WT-dCas9	pSD1 with non-specific sgRNA	This study
Primer name	Oligonucleotide (5′-3′)	Application
PBts-tcaA-F-KpnI	GCGGGTACCAGAGCAGTTTATAAATAACG	tcaA knockout
PBts-tcaA -R-0	TGTACAGATATGTACACAATGGTGATAAGATTACCGCAAC	tcaA knockout
PBts-tcaA -F-0	GTTGCGGTAATCTTATCACCTTGTGTACATATCTGTACAT	tcaA knockout
PBts-tcaA-R-SacI	GCGGAGCTCAGAAATCTTAATGCAACCAT	tcaA knockout
pRMC-tcaA-F-KpnI	GCGGGTACCAGGTGAAAGTATGAAATC	tcaA complementation
pRMC-tcaA-R-SacI	GCGGAGCTCCTATTTTTCTGATGTCTTG	tcaA complementation
RT-tcaA-F	GCGAAAGTATACATTAAC	qRT-PCR
RT-tcaA-R	GATATAACGCGTGCCATT	qRT-PCR
tcaA-oligo1	CTAAGAAACTTCAATTCACCTGAAGCGC	SgRNA1
tcaA-oligo2	AACGCGCTTCAGGTGAATTGAAGTTTCT	sgRNA1
tcaB-oligo3	CTAGGTAATTTGTTTGCTGGTCCAATTTC	sgRNA2
tcaB-oligo4	AACGAAATTGGACCAGCAAACAAATTACC	sgRNA2
tcaR-oligo5	CTAAGAGCAGTTTATAAATAACGTTAAC	sgRNA3
tcaR-oligo6	AACGTTAACGTTATTTATAAACTGCTCT	sgRNA3
RT-F-tcaB	CCTGGATTACCAGATATTAG	qRT-PCR
RT-R-tcaB	AAACAATACCTAAACTTGCT	qRT-PCR
RT-F-tcaR	AACTGCAAAAATGTTGAAAG	qRT-PCR
RT-R-tcaR	TTAACTAATTTAGCATCGAT	qRT-PCR
RT-F-clpP	CTAGGAGACATCAGTGAA	qRT-PCR
RT-R-clpP	CACTCATAGCGATAACAC	qRT-PCR
RT-F-gylS	ACAGAGGTTTTGTGTTCCC	qRT-PCR
RT-R-gylS	ATACTTTTGGATTCATTAAG	qRT-PCR
RT-F-sgtB	GCGATAGGTACTCAAACTCA	qRT-PCR
RT-R-sgtB	GATACCAATAAACAATGCG	qRT-PCR
RT-F-vWbp	AGAAGACTTAGAAACCAT	qRT-PCR

(Continued)

TABLE 1 (Continued)

Strain	Relevant genotype	Source
RT-R-vWbp	TGATTCATCACTTTTTGCTG	qRT-PCR
RT-F-ddl	GTGCAGAACACGAAGTATCG	qRT-PCR
RT-R-ddl	TGTGAAATCTCAAGCGCCTC	qRT-PCR
RT-F-Cro	GGCATTTCGATTTCTGATA	qRT-PCR
RT-R-Cro	GTGGTAATTCTAACACTTCA	qRT-PCR
RT-F-essC	ACAGGCAGATGATTACAA	qRT-PCR
RT-R-essC	CGCCATATCACTGTATTG	qRT-PCR
RT-F-EsxB	ATGGGTGGATATAAAGGTA	qRT-PCR
RT-R-EsxB	TGCCATAATGAGTAACAC	qRT-PCR
RT-F-guaA	ACTTTGGTAGCCAATACAAC	qRT-PCR
RT-R-guaA	CCGGATCAATTGTAAATG	qRT-PCR
RT-F-EsxA	TCCAGAGGAAATCAGAGCAA	qRT-PCR
RT-R-EsxA	GGACTAAGTTGTTGGAATTGC	qRT-PCR
RT-F-FtsL	CCAACCATATGACGAACAAG	qRT-PCR
RT-R-FtsL	CATAGCAATTACAGTAATC	qRT-PCR
RT-F-alr2	GCTGTAACTCAGTTTATCCA	qRT-PCR
RT-R-alr2	GTAATATGTCAACGACGGC	qRT-PCR

2.2. Whole genome RNA sequencing procedure

We performed RNA sequencing to study the transcriptome of the resistant isolate. *S. aureus* 24 h culture was 100 times diluted and was grown for 2 h (OD_{600} = 2) in TSB medium, cells were harvested, washed, and dissolved in RNAiso Plus (Takara Tokyo, Japan) and preserved at −80°C. RNA sequencing was performed in two biological replicates by the core sequence facility of the Institute of Microbiology Chinese Academy of Sciences. A NanoPhotometer® (Implen CA, USA), the Qubit® RNA Assay Kit and Fluorometer (Life Technologies CA, USA), and the RNA Nano 6,000 Assay Kit from the Agilent Bioanalyzer 2,100 system were used to measure RNA purity, concentration, and integrity, respectively. RNA degradation or contamination was checked by agarose gel (1%).

2.2.1. Library construction for transcriptome sequencing

The NEBNext®UltraTM RNA Library Prep Kit from Illumina®(USA) was used for library preparation, and index codes were added to attribute sequences to each sample. Briefly, poly-T oligo-attached magnetic beads were used to purify mRNA from total RNA, then fragmentation was performed using divalent cations under high temperature in NEBNext First-Strand Synthesis Reaction Buffer (5X). Next, the first-strand cDNA strand was synthesized by M-MuLV Reverse Transcriptase and random hexamer primer, whereas the second-strand cDNA strand was synthesized by DNA Polymerase I and RNase H, and the ends of the remaining overhang were converted into blunt ends by the polymerase activity. Further, adenylation of 3′ ends of DNA fragments was performed, and an adaptor (NEBNext) with a hairpin loop structure was ligated, and cDNA fragments of 200-250 bp were preferentially purified by the AMPure XP system (Beckman Coulter, Beverly, USA). The size-selected, adaptor-ligated cDNA was treated with 3 µL USER Enzyme (NEB, USA) at 37°C for 15–20 min and followed by 5 min at 95°C. Next, PCR was performed with Phusion High-Fidelity DNA polymerase, Index (X) Primer, and Universal PCR primers. The PCR product was purified by the AMPure XP system. The Agilent Bioanalyzer 2,100 system was used to assess the quality of the constructed library (Wang Q. et al., 2017).

2.2.2. Clustering and sequencing

The index-coded sample clustering was done on the Illumina cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS protocols. After cluster generation, the Illumina Hiseq 2,500 platform was used for library sequencing, and paired-end reads were generated.

2.2.3. Quality assessment and comparative analysis

Raw reads (raw data) of fastq format were processed through in-house Perl scripts and the clean data was obtained by removing reads containing adapter and ploy-N. In addition, the low-quality reads from raw data were also removed, and the clean data Q20, Q30, GC-content, and sequence duplication levels were calculated. Clean data with high quality were used for all the downstream analyses. During data processing, the adaptor sequences and low-quality reads were removed, and clean reads were generated that were mapped to the reference genome sequence. The reads with a perfect match were annotated based on the reference genome. Hisat2 software was used to annotate the reads with the reference genome.

2.2.4. Gene functional and differential expression analysis

Gene functions were annotated with the help of the following databases: Nt (NCBI non-redundant nucleotide sequences); Nr (NCBI non-redundant protein sequences); Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot (protein sequence database); KO (KEGG Orthologue database); GO (Gene Ontology). Differential expression analysis was performed using the DESeq R package (1.10.1). DESeq is used for determining differential expression in digital gene expression data using the model based on the negative binomial distribution (Anders and Huber, 2010). Benjamini and Hochberg's approach was used to adjust the *p*-values to control for the false discovery rate. Genes with an adjusted $p < 0.05$ found by DESeq were assigned as differentially expressed.

2.2.5. Gene Ontology (GO) enrichment analysis

For GO enrichment analysis of the differentially expressed genes (DEGs), the GOstats and topGO packages based on Wallenius non-central hypergeometric distribution were applied (Young et al., 2010).

2.2.6. KEGG enrichment analysis

The KEGG database is a set of processes to map genes and proteins, etc. to molecular interaction and relation networks, and is used for large-scale molecular datasets analysis generated by genome sequencing[1] (Kanehisa et al., 2007). We used KOBAS software to test the statistical enrichment of differential expression genes in KEGG pathways (Mao et al., 2005).

2.3. RNA isolation and RT-qPCR

Staphylococcus aureus cells were collected and dissolved in 1 mL of RNAiso Plus. The cell lysis was performed with the help of 0.1-mm silica beads in the FastPrep-24 automated system, and then the lysate was treated with DNase I to remove the remaining DNA. For reverse transcription and qPCR, the PrimeScript cDNA synthesis kit and SYBR Premix Ex Taq reagent kit (Takara Tokyo, Japan) were used, respectively. The StepOne real-time PCR system was used for RT-qPCR analysis. The *hu* gene cDNA abundance was used for normalization (Valihrach and Demnerova, 2012).

2.4. Gene knockdown vector pSD1

For gene knockdown, the anhydrotetracycline (ATC) inducible vector pSD1 was used which expresses dcas9 and custom-designed sgRNA. The pSD1 vector was derived from *Streptococcus pyogenes* cas9 and was used as a CRISPR interference system (CRISPRi) (Zhao et al., 2017). From the vector design, the dcas9 and sgRNA are under the control of the P_{tetO} and P_{pflB} inducible and constitutive promoters, respectively. For *tcaA*/*tcaB*/*tcaR* knockdown, gene specific complementary oligonucleotide sequences were synthesized and cloned into the SapI-digested pSD1 site. The sgRNA 5′ variable region binds to the target gene while the 3′ constant region binds to dCas9. The dCas9 functions as a DNA-binding protein guided by sgRNA which is complementary to each target gene. The pSD1 plasmid without the target gene (WT-dCas9) was used as a negative control that can also express the sgRNA which has no specific target site in *S. aureus*.

2.5. Construction of the *tcaA* mutant strain

To create the *tcaA* mutant, the upstream and downstream regions were amplified by primer pairs PBts-tcaA-F-KpnI and PBts-tcaA-R-0, and PBts-tcaA-F-0 and PBts-tcaA-R-SacI from *S. aureus* WT genome, respectively, (Table 1). The PBts-tcaA-R-0 and PBts-tcaA-F-0 are the overlap primers that contain the overlap segments, and a 1,400-bp fragment was generated by PBts-tcaA-F-KpnI and PBts-tcaA-R-SacI in Prime Star PCR conditions: 95°C for 5 min, 95°C for 30 s, 55°C for 1.5 min, 72°C for 1 min, 35 cycles, and 72°C for 10 min. A 1400-bp joint fragment was constructed, sequenced, and purified to clone into pBTs, a modified vector of pBT2 (Brückner, 1997; Bae and Schneewind, 2006) via restriction enzymes. The 1,400 bp fragment and pBTs vector were digested with KpnI and SacI for 1 h at 37°C, the mixture was purified by a DNA purification kit separately and ligated into pBTs by DNA ligase. The constructed recombinant vector was initially transferred to DH5α, then transferred to RN4220 for genomic modification, and lastly to *S. aureus* WT. *S. aureus* colonies carrying the recombinant vector were selected by tryptic soy agar plus chloramphenicol (20 μg/mL) at 37°C. *S. aureus* carrying the recombinant vector pBTs-tcaA was grown at 30°C with shaking at 220 rpm for 24–72 h. Daily screening was performed on ATC inducible tryptic soy agar plates at 37°C. PCR was performed to screen the mutant colonies. The *tcaA* markerless mutant was confirmed by DNA sequencing.

2.6. Antibiotic susceptibility and MIC testing

We followed our previous method of antibiotic susceptibility testing in *S. aureus* (Habib et al., 2020). Initially, the *tcaA* complementary strain (C-tcaA) was constructed by amplifying the *tcaA* gene through primer pairs pRMC-tcaA-F-KpnI and pRMC-tcaA-R-SacI, and the vector WTpRMC-tcaA was constructed and transferred to *S. aureus* WT. The 24 h fresh culture of *S. aureus*, ΔtcaA, and C-tcaA (complementary strain) was grown for 2 h in TSB ($OD_{600} = 2.0$) and 2×10^9 CFU/mL were challenged with 100 fold MIC of teicoplanin, vancomycin, and azithromycin at 37°C. The inhibition zones were mapped after 24–36 h. MIC was determined in MHB medium using the two-fold broth microdilution method (Clinical and Laboratory Standards Institute, 2018). Briefly, the double dilutions of antibiotics were distributed into microtiter plates wells, and freshly prepared bacterial suspension (5×10^5 CFU/mL) was added to each well, and plates were incubated at 35°C. *S. aureus* colonies suspension with a density of 5×10^5 CFU/mL required a transfer of 100 μL of the 0.5 McFarland equivalent suspension to 10 mL of broth. Of note, CLSI does not recommend performing vancomycin susceptibility testing using the disk diffusion method.

1 http://www.genome.jp/kegg/

2.7. Western blot assay

The protein expression levels of *tcaA*, *tcaB,* and *tcaR* were detected by specific antisera for *tcaA*, *tcaB*, and *tcaR* through Western blotting. The dCas9-tcaA, dCas9-tcaB, and dCas9-tcaR were induced at OD_{600} = 0.4 with ATC 100 ng/mL for 1 h. The cells were collected, and media were removed and washed twice with ddH_2O. An equal number of cells were taken and lysed by lysostaphin for 1 h at 37°C. The mixture was washed with normal saline and total proteins were collected in lysis buffer. A 12% SDS-PAGE was used for whole-cell protein separation and proteins were electrotransferred to a polyvinylidene difluoride membrane. The tcaA, tcaB, and tcaR proteins were detected with rabbit anti-tcaA, anti-tcaB, and anti-tcaR antibodies (1:1000) followed by horseradish-peroxidase conjugated antibody (1:10,000 dilution). The membrane was analyzed using a Thermo Fisher chemiluminescent detection kit and spots were detected using an ImageQuant LAS 4000.

2.8. Persister assay

A persistence assay was adopted from our previous protocol (Habib et al., 2020). The cells were grown to OD_{600} = 0.40 in the TSB medium. The pSD1 containing dCas9-tcaA and dCas9-tcaB were induced with ATC 100 ng/mL for 1 h, the medium was removed, and fresh TSB and antibiotic were added whereas ΔtcaA cells were challenged with antibiotic at OD_{600} = 0.40. After 12 h, a 200 μL sample was taken and CFU counting was performed. Each antibiotic concentration was 10 fold of MIC, and *S. aureus* WT and *S. aureus* WT-dCas9 were used as controls.

2.9. Fluorescence microscopy

Fluorescence microscopy was used to observe the persister cell resuscitation ability of the whole population. *S. aureus* WT and ΔtcaA were transferred with pALC fluorescence shuttle plasmid with GFP and challenged with 20-fold MIC for 48 h. The cells were harvested, washed with ddH_2O, and resuspended in TSB media. The cells were grown for 2 h at 37°C and the *S. aureus* WT and ΔtcaA were observed under the fluorescence microscope.

2.10. Growth curve analysis

The overnight culture was 100 times diluted in TSB medium and a 200 μL was inoculated into a 96-well microtiter plate. The plates were incubated at 37°C with shaking at 200 rpm. At different time intervals (1–18 and 24 h), OD_{600} was measured using a microplate reader (Thermo Fisher, Waltham, MA, USA).

2.11. Statistical analyses

Experiments were performed in biological triplicates unless otherwise stated. The data values were analyzed by Student's *t*-test for two groups (unpaired, two-tailed) and a one-way analysis of variance for more than two groups. The gene sequences were analyzed by vector NTI advance and data were analyzed by GraphPad prism 8. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

3. Results

3.1. Characterization of glycopeptide intermediate resistant *Staphylococcus aureus* (WT)

Staphylococcus aureus isolated from a COVID-19 patient was resistant to methicillin, teicoplanin, and vancomycin, while sensitive to azithromycin and ciprofloxacin. The MIC of methicillin was >512 μg/mL, teicoplanin was 16 μg/mL, and vancomycin was 10 μg/mL (Table 2). RNA sequencing results of WT revealed that multiple genes and pathways have been affected, comprising 62 differentially expressed genes. The differential expression of highly affected genes is shown by a heat map (Figure 1A). Among the differential expressed genes, Clusters of Orthologous Genes (COG) involved in cell motility (N), extracellular structures (W), prophages and transposons (V), cell division, and chromosome partitioning (D) were marginally expressed whereas amino acid transport and metabolism (E), inorganic ion transport and metabolism (P), and general function prediction (R) were highly expressed (Figure 1B). The COG group associated with nucleotide transport and metabolism (F), translation (J), transcription (K), secondary metabolites biosynthesis (Q), intracellular trafficking (U), defense mechanism (V), cell wall/membrane/envelope biogenesis (M) were slightly expressed (Figure 1B). Differentially expressed genes such as *ClpP*, *EsxB*, *EsxA,* toxin-like hypothetical protein, *glyS*, *Cro*, *sgtB*, *ddl*, *alr2*, and *vWbp* genes expression was high whereas *tcaA*, *essC*, *tcaB*, *FtsL*, and ABC transporter permease gene expression was low (Table 3). We screened out the genes responsible for glycopeptide resistance in *S. aureus* which is reported to be the tcaRAB operon. The tcaRAB is stimulated when *S. aureus* is exposed to a minimum inhibitory concentration of glycopeptides. In the tcaRAB operon, the *tcaA* is a 454-residues zinc ribbon domain-containing protein, the *tcaB* is a 402-residues multidrug efflux MFS transporter, and *tcaR* is a 151-residues MarR family transcriptional regulator. The WT strain RNA sequencing data revealed a significant decrease in *tcaA*

TABLE 2 MICs of antibiotics.

Antibiotics	*S. aureus* WT	ΔtcaA	
Methicillin	≥512 μg/mL	≥256 μg/mL	
Teicoplanin	16 μg/mL	18 μg/mL	
Vancomycin	10 μg/mL	10 μg/mL	
Azithromycin	≤8 μg/mL	≤8 μg/mL	
Ciprofloxacin	≤2 μg/mL	≤2 μg/mL	
CLSI criteria for MIC testing (μg/ml)	**Susceptible**	**Intermediate**	**Resistance**
Vancomycin (*S. aureus*)	≤2	4–8	≥16
Teicoplanin (All Staphylococci)	≤8	16	≥32

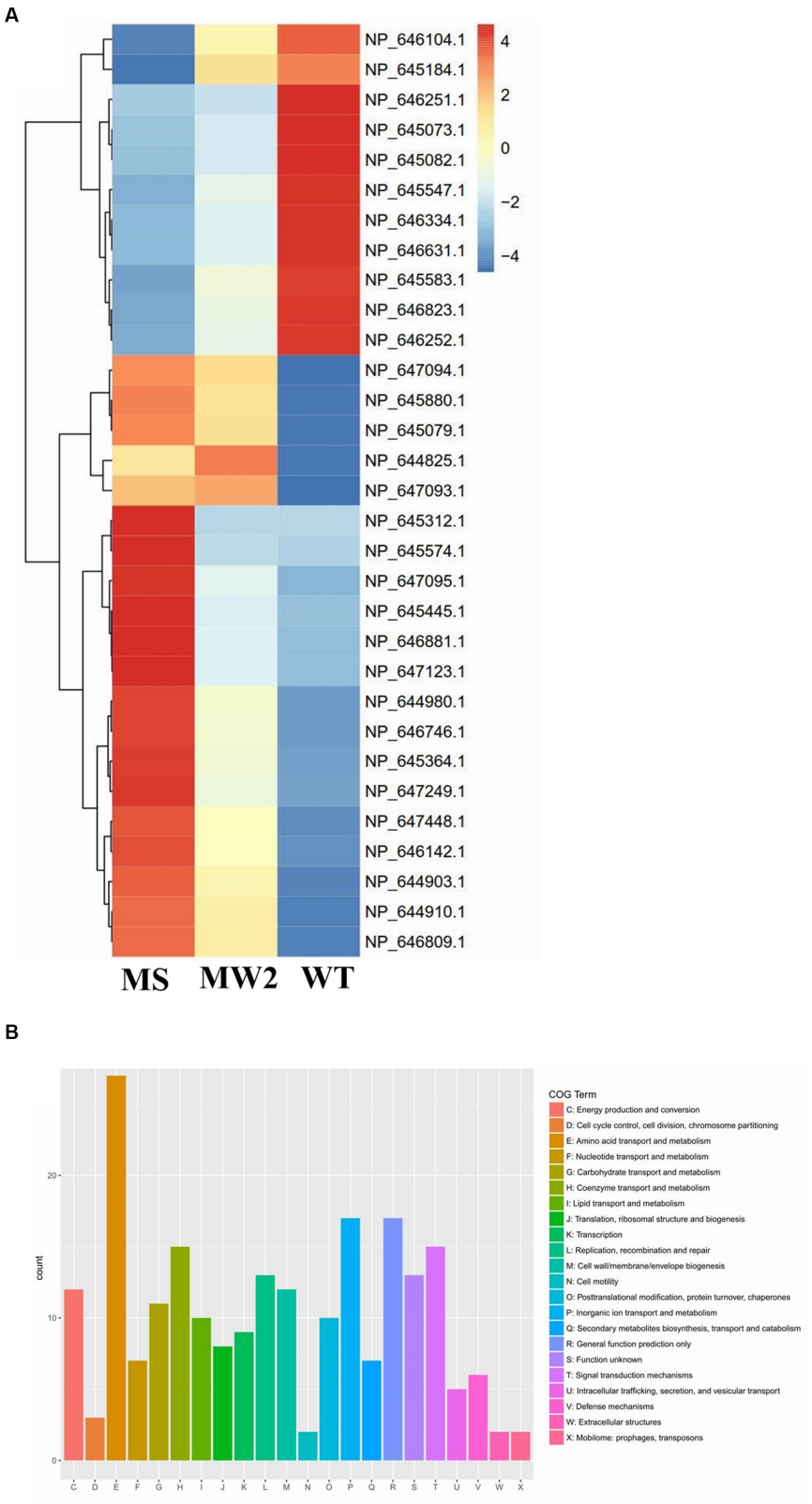

FIGURE 1
RNA-seq analysis. **(A)** The heat map of differentially expressed genes. Red represents upregulated genes and blue indicates downregulated genes. The tcaRAB operon genes *tcaA* (NP_647094.1) and *tcaB* (NP_647093.1) were significantly downregulated. The differentially expressed genes of *S. aureus* WT were compared with MS (methicillin-sensitive *S. aureus*) and MRSA MW2 as reference strains. **(B)** The COG function classification of differentially expressed genes was performed in *S. aureus* WT vs. MRSA MW2.

TABLE 3 RNA-seq data of differentially expressed genes in *S. aureus* WT.

Accession number (Reference genome)	Genes / Description	*S. aureus* WT	*p*-value
NP_646251.1	Toxin like hypothetical protein	4.64 ± 0.40	0.006
NP_645073.1	T7SS *EsxA*	4.34 ± 0.32	0.007
NP_645082.1	T7SS *EsxB*	4.24 ± 0.27	0.008
NP_645547.1	*ClpP*	4.25 ± 0.20	0.006
NP_646334.1	*glyS*	4.19 ± 0.25	0.040
NP_646631.1	*sgtB*	4.16 ± 0.26	0.030
NP_645583.1	*vWbp*	4.14 ± 0.20	0.030
NP_646823.1	*ddl*	4.10 ± 0.25	0.050
NP_646252.1	*Cro*	4.06 ± 0.20	0.040
NP_646104.1	*alr2*	4.04 ± 0.20	0.050
NP_645184.1	*guaA*	4.10 ± 0.35	0.060
NP_645762.1	*Chitinase B*	4.03 ± 0.20	0.060
NP_645294.1	*ctsR*	4.02 ± 0.20	0.070
NP_644846.1	*mecA*	3.25 ± 0.45	0.120
NP_647094.1	*tcaA*	−4.53 ± 0.15	0.008
NP_645880.1	*FtsL*	−4.30 ± 0.20	0.030
NP_647093.1	*tcaB*	−4.25 ± 0.20	0.040
NP_645079.1	*essC*	−4.20 ± 0.20	0.050
NP_644825.1	ABC transporter like hypothetical protein	−4.15 ± 0.20	0.050
NP_646142.1	Facilitator transporter protein	−3.80 ± 0.30	0.050
NP_647448.1	*Ribonuclease P*	−3.70 ± 0.40	0.080
NP_644903.1	*sirA*	−4.22 ± 0.11	0.230
NP_646809.1	*MazF*	−4.17 ± 0.26	0.160
NP_644910.1	*SbnH*	−4.15 ± 0.20	0.110
NP_644980.1	*murQ*	−4.10 ± 0.30	0.250
NP_647095.1	*tcaR*	−3.00 ± 0.25	0.080

($p = 0.008$) and *tcaB* ($p = 0.04$) expression while *tcaR* was not significantly altered ($p = 0.08$) (Figure 1A and Table 3). We validated the RNA sequence data by RT-qPCR which confirmed that *tcaAB* and *essC* genes were significantly suppressed whereas *EsxAB*, *ClpP*, *glyS*, and *sgtB* were significantly upregulated (Figure 2 and Table 3). The EsxAB are the secretory protein of type seven secretion system (T7SS), ClpP is involved in proteostasis, glyS is glycine tRNA synthetase, sgtB is mono-functional peptidoglycan glycosyltransferase, ddl codes for D-alanine-D-alanine ligase/synthetase, and alr2 is alanine racemase. From this data, we proposed that *EsxAB*, *ClpP*, *glyS*, and *sgtB* expression was upregulated when *tcaA* was suppressed which might be linked with cell wall protection against wall-damaging agents such as glycopeptides and viruses. Besides, the T7SS ATP synthesis machinery gene *essC* was significantly downregulated (Figure 2) which is associated with *S. aureus* survival during host infection. Collectively, these results corroborated the RNA sequencing data and confirmed that *tcaAB* suppression is associated with glycopeptides resistance either to protect the cell wall against wall-piercing agents or to increase survival during host infection.

3.2. Knockdown of *tcaA*, *tcaB*, and *tcaR* By CRISPR-dCas9

We performed CRISPRi-mediated *tcaA*, *tcaB*, and *tcaR* knockdown by dCas9 in *S. aureus*. Gene-specific sgRNAs were designed that can bind to the target site of *tcaA*, *tcaB*, and *tcaR* (Table 1). Media were supplied with 100 ng/mL of ATC to induce the CRISPR-dCas9 to suppress gene expression. Initially, the dCas9 repression efficiency was determined in both the knockdown and WT strain by RT-qPCR. The data revealed that the strains expressing sgRNA1, sgRNA2, and sgRNA3 exhibited a 22-fold, 20-fold, and 26-fold decrease in the *tcaA*, *tcaB*, and *tcaR* mRNA levels, respectively, (Figure 3A). Further, we analyzed the dCas-tcaA, dCas-tcaB, and dCas-tcaR protein expression level by rabbit anti-tcaA, anti-tcaB, and anti-tcaR antibodies through Western blotting. The immunoblots showed brighter tcaA, tcaB, and tcaR bands in *S. aureus* WT whereas suppression was seen in dCas-tcaA, dCas-tcaB, and dCas-tcaR carrying *S. aureus* (Figures 3B–D). The dCas9 was used as a negative control to check the inhibitory effects of the dCas-9 vector on tcaRAB proteins. From Figures 3B–D, it is confirmed that CRISPRi-mediated *tcaA*, *tcaB*, and *tcaR* knockdown was successful and can be used for persister assay.

3.3. The ΔtcaA increased persistence to glycopeptides antibiotics

We performed a persistence assay with the WT, WT-dCas9, dCas9-tcaA, ΔtcaA, and dCas9-tcaB strains. The WT-dCas9 was the *S. aureus* WT strain expressing target unspecific sgRNA (control), dCas9-tcaA was the WT strain expressing sgRNA binding to *tcaA* (sgRNA1), dCas9-tcaB was WT expressing sgRNA binding to *tcaB* (sgRNA2), and ΔtcaA was *tcaA* mutant in *S. aureus* WT. All the strains were challenged with 10-fold MIC of teicoplanin, vancomycin, ciprofloxacin, and azithromycin which revealed that the dCas9-tcaA and ΔtcaA had a higher number of persisters compared to WT-dCas9 and WT strain. After 12 h of teicoplanin treatment, the surviving fraction of cells of the dCas9-tcaA and ΔtcaA strains were 17 and 18 times more than control strains WT-dCas9 and WT, respectively, (Figure 4A). Similarly, upon vancomycin treatment, the dCas9-tcaA and ΔtcaA showed a 15- and 16-fold increase relative to controls, respectively, (Figure 4B). After 48 h, the dCas9-tcaA and ΔtcaA showed a 13- and 14-fold increase in persister cells in response to teicoplanin and a 10- and 11-fold increase toward vancomycin relative to controls, respectively, (Figures 4A,B). The dCas9-tcaB did not induce persister cell formation and was similar to WT-dCas9 and WT strain (Figures 4A,B). When the cells were challenged with azithromycin and ciprofloxacin, the dCas9-tcaA, ΔtcaA, and dCas9-tcaB showed similar results with controls (Figures 4C,D). Overall, neither of the strains showed persister cell formation toward azithromycin and ciprofloxacin that confirmed the emergence of glycopeptides persisters due to *tcaA* suppression and deletion. Further, we transferred the pALC shuttle vector (with GFP) to *S. aureus* WT

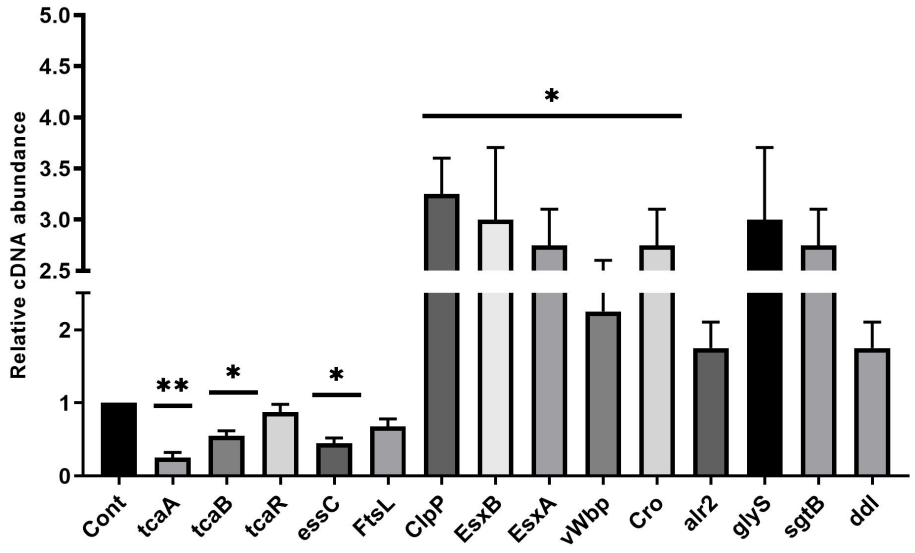

FIGURE 2
The up and downregulated genes. The RT-qPCR analysis revealed the transcript level of tcaRAB, *essC*, *FtsL*, *ClpP*, *EsxB*, *EsxA*, *glyS*, *alr2*, *sgtB*, *ddl*, *Cro*, and *vWbp* in *S. aureus* WT strain. * $p < 0.05$; ** $p < 0.01$.

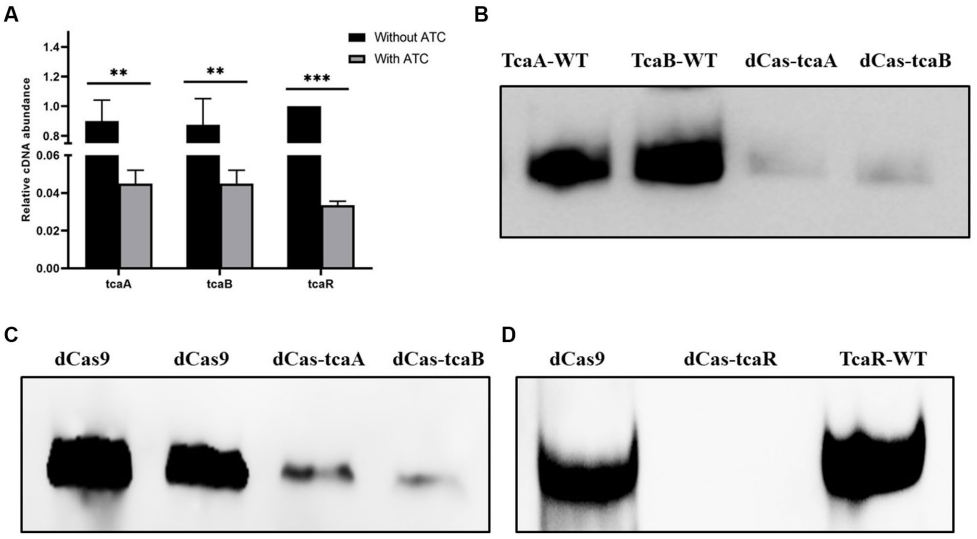

FIGURE 3
CRISPR-dCas9 mediated suppression. **(A)** The transcript level of tcaRAB operon before and after ATC induction (100 ng/mL). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. **(B)** The protein expression level was detected by Western blot before and after ATC induction (100 ng/mL). TcaA-WT and TcaB-WT bands are brighter, whereas dCas-TcaA and dCas-TcaB bands are dim, indicating *tcaA* and *tcaB* suppression. **(C)** The dCas9 was used as negative control (blank vector) and brighter bands were detected, while the dCas-TcaA and dCas-TcaB showed tcaA and tcaB protein suppression. **(D)** The suppression of tcaR protein was confirmed by dCas-tcaR where no protein band was detected whereas TcaR-WT and dCas9 have clear tcaR protein bands.

and ΔtcaA competent cells and challenged them with a 20-fold MIC of teicoplanin and vancomycin. After 48 h, the cells were washed and resuspended in a fresh TSB medium without antibiotics, and fluorescence microscopy was performed. The microscopy revealed a high number of cells expressing GFP in ΔtcaA cells compared to WT (Figure 5). This confirmed that persister cells tolerated a high concentration of teicoplanin and vancomycin (20 fold MIC) for 48 h and resuscitated the whole population when the antibiotic was removed. Collectively, the *tcaA* gene inactivation developed persistence to glycopeptide antibiotics while the *tcaB* did not influence the persistence phenotype. This data disclosed that *S. aureus* ΔtcaA formed persister cells and confirmed the RNA-seq and RT-qPCR results where suppression of *tcaA* was associated with the development of glycopeptides resistance. To date, Brandenberger et al. (2000) and Maki et al. (2004) reported ΔtcaA involvement in glycopeptides resistance, and the present data corroborated their results and reproduced the findings that the ΔtcaA showed resistance to teicoplanin and vancomycin and C-ΔtcaA strain restored the

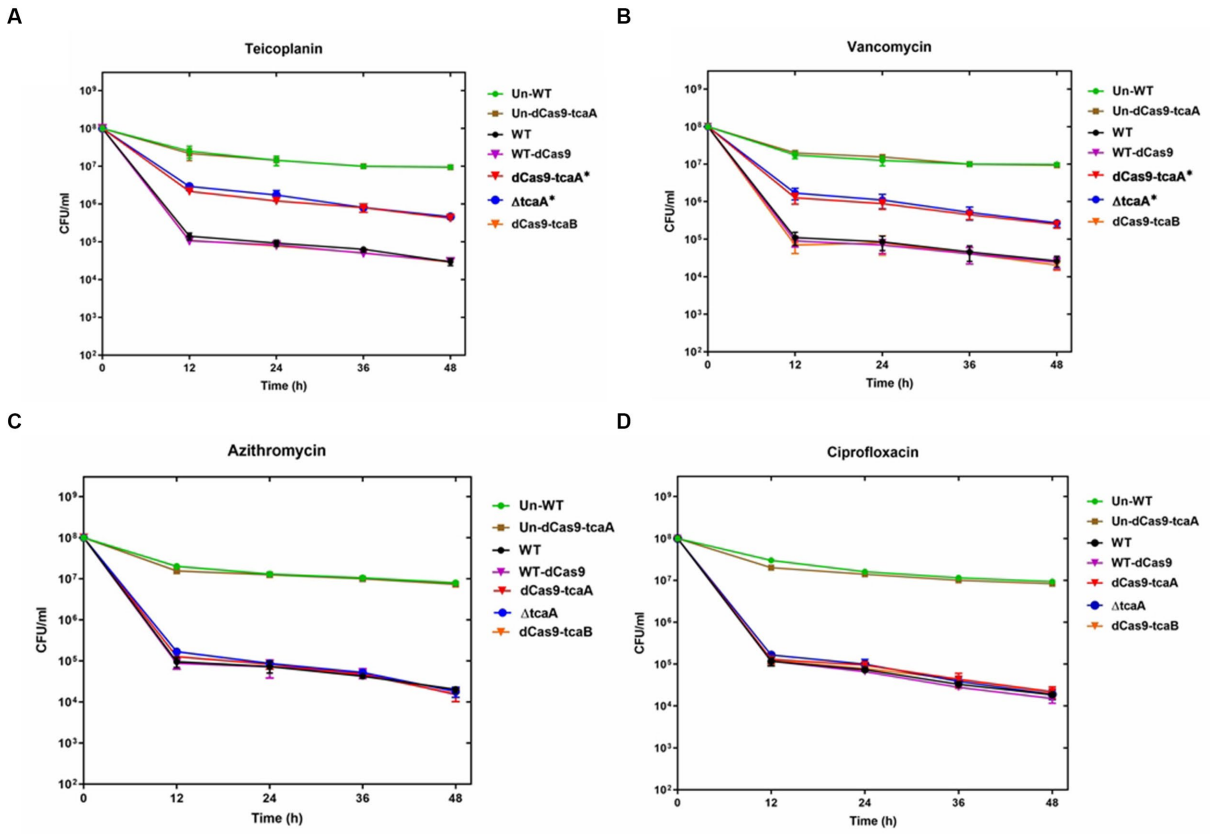

FIGURE 4
Persister assay. **(A–D)** The WT, WT-dCas9, dCas9-tcaA, dCas9-tcaB, and ΔtcaA were challenged with 10-fold MIC of teicoplanin for 48 h. The sample was taken after 12 h and CFU counting was performed. The dCas9-tcaA and ΔtcaA significantly increased the number of persister cells in panel **(A,B)**. No significant changes were observed in azithromycin and ciprofloxacin persister assays **(C,D)**. Un-WT and the Un-dCas9-tcaA were used as untreated antibiotic control strains. *S. aureus* WT and WT-dCas9 were used as controls. Experiments were performed in triplicates and error bars denote standard deviation. Statistical significance was determined using Student's *t*-test (control versus treatment). * $p < 0.05$.

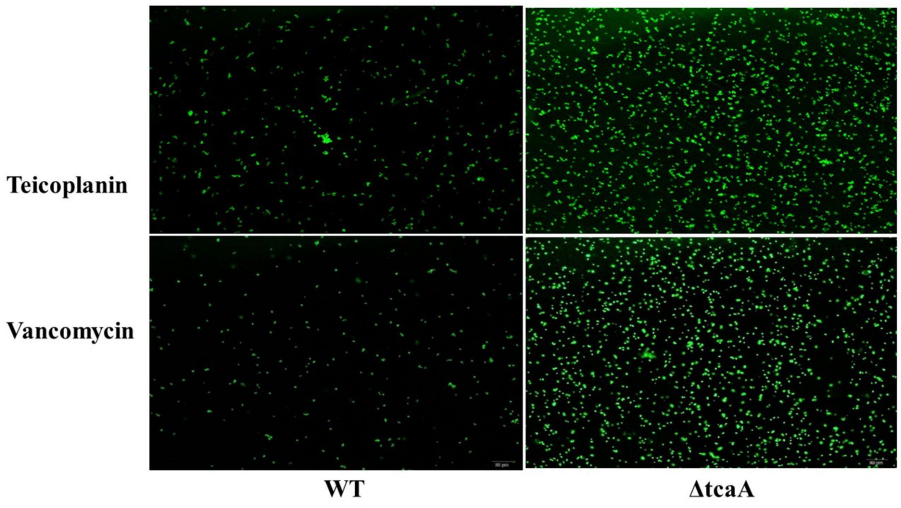

FIGURE 5
The fluorescence microscopy. *S. aureus* WT showed very dim GFP expression that revealed a low number of persisters. The ΔtcaA cells have brighter GFP expression that showed a higher number of persister and resuscitation of the bacterial population.

phenotype by expressing the *tcaA* gene via WT-pRMC-tcaA (Supplementary Figure S1).

3.4. The ΔtcaA growth analysis

We tested cell growth in the TSB medium with 1–2 mg/L of teicoplanin and vancomycin. The results showed that the MRSA MW2 reference strain displayed slow growth rates at 1 mg/L, whereas the WT, ΔtcaA, and C-ΔtcaA strains displayed fast and steady growth (Figures 6A,B). At 2 mg/L, MRSA MW2 did not show any growth whereas WT, ΔtcaA, and ΔtcaA complementary strain displayed slow growth (Figures 6C,D). These results confirmed a decrease in susceptibility of the WT, C-ΔtcaA, and ΔtcaA to teicoplanin and vancomycin compared to MW2. We conclude that *S. aureus* WT, ΔtcaA, and C-ΔtcaA strains grow at 1–2 mg/L concentration of glycopeptides which supports the fact that *tcaA* is involved in the emergence of glycopeptides resistance.

3.5. Inactivation of the *tcaA* influences the expression of cell wall biosynthesis genes

The present data indicated that *tcaA* is involved in cell wall-associated glycopeptide resistance and persistence. From RNA-seq data, the *tcaA* was significantly suppressed and *tcaA* deletion increased persister cell formation. During *tcaA* suppression, the *glyS*, *sgtB*, *ddl*, and *alr2* transcript level was high. We hypothesized that evaluating these gene expressions in ΔtcaA would validate the RNA-seq data and disclose the correlation. From RT-qPCR analysis, the *glyS* and *sgtB* expression was significant in the *tcaA* mutant while *ddl* and *alr2* transcript level was not significant (Figure 7). The high expression of *glyS* and *sgtB* might help in cell wall biogenesis because *glyS* is glycine tRNA synthetase which is involved in the supply of glycine for incorporation into nascent polypeptides during bacterial cell wall synthesis (Schneider et al., 2004; Giannouli et al., 2009) whereas *sgtB* is a mono-functional peptidoglycan glycosyltransferase which is involved in peptidoglycan synthesis and also supports the growth of *S. aureus* in the absence of the main glycosyltransferase pbp2 (Wang et al., 2001; Reed et al., 2015). Collectively, this data revealed that the *tcaA* inactivation altered the expression of cell wall-associated genes, probably allowing the cell wall to better withstand external pressures, and it would be interesting to explore *glyS* and *sgtB* involvement in cell wall biogenesis during glycopeptide treatment or in the persister cell formation.

4. Discussion

Glycopeptide resistance in MRSA is a global problem and vancomycin- and teicoplanin-resistant strains are of major clinical relevance. Several studies have reported the emergence of

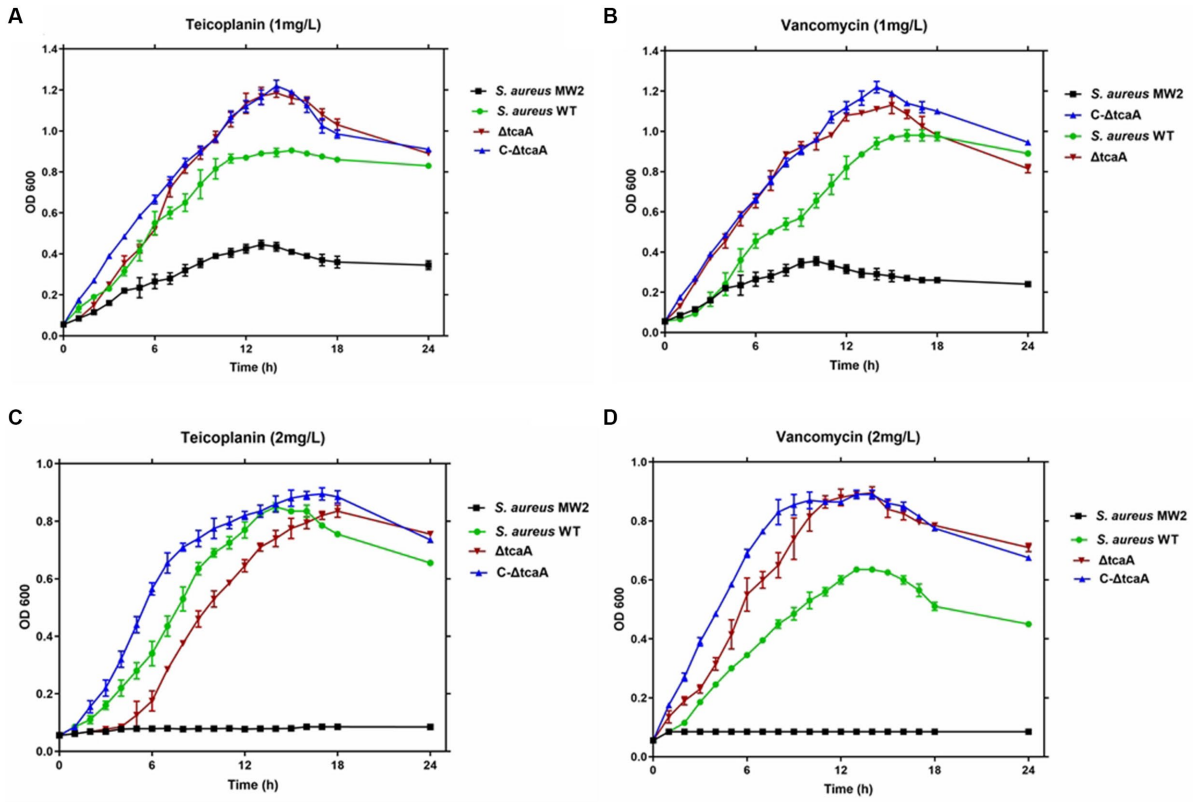

FIGURE 6
Growth curves. The *S. aureus* MW2, *S. aureus* WT, ΔtcaA, and C-ΔtcaA were grown in the TSB medium containing 1 mg/L and 2 mg/L of teicoplanin and vancomycin. **(A,B)** The growth curves at 1 mg/L of teicoplanin and vancomycin showed slow growth by MW2 and fast by *S. aureus* WT, ΔtcaA, and C-ΔtcaA. **(C,D)** At 2 mg/L of teicoplanin and vancomycin, *S. aureus* MW2 showed no growth, whereas *S. aureus* WT, ΔtcaA, and C-ΔtcaA displayed a slow growth.

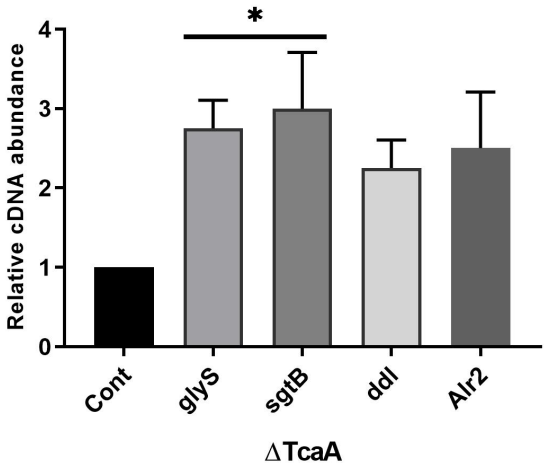

FIGURE 7
The cell wall-associated genes analysis in ΔtcaA. The *glyS* and *sgtB* showed significant expression in the mutant strain relative to *S. aureus* WT, whereas *ddl* and *Alr2* were not significant. *$p<0.05$.

teicoplanin and vancomycin resistance in *S. aureus* (Wang Y. et al., 2017; Szymanek-Majchrzak et al., 2018; Wu et al., 2021). Typically, teicoplanin and vancomycin bind to D-alanine-D-alanine subunits of the murein monomer and cross resistance could develop between teicoplanin and vancomycin (Bakthavatchalam et al., 2019). The cell wall thickness also contributed to the development of vancomycin and teicoplanin resistance in *S. aureus* (Cui et al., 2006; Bakthavatchalam et al., 2019). Two-component systems, such as walKR, vraSR, graSR, and tcaRAB operon, are linked to the development of teicoplanin and vancomycin resistance in *S. aureus* (Howden et al., 2010). Also, *vanA* and *tcaA* are considered to give resistance to vancomycin and teicoplanin in *S. aureus* (Chang et al., 2003; Maki et al., 2004), while a report also claimed that the effects of the *tcaA* deletion on resistance were strain specific because the *tcaA* mutant in the *S. aureus* Col strain had higher MIC than the *tcaA* mutant in the BB1372 strain (Brandenberger et al., 2000). *tcaA* has been reported to be upregulated by vancomycin (Kuroda et al., 2003), oxacillin, and teicoplanin treatment (Utaida et al., 2003).The *tcaA* gives strong resistance when inactivated while its overexpression from an inducible promoter was effective in lowering teicoplanin resistance (Maki et al., 2004). A study reported that pbp2 and pbp4 lead to *S. aureus* cell wall thickening that reduced vancomycin susceptibility (Sieradzki and Tomasz, 2003). The pbp2 upregulation promoted cell wall synthesis, while pbp4 downregulation resulted in a decrease in murein cross-linking that increased D-alanine-D-alanine production (Gardete and Tomasz, 2014). Importantly, mutations in different genes such as *yvqF* (Elsaghier et al., 2002), *vraSR* (Yoo et al., 2013), and *rpoB* were also associated with teicoplanin and vancomycin resistance (Watanabe et al., 2011). In the present investigation, we detected higher MICs of 16 μg/mL and 10 μg/mL for teicoplanin and vancomycin, respectively, which is in accordance with previous reports where teicoplanin and vancomycin MICs of 16 μg/mL and 2 μg/mL were reported for resistant isolates (Wang Y. et al., 2017). Previously, it was shown that the overexpression of *tcaA* in clinical strains decreased glycopeptide MICs while its inactivation resulted in glycopeptide resistance (Maki et al., 2004). Here, the data revealed that *S. aureus* suppressed the *tcaA* expression under the influence of COVID-19 infection and developed glycopeptide resistance. The results were derived from RNA-seq, RT-qPCR, and Western blot analysis that disclosed *tcaA* suppression and inactivation. The results were corroborated by creating a *tcaA* mutant which revealed that *tcaA* is solely responsible for the emergence of glycopeptide resistance and persistence. The *tcaA* persistence assay revealed a 10–11 fold and 13–14 fold increase in persister cells toward vancomycin and teicoplanin treatment, respectively. The *tcaA*-dependent persisters were not detected in azithromycin and ciprofloxacin challenge assays that indicated persisters might be dependent on specific gene function or dysfunction in a specific environment. We also confirmed that *tcaA* deletion did not affect azithromycin resistance whereas vancomycin and teicoplanin susceptibility was decreased as previously reported (Brandenberger et al., 2000; Maki et al., 2004). The study of Vogwill et al. (2016) investigated the coevolution of resistance and persistence to ciprofloxacin and rifampicin across the genus *Pseudomonas* and concluded that persistence correlates positively to antibiotic resistance across *Pseudomonas* strains (Vogwill et al., 2016). From theoretical and experimental analysis, Windels et al. (2019) proposed that persisters facilitated genetic resistance and increased survival and mutation rates that might affect the evolution of clinical resistance in *E. coli* (Windels et al., 2019). To date, *S. aureus* persisters increased with the decrease in intracellular ATP (Conlon et al., 2016), while decreasing upon deletion of the msaABCR operon (Pandey et al., 2021). Moreover, phenol-soluble modulin toxins expression reduced persisters in *S. aureus* (Baldry et al., 2020), and the expression of T7SS facilitated *S. aureus* survival in persistent infection. *S. aureus* T7SS is crucial for its virulence and the *essC* is thought to be a central membrane transporter (Burts et al., 2005; Jäger et al., 2018). Studies have shown that deletion of the entire T7SS or its components (EsxA, EssC, EsxB, EsaD, etc.) decreased *S. aureus* virulence (Burts et al., 2005, 2008; Anderson et al., 2017). EsxA and EsxB are the secretory proteins of T7SS and are required for establishing *S. aureus* infection in the host and the *EsxAB* mutants caused a decrease in abscess formation in mice (Burts et al., 2005). Currently, a high expression of *EsxAB* and a low expression of *essC* were detected that might facilitate *S. aureus* survival during host infection, however, the mechanism behind this phenomenon remained elusive. Even though the *EsxAB* genes are involved in *S. aureus* persistence and *essC* is required for establishing *S. aureus* infection during lung infection (Ishii et al., 2014), details of their altered chemistry in host infection need further investigation. From current data, we conclude that *tcaA* suppression and deletion induced persistence in *S. aureus*, and *glyS* and *sgtB* showed increased expression in the *tcaA* mutant strain that might indicate a cumulative approach toward cell wall protection. Although *glyS* and *sgtB* roles in cell wall synthesis in *S. aureus* are well defined (Wang et al., 2001; Schneider et al., 2004; Giannouli et al., 2009; Reed et al., 2015), their involvement in cell wall protection during host infection and persister cell formation remained obscure. Altogether, this is the first study to reveal persister cell formation due to *tcaA* inactivation, and conclude that the emergence of resistance might reflect the adaptation mechanism of persister cell genotype in future.

5. Conclusion

It is well known that *S. aureus* can change its fitness during infection to increase its survival in a hostile environment. In the present study, *S. aureus,* showing an intermediate level of glycopeptide resistance, was recovered from a COVID-19 patient. Genome analysis revealed a high expression of genes involved in cell wall biosynthesis and a low expression of virulence regulatory genes. The data summarize that *tcaA* inactivation gave rise to persisters that tolerated a high concentration of glycopeptides and resuscitated the bacterial population. This showed the involvement of genetic determinants in the development of persisters. Here, we conclude that if bacterial genes remain the main culprit of persister cell formation, then there would be concern regarding the potential spread of the persistence-associated genes. Although more remains to be explored concerning the genetic basis of persisters, our data will increase the understanding of the mechanism of persister cell formation during host infection.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

GH and HG: conceptualization and writing-original draft. GH, HG, MR, and AH: data curation and formal analysis. GH, HG, HE, PA, and IM: methodology and software. GH, MR, PA, HE, and IM: resources and funding acquisition. HG, MR and AH: supervision and project administration: AH, MR, HG, PA, HE, and IM: review and editing. All authors proofread the article and approved the current version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1241995/full#supplementary-material

References

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Nat. Prec.*:1. doi: 10.1038/npre.2010.4282.2

Anderson, M., Ohr, R. J., Aly, K. A., Nocadello, S., Kim, H. K., and Schneewind, C. E. (2017). EssE promotes *Staphylococcus aureus* ESS-dependent protein secretion to modify host immune responses during infection. *J. Bacteriol.* 199:e00527-16. doi: 10.1128/JB.00527-16

Appelbaum, P. (2006). The emergence of vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus*. *Clin. Microbiol. Infect.* 12, 16–23. doi: 10.1111/j.1469-0691.2006.01344.x

Aqib, A., and Rodriguez-Morales, A.J. (2021). *Insights into drug resistance in Staphylococcus aureus*. London. IntechOpen.

Bae, T., and Schneewind, O. (2006). Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid* 55, 58–63. doi: 10.1016/j.plasmid.2005.05.005

Bakthavatchalam, Y. D., Babu, P., Munusamy, E., Dwarakanathan, H. T., Rupali, P., Zervos, M., et al. (2019). Genomic insights on heterogeneous resistance to vancomycin and teicoplanin in methicillin-resistant *Staphylococcus aureus*: a first report from South India. *PLoS One* 14:e0227009. doi: 10.1371/journal.pone.0227009

Baldry, M., Bojer, M. S., Najarzadeh, Z., Vestergaard, M., Meyer, R. L., Otzen, D. E., et al. (2020). Phenol-soluble modulins modulate persister cell formation in *Staphylococcus aureus*. *Front. Microbiol.* 11:573253. doi: 10.3389/fmicb.2020.573253

Bao, Y., Li, Y., Jiang, Q., Zhao, L., Xue, T., Hu, B., et al. (2013). Methylthioadenosine/S-adenosylhomocysteine nucleosidase (Pfs) of *Staphylococcus aureus* is essential for the virulence independent of LuxS/AI-2 system. *Int. J. Med. Microbiol.* 303, 190–200. doi: 10.1016/j.ijmm.2013.03.004

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., and Piddock, L. J. (2015). Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* 13, 42–51. doi: 10.1038/nrmicro3380

Brandenberger, M., Tschierske, M., Giachino, P., Wada, A., and Berger-Bächi, B. (2000). Inactivation of a novel three-cistronic operon tcaR-tcaA-tcaB increases teicoplanin resistance in *Staphylococcus aureus*. *Biochim. Biophys. Acta* 1523, 135–139. doi: 10.1016/S0304-4165(00)00133-1

Brauner, A., Fridman, O., Gefen, O., and Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* 14, 320–330. doi: 10.1038/nrmicro.2016.34

Brückner, R. (1997). Gene replacement in Staphylococcus carnosus and *Staphylococcus xylosus*. *FEMS Microbiol. Lett.* 151, 1–8. doi: 10.1016/S0378-1097(97)00116-x

Brunet, F., Vedel, G., Dreyfus, F., Vaxelaire, J., Giraud, T., and Schremmer, B. (1990). Failure of teicoplanin therapy in two neutropenic patients with staphylococcal septicemia who recovered after administration of vancomycin. *Eur. J. Clin. Microbiol. Infect. Dis.* 9, 145–147. doi: 10.1007/BF01963643

Burts, M. L., DeDent, A. C., and Missiakas, D. M. (2008). EsaC substrate for the ESAT-6 secretion pathway and its role in persistent infections of *Staphylococcus aureus*. *Mol. Microbiol.* 69, 736–746. doi: 10.1111/j.1365-2958.2008.06324.x

Burts, M. L., Williams, W. A., DeBord, K., and Missiakas, D. M. (2005). EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. *Proc. Natl. Acad. Sci.* 102, 1169–1174. doi: 10.1073/pnas.0405620102

Cameron, D. R., Shan, Y., Zalis, E. A., Isabella, V., and Lewis, K. (2018). A genetic determinant of persister cell formation in bacterial pathogens. *J. Bacteriol.* 200:e00303-18. doi: 10.1128/JB.00303-18

Chang, S., Sievert, D. M., Hageman, J. C., Boulton, M. L., Tenover, F. C., Downes, F. P., et al. (2003). Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. *N. Engl. J. Med.* 348, 1342–1347. doi: 10.1056/NEJMoa025025

Chen, K.-Y., Chang, H.-J., Hsu, P.-C., Yang, C.-C., Chia, J.-H., Wu, T.-L., et al. (2013). Relationship of teicoplanin MICs to treatment failure in teicoplanin-treated patients with methicillin-resistant *Staphylococcus aureus* pneumonia. *J. Microbiol. Immunol. Infect.* 46, 210–216. doi: 10.1016/j.jmii.2012.06.010

Clinical and Laboratory Standards Institute (2018). "Performance standards for antimicrobial susceptibility testing" in *CLSI supplement M100. 28th edn.* (Wayne, PA, USA: Clinical and Laboratory Standards Institute). Available at: https://clsi.org/media/1930/m100ed28_sample.pdf

Conlon, B. P., Rowe, S. E., Gandt, A. B., Nuxoll, A. S., Donegan, N. P., Zalis, E. A., et al. (2016). Persister formation in *Staphylococcus aureus* is associated with ATP depletion. *Nat. Microbiol.* 1, 1–7. doi: 10.1038/nmicrobiol.2016.51

Corrigan, R. M., and Foster, T. J. (2009). An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. *Plasmid* 61, 126–129. doi: 10.1016/j.plasmid.2008.10.001

Cui, L., Iwamoto, A., Lian, J.-Q., Neoh, H. M., Maruyama, T., and Horikawa, Y. (2006). Novel mechanism of antibiotic resistance originating in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrob. agents chemother.* 50, 428–438. doi: 10.1128/AAC.50.2.428-438.2006

Eisenreich, W., Rudel, T., Heesemann, J., and Goebel, W. (2021). Persistence of intracellular bacterial pathogens—with a focus on the metabolic perspective. *Front. Cell. Infect. Microbiol.* 10:615450. doi: 10.3389/fcimb.2020.615450

Elsaghier, A. A., Aucken, H. M., Hamilton-Miller, J. M., Shaw, S., and Kibbler, C. C. (2002). Resistance to teicoplanin developing during treatment of methicillin-resistant *Staphylococcus aureus* infection. *J. Antimicrob. Chemother.* 49, 423–424. doi: 10.1093/jac/49.2.423

Gardete, S., and Tomasz, A. (2014). Mechanisms of vancomycin resistance in *Staphylococcus aureus*. *J. Clin. Invest.* 124, 2836–2840. doi: 10.1172/JCI68834

Ghanizadeh, A., Najafizade, M., Rashki, S., Marzhoseyni, Z., and Motallebi, M. (2021). Genetic diversity, antimicrobial resistance pattern, and biofilm formation in *Klebsiella pneumoniae* isolated from patients with coronavirus disease 2019 (COVID-19) and ventilator-associated pneumonia. *Biomed. Res. Int.* 2021, 1–11. doi: 10.1155/2021/2347872

Giannouli, S., Kyritsis, A., Malissovas, N., Becker, H. D., and Stathopoulos, C. (2009). On the role of an unusual tRNAGly isoacceptor in *Staphylococcus aureus*. *Biochimie* 91, 344–351. doi: 10.1016/j.biochi.2008.10.009

Habib, G., Mahmood, K., Ahmad, L., Gul, H., Hayat, A., and Rehman, M. U. (2023). Clinical manifestations of active tuberculosis patients coinfected with severe acute respiratory syndrome coronavirus-2. *J. Clin. Tuberc. Other Mycobact. Dis.* 31:100359. doi: 10.1016/j.jctube.2023.100359

Habib, G., Mahmood, K., Gul, H., Tariq, M., Ain, Q. U., Hayat, A., et al. (2022). Pathophysiology of methicillin-resistant *Staphylococcus aureus* superinfection in COVID-19 patients. *Pathophysiology* 29, 405–413. doi: 10.3390/pathophysiology29030032

Habib, G., Zhu, J., and Sun, B. (2020). A novel type I toxin-antitoxin system modulates persister cell formation in *Staphylococcus aureus*. *Int. J. Med. Microbiol.* 310:151400. doi: 10.1016/j.ijmm.2020.151400

Handwerger, S., and Tomasz, A. (1985). Antibiotic tolerance among clinical isolates of bacteria. *Annu. Rev. Pharmacol. Toxicol.* 25, 349–380. doi: 10.1146/annurev.pa.25.040185.002025

Hiramatsu, K. (2001). Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance. *Lancet Infect. Dis.* 1, 147–155. doi: 10.1016/S1473-3099(01)00091-3

Howden, B. P., Davies, J. K., Johnson, P. D., Stinear, T. P., and Grayson, M. L. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clin. Microbiol. Rev.* 23, 99–139. doi: 10.1128/CMR.00042-09

Howden, B. P., Stinear, T. P., Allen, D. L., Johnson, P. D., Ward, P. B., and Davies, J. K. (2008). Genomic analysis reveals a point mutation in the two-component sensor gene graS that leads to intermediate vancomycin resistance in clinical *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 52, 3755–3762. doi: 10.1128/AAC.01613-07

Hu, J., Zhang, X., Liu, X., Chen, C., and Sun, B. (2015). Mechanism of reduced vancomycin susceptibility conferred by walK mutation in community-acquired methicillin-resistant *Staphylococcus aureus* strain MW2. *Antimicrob. Agents Chemother.* 59, 1352–1355. doi: 10.1128/AAC.04290-14

Hu, Q., Peng, H., and Rao, X. (2016). Molecular events for promotion of vancomycin resistance in vancomycin intermediate *Staphylococcus aureus*. *Front. Microbiol.* 7:1601. doi: 10.3389/fmicb.2016.01601

Ishii, K., Adachi, T., Yasukawa, J., Suzuki, Y., Hamamoto, H., and Sekimizu, K. (2014). Induction of virulence gene expression in *Staphylococcus aureus* by pulmonary surfactant. *Infect. immun.* 82, 1500–1510. doi: 10.1128/IAI.01635-13

Jäger, F., Kneuper, H., and Palmer, T. (2018). EssC is a specificity determinant for *Staphylococcus aureus* type VII secretion. *Microbiology* 164, 816–820. doi: 10.1099/mic.0.000650

Jia, G., Rao, Z., Zhang, J., Li, Z., and Chen, F. (2013). Tetraether biomarker records from a loess-paleosol sequence in the western Chinese loess plateau. *Front. Microbiol.* 4:199. doi: 10.3389/fmicb.2013.00199

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., et al. (2007). KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* 36, D480–D484. doi: 10.1093/nar/gkm882

Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., and Hiramatsu, K. (2003). Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Mol. Microbiol.* 49, 807–821. doi: 10.1046/j.1365-2958.2003.03599.x

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., and Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science* 355, 826–830. doi: 10.1126/science.aaj2191

Levin, B. R., McCall, I. C., Perrot, V., Weiss, H., Ovesepian, A., and Baquero, F. (2017). A numbers game: ribosome densities, bacterial growth, and antibiotic-mediated stasis and death. *MBio* 8:e02253-16. doi: 10.1128/mBio.02253-16

Lewis, K. (2010). Persister cells. *Annu. Rev. Microbiol.* 64, 357–372. doi: 10.1146/annurev.micro.112408.134306

Lin, L., Wang, X., Wang, W., Zhou, X., and Hargreaves, J. R. (2020). Cleaning up China's medical cabinet—an antibiotic take-back programme to reduce household antibiotic storage for unsupervised use in rural China: a mixed-methods feasibility study. *Antibiotics* 9:212. doi: 10.3390/antibiotics9050212

Maki, H., McCallum, N., Bischoff, M., Wada, A., and Berger-Bächi, B. (2004). tcaA inactivation increases glycopeptide resistance in *Staphylococcus aureus*. *Antimicrob. agents chemother.* 48, 1953–1959. doi: 10.1128/AAC.48.6.1953-1959.2004

Mao, X., Cai, T., Olyarchuk, J. G., and Wei, L. (2005). Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics* 21, 3787–3793. doi: 10.1093/bioinformatics/bti430

Mohiuddin, S. G., Ghosh, S., Ngo, H. G., Sensenbach, S., Karki, P., Dewangan, N. K., et al. (2021). Cellular self-digestion and persistence in Bacteria. *Microorganisms* 9:2269. doi: 10.3390/microorganisms9112269

Pandey, S., Sahukhal, G. S., and Elasri, M. O. (2021). The msaABCR operon regulates persister formation by modulating energy metabolism in *Staphylococcus aureus*. *Front. Microbiol.* 12:657753. doi: 10.3389/fmicb.2021.657753

Personnic, N., Doublet, P., and Jarraud, S. (2023). Intracellular persister: a stealth agent recalcitrant to antibiotics. *Front. Cell. Infect. Microbiol.* 13:1141868. doi: 10.3389/fcimb.2023.1141868

Reed, P., Atilano, M. L., Alves, R., Hoiczyk, E., Sher, X., Reichmann, N. T., et al. (2015). *Staphylococcus aureus* survives with a minimal peptidoglycan synthesis machine but sacrifices virulence and antibiotic resistance. *PLoS Pathog.* 11:e1004891. doi: 10.1371/journal.ppat.1004891

Renzoni, A., Kelley, W. L., Barras, C., Monod, A., Huggler, E., and François, P. (2009). Identification by genomic and genetic analysis of two new genes playing a key role in intermediate glycopeptide resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 53, 903–911. doi: 10.1128/AAC.01287-08

Schneider, T., Senn, M. M., Berger-Bächi, B., Tossi, A., Sahl, H. G., and Wiedemann, I. (2004). In vitro assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly5) of *Staphylococcus aureus*. *Mol. Microbiol.* 53, 675–685. doi: 10.1111/j.1365-2958.2004.04149.x

Schulthess, B., Meier, S., Homerova, D., Goerke, C., Wolz, C., Kormanec, J., et al. (2009). Functional characterization of the σB-dependent yabJ-spoVG operon in *Staphylococcus aureus*: role in methicillin and glycopeptide resistance. *Antimicrob. Agents Chemother.* 53, 1832–1839. doi: 10.1128/AAC.01255-08

Shang, Y., Wang, X., Chen, Z., Lyu, Z., Lin, Z., Zheng, J., et al. (2020). *Staphylococcus aureus* PhoU homologs regulate persister formation and virulence. *Front. Microbiol.* 11:865. doi: 10.3389/fmicb.2020.00865

Shariati, A., Dadashi, M., Moghadam, M. T., van Belkum, A., Yaslianifard, S., and Darban-Sarokhalil, D. (2020). Global prevalence and distribution of vancomycin resistant, vancomycin intermediate and heterogeneously vancomycin intermediate *Staphylococcus aureus* clinical isolates: a systematic review and meta-analysis. *Sci. Rep.* 10:12689. doi: 10.1038/s41598-020-69058-z

Sieradzki, K., and Tomasz, A. (2003). Alterations of Cell Wall structure and MetabolismAccompany reduced susceptibility to vancomycin in an IsogenicSeries of clinical isolates of Staphylococcusaureus. *J. Bacteriol.* 185, 7103–7110. doi: 10.1128/JB.185.24.7103-7110.2003

Sieradzki, K., Villari, P., and Tomasz, A. (1998). Decreased susceptibilities to teicoplanin and vancomycin among coagulase-negative methicillin-resistant clinical isolates of staphylococci. *Antimicrob. Agents Chemother.* 42, 100–107. doi: 10.1128/AAC.42.1.100

Song, K.-H., Kim, M., Kim, C. J., Cho, J. E., Choi, Y. J., Park, J. S., et al. (2017). Impact of vancomycin MIC on treatment outcomes in invasive *Staphylococcus aureus* infections. *Antimicrob. Agents Chemother.* 61, e01845–e01816. doi: 10.1128/AAC.01845-16

Szymanek-Majchrzak, K., Mlynarczyk, A., and Mlynarczyk, G. (2018). Characteristics of glycopeptide-resistant *Staphylococcus aureus* strains isolated from inpatients of three teaching hospitals in Warsaw, Poland. *Antimicrob. Resist. Infect. Control* 7, 1–6. doi: 10.1186/s13756-018-0397-y

Tariq, F. N., Shafiq, M., Khawar, N., Habib, G., Gul, H., Hayat, A., et al. (2023). The functional repertoire of AmpR in the AmpC β-lactamase high expression and decreasing

β-lactam and aminoglycosides resistance in ESBL *Citrobacter freundii*. *Heliyon* 9:e19486. doi: 10.1016/j.heliyon.2023.e19486

Utaida, S., Dunman, P., Macapagal, D., Murphy, E., Projan, S., and Singh, V. (2003). Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. *Microbiology* 149, 2719–2732. doi: 10.1099/mic.0.26426-0

Valihrach, L., and Demnerova, K. (2012). Impact of normalization method on experimental outcome using RT-qPCR in *Staphylococcus aureus*. *J. Microbiol. Methods* 90, 214–216. doi: 10.1016/j.mimet.2012.05.008

Vogwill, T., Comfort, A., Furió, V., and MacLean, R. (2016). Persistence and resistance as complementary bacterial adaptations to antibiotics. *J. Evol. Biol.* 29, 1223–1233. doi: 10.1111/jeb.12864

Wang, Q., Lu, F., and Lan, R. (2017). RNA-sequencing dissects the transcriptome of polyploid cancer cells that are resistant to combined treatments of cisplatin with paclitaxel and docetaxel. *Mol. BioSyst.* 13, 2125–2134. doi: 10.1039/C7MB00334J

Wang, Q. M., Peery, R. B., Johnson, R. B., Alborn, W. E., Yeh, W.-K., and Skatrud, P. L. (2001). Identification and characterization of a monofunctional glycosyltransferase from *Staphylococcus aureus*. *J. Bacteriol.* 183, 4779–4785. doi: 10.1128/JB.183.16.4779-4785.2001

Wang, W., Chen, J., Chen, G., Du, X., Cui, P., and Wu, J. (2015). Transposon mutagenesis identifies novel genes associated with *Staphylococcus aureus* persister formation. *Front. Microbiol.* 6:1437. doi: 10.3389/fmicb.2015.01437

Wang, Y., Bojer, M. S., George, S. E., Wang, Z., Jensen, P. R., Wolz, C., et al. (2018). Inactivation of TCA cycle enhances *Staphylococcus aureus* persister cell formation in stationary phase. *Sci. Rep.* 8:10849. doi: 10.1038/s41598-018-29123-0

Wang, Y., Li, X., Jiang, L., Han, W., Xie, X., Jin, Y., et al. (2017). Novel mutation sites in the development of vancomycin-intermediate resistance in *Staphylococcus aureus*. *Front. Microbiol.* 7:2163. doi: 10.3389/fmicb.2016.02163

Watanabe, Y., Cui, L., Katayama, Y., Kozue, K., and Hiramatsu, K. (2011). Impact of rpoB mutations on reduced vancomycin susceptibility in *Staphylococcus aureus*. *J. Clin. Microbiol.* 49, 2680–2684. doi: 10.1128/JCM.02144-10

Windels, E. M., Michiels, J. E., Fauvart, M., Wenseleers, T., Van den Bergh, B., and Michiels, J. (2019). Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. *ISME J.* 13, 1239–1251. doi: 10.1038/s41396-019-0344-9

Wu, Q., Sabokroo, N., Wang, Y., Hashemian, M., Karamollahi, S., and Kouhsari, E. (2021). Systematic review and meta-analysis of the epidemiology of vancomycin-resistance *Staphylococcus aureus* isolates. *Antimicrob. Resist. Infect. Control* 10, 1–13. doi: 10.1186/s13756-021-00967-y

Yoo, J. I., Kim, J. W., Kang, G. S., Kim, H. S., Yoo, J. S., and Lee, Y. S. (2013). Prevalence of amino acid changes in the yvqF, vraSR, graSR, and tcaRAB genes from vancomycin intermediate resistant *Staphylococcus aureus*. *J. Microbiol.* 51, 160–165. doi: 10.1007/s12275-013-3088-7

Young, M. D., Wakefield, M. J., Smyth, G. K., and Oshlack, A. (2010). Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol.* 11, R14–R12. doi: 10.1186/gb-2010-11-2-r14

Zhao, C., Shu, X., and Sun, B. (2017). Construction of a gene knockdown system based on catalytically inactive ("dead") Cas9 (dCas9) in *Staphylococcus aureus*. *Appl. Environ. Microbiol.* 83, e00291–e00217. doi: 10.1128/AEM.00291-17

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Comparative analysis of the biological characteristics and mechanisms of azole resistance of clinical *Aspergillus fumigatus* strains

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Aspergillus fumigatus is a common causative pathogen of aspergillosis. At present, triazole resistance of *A. fumigatus* poses an important challenge to human health globally. In this study, the biological characteristics and mechanisms of azole resistance of five *A. fumigatus* strains (AF1, AF2, AF4, AF5, and AF8) were explored. There were notable differences in the sporulation and biofilm formation abilities of the five test strains as compared to the standard strain AF293. The ability of strain AF1 to avoid phagocytosis by MH-S cells was significantly decreased as compared to strain AF293, while that of strains AF2, AF4, and AF5 were significantly increased. Fungal burden analysis with *Galleria mellonella* larvae revealed differences in pathogenicity among the five strains. Moreover, the broth microdilution and *E*-test assays confirmed that strains AF1 and AF2 were resistant to itraconazole and isaconazole, while strains AF4, AF5, and AF8 were resistant to voriconazole and isaconazole. Strains AF1 and AF2 carried the *cyp51A* mutations TR34/L98H/V242I/S297T/F495I combined with the *hmg1* mutation S541G, whereas strains AF4 and AF8 carried the *cyp51A* mutation TR46/Y121F/V242I/T289A, while strain AF5 had no *cyp51A* mutation. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis revealed differences in the expression levels of genes associated with ergosterol synthesis and efflux pumps among the five strains. In addition, transcriptomics, RT-qPCR, and the NAD^+/NADH ratio demonstrated that the mechanism of voriconazole resistance of strain AF5 was related to overexpression of genes associated with energy production and efflux pumps. These findings will help to further elucidate the triazole resistance mechanism in *A. fumigatus*.

KEYWORDS

biological characteristics, azole resistance, virulence, *cyp51A*, *Aspergillus fumigatus*

Introduction

Aspergillus fumigatus is a saprophytic fungus that widely exists in hospitals, gardens, and fields (Burks et al., 2021; Zhou et al., 2022), and the most common filamentous pathogenic fungus encountered in clinical practice, followed by *A. flavus*, *A. niger*, and *A. terreus* (Cadena et al., 2021). Aspergillosis is a spectrum of infections typically caused by *A. fumigatus*, which is traditionally classified as allergic bronchitis pulmonary aspergillosis (ABPA) (Eraso et al., 2020), chronic pulmonary aspergillosis (CPA) (Sehgal et al., 2020), and invasive aspergillosis (IA) (Snelders et al., 2011). ABPA is a hypersensitive lung disease that mainly occurs in patients with asthma and cystic fibrosis (El-Baba et al., 2020). CPA usually occurs in immunocompetent individuals with lung diseases, such as cavitary pulmonary tuberculosis, chronic obstructive pulmonary disease, and sarcoidosis (Smith and Denning, 2011). Pulmonary aspergillus disease is commonly encountered in clinical practice. IA is the most severe form of pulmonary aspergillus with a mortality rate exceeding 50% (Brown et al., 2012). Recent global estimates have shown that aspergillosis accounts for about 5 million cases of ABPA (Agarwal et al., 2020), 3 million cases of CPA, and one-quarter million cases of IA annually (Bongomin et al., 2017).

At present, antifungal drugs for the treatment of aspergillosis mainly include azoles, echinocandins, and polyenes (Galiger et al., 2013). Azoles, the most commonly used first-line drugs against aspergillosis, include isavuconazole (ISA), itraconazole (ITR), posaconazole (POS), and voriconazole (VRC). Cyp51 (cytochrome P450 family 51 subfamily A member 1) is an enzyme encoded by *cyp51A* and *cyp51B*, which plays an important role in sterol biosynthesis. By binding with Cyp51, azoles inhibit the synthesis of ergosterol on the fungal cell membrane, thereby inhibiting synthesis of sterol 14α-demethylase as an antifungal effect (Mellado et al., 2001; Parker et al., 2014; Peyton et al., 2015). At present, the high death rate of aspergillosis is mainly due to the limited choice of antifungal drugs and the increasing rate of resistance of clinical *A. fumigatus* strains. With the exception of Antarctica, azole-resistant *A. fumigatus* has been reported worldwide (Burks et al., 2021). According to data released from Netherlands, the prevalence of azole-resistant *A. fumigatus* increased from 0.79% in 1996–2001 to 11.30% in 2013–2018 (Buil et al., 2019; Lestrade et al., 2020). Recently, 21 research centers worldwide reported that the incidence of triazole-resistant *A. fumigatus* ranged from 3.2 to 26.1% (van der Linden et al., 2015), while the prevalence of *Aspergillus* is increasing in some parts of the world (Yang et al., 2021). The increased prevalence of drug-resistant *A. fumigatus* has led to increased treatment failure and healthcare costs, thus, emerging as public health concern globally. An insertion mutation of a tandem repeat sequence in the promoter region of *cyp51A* was confirmed to change to the amino acid coding sequence resulting in resistance to azoles (Snelders et al., 2010). The resulting mutations mainly include TR34/L98, TR46/Y121F/T289A, TR53, and TR120 (Alvarez-Moreno et al., 2017; Hare et al., 2019). Of these mutations, TR34/L98 and TR46/Y121F/T289A are the most common in clinical strains. Additional mutations to the Cyp51A amino acid coding sites include G54, Y121, G138, P216, F219, M220, A284, Y431, G432, G434, and G448 (Pérez-Cantero et al., 2020). Besides changes to the amino acid sequence, overexpression of genes associated with drug efflux pumps has been implicated in azole resistance of *A. fumigatus*. The adenosine triphosphate (ATP)-binding cassette (ABC) efflux pump and major facilitator superfamily (MFS) transporters play key roles in drug efflux. A recent investigation found that the *A. fumigatus* genome has 49 and 278 genes coding for proteins that comprise ABC efflux pumps and MFS transporters, respectively (Arastehfar et al., 2021). However, relatively few genes coding for drug efflux pumps have been confirmed, as most other members are redundant. Nonetheless, the mechanisms underlying *A. fumigatus* resistance to azoles remain unclear.

In this study, five strains of *A. fumigatus* (AF1, AF2, AF4, AF5, and AF8) were identified by morphological and molecular analyses. In addition, the biological characteristics of sporulation, biofilm formation, evasion of phagocytosis, virulence, and drug sensitivity of the five strains were investigated. Sanger sequencing, three-dimensional (3D) protein construction, high-performance liquid chromatography (HPLC), and real-time quantitative polymerase chain reaction (RT-qPCR) were used to clarify the mechanisms underlying drug resistance of the five strains. Furthermore, transcriptomics, RT-qPCR, and biochemical analyses were employed to explore the specific drug resistance mechanism of the AF5 strain.

Materials and methods

Strains and cell line

In total, 45 *A. fumigatus* strains from different clinical specimens were collected from May 2019 to April 2021. The patient information related to these strains in the study was showed in **Supplementary Table 1**. The AF293 strain was used as a control. All strains were stored in sterile ddH_2O at room temperature (Hartung de Capriles et al., 1989). Prior to analysis, the strains were cultured on PDA at 37°C for 3–7 days. Mouse alveolar macrophages (MH-S cells) were donated by Professor Jia Jing of the Affiliated Hospital of Southwest Medical University (Luzhou, China).

Identification of strains and spot assay

The AF1, AF2, AF4, AF5, AF8, and AF293 strains were cultured on PDA at 37°C for 5 days. Colonies were selected and stained with lactophenol cotton blue stain solution. Morphological characteristics of conidia, conidiophores, and mycelia were observed under a microscope.

Each strain was cultured in Roswell Park Memorial Institute (RPMI) 1640 liquid medium (Cytiva, Marlborough, MA, USA) at 37°C for 48 h. Afterward, the mycelia were collected. Genomic DNA was extracted using a commercial DNA extraction kit (Sangon Biotech Co. Ltd., Shanghai, China) in accordance with the manufacturer's instructions. The internal transcribed spacer (*ITS*) sequences of the test strains were amplified by RT-qPCR with the primers listed in **Supplementary Table 2**. The Sanger sequencing results were compared with the Basic Local

Alignment Search Tool.[1] An evolutionary tree of the *ITS* sequences was generated with Molecular Evolutionary Genetics Analysis software.[2]

Conidia of *A. fumigatus*, cultured for 5 days, were eluted with 0.05% Tween 80 and diluted to 1×10^6 cells/mL. Then, 3 μL of diluted conidia were spotted in the center of PDA plates (diameter, 9 cm) and incubated at 37°C for 36 and 120 h, respectively. Then, all conidia were eluted with 0.05% Tween 80 in sterile water and counted under a light microscope (magnification, 400 ×).

Observation and detection of biofilm formation

The biofilm formation assay was performed as described in a previous report (Zhang et al., 2022). Briefly, 2 mL of conidia (1.0×10^5 cells/mL) were added to the wells of a 12-well cell culture plate, covered with round coverslips, and cultured at 37°C for 24 h. Then, the unattached spores and mycelia were washed three times with PBS. Afterward, 1 mL of calcofluor white stain (CFW) (Beijing Solarbio Science & Technology Co., Ltd.) and 20 μL of 10% KOH were added to each well and the plate was incubated at room temperature for 3–5 min. Biofilm formation was observed with a confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan).

To further confirm the biofilm formation ability of the test strains, 2 mL of spore suspensions (1.0×10^5 cells/mL) were added into the wells of 12-well cell culture plates and cultured at 37°C for 24 h. Afterward, the unattached spores and mycelia were washed three times with PBS. Then, 200 μL of a mixture of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide sodium salt and menadione were added to each well and the plate was incubated at 37°C for 2 h in the dark. Finally, the supernatant was transferred to the wells of a new 96-well plate and absorbance was measured at 490 nm.

Phagocytosis assay

The phagocytosis assay was performed as previously described (Lim et al., 2022). Briefly, MH-S cells were cultured in complete Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Then, 1 mL of the cultured MH-S cells (1×10^5 cells/mL) was added to each well of a 12-well plate, covered with round coverslips, and cultured overnight at 37°C under an atmosphere of 5% CO_2/95% air. Afterward, 1 mL of conidia (1×10^6 cells/mL) was added to each well and the plate was cultured for 2 h under an atmosphere of 5% CO_2/95% air. The 12-well plate was washed three times with PBS pre-cooled at 4°C. Then, the round coverslips were stained with 50 μg/mL of CFW and observed under a microscope. The phagocytosis percentage (Eq. 1) and phagocytosis index (Eq. 2) were calculated in reference to the microscope images and repeated independently three times. In total, 200 MH-S cells were randomly counted.

Phagocytosis rate = number of MH-S cells of phagocytizing conidia/200 × 100% (Eq. 1).

Phagocytosis index = total number of phagocytic conidia/number of MH-S cells of phagocytizing conidia (Eq. 2).

Infection of the *Galleria mellonella*

G. mellonella larvae (n = 10/group) were injected with 10 μL of the test strain in suspension through the last pro-leg into the hemocoel using a 50-μL microinjector (Shanghai Gaoge Industry and Trade Co., Ltd., Shanghai, China) (Özkan and Coutts, 2015). Untreated, pierced only, and PBS-injected groups were used as controls. The experimental groups were injected with 10 μL of conidia (1×10^7 cells/mL). After injection, each group of larvae was divided into two petri dishes and cultured at 37°C in the dark. The larvae were photographed and morphological changes were recorded every 24 h. Each experiment was repeated three times.

Different groups of infected larvae were sacrificed at 24 or 72 h after infection. The whole larvae were macerated in microtubes with 1 mL of ddH_2O. Afterward, 50 μL of the dilution were seeded on PDA medium and the plates were incubated at 37°C. Each experiment was repeated three times.

Antifungal susceptibility testing

Drug sensitivity of the five strains was determined in accordance with Clinical and Laboratory Standards Institute (CLSI) document M38-A2—Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi (Wayne, 2008). The antifungal agents were used at the following concentrations: fluconazole (FLC), 64–0.0625 μg/mL; anidulafungin (ANI), amphotericin B (AmB), caspofungin (CAS), ISA, micafungin (MF), POS, and VRC, 32–0.03125 μg/mL. The conidia (1.0×10^5 cells/mL) were dispensed into triplicate wells of 96-well microtiter plates and incubated at 37°C for 48 h. Conidia in drug-free wells were used as growth controls. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentrations that caused complete visible inhibition of growth after incubation at 37°C for 48 h. The minimum effective concentration (MEC) was defined as the lowest drug concentration causing the mycelia to become shorter and thicker, as observed under a microscope, after incubation at 37°C for 48 h (Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing, 2008).

For the strip tests, the conidia (1.0×10^6 cells/mL) were evenly coated on RPMI 1640 solid medium using cotton swabs. Once the plate was dry, an *E*-test strip (Liofilchem s.r.l., Roseto degli Abruzzi, Italy) was attached to the center of the medium with sterile tweezers. The results were read after incubation at 37°C for 24 or 48 h. The MIC was determined in reference to the scale at the intersection of the inhibition zone and the *E*-test strip. For both methods, strain AF293 was used for quality control. Each experiment was repeated three times.

1 https://blast.ncbi.nlm.nih.gov/Blast.cgi

2 https://www.megasoftware.net/

Sequencing of *cyp51A*, *cyp51B*, and *hmg1*

Sanger sequencing of *cyp51A*, *cyp51B*, and *hmg1*, in addition to the promoter region of *cyp51A*, was conducted. The sequences were compared using DNAMAN software (Lynnon Biosoft, San Ramon, CA, USA) to identify short tandem repeats and amino acid mutations. The 3D structures of the Cyp51A proteins of the test strains were generated and analyzed with Discovery Studio software (BIOVIA, San Diego, CA, USA).

Analysis of ergosterol content

The ergosterol content was determined by HPLC (1260 infinity II; Agilent Technologies, Inc., Santa Clara, CA, USA) as described in a previous report (Zhang et al., 2022). Briefly, conidia (1×10^6 cells/mL) were inoculated into 50 mL of RPMI 1640 medium and cultured at 37°C for 48 h at 200 rpm. The samples were exposed to ultrasonic waves at 40 kHz. Following the addition of methanol (w/w), the supernatant was collected by centrifugation as the sample to be tested. An ergosterol standard (Shanghai Macklin Biochemical Co., Ltd., Shanghai, China) was used as a reference and 100% methanol as a negative control. The concentration of ergosterol = (peak area of experimental group × concentration of ergosterol standard group)/peak area of ergosterol standard product. Each experiment was repeated three times.

RT-qPCR and transcriptomic analysis

Conidia (1.0×10^6 cells/mL) of the test strain were inoculated into RPMI 1640 medium and cultured at 37°C for 16 h at 200 rpm, and then co-cultured with or without drugs for 0, 4, 8, or 12 h. The mycelia were collected and frozen in liquid nitrogen until assayed. Total RNA extraction, reverse transcription, and RT-qPCR reactions were performed using commercial kits (Takara Bio, Inc., Shiga, Japan) in accordance with the manufacturer's instructions. Transcript levels were determined by a ABI 7500Fast Real-Time PCR Detection System (Thermo Fisher Scientific, Waltham, MA, USA) and measured with the $2^{-\Delta\Delta CT}$ method against β-*tubulin* as an internal control (Vandesompele et al., 2002).

To further clarify the azole resistance mechanism, conidia of strain AF5 prepared in RPMI 1640 medium were treated with or without VRC (MIC = 0.5) and incubated at 37°C for 8 h. Total RNA was collected and sequenced by Biomarker Technologies (Qingdao, China) with an Illumina RNA sequencing system (Illumina, Inc., San Diego, CA, USA). Then, quality control detection of the raw sequence data was performed on the Biomarker platform.[3] Sequencing alignment was performed using the Hisat2 (version 2.2.1, and the reference genome is *Aspergillus_fumigatus*.GCF_000002655.1_ASM265v1.genome.fa). After that the samtools (version 1.9) was used to sort the sam files to bam files, and then matched Reads were assembled and quantified using the StringTie tool (version 1.3.4d). The tool for differentially expressed genes (DEGs) analysis is DESeq2 (version 1.6.3). Further gene function annotation was included COG (Cluster of Orthologous Groups of proteins), KO (Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog database), and GO (Gene Ontology). The time-specific expression patterns of genes were determined by RT-qPCR analysis. Raw sequence data were deposited in the Genome Sequence Archive of the Beijing Institute of Genomics (Chinese Academy of Sciences, Beijing, China; accession number CRA011198).

3 www.biocloud.net

Determination of nicotinamide adenine dinucleotide (NAD^+) and NADH contents

The contents of NAD^+ and NADH were measured using a commercial kit (Beyotime Institute of Biotechnology, Shanghai, China) as described in a previous report (Hsieh et al., 2020). Briefly, the conidia (1.0×10^6 cells/mL) of strain AF5 were inoculated into RPMI 1640 medium and cultured at 37°C for 16 h, and then co-cultured with VRC (MIC = 0.5) for an additional 0, 4, 8, or 12 h. Afterward, the mycelia were collected and frozen in liquid nitrogen until assayed. Following the addition of working solution, the supernatant of each sample was collected and divided into two aliquots. One aliquot was directly transferred to the wells of a 96-well plate and the other was incubated at 60°C for 30 min to decompose NAD^+ into NADH, and then transferred to the wells of a 96-well plate to determine the intracellular content of NADH.

Statistical analysis

Data analyses were conducted using GraphPad Prism software v9.0 (GraphPad Software, Inc., San Diego, CA, USA). Group comparisons were performed with the Student's *t*-test or one-way analysis of variance. Mortality curves were generated by the Kaplan–Meier method and compared with the log-rank test. Each experiment was repeated three times. A probability (p) value < 0.05 was considered statistically significant.

Results

Identification of strains and comparisons of sporulation ability

The conidiophores of *Aspergillus* strains AF1, AF2, AF4, AF5, and AF8 were stained with lactophenol cotton blue stain solution and observed under a microscope (**Figure 1A**). Phylogenetic tree analysis of the *ITS* sequences revealed that all five test strains (AF1, AF2, AF4, AF5, and AF8) were clustered with strain AF293 (**Figure 1B**), confirming that all five test strains were *A. fumigatus*.

All five test strains were cultured on PDA medium for 5 days. As compared to strain AF293, there were no significant differences in the colony color and morphology of the five test strains (**Figure 1C**). As compared to strain AF293, the conidia yields of strains AF1 and AF2 were significantly increased by incubation for 36 and 120 h, while there was no significant difference among strains AF4, AF5, and AF8 (**Figures 1D, E**).

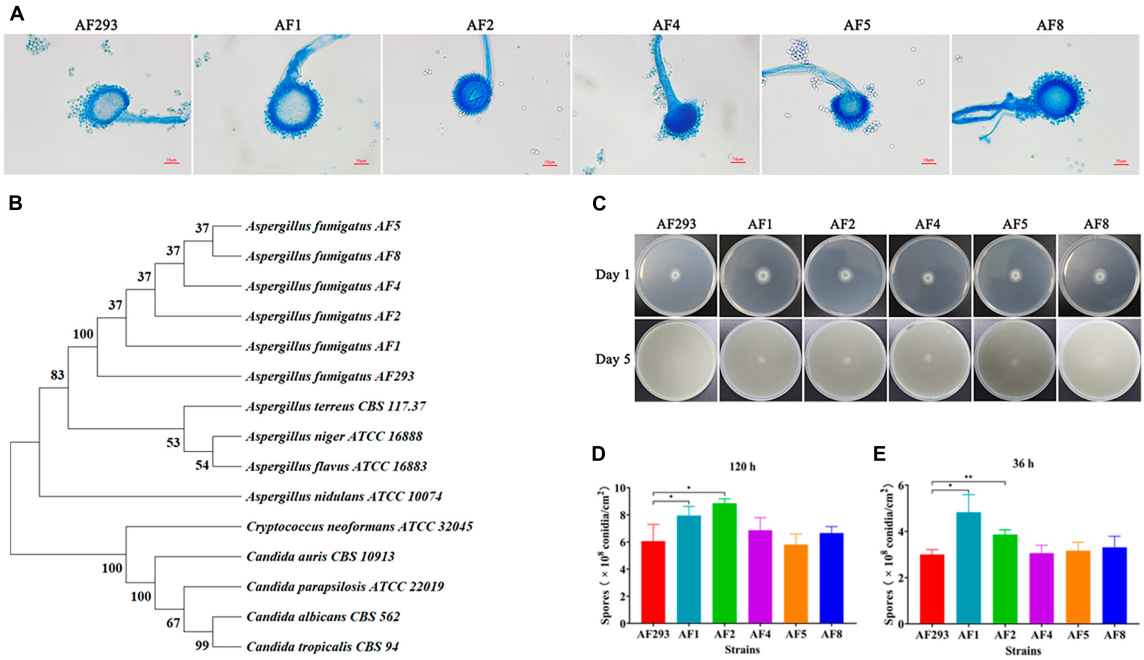

FIGURE 1
Identification of isolates and analysis of sporulation ability. **(A)** Morphological observation of conidiophores (magnification, 40 ×). Scale bar = 10 μm. **(B)** Phylogenetic analysis of ITS sequences. **(C)** Observations of colony color and morphology. Test strains were cultured on PDA medium at 37°C. **(D,E)** Sporulation statistics at 36 and 120 h. *$p < 0.05$ and **$p < 0.01$ vs. strain AF293.

Differences in biofilm formation and evasion of phagocytosis among the test strains

To explore the differences biofilm formation ability among test strains, as compared to strain AF293, strains AF1, AF2, AF4, AF5, and AF8 formed more and denser biofilms (Figure 2A), as confirmed by significantly increased optical density at 490 nm (Figure 2B).

To explore the interaction between the *A. fumigatus* conidia and MH-S cells, the conidia of the test strains were phagocytosed by MH-S cells after co-culture for 2 h, as observed with a fluorescence microscope (Figure 3A). As compared to strain AF293, phagocytosis of the conidia of strains AF2, AF4, AF5, and AF8 was significantly increased, while that of strain AF1 conidia was significantly decreased (Figure 3B). Further phagocytic index analysis, representing the average number of conidia phagocytosed per MH-S cell, showed that the phagocytosis index of MH-S cells to strain AF1 was significantly decreased, while that of strain AF4 was significantly increased (Figure 3C). These results indicate enhanced ability of strain AF1 to avoid phagocytosis, while the ability of the other strains to avoid phagocytosis was decreased.

Differences in virulence among the test strains

The *G. mellonella* larvae infection models were subsequently opted to explore variations in virulence among test strains. *G. mellonella* larvae infected with *A. fumigatus* conidia begin to melanize and die after 24 h. However, there was no significant correlation between melanization and strains (Supplementary Figure 1). As compared to strain AF293, the larvae survival rate was significantly decreased in the AF1, AF2, AF4, and AF8 model groups at 120 h after inoculation with 10^7 cells/mL (Figure 4A). The fungal burden was significantly decreased in the AF1 and AF4 groups at 24 h (Figure 4B). At 72 h, the fungal burden was significantly decreased in the AF1 and AF5 groups, while significantly increased in the AF2 and AF8 groups (Figure 4C).

Susceptibility to AmB, echinocandins, and triazoles

The MIC_{90} and MEC of azoles, AmB, and polyenes against the test strains are shown in Table 1. All test strains were resistant to FLC, as determined by the broth microdilution method, while strains AF1 and AF2 were resistant to ITR and ISA, and sensitive to AmB, ANI, CAS, MF, POS, and VRC. Meanwhile, strains AF4, AF5, and AF8 were resistant to VRC and ISA, and sensitive to AmB, ANI, CAS, MF, ITR, and POS. In addition, the *E*-test results were consistent with the results of the broth microdilution method (Supplementary Figure 2).

cyp51A-mediated mechanism of azoles resistance

Sanger sequencing and comparison analysis showed that strains AF1 and AF2 carried the *cyp51A* mutations

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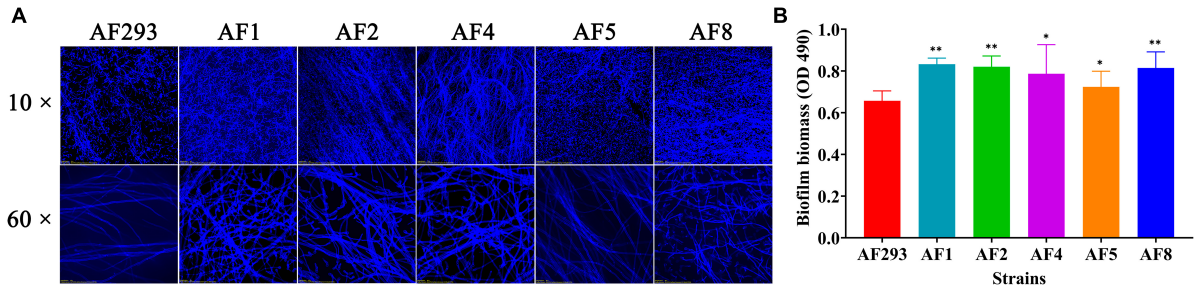

FIGURE 2
Biofilm formation ability of the test strains. The test strains were cultured at 37°C for 24 h. **(A)** Biofilm formation of the test strains after staining with CFW was observed with a confocal laser scanning microscope. Scale bar = 100 μm (magnification, 10 ×) or 20 μm (magnification, 60 ×). **(B)** Quantification of biofilm formation of the test strains using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide sodium salt assay. *$p < 0.05$ and **$p < 0.01$ vs. strain AF293.

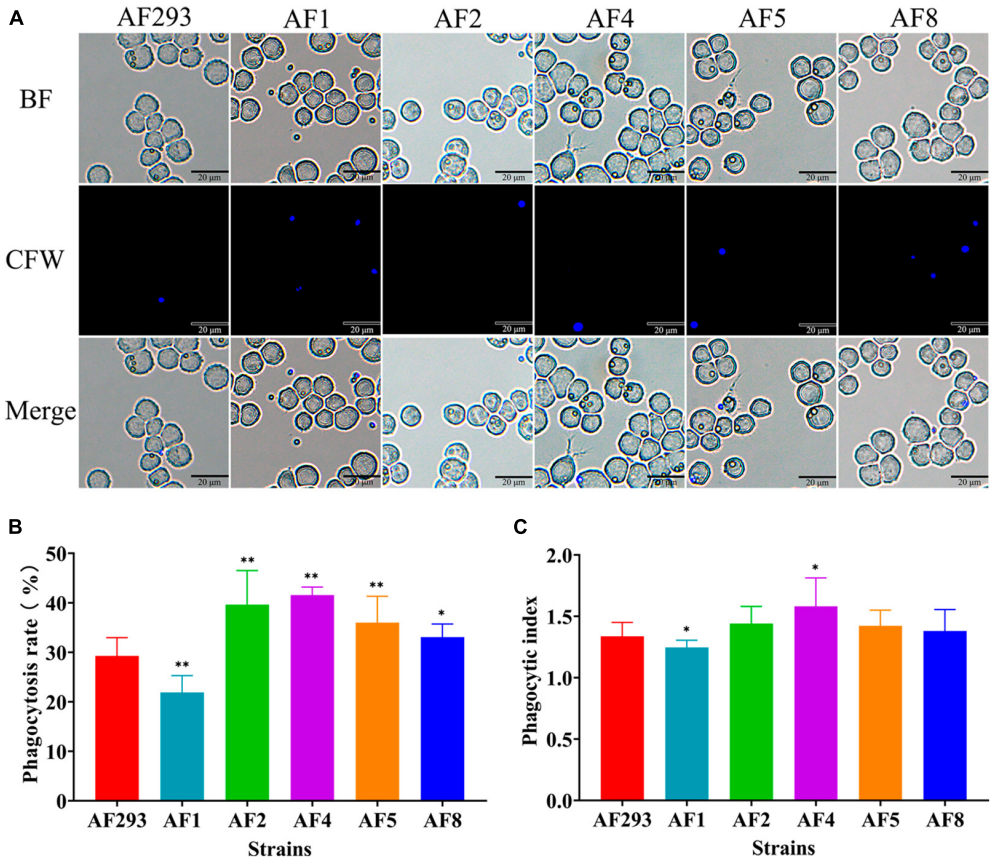

FIGURE 3
Comparative analysis of anti-phagocytosis ability of the test strains. MH-S cells were challenged with fivefold *A. fumigatus* conidia and then incubated for at 37°C for 2 h under an atmosphere of 5% CO_2/95% air. **(A)** Phagocytosis to *A. fumigatus* conidia by MH-S cells was observed with a fluorescence microscope. Scale bar = 20 μm. **(B)** Statistical analysis of the phagocytosis rate. **(C)** Statistical analysis of the phagocytic index. *$p < 0.05$ and **$p < 0.01$ vs. strain AF293.

TR34/L98H/S297T/F495I, strains AF4 and AF8 carried the *cyp51A* mutations TR46/Y121F/T289A, and strain AF5 carried no mutation (data not shown). Further analysis with Discovery Studio software showed that there was no difference in the 3D structure of Cyp51A between strains AF293 and AF5. Moreover, although there were small spatial differences in the branch chain of Cyp51A among the other four strains, the spatial structure of the main chain conformation was consistent with that of strain AF293 (Supplementary Figure 3). HPLC analysis found that there was no significant difference in ergosterol content (data not shown). In addition, Sanger sequencing and comparison analysis showed that none of the five test strains carried a *cyp51B* mutation, while strains AF1 and AF2 carried the *hmg1* mutation S541G (data not shown).

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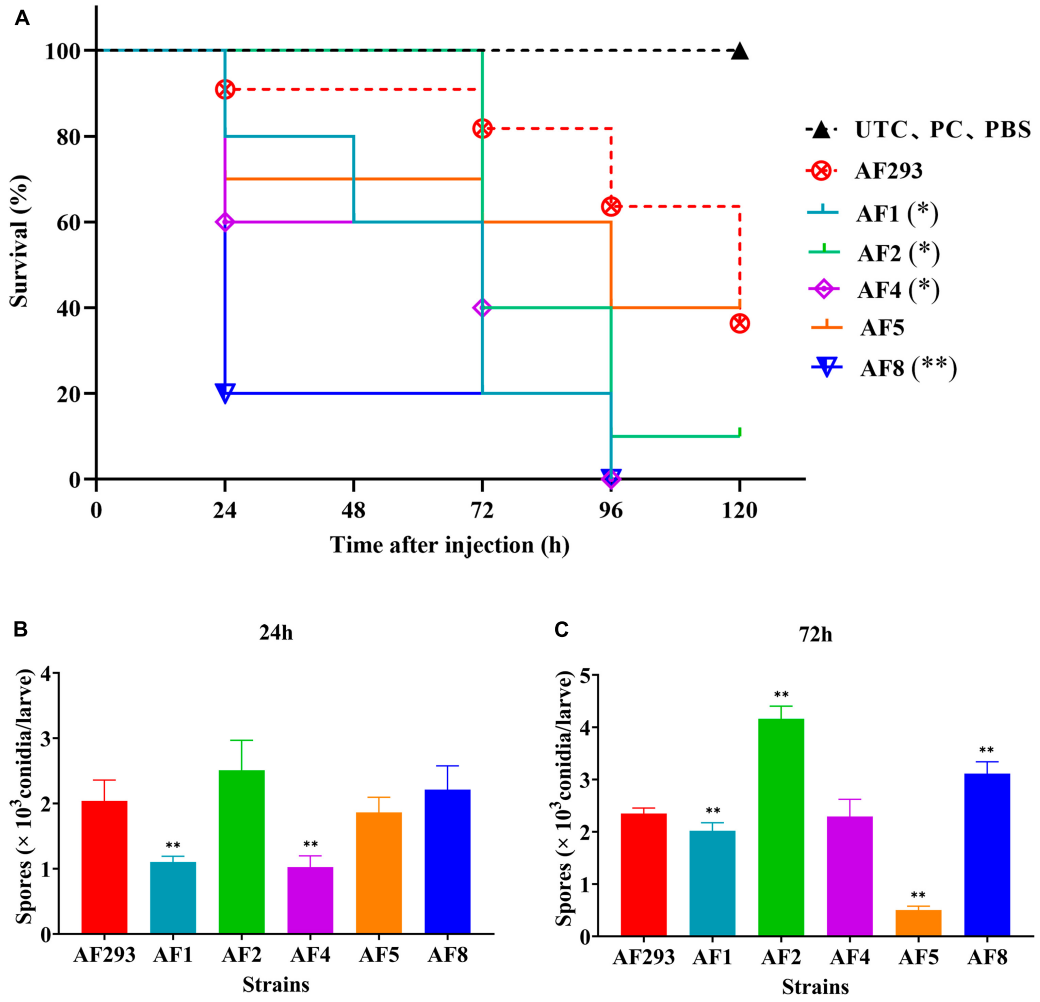

FIGURE 4
Survival and fungal burden analysis of *G. mellonella* larvae. **(A)** Survival curves of the model groups injected with *A. fumigatus* conidia. UTC: blank control group, PC: puncture without injection group, PBS: PBS injection group. Fungal burden of *G. mellonella* larvae at 24 h **(B)** and 72 h **(C)** after infection. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. strain AF293.

TABLE 1 Sensitivity of antifungal drugs of test strains.

Strains	MIC_{90} (μg/mL)					MEC (μg/mL)		
	FLC	ITR	ISA	VRC	AmB	ANI	CAS	MF
AF1	>64	>32	8	0.25	0.25	<0.0625	0.125	<0.0625
AF2	>64	>32	16	0.5	<0.25	0.0625	0.125	0.0625
AF4	>64	2	16	32	0.5	<0.0625	0.125	<0.0625
AF5	>64	2	>16	16	0.5	<0.0625	0.25	0.25
AF8	>64	2	>16	32	0.5	<0.0625	<0.0625	<0.0625
AF293	>64	0.25	0.125	0.125	0.25	0.0625	0.125	0.0625

ANI, anidulafungin; AmB, amphotericin B; CAS, caspofungin; FLC, fluconazole; ITR, itraconazole; ISA, isavuconazole; MF, micafungin; VRC, voriconazole; MIC_{90}, minimal drug concentration that inhibits the growth of 90%; MEC, minimum effective concentration.

Differences in the expression levels of genes associated with ergosterol synthesis, including *cyp51A*, *cyp51B*, *erg1*, *erg4*, *erg24*, and *hmg1*, were detected by RT-qPCR analysis. As compared to strain AF293, *cyp51A* expression was significantly up-regulated, while the expression levels of *erg1*, *erg24*, and *hmg1* genes were significantly down-regulated in strain AF1 (Figure 5A). Treatment of strain AF1 with ITR (32 μg/mL for 8 h) significantly up-regulated the expression levels of *cyp51A*, *cyp51B*, *erg1*, *erg24*, and *hmg1* (Figure 5B). As compared to strain AF293, *cyp51A* and *erg24* were significantly up-regulated, while *erg4* and *hmg1* were significantly down-regulated in strain AF2 (Figure 5C). Treatment of strain AF2 with ITR (32 μg/mL for 8 h) significantly

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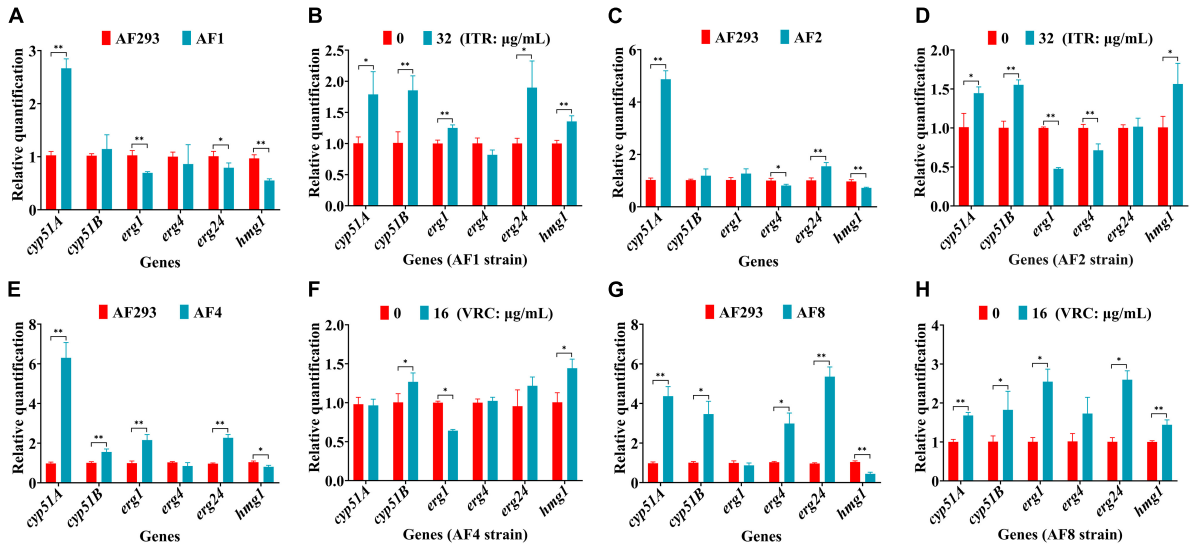

FIGURE 5
Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of genes associated with ergosterol synthesis. After incubation at 37°C for 16 h, the culture was continued for another 8 h with or without the addition of ITR or VRC. **(A,C,E,G)** Changes in gene expression levels of the four strains. **(B,D,F,H)** Gene expression levels following administration of ITR (32 μg/mL) or VRC (16 μg/mL) (MIC = 0.5). *$p < 0.05$ and **$p < 0.01$ vs. untreated strain AF293.

up-regulated *hmg1* and significantly down-regulated *cyp51A* and *cyp51B* (Figure 5D). As compared to strain AF293, *cyp51A*, *cyp51B*, *erg1*, and *erg24* were significantly up-regulated, while *hmg1* was significantly down-regulated in strain AF4 (Figure 5E). Treatment of strain AF4 with VRC (16 μg/mL for 8 h) significantly up-regulated *cyp51A* and *hmg1*, and significantly down-regulated *erg1* (Figure 5F). As compared to strain AF293, *cyp51A*, *cyp51B*, *erg4*, and *erg24* were significantly up-regulated, while *hmg1* was significantly down-regulated in strain AF8 (Figure 5G). Treatment of strain AF8 with VRC (16 μg/mL for 8 h) significantly up-regulated *cyp51A*, *cyp51B*, *erg1*, *erg24*, and *hmg1* (Figure 5H). These results indicate that the resistance mechanism of strains AF1, AF2, AF4, and AF8 is related to *cyp51* mutation or overexpression.

Non-*cyp51A*-mediated mechanism of azole resistance

Changes to the expression levels of genes associated with drug efflux pumps (e.g., *atrF*, *cdr1B*, *mdr1*, *mdr2*, *mdr4*, *mdrA*, and *mfsB*) were further confirmed. As compared to strain AF293, *mdr2*, and *mdrA* were significantly up-regulated, while *atrF*, *mdr1*, and *mfsB* were significantly down-regulated in strain AF1 (Figure 6A). Treatment of strain AF1 with ITR (32 μg/mL for 8 h) significantly up-regulated *cdr1B* and *mdr1* (Figure 6B). As compared to strain AF293, *mdr4* was significantly up-regulated, while *atrF*, *mdr1*, and *mfsB* were significantly down-regulated in strain AF2 (Figure 6C). Treatment of strain AF2 with ITR (32 μg/mL for 8 h) significantly up-regulated *atrF*, *mdr1*, *mdr4*, and *mdrA*, and significantly down-regulated *mfsB* (Figure 6D). As compared to strain AF293, *cdr1B*, *mdr2*, *mdr4*, and *mdrA* were significantly up-regulated, while *atrF*, *mdr1*, and *mfsB* were significantly down-regulated in strain AF4 (Figure 6E). Treatment of strain AF4 with VRC (16 μg/mL for 8 h) significantly up-regulated *atrF*, *mdr1*, *mdr4*, and *mfsB*, and significantly down-regulated *cdr1B* and *mdrA* (Figure 6F). As compared to strain AF293, *mdr1*, *mdr2*, *mdr4*, and *mdrA* were significantly up-regulated, while *atrF*, *cdr1B*, and *mfsB* were significantly down-regulated in strain AF8 (Figure 6G). Treatment of strain AF8 with VRC (16 μg/mL for 8 h) significantly up-regulated *cdr1B*, *mdr1*, *mdr2*, *mdr4*, and *mdrA*, with no significant difference in expression of *atrF* and *mfsB* (Figure 6H). These results indicate that the azole resistance mechanisms of strains AF1, AF2, AF4, and AF8 are related to overexpression of genes associated with efflux pumps.

The mechanism of VRC resistance in AF5 strain

The results of previous experiments found that *cyp51A*, *cyp51B*, and *hmg1* of strain AF5 carried no mutations, and there was no change in the ergosterol content. As compared to strain AF293, the expression levels of genes associated with ergosterol synthesis (i.e., *cyp51A*, *cyp51B*, *erg1*, *erg4*, and *hmg1*) and efflux pumps (i.e., *cdr1B*, *mdr1*, *mdr4*, and *mdrA*) were significantly up-regulated in strain AF5. After treatment with VRC (8 μg/mL for 8 h), the expression levels of genes associated with ergosterol synthesis (i.e., *cyp51B*, *erg24*, and *hmg1*) and efflux pumps (i.e., *atrF*, *mdr1*, *mdrA*, and *mfsB*) were significantly down-regulated in strain AF5 (Figures 7A, B). Transcriptomics sequencing was conducted to further explore the mechanism of VRC resistance of strain AF5. The results showed that among these DEGs, 73 genes were significantly up-regulated and 147 genes were significantly down-regulated (Figure 7C). DEGs were functionally grouped into GO classes comprising 40 functional categories (Supplementary Figure 4), and the DEGs were involved in biological processes including drug efflux, glucose

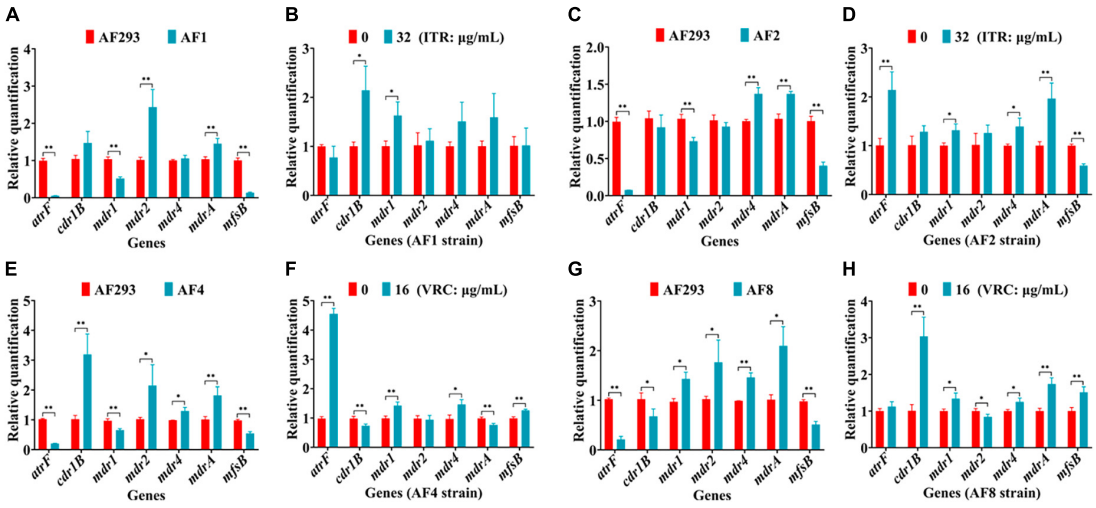

FIGURE 6
Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of genes associated with drug efflux pumps. After incubation at 37°C for 16 h, the culture was continued for another 8 h with or without administration of ITR or VRC. **(A,C,E,G)** Changes to gene expression levels of the four strains. **(B,D,F,H)** Gene expression levels following administration of ITR (32 μg/mL) or VRC (16 μg/mL) (MIC = 0.5). *$p < 0.05$ and **$p < 0.01$ vs. untreated strain AF293.

metabolism, and ribosome metabolism (**Supplementary Figure 5**). Further analysis found that there are 32 DEGs of up-regulated genes were assigned to 20 KEGG enrichment pathways, the top one pathway is ABC efflux pumps (4.1%), and another interesting pathway is the glycolysis pathway (2.74%), whereas there are 144 DEGs of the down-regulated genes were assigned to 20 KEGG enrichment pathways, and the top one pathway is ribosome metabolic pathway (50.34%) (**Figures 7D, E**), which are consistent with the GO analysis.

To confirm the results of transcriptome analysis, RT-qPCR analysis of genes associated with efflux pumps and energy metabolism was performed. The results showed that the expression levels of genes associated with effluent pumps (i.e., *abcA*, *abcC*, *atrA*, *atrF*, *mdr1*, *mdrA*, and *mfsB*) were significantly up-regulated (**Figure 7F**), in addition to genes associated with glycolysis (i.e., *hxkA* and *PK*), the tricarboxylic acid cycle (i.e., *IDH*, *DLST*, and *MDH*), the electron transport chain (i.e., *ND5*, *CBR*, and *COX5*), the pentose phosphate pathway rate-inhibiting enzyme (i.e., *PRPS1*), the malic acid cycle (i.e., *Pdc*), and the cholesterol and fatty acid anabolic pathways (i.e., *HMG-COA* and *ACC*). Meanwhile, the expression levels of *Sui* (a key gene of ATP synthase) and ATPase-related genes were also significantly up-regulated (**Figure 7G**). Further biochemical analysis showed that the NAD^+/NADH ratio was significantly increased in strain AF5 at 4, 8, and 12 h after administration of VRC (**Figure 7H**). These results suggest that strain AF5 responds to VRC by increasing energy production and overexpression of genes associated with efflux pumps.

Discussion

Lactophenol cotton blue staining is a common and rapid method for identification of filamentous fungi (Thomas et al., 1991). However, the accuracy of this method for species identification is poor. Thus, molecular techniques can be added to address this shortcoming. Common genetic markers for identification of *Aspergillus* spp. include *ITS*, β-*tubulin*, *actin*, and *calmodulin* (Hong et al., 2005; Samson et al., 2007). In the present study, morphological and molecular techniques were used to assess the biological characteristics and mechanisms of azole resistance of five commonly encountered clinical *A. fumigatus* strains.

Conidia and biofilm are related to the virulence of *A. fumigatus*. The conidia of *A. fumigatus* are ubiquitous in the environment and each person inhales at least 100 conidia/day (Nywening et al., 2020). Because of the small diameter, conidia easily enter the lungs and can also be easily cleared by alveolar macrophages. However, in immunocompromised individuals, a small portion of conidia will swell, germinate, and form mycelia, which will eventually cause invasive tissue damage and lead to IA (Zhang et al., 2021). Meanwhile, the formation of biofilm by *A. fumigatus* diminishes susceptibility to antifungal drugs, thereby frequently resulting in treatment failure (Kowalski et al., 2020; Morelli et al., 2021). Biofilm is a network structure mainly composed of conidia, mycelia, and extracellular matrix. As compared to planktonic cells of the same organism, microbial cells within a biofilm are highly resistant to current antifungal drugs and have become a source of persistent infection and high pathogenicity, especially in immunocompromised patients (Beauvais and Latgé, 2015; Borghi et al., 2016; Tits et al., 2020). In this study, there were notable differences in conidia production and biofilm formation between the two azole-resistant test strains (**Figures 1**, **2**), suggesting differences in the degrees of virulence. As a model organism, *G. mellonella* provides a reliable means to assess the virulence potential of *A. fumigatus* (Durieux et al., 2021). The process of melanization serves as a crucial indicator for *G. mellonella* in its defense against pathogens (Kavanagh and Reeves, 2004). The phagocytosis assay and *G. mellonella* infection study also confirmed differences in pathogenicity (**Figures 3**, **4**). In addition, the prognosis of patients infected with different azole resistant

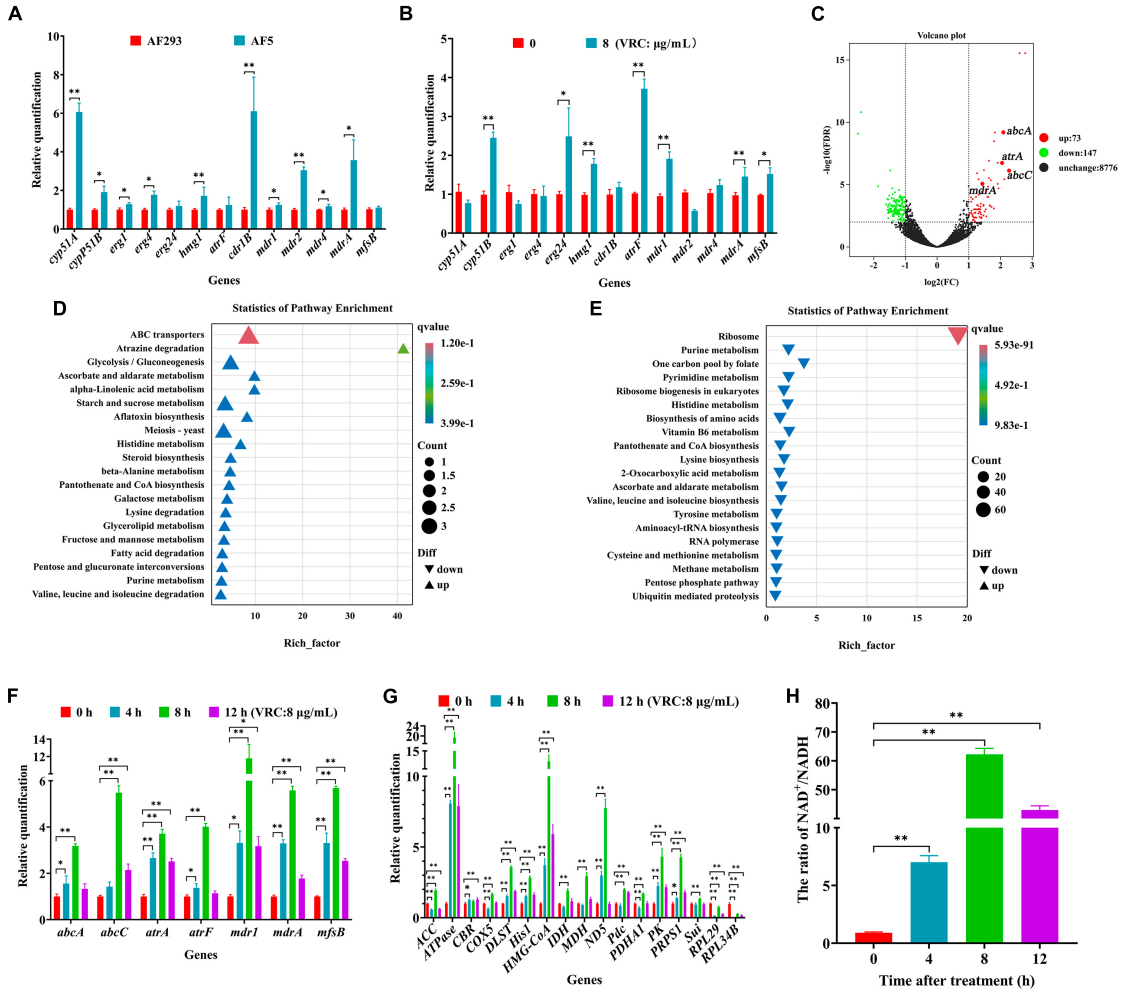

FIGURE 7
The mechanism of VRC resistance in AF5 strain. **(A)** Expression levels of genes associated with ergosterol synthesis and drug efflux pumps in strain AF5 vs. strain AF293. **(B)** Expression levels of gens associated with ergosterol syntheses and drug efflux pumps in strain AF5 treated with VRC at 8 μg/mL (MIC = 0.5). **(C)** Volcano map of differentially expressed genes. **(D,E)** Scatter plot of enriched pathways of the up- and down-regulated genes. Strain AF5 was incubated in RPIM 1640 medium for 16 h at 37°C and then treated with VRC (MIC = 0.5) for another 8 h. **(F,G)** Expression levels of gens associated with efflux pumps and energy metabolism. **(H)** Bar chart of NAD^+/NADH. Strain AF5 was incubated in RPIM 1640 medium for 16 h at 37°C and then treated with VRC (MIC = 0.5) for another 0, 4, 8, and 12 h. $^*p < 0.05$ and $^{**}p < 0.01$ vs. 0 h.

strains showed that the virulence of strains on the host body was also closely related to the host's own underlying diseases (**Supplementary Table 2**). However, further *in vivo* studies are needed to clarify the specific differences in the disease processes of the two azole-resistant strains.

Ergosterol stabilizes the fungal cell membrane by directly binding to phospholipids and plays important roles in cell membrane fluidity, cell cycle progression, cell morphology, and substance transport (Banerjee et al., 2014; Rana et al., 2019). Ergosterol synthesis involves the participation of many enzymes and genes (i.e., *cyp51A*, *cyp51B*, *erg1*, *erg4*, and *hmg1*) (Lan et al., 2021). Azole resistance of *A. fumigatus* is associated with a *cyp51A* mutation (Gonzalez-Lara et al., 2019), which is reported to decrease affinity of azoles and counteract the antifungal effects (van Ingen et al., 2015; Pérez-Cantero et al., 2020). Azoles target *cyp51A*, which codes for the key enzyme in ergosterol synthesis (Pontes et al., 2020). Common *cyp51A* mutations include TR34/L98, TR46/Y121F/T289A, TR53, and TR120 (Alvarez-Moreno et al., 2017; Hare et al., 2019). The results of the present study confirmed that the *cyp51A* mutations TR34/L98H/S297T/F495I conferred resistance to ITR in strains AF1 and AF2, while the *cyp51A* mutations TR46/Y121F/T289A conferred resistance to VRC in strains AF4 and AF8. In addition, Cyp51B, as a homologous protein of Cyp51A, has been confirmed in Saccharomyces cerevisiae by allogeneic expression of defective *cyp51* of *A. fumigatus*. Cyp51A and Cyp51B effectively complement each other in terms of ergosterol content and tolerance to azoles (Martel et al., 2010). In this study, there was no Cyp51B mutation to in four of the test strains. Moreover, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme in the first step of ergosterol synthesis, thus change to the expression of the HMG-CoA-coding gene hmg1 will directly affect ergosterol synthesis (Liang et al., 2021). The different mutation sites of *hmg1* confer different patterns of resistance to triazoles by *A. fumigatus*. For example, the *hmg1* mutation S541G had no effect on azole resistance. However, the combination of *cyp51A* and

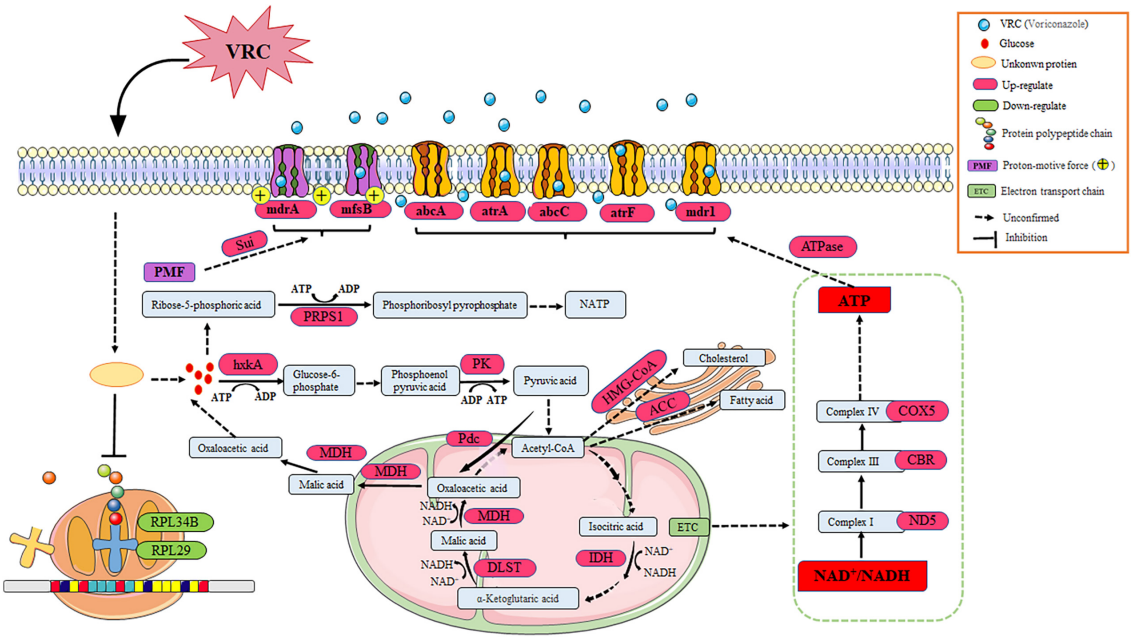

FIGURE 8
The comprehensive mechanism of resistance to VRC in AF5 strain. VRC reacts with unknown proteins to activate glycolysis, the tricarboxylic acid cycle, and the electron transport chain. Meanwhile, the ribosome metabolic pathway was inhibited, which prevented energy production for the ABC efflux pumps.

hmg1 mutations increased azole resistance (Arai et al., 2021). For example, the *hmg1* mutation S541G combined with the *cyp51A* mutation W273S or TR34/L98H leads to drug resistance (Resendiz-Sharpe et al., 2020). In the present study, the *hmg1* mutation S541G was detected in strains AF1 and AF2. However, the effect of the combination of the *hmg1* mutation S541G with the *cyp51A* mutations TR34/L98H/S297T/F495I on azole resistance remains unclear.

Besides the mutation sites of genes associated with ergosterol synthesis, overexpression of these genes also plays an important role in azole resistance (Handelman et al., 2021). The results of this study also confirmed that the genes associated with ergosterol synthesis were mainly up-regulated in strains AF1, AF2, AF4, and AF8 after azole treatment (**Figure 5**). In addition, multidrug efflux pumps are composed of multilayer transmembrane structures, which play important roles in expulsion of drugs and various molecules from the cell (Rajendran et al., 2011). Overexpression of genes associated with efflux pump transporters has been linked to multidrug resistance in *A. fumigatus* (Costa et al., 2014). A previous study confirmed that genes associated with ABC drug efflux pumps (e.g., *atrF*, *cdr1B*, and *mdr1*) are related to azole resistance of *A. fumigatus* (Paul et al., 2018). Moreover, deletion of the MFS-related genes *mdrA* and *mfsB* significantly increased susceptibility to ITR and VRC (Meneau et al., 2016). In the present study, as compared to strain AF293, the ABC efflux pump-related gene *atrF* was down-regulated, while the MFS efflux pump-related gene *mdrA* was up-regulated and *mfsB* was down-regulated in strains AF1, AF2, AF4, and AF8 (**Figure 6**). In addition, azole treatment influenced the expression levels of genes associated with efflux pump transporters. These results suggest that azole resistance of the four strains is not only related to mutations and overexpression of genes associated with ergosterol synthesis, but also genes associated with efflux pumps.

The molecular mechanisms of azole resistance in *A. fumigatus* primarily involve mutations in genes associated with ergosterol synthesis, upregulation of drug efflux pumps, and activation of cellular stress responses. Interestingly, *cyp51* of strain AF5 carried no mutation, indicating that *cyp51* does not participate in the mechanism of azole resistance of strain AF5. Furthermore, transcriptome sequencing found that after treatment of VRC, the expression of ribosome-related genes in AF5 strain was down-regulated, and the expression of drug-efflux pump related genes was up-regulated, which indicate that the non-*cyp51* resistance mechanism of AF5 strain was mainly related to activation of ABC efflux pumps, the glycolysis pathway, and the ribosome metabolic pathway. Our findings further validated that the application of VRC induces up-regulation of genes, associated with energy production pathways, down-regulation of genes, related to energy expenditure pathways, and an increase in the NAD^+/NADH ratio in the AF5 strain (**Figures 7G, H**), these changes in the AF5 strain will reduce its own energy consumption and make the energy supply to the membrane transport pumps more adequate. It is known that ABC efflux pumps are mainly powered by energy from ATP (Biemans-Oldehinkel et al., 2006). Therefore, we postulated that under the pressure of VRC, AF5 strain can rapidly regulate intracellular energy supply and mobilize more energy to supply ABC efflux pumps, thereby facilitating drug efflux and reducing intracellular drug concentration. Based on these findings, the molecular mechanism of VRC resistance of strain AF5 is related to increased glycolysis, the tricarboxylic acid cycle, fatty acid and cholesterol synthesis, and inhibition of ribosomal protein synthesis to increase energy production for drug efflux pumps (**Figure 8**).

In summary, there were notable differences in the biological characteristics and pathogenic ability among the five test strains assessed in this study. The *cyp51A* mutations TR34/L98H/V242I/S297T/F495I combined with the *hmg1* mutation S541G were associated with the mechanism of ITR resistance of strains AF1 and AF2. In addition, the *cyp51A* mutations TR46/Y121F/V242I/T289A confer VRC resistance to strains AF4 and AF8. However, the VRC resistance mechanism of strain AF5 was related to changes in energy production and increased expression of genes associated with drug efflux pumps. This study provides reference for the discovery of a new mechanism of resistance to azoles by *A. fumigatus* and the development of drug targets.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary material**.

Ethics statement

The studies involving humans were approved by Clinical Trial Ethics Committee, Southwest Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

ZS conceived and designed the research. FL, MZ, XZ, and CY conducted the experiments. YL and JZ contributed new reagents or analytical the tools. FL, CX, and GQ analyzed the data. MZ and ZS wrote the manuscript. All authors read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1253197/full#supplementary-material

References

Agarwal, R., Sehgal, I. S., Dhooria, S., Muthu, V., Prasad, K. T., Bal, A., et al. (2020). Allergic bronchopulmonary aspergillosis. *Indian J. Med. Res.* 151, 529–549. doi: 10.4103/ijmr.IJMR_1187_19

Alvarez-Moreno, C., Lavergne, R. A., Hagen, F., Morio, F., Meis, J. F., and Le Pape, P. (2017). Azole-resistant *Aspergillus fumigatus* harboring TR(34)/L98H, TR(46)/Y121F/T289A and TR(53) mutations related to flower fields in Colombia. *Sci. Rep.* 7:45631. doi: 10.1038/srep45631

Arai, T., Umeyama, T., Majima, H., Inukai, T., Watanabe, A., Miyazaki, Y., et al. (2021). Hmg1 mutations in *Aspergillus fumigatus* and their contribution to triazole susceptibility. *Med. Mycol.* 59, 980–984. doi: 10.1093/mmy/myab026

Arastehfar, A., Carvalho, A., Houbraken, J., Lombardi, L., Garcia-Rubio, R., Jenks, J. D., et al. (2021). *Aspergillus fumigatus* and aspergillosis: From basics to clinics. *Stud. Mycol.* 100:100115. doi: 10.1016/j.simyco.2021.100115

Banerjee, D., Burkard, L., and Panepinto, J. C. (2014). Inhibition of nucleotide biosynthesis potentiates the antifungal activity of amphotericin B. *PLoS One* 9:e87246.

Beauvais, A., and Latgé, J. P. (2015). Aspergillus biofilm *in vitro* and *in vivo*. *Microbiol. Spectr.* 3:10. doi: 10.1128/microbiolspec.MB-0017-2015

Biemans-Oldehinkel, E., Doeven, M. K., and Poolman, B. (2006). ABC transporter architecture and regulatory roles of accessory domains. *FEBS Lett.* 580, 1023–1035. doi: 10.1016/j.febslet.2005.11.079

Bongomin, F., Gago, S., Oladele, R. O., and Denning, D. W. (2017). Global and multi-national prevalence of fungal diseases-estimate precision. *J. Fungi* 3:57. doi: 10.3390/jof3040057

Borghi, E., Borgo, F., and Morace, G. (2016). Fungal biofilms: Update on resistance. *Adv. Exp. Med. Biol.* 931, 37–47. doi: 10.1007/5584_2016_7

Brown, G. D., Denning, D. W., Gow, N. A., Levitz, S. M., Netea, M. G., and White, T. C. (2012). Hidden killers: Human fungal infections. *Sci. Transl. Med.* 4:165rv113. doi: 10.1126/scitranslmed.3004404

Buil, J. B., Snelders, E., Denardi, L. B., Melchers, W. J. G., and Verweij, P. E. (2019). Trends in azole resistance in *Aspergillus fumigatus*, the Netherlands, 1994-2016. *Emerg. Infect. Dis.* 25, 176–178. doi: 10.3201/eid2501.171925

Burks, C., Darby, A., Gomez Londono, L., Momany, M., and Brewer, M. T. (2021). Azole-resistant *Aspergillus fumigatus* in the environment: Identifying key reservoirs and hotspots of antifungal resistance. *PLoS Pathog* 17:e1009711. doi: 10.1371/journal.ppat.1009711

Cadena, J., Thompson, G. R. III, and Patterson, T. F. (2021). Aspergillosis: Epidemiology, diagnosis, and treatment. *Infect. Dis. Clin. North Am.* 35, 415–434. doi: 10.1016/j.idc.2021.03.008

Costa, C., Dias, P. J., Sá-Correia, I., and Teixeira, M. C. (2014). MFS multidrug transporters in pathogenic fungi: Do they have real clinical impact? *Front. Physiol.* 5:197. doi: 10.3389/fphys.2014.00197

Durieux, M. F., Melloul, É, Jemel, S., Roisin, L., Dardé, M. L., Guillot, J., et al. (2021). Galleria mellonella as a screening tool to study virulence factors of *Aspergillus fumigatus*. *Virulence* 12, 818–834. doi: 10.1080/21505594.2021.1893945

El-Baba, F., Gao, Y., and Soubani, A. O. (2020). Pulmonary aspergillosis: What the generalist needs to know. *Am. J. Med.* 133, 668–674. doi: 10.1016/j.amjmed.2020.02.025

Eraso, I. C., Sangiovanni, S., Morales, E. I., and Fernández-Trujillo, L. (2020). Use of monoclonal antibodies for allergic bronchopulmonary aspergillosis in patients with asthma and cystic fibrosis: Literature review. *Ther. Adv. Respir. Dis.* 14:1753466620961648. doi: 10.1177/1753466620961648

Galiger, C., Brock, M., Jouvion, G., Savers, A., Parlato, M., and Ibrahim-Granet, O. (2013). Assessment of efficacy of antifungals against *Aspergillus fumigatus*: Value of real-time bioluminescence imaging. *Antimicrob. Agents Chemother.* 57, 3046–3059. doi: 10.1128/aac.01660-12

Gonzalez-Lara, M. F., Roman-Montes, C. M., Diaz-Lomeli, P., Rangel-Cordero, A., Valenzuela, M. O., Ponce-de-Leon, A., et al. (2019). Azole resistance and *cyp51A* mutation screening in *Aspergillus fumigatus* in Mexico. *J. Antimicrob. Chemother.* 74, 2047–2050. doi: 10.1093/jac/dkz121

Handelman, M., Meir, Z., Scott, J., Shadkchan, Y., Liu, W., Ben-Ami, R., et al. (2021). Point Mutation or Overexpression of *Aspergillus fumigatus cyp51B*, encoding lanosterol 14α-sterol demethylase, leads to triazole resistance. *Antimicrob. Agents Chemother.* 65:e0125221. doi: 10.1128/aac.01252-21

Hare, R. K., Gertsen, J. B., Astvad, K. M. T., Degn, K. B., Løkke, A., Stegger, M., et al. (2019). In vivo selection of a unique tandem repeat mediated azole resistance mechanism (TR(120) in *Aspergillus fumigatus cyp51A*, Denmark. *Emerg. Infect. Dis.* 25, 577–580. doi: 10.3201/eid2503.180297

Hartung de Capriles, C., Mata, S., and Middelveen, M. (1989). Preservation of fungi in water (Castellani): 20 years. *Mycopathologia* 106, 73–79. doi: 10.1007/bf00437084

Hong, S. B., Go, S. J., Shin, H. D., Frisvad, J. C., and Samson, R. A. (2005). Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* 97, 1316–1329. doi: 10.3852/mycologia.97.6.1316

Hsieh, C. L., Hsieh, S. Y., Huang, H. M., Lu, S. L., Omori, H., Zheng, P. X., et al. (2020). Nicotinamide increases intracellular NAD(+) content to enhance autophagy-mediated group A *Streptococcal* clearance in endothelial cells. *Front. Microbiol.* 11:117. doi: 10.3389/fmicb.2020.00117

Kavanagh, K., and Reeves, E. P. (2004). Exploiting the potential of insects for *in vivo* pathogenicity testing of microbial pathogens. *FEMS Microbiol. Rev.* 28, 101–112. doi: 10.1016/j.femsre.2003.09.002

Kowalski, C. H., Morelli, K. A., Schultz, D., Nadell, C. D., and Cramer, R. A. (2020). Fungal biofilm architecture produces hypoxic microenvironments that drive antifungal resistance. *Proc. Natl. Acad. Sci. U. S. A.* 117, 22473–22483. doi: 10.1073/pnas.2003700117

Lan, Q., Li, Y., Wang, F., Li, Z., Gao, Y., Lu, H., et al. (2021). Deubiquitinase Ubp3 enhances the proteasomal degradation of key enzymes in sterol homeostasis. *J. Biol. Chem.* 296:100348. doi: 10.1016/j.jbc.2021.100348

Lestrade, P. P. A., Buil, J. B., van der Beek, M. T., Kuijper, E. J., van Dijk, K., Kampinga, G. A., et al. (2020). Paradoxal trends in azole-resistant *Aspergillus fumigatus* in a national multicenter surveillance program, the Netherlands, 2013-2018. *Emerg. Infect. Dis.* 26, 1447–1455. doi: 10.3201/eid2607.200088

Liang, T., Yang, X., Li, R., Yang, E., Wang, Q., Osherov, N., et al. (2021). Emergence of W272C substitution in hmg1 in a triazole-resistant isolate of *Aspergillus fumigatus* from a Chinese patient with chronic cavitary pulmonary aspergillosis. *Antimicrob. Agents Chemother.* 65:e0026321. doi: 10.1128/AAC.00263-21

Lim, J. Y., Kim, Y. J., and Park, H. M. (2022). The Gβ-like protein AfCpcB affects sexual development, response to oxidative stress and phagocytosis by alveolar macrophages in *Aspergillus fumigatus*. *J. Fungi* 8, 56. doi: 10.3390/jof8010056

Martel, C. M., Parker, J. E., Warrilow, A. G., Rolley, N. J., Kelly, S. L., and Kelly, D. E. (2010). Complementation of a *Saccharomyces cerevisiae* ERG11/CYP51 (sterol 14α-demethylase) doxycycline-regulated mutant and screening of the azole sensitivity of *Aspergillus fumigatus* isoenzymes CYP51A and CYP51B. *Antimicrob. Agents Chemother.* 54, 4920–4923. doi: 10.1128/aac.00349-10

Mellado, E., Diaz-Guerra, T. M., Cuenca-Estrella, M., and Rodriguez-Tudela, J. L. (2001). Identification of two different 14-alpha sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. *J. Clin. Microbiol.* 39, 2431–2438. doi: 10.1128/JCM.39.7.2431-2438.2001

Meneau, I., Coste, A. T., and Sanglard, D. (2016). Identification of *Aspergillus fumigatus* multidrug transporter genes and their potential involvement in antifungal resistance. *Med. Mycol.* 54, 616–627. doi: 10.1093/mmy/myw005

Morelli, K. A., Kerkaert, J. D., and Cramer, R. A. (2021). *Aspergillus fumigatus* biofilms: Toward understanding how growth as a multicellular network increases antifungal resistance and disease progression. *PLoS Pathog.* 17:e1009794. doi: 10.1371/journal.ppat.1009794

Nywening, A. V., Rybak, J. M., Rogers, P. D., and Fortwendel, J. R. (2020). Mechanisms of triazole resistance in *Aspergillus fumigatus*. *Environ. Microbiol.* 22, 4934–4952. doi: 10.1111/1462-2920.15274

Özkan, S., and Coutts, R. H. (2015). Aspergillus fumigatus mycovirus causes mild hypervirulent effect on pathogenicity when tested on *Galleria mellonella*. *Fungal Genet. Biol.* 76, 20–26. doi: 10.1016/j.fgb.2015.01.003

Parker, J. E., Warrilow, A. G., Price, C. L., Mullins, J. G., Kelly, D. E., and Kelly, S. L. (2014). Resistance to antifungals that target CYP51. *J. Chem. Biol.* 7, 143–161. doi: 10.1007/s12154-014-0121-1

Paul, R. A., Rudramurthy, S. M., Dhaliwal, M., Singh, P., Ghosh, A. K., Kaur, H., et al. (2018). Magnitude of voriconazole resistance in clinical and environmental isolates of *Aspergillus flavus* and investigation into the role of multidrug efflux pumps. *Antimicrob. Agents Chemother.* 62, e1022–e1018. doi: 10.1128/aac.01022-18

Pérez-Cantero, A., López-Fernández, L., Guarro, J., and Capilla, J. (2020). Azole resistance mechanisms in *Aspergillus*: Update and recent advances. *Int J Antimicrob Agents* 55:105807. doi: 10.1016/j.ijantimicag.2019.09.011

Peyton, L. R., Gallagher, S., and Hashemzadeh, M. (2015). Triazole antifungals: A review. *Drugs Today* 51, 705–718. doi: 10.1358/dot.2015.51.12.2421058

Pontes, L., Beraquet, C. A. G., Arai, T., Pigolli, G. L., Lyra, L., Watanabe, A., et al. (2020). *Aspergillus fumigatus* clinical isolates carrying CYP51A with TR34/L98H/S297T/F495I substitutions detected after four-year retrospective azole resistance screening in Brazil. *Antimicrob. Agents Chemother.* 64, e2059–e2019. doi: 10.1128/aac.02059-19

Rajendran, R., Mowat, E., McCulloch, E., Lappin, D. F., Jones, B., Lang, S., et al. (2011). Azole resistance of *Aspergillus fumigatus* biofilms is partly associated with efflux pump activity. *Antimicrob. Agents Chemother.* 55, 2092–2097. doi: 10.1128/aac.01189-10

Rana, R., Sharma, R., and Kumar, A. (2019). Repurposing of fluvastatin against *Candida albicans* CYP450 lanosterol 14 α-demethylase, a target enzyme for antifungal therapy: An in silico and *in vitro* study. *Curr. Mol. Med.* 19, 506–524. doi: 10.2174/1566524019666190520094644

Resendiz-Sharpe, A., Hokken, M. W. J., Mercier, T., Merckx, R., Verhagen, K., Dewitte, L., et al. (2020). *Hmg1* gene mutation prevalence in triazole-resistant *Aspergillus fumigatus* clinical isolates. *J. Fungi* 6:227. doi: 10.3390/jof6040227

Samson, R. A., Hong, S., Peterson, S. W., Frisvad, J. C., and Varga, J. (2007). Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. *Stud. Mycol.* 59, 147–203. doi: 10.3114/sim.2007.59.14

Sehgal, I. S., Dhooria, S., Muthu, V., Prasad, K. T., and Agarwal, R. (2020). An overview of the available treatments for chronic cavitary pulmonary aspergillosis. *Expert. Rev. Respir. Med.* 14, 715–727. doi: 10.1080/17476348.2020.1750956

Smith, N. L., and Denning, D. W. (2011). Underlying conditions in chronic pulmonary aspergillosis including simple aspergilloma. *Eur. Respir. J.* 37, 865–872. doi: 10.1183/09031936.00054810

Snelders, E., Karawajczyk, A., Schaftenaar, G., Verweij, P. E., and Melchers, W. J. (2010). Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. *Antimicrob. Agents Chemother.* 54, 2425–2430. doi: 10.1128/AAC.01599-09

Snelders, E., Melchers, W. J., and Verweij, P. E. (2011). Azole resistance in *Aspergillus fumigatus*: A new challenge in the management of invasive aspergillosis? *Future Microbiol.* 6, 335–347. doi: 10.2217/fmb.11.4

Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing (2008). EUCAST technical note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia-forming moulds. *Clin. Microbiol. Infect.* 14, 982–984. doi: 10.1111/j.1469-0691.2008.02086.x

Thomas, P. A., Kuriakose, T., Kirupashanker, M. P., and Maharajan, V. S. (1991). Use of lactophenol cotton blue mounts of corneal scrapings as an aid to the diagnosis of mycotic keratitis. *Diagn. Microbiol. Infect. Dis.* 14, 219–224. doi: 10.1016/0732-8893(91)90035-e

Tits, J., Cammue, B. P. A., and Thevissen, K. (2020). Combination therapy to treat fungal biofilm-based infections. *Int. J. Mol. Sci.* 21:8873. doi: 10.3390/ijms21228873

van der Linden, J. W., Arendrup, M. C., Warris, A., Lagrou, K., Pelloux, H., Hauser, P. M., et al. (2015). Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerg. Infect. Dis.* 21, 1041–1044. doi: 10.3201/eid2106.140717

van Ingen, J., van der Lee, H. A., Rijs, T. A., Zoll, J., Leenstra, T., Melchers, W. J., et al. (2015). Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and TR46/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. *J. Antimicrob. Chemother.* 70, 178–181. doi: 10.1093/jac/dku364

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:Research0034. doi: 10.1186/gb-2002-3-7-research0034

Wayne, P. (2008). *CLSI, reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved Standard: CLSI document M38–A2*, 2nd Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

Yang, X., Chen, W., Liang, T., Tan, J., Liu, W., Sun, Y., et al. (2021). A 20-year antifungal susceptibility surveillance (from 1999 to 2019) for *Aspergillus* spp. and proposed epidemiological cutoff values for *Aspergillus fumigatus* and *Aspergillus flavus*: A study in a tertiary hospital in China. *Front. Microbiol.* 12:680884. doi: 10.3389/fmicb.2021.680884

Zhang, J., Debets, A. J. M., Verweij, P. E., and Snelders, E. (2021). Azole-resistance development; how the *Aspergillus fumigatus* lifecycle defines the potential for adaptation. *J. Fungi* 7, 599. doi: 10.3390/jof7080599

Zhang, Q., Liu, F., Zeng, M., Zhang, J., Liu, Y., Xin, C., et al. (2022). Antifungal activity of sodium new houttuyfonate against *Aspergillus fumigatus in vitro* and *in vivo*. *Front. Microbiol.* 13:856272. doi: 10.3389/fmicb.2022.856272

Zhou, X., Yang, G., Li, C., Yang, F., and Chang, X. (2022). Requirement of a putative mitochondrial GTPase, GemA, for azole susceptibility, virulence, and cell wall integrity in *Aspergillus fumigatus*. *Front. Microbiol.* 13:957857. doi: 10.3389/fmicb.2022.957857

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Occurrence and characterization of NDM-5-producing *Escherichia coli* from retail eggs

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The New Delhi Metallo-β-lactamase (NDM) producing Enterobacterales has been detected from diverse sources but has rarely been reported in retail eggs. In this study, 144 eggshell and 96 egg content samples were collected in 2022 from Guangdong province and were screened for NDM-producing strains. Four *Escherichia coli* strains (ST3014, ST10, ST1485, and ST14747) recovered from two (1.39%, 2 of 144) eggshells and two (2.08%, 2 of 96) egg content samples were identified as bla_{NDM-5}-positive strains. Oxford Nanopore MinION sequencing and conjugation assays revealed that the bla_{NDM-5} gene was carried by IncX3 ($n = 1$), IncI1 ($n = 1$), and IncHI2 ($n = 2$). The IncI1-plasmid-carrying bla_{NDM-5} displayed high homology with one plasmid pEC6563-NDM5 from the human clinic, while the IncHI2 plasmid harboring bla_{NDM-5} shared highly similar structures with plasmids of animal origin. To the best of our knowledge, this is the first report on the identification of bla_{NDM-5}-positive bacteria in retail eggs. NDM-producing *E. coli* could be transmitted to humans by the consumption of eggs or direct contact, which could pose a potential threat to human health.

KEYWORDS

resistance, food, carbapenemase, egg, plasmid

1 Introduction

Carbapenemase-resistant Enterobacterales (CRE) have increased rapidly over the last decades and have become an urgent public health threat (El-Gamal et al., 2017). Carbapenem resistance in Enterobacteriaceae is attributed to three dominant carbapenemase enzymes, including New Delhi metallo-beta-lactamases (NDM), *Klebsiella pneumoniae* carbapenemases (KPC), and carbapenem-hydrolyzing oxacillinase-48-type β-lactamases (OXA-48) (Iovleva and Doi, 2017). Among these CRE, NDM-producing strains are highly prevalent around the world, especially in China and South Asia (Wu et al., 2019). At present, 47 variants of NDM have been identified (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#NDM); of these, NDM-1 and NDM-5 remain the most prevalent carbapenemases (Shen et al., 2022; Saravanan et al., 2023). The NDM-5 possesses higher carbapenemase activity than NDM-1 (Hornsey et al., 2011). Currently, the bla_{NDM-5} gene has been disseminated to various bacterial species (e.g., *Escherichia coli, K. pneumoniae*, and *Klebsiella aerogenes*), with *E. coli* as the main bacterial host (Nordmann and Poirel, 2019; Jean et al., 2022; Ma et al., 2023).

Plasmid-mediated transmission has facilitated the widespread distribution of the bla_{NDM-5} gene among bacteria from various environmental sources and geographical

regions. The bla_{NDM-5} gene has been found in an array of plasmid replicon types, such as IncX3, IncFII, IncF, IncN, and IncHI2 (Nordmann and Poirel, 2019; Jean et al., 2022; Lv et al., 2022). The IncX3 has long been recognized as the primary carrier for transmission of the bla_{NDM-5} gene (Shen et al., 2022); however, recently, there is an increase in IncHI2 plasmid as a carrier of bla_{NDM-5} in China (Ma et al., 2021; Zhao Q. et al., 2021; Lv et al., 2022; Wang et al., 2022; He et al., 2023). Alarmingly, IncHI2 has also been found to carry multiple antibiotic resistance genes, including colistin resistance gene (*mcr*), extended-spectrum beta-lactamase, and quinolone resistance genes (Webb et al., 2016; Mmatli et al., 2022).

Although carbapenems have not been approved for food animals, CRE has been continuously detected in pigs, poultry, and animal-derived foods, especially from chickens and poultry products. Eggs are important poultry products that play an essential role in the daily healthy diet of human beings and are the most consumed food all over the world [https://www.who.int/zh/news-room/fact-sheets/detail/salmonella-(non-typhoidal)]. Poultry eggs are also considered as reservoirs and transmission vectors of resistance genes, such as *mcr-1*, *fosA*, *qnrS1*, $bla_{CTX-M-1}$, bla_{IMP}, and $bla_{OXA-48-like}$ (Benameur et al., 2018; Kapena et al., 2020; Zhang et al., 2021, 2022b; Kanaan et al., 2022; Li et al., 2022). Resistant bacteria and genes in eggs have the risk of spreading to humans through various ways, such as hand-to-egg contact and storing unwashed eggs in fridge. However, the occurrence of clinically important resistant bacteria, NDM-producing Enterobacterales, in eggs has rarely been studied. Hence, we investigated the prevalence of NDM-producing Enterobacterales among egg samples recovered from markets in Guangzhou and characterized the molecular traits of bla_{NDM}-positive isolates.

2 Methods

2.1 Sampling

From June to September 2022, 144 non-repetitive egg samples were randomly collected from 29 farmer markets located in four districts (Tianhe, Baiyun, Yuexiu, and Haizhu) of Guangzhou. To ensure diversity in the sampling and prevent repeated sampling from a singular supplier, we selected different stalls within each market, with a maximum of three eggs procured from any single stall. Each sample was placed in a separate sterile sample bag, and all samples were transported to the laboratory in a cool box within 8 h.

2.2 Bacterial isolation and detection of carbapenemase-encoding genes

For the isolation of bacteria from eggshells, the surface of eggs was wiped with a sterile swab, and then, the swab was placed into 4 ml sterilized Luria–Bertani (LB) broth medium for enrichment cultivation at 37°C overnight. For the isolation of bacteria from egg content, the eggshell was wiped with gauze with 70% ethanol, followed by being homogenized. During the processing of the first batch, 48 cracked eggs were collided and discarded to avoid cross-contamination (detailed information about all the samples is shown in Supplementary Table S1), and then, the remaining eggs were opened to extract the whole egg content. In total, 1 ml of egg content was dispensed into 4 ml sterilized Luria–Bertani (LB) broth medium and enriched at 37°C overnight with shaking. The overnight cultures of each sample were incubated on MacConkey agar plates supplemented with 0.5 mg/L meropenem, and the plates were incubated at 37°C for 16 h. One to three colonies with different morphologies in each plate were selected for the detection of carbapenemase-encoding genes, bla_{NDM}, using PCR and DNA sequencing (primers are shown in Supplementary Table S2). All bla_{NDM}-positive isolates were collected for species identification by direct smear and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonik GmbH, Bremen, Germany).

2.3 Antimicrobial susceptibility testing

According to the recommendations of the Clinical and Laboratory Standards Institute, the minimal inhibitory concentrations (MICs) of 19 antimicrobials against NDM-positive isolates were determined using the agar dilution method or broth microdilution (colistin and tigecycline) method. *E. coli* ATCC 25922 was used as a quality control strain. The results of MICs were interpreted according to CLSI (M100-S30) criteria and EUCAST (http://www.eucast.org/clinical_breakpoints/).

2.4 Plasmid transferability and stability

Conjugation experiments were performed by broth mating using bla_{NDM}-positive strains as the donor and a sodium azide-resistant (MIC > 2,000 μg/ml) *E. coli* J53 strain as the recipient. In detail, the donor and recipient strains were incubated separately in LB broth for 4 h, followed by mating the bacterial cultures with a ratio of 1:1 and incubating at 37°C without shaking overnight. A 50-μL overnight mixture was plated onto MacConkey agar plates containing 0.5 mg/L meropenem and 150 mg/L sodium azide, and incubated for 18 h to count and select transconjugants. Conjugation frequency was calculated as the number of transconjugants per recipient. Chemical transformation experiments were performed in those cases that bla_{NDM}-positive strains failed to conjugate. All transconjugants and transformants were confirmed by PCR (primers are shown in Supplementary Table S2) and antimicrobial susceptibility testing.

The stability of bla_{NDM-5}-carrying plasmids in host bacteria was performed by a passage in the absence of antibiotic Luria broth (LB). Three single clones of each bla_{NDM-5}-positive strain were grown in 3 ml LB without antibiotic treatment overnight at 37°C. The overnight culture was daily diluted 1:100 in fresh LB broth for 15 days. Cultures were collected at the end of each of 3 days for streaking on antibiotic-free MacConkey agar plates. Then, 100 colonies were selected, and the presence of bla_{NDM-5} and the corresponding plasmids was verified by PCR amplification of bla_{NDM-5} and *repA* (primers are shown in Supplementary Table S2). Plasmid retention was calculated as the ratio of strains with bla_{NDM-5} and *repA* and over 100 colonies.

2.5 Whole-genome sequencing and bioinformatics analysis

Genomic DNAs of four NDM-positive isolates were extracted by using HiPure Bacterial DNA Kit (Magen, Beijing, China), according to the manufacturer's instructions. Whole genomic DNA was sequenced using the Illumina NovaSeq 6000 and MinION platform (Nanopore, UK). Hybrid assembly of complete genomes was carried out using the Unicycler version 0.4.8 (Wick et al., 2017). MLST v2.19 (https://github.com/tseemann/mlst) was applied to the verified sequence type (ST). Center for Genomic Epidemiology (CGE) (http://genomicepidemiology.org/services/) and PubMLST were used to identify antimicrobial resistance genes (ARGs) and plasmid replication types. Prokka software was used to annotate the draft genome (Seemann, 2014). EasyFig tool (http://mjsull.github.io/Easyfig/) was used to draw the genetic context of bla_{NDM} and plasmid alignments and comparisons (Sullivan et al., 2011).

2.6 Accession numbers

The complete sequences of NDM-positive isolates have been deposited in the GenBank database under accession numbers PRJNA983957.

3 Results

3.1 Prevalence of NDM-producing enterobacterales in egg samples

From the 144 eggs, 144 eggshell and 96 egg content samples were collected, and 4 bla_{NDM}-positive isolates were recovered from 2 (1.39%, 2/144) eggshell samples (GD22SC3180PM and GD22SC3312PM) and 2 (2.08%, 2/96) egg content samples (GD22SC3148PM and GD22SC4181PM). These strains were further identified as *E. coli* by MALDI-TOF MS. All the bla_{NDM} genes were identified as bla_{NDM-5}.

3.2 Antimicrobial resistance patterns of bla_{NDM-5}-positive *E. coli* strains

Antimicrobial susceptibility testing showed that all four isolates were resistant to most of the antimicrobials, including β-lactams (ampicillin, cefoxitin, cefotaxime, ceftazidime, cefquinome, and imipenem), aminoglycosides (apramycin, neomycin, and streptomycin), fosfomycin, florfenicol, tetracycline, and sulfamethoxazole. However, all isolates remained susceptible to amikacin, colistin, and tigecycline (Supplementary Table S3).

3.3 Genotyping and genetic background of bla_{NDM-5}-positive *E. coli* strains

The four bla_{NDM-5}-positive *E. coli* strains were subjected to short- and long-read sequencing to acquire complete genomes. Sequence analysis revealed that GD22SC3180PM, GD22SC3312PM, GD22SC3148PM, and GD22SC4181PM belonged to ST14747, ST10, ST3014, and ST1485, respectively (Table 1). All bla_{NDM}-positive isolates harbored multiple antimicrobial resistance genes (ARGs) and plasmid replicon types (Table 1). Aminoglycoside resistance gene, β-lactam resistance gene, florfenicol resistance gene *floR*, macrolide resistance gene *mdf(A)*, and sulfonamide resistance gene *dfrA* were detected in all the bla_{NDM-5}*-positive E. coli strains*. In addition, the fluoroquinolone resistance gene *qnrS* and rifampicin resistance gene *arr-3* were identified in two isolates (GD22SC3312PM and GD22SC4181PM). GD22SC3180PM additionally harbored tetracycline resistance gene *tet*(A), fosfomycin resistance gene *fosA3*, and lincomycin resistance gene *lnu(F)*.

3.4 Characterization of bla_{NDM-5}-carrying plasmids

Bioinformatics analysis revealed that there were three types of replicons carrying the bla_{NDM-5} gene, namely, IncX3 (pHN22SC3148), IncI1 (pHN22SC3180), and IncHI2 (pHN22SC3312 and pHN22SC4181) (Table 1). The complete sequence of IncI1 plasmid pHN22SC3180 is a 107,617 circular molecule with a GC content of 47% (Figure 1A). The backbone regions of the pHN22SC3180 displayed the highest similarity to plasmid pEC6563-NDM5 (CP095858.1, urine, Homo sapiens, *E. coli*, Zhejiang, China), with 100% identity and 99.0% coverage (Figure 1A) (Zhang et al., 2022a), but had low similarity to the other four bla_{NDM-5}-bearing IncI1 plasmids deposited in the NCBI database (Supplementary Table S4). The plasmid pHN22SC3312 (IncHI2) was 263,640 bp in size with a GC content of 54% (Figure 1B) and had a high degree of homology with pHNGD64-NDM (MW296099.1, pig, *E. coli*, Guangdong, China) with 100% identity and 98.0% coverage, pNDM33-1 (CP076648.1, duck, *E. coli*, Guangdong, China) with 99.99% identity and 99.0% coverage, and pHNBYF33-1 (CP101733.1, fish, *E. coli*, Guangdong, China) with 99.99% identity and 98.0% coverage (Figure 1B). Furthermore, in addition to bla_{NDM-5}, the variable region of pHN22SC3312 contained several antibiotic resistance genes [e.g., bla_{OXA-10}, tet*(A)*, *qnrS1*, *floR*, *sul3*, *aadA2*, and *aph(4)-Ia*].

3.5 The biological features of bla_{NDM-5}-carrying plasmids

To evaluate the transferability of the bla_{NDM-5} gene, all four bla_{NDM-5} positive strains were conducted on a conjugation assay. The bla_{NDM-5}-carrying plasmids were successfully transferred to recipients *E. coli* J53 at a frequency of 10^{-5}-10^{-6}. The imipenem MICs of the transconjugants were 2–4 μg/ml, which were 32–64-fold the MICs of the recipient (Supplementary Table S3). To evaluate the stability of bla_{NDM-5}-carrying plasmids, we performed passage with the four bla_{NDM-5}-positive strains in antibiotic-free Luria broth. The stability of the IncX3 plasmid pHN22SC3148 was 100% in the absence of antibiotic after 15 days (i.e., ~150 generations) in the natural host GD22SC3148PM, while IncI1

TABLE 1 Characteristics of *bla*$_{NDM-5}$-positive isolates.

Isolates	Source	Organism	Sequence type	Resistance genes	Plasmid types	Plasmid size (bp)	Imipenem MIC (μg/mL)	Conjugation frequency[b]
GD22SC3148PM	Egg content FM16[a]	*E. coli*	ST3014	*aadA2*, ***bla***$_{NDM-5}$, *cmlA1*, *dfrA12*, *floR*, *mdf(A)*, *sitABCD*, *sul2*, *sul3*	IncFIA, IncFIB(AP001918), IncFIC(FII), **IncX3**	–	4	N.D.
GD22SC3180PM	Egg shells FM17	*E. coli*	ST14747	*aac(3)-IId*, *aac(3)-IVa*, *aadA17*, *aadA5*, *aph(3″)-Ib*, *aph(3′)-Ia*, *aph(6)-Id*, *bla*$_{CTX-M-55}$, ***bla***$_{NDM-5}$, *dfrA17*, *floR*, *fosA3*, *lnu(F)*, *mdf(A)*, *mph(A)*, *sitABCD*, *sul1*, *sul2*, *sul3*, *tet*(A)	IncFIB(AP001918), IncFII(pHN7A8), **IncI1**, p0111	107,617	8	$(3.75 \pm 1.33) \times 10^{-6}$
GD22SC3312PM	Egg shells FM20	*E. coli*	ST10	***arr-3***, ***aac(3)-IV***, ***aph(3″)-Ib***, *aph(3′)-Ia*, *aph(4)-Ia*, ***aph(6)-Id***, ***bla***$_{CTX-M-55}$, ***bla***$_{NDM-5}$, ***bla***$_{OXA-10}$, *aadA2*, *dfrA12*, ***dfrA14***, ***floR***, *lnu(F)*, ***mdf(A)***, ***qnrS1***, ***sitABCD***, ***sul1***, ***sul3***	Col156, IncB/O/K/Z, IncFIB(AP001918), IncFIC(FII), **IncHI2**, IncHI2A, p0111	263,640	4	$(1.76 \pm 0.16) \times 10^{-5}$
GD22SC4181PM	Egg content FM25	*E. coli*	ST1485	***arr-3***, *aac(3)-IVa*, *aph(3′)-Ia*, *aph(4)-Ia*, ***bla***$_{NDM-5}$, *bla*$_{OXA-10}$, *bla*$_{TEM-1B}$, *cmlA1*, *dfrA14*, *floR*, *mdf(A)*, *qnrS1*, *sitABCD*	IncFIA, IncFIB(AP001918), IncFIC(FII), IncFII, IncHI2A, **IncHI2**, p0111	–	4	N.D.

Bold, *bla*$_{NDM-5}$-positive plasmids and their resistance genes.
[a]FM, Famers' market.
[b]Average ± Standard error (SE).
N.D. means that conjugation frequency cannot be measured.

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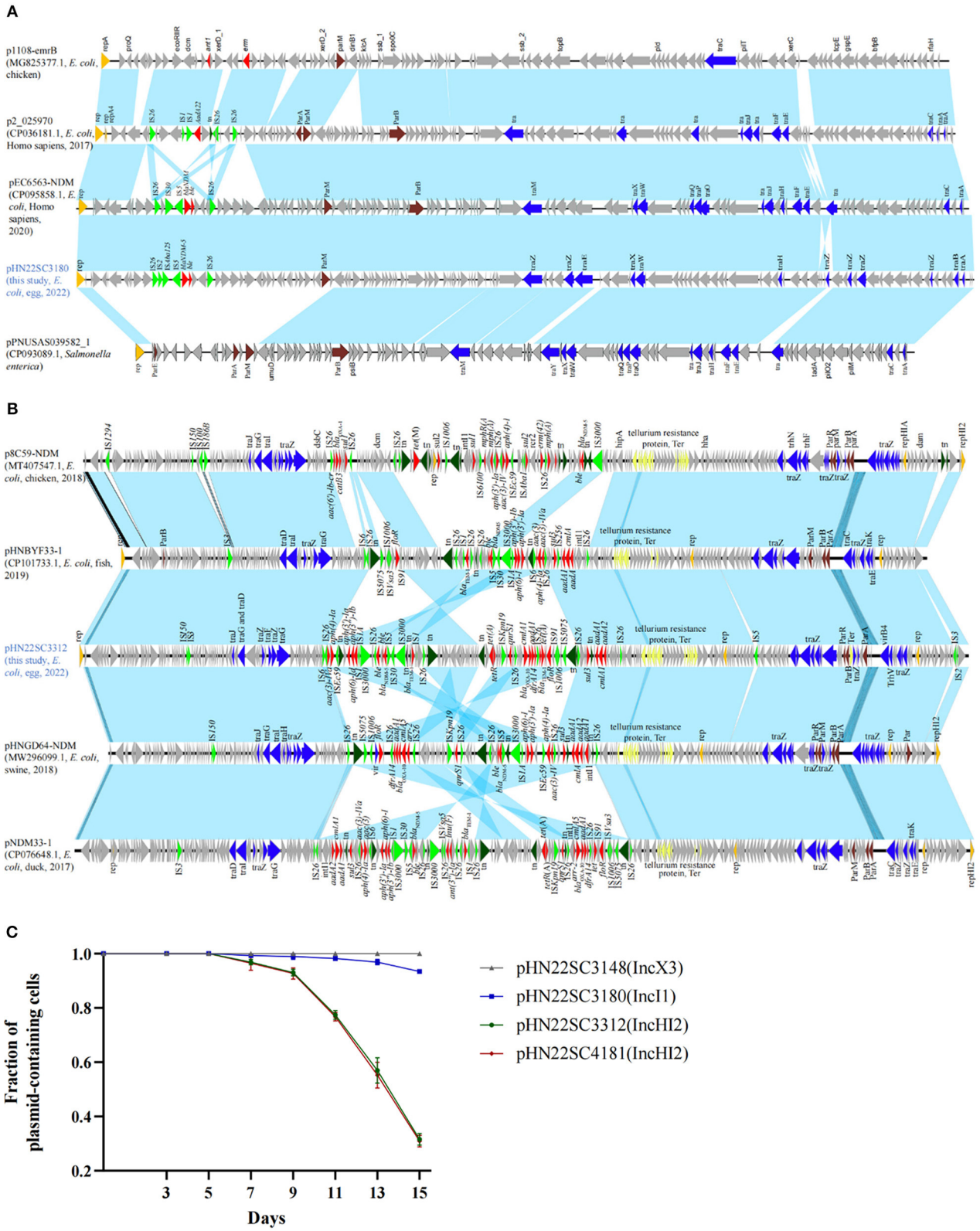

FIGURE 1
Comparison and stability of bla_{NDM-5}-carrying plasmids. **(A)** bla_{NDM-5}-harboring IncI1 plasmids in this study with other similar plasmids. p1108-emrB (NZ_MG825377.1), p2_025970 (CP036181.1), pPNUSAS039582_1 (CP093089.1), and pEC6563-NDM5 (CP095858.1). **(B)** bla_{NDM-5}-harboring IncHI2 plasmids in this study with other similar plasmids. p8C59-NDM (MT407547.1), pHNBYF33-1 (CP101733.1), pHNGD64-NDM (MW296099.1), and pNDM33-1 (CP076648.1). **(C)** Stability of bla_{NDM-5}-carrying plasmids in their corresponding host bacteria. Error bars represent standard deviations ($n = 3$).

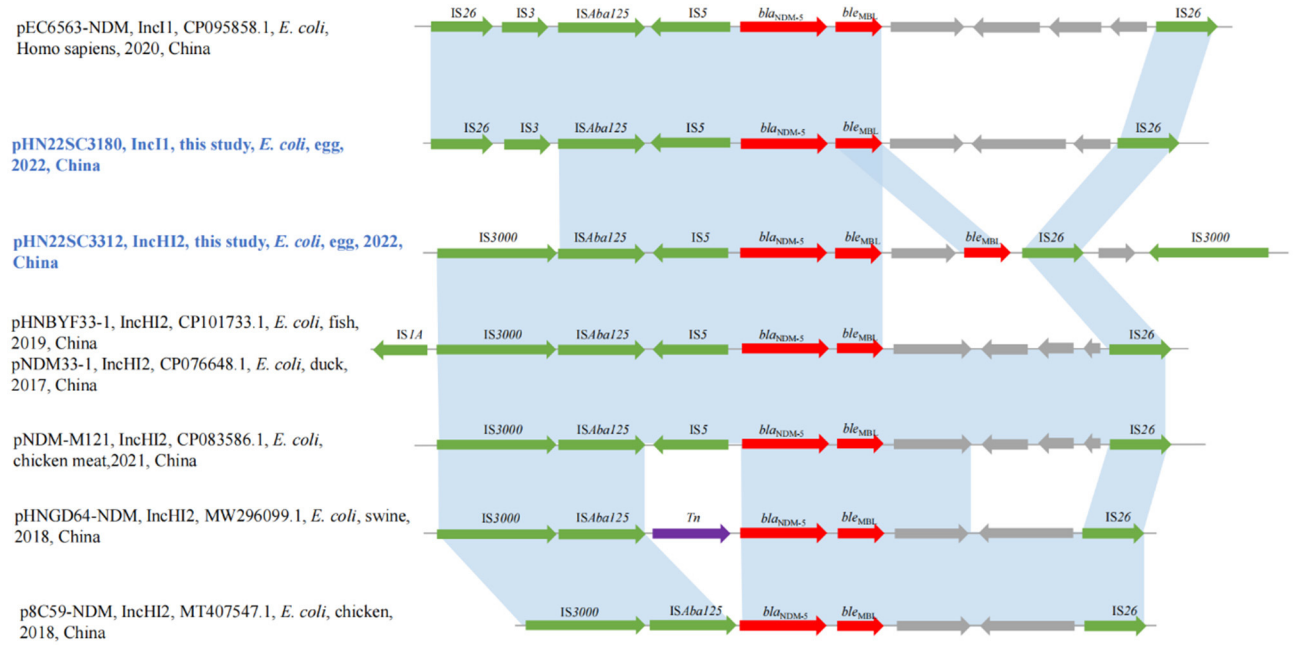

FIGURE 2
The genetic environment of bla_{NDM-5} genes.

plasmid pHN22SC3180 and IncHI2 plasmids, pHN22SC3312 and pHN22SC4181, were gradually lost from their corresponding host strains after 7 days of passage, with 93.40, 31.60, and 30.90% retention after 15 days, respectively (Figure 1C). Thus, in the absence of antibiotic selection, IncX3 plasmid pHN22SC3148 is stable in the original isolate, and the other three plasmids (IncHI2 and IncI1) are less stable in the host strains.

3.6 Genetic environments of bla_{NDM-5} genes in IncI1 and IncHI2

The genetic context of the bla_{NDM-5} gene in IncI1 plasmid pHN22SC3180 was IS*26*-IS3-IS*Aba125*-IS*5*-bla_{NDM-5}-ble_{MBL}-trpF-*dsbD*-IS*26*, which was similar to the genetic environment recently discovered in IncI1 plasmid pEC6563-NDM5-carrying bla_{NDM-5} (GenBank accession no. CP095858.1) (Figure 2). Although the bla_{NDM-5} region (IS*26*-IS3-IS*Aba125*-IS*5*-bla_{NDM-5}-ble_{MBL}-trpF-*dsbD*-IS*26*) was surrounded by two copies of IS*26* with the same direction, no circular intermediate was obtained in this study, similar to previous report (Zhang et al., 2022a). Moreover, the genetic contexts of bla_{NDM-5} in IncHI2 plasmid pHN22SC3312, IS*3000*-IS*Aba125*-IS*5*-bla_{NDM-5}-ble_{MBL}-trpF-ble_{MBL}-IS*26*-*dsbD*-IS*3000*, were highly similar to other bla_{NDM-5}-harboring IncHI2 plasmids, pNDM-M121 (GenBank accession no. CP083586.1), pHNBYF33-1 (GenBank accession no. CP101733.1), and pNDM33-1 (GenBank accession no. CP076648.1), except for the presence of two copies of ble_{MBL} downstream of bla_{NDM-5} in pHN22SC3312 in this study (the information of all egg samples is shown in Supplementary Table S1).

4 Discussion

To date, bla_{NDM}-positive Enterobacterales have been identified in various sources, including food animals, pets, human beings, animal foods, vegetables, and the environment (Zhai et al., 2020; Huang et al., 2023; Ma et al., 2023). However, there are few reports of NDM-producing bacteria in egg sources, except for one study, which reported the presence of bla_{NDM}-positive *Salmonella enterica* in eggs from Iraq (Kanaan et al., 2022). To the best of our knowledge, this is the first report of NDM-5-producing Enterobacterales in retail egg samples from China. As eggs are an important food in the human diet and its consumption continues to increase, the NDM-positive Enterobacterales in eggs have the risk of spreading to humans via the food chain and even hand–egg contact.

Previous studies revealed that IncX3 is the most epidemiologically successful vehicle for spreading bla_{NDM}-5 (Zhang et al., 2019; Zhao Q. Y. et al., 2021; Ma et al., 2023). bla_{NDM-5}-bearing IncX3 plasmids are widely distributed in animals, human beings, and environments worldwide (Lv et al., 2022; Ma et al., 2023). The IncX3 plasmids carrying bla_{NDM-5} in this study further confirmed the importance of the IncX3 plasmid by acting as a vehicle for bla_{NDM-5} transfer. The bla_{NDM-5}-positive IncX3 plasmids can be stably inherited in the original isolate (Figure 1C), which may partly explain the rapid global dissemination of bla_{NDM-5}-bearing IncX3 plasmids (Ma et al., 2020).

In this study, we also detected the IncI1 plasmid (pHN22SC3180) and IncHI2 plasmid (pHN22SC3312) carrying the bla_{NDM-5} gene. IncI1 is an epidemic plasmid and can carry many resistance genes, especially the extended-spectrum beta-lactamase gene bla_{CTX}, which has widely spread in patients

and animals (Yang et al., 2014; Chong et al., 2018; Carattoli et al., 2021; Liu et al., 2021). However, the reports of the bla_{NDM-5}-bearing IncI1 plasmid are few, and bla_{NDM-5}-bearing IncI1 plasmid has just been detected in isolates from clinical and duck samples in China (Zhao Q. Y. et al., 2021; Dong et al., 2022; Zhang et al., 2022a). Of note, by searching through the NCBI database, we found only five bla_{NDM-5}-bearing IncI1 plasmids, four of which were clinical samples isolated from China in recent years, implying that the prevalence and risk of bla_{NDM-5}-bearing IncI1 in the clinic might be underestimated and need further investigation.

IncHI2 is a wide host plasmid and acts as an important vector for the dissemination of multiple ARGs, especially *mcr-1* (Webb et al., 2016; Liu and Liu, 2018; Wu et al., 2018; Cao et al., 2020). To date, IncHI2-type plasmids carrying bla_{NDM-5} have only been detected in strains recovered from chicken, duck, pig feces, and freshwater fish (Ma et al., 2021; Zhao Q. Y. et al., 2021; Lv et al., 2022). These IncHI2-bla_{NDM-5} plasmids mainly spread regionally in Guangdong province in China but have also spread to other regions (Wang et al., 2022; He et al., 2023). The high similarity of the bla_{NDM-5}-bearing IncHI2 plasmids in eggs and other origins suggested that these plasmids are spreading. However, IncHI2-type plasmids carrying bla_{NDM-5} are not stable in bacterial hosts (Figure 1C). The increasing occurrence of IncHI2-type plasmids carrying bla_{NDM-5} in China might be associated with the co-selection by other antimicrobials as IncHI2 plasmids usually carry various antimicrobial resistance genes.

While our findings indicate a slightly higher prevalence of bla_{NDM}-positive isolates in egg contents (2.08%, 2/96) compared with eggshells (1.39%, 2/144), this study is not without limitations. The exclusion of 48 egg content samples may influence the overall contamination rates. Furthermore, the sample size, hovering around a hundred, does not offer a comprehensive representation of the prevalence of bla_{NDM} in egg samples, highlighting the need for continuous surveillance.

5 Conclusion

In summary, to the best of our knowledge, we report the first case of Enterobacterales carrying bla_{NDM-5} of retail eggs in China. The bla_{NDM-5}-bearing plasmids displayed high homology with those of plasmids from other sources. Of note, the IncHI2 plasmids carrying both carbapenem and multiple resistance genes showed an increasing trend that pose another threat to human health. Considering the clinical importance of carbapenem together with the fact that the consumption of eggs is substantial in our diet, the carbapenem resistance in eggs has the risk to spread to humans through the food chain or contact with the contaminant. Continued monitoring of carbapenem resistance in eggs is urgently needed.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

Y-YL: Investigation, Writing—original draft, Writing—review & editing. TL: Formal analysis, Investigation, Writing—original draft. HY: Investigation, Writing—original draft. CY: Investigation, Writing—original draft. LL: Formal analysis, Writing—original draft. JC: Formal analysis, Writing—original draft. HD: Formal analysis, Writing—original draft. XG: Formal analysis, Writing—original draft. J-HL: Conceptualization, Funding acquisition, Writing—review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1281838/full#supplementary-material

References

Benameur, Q., Tali-Maamar, H., Assaous, F., Guettou, B., Tahrat, N., and Aggoune, N., et al. (2018). Isolation of *Escherichia coli* carrying the *bla*(CTX-M-1) and *qnrS1* genes from reproductive organs of broiler breeders and internal contents of hatching eggs. *J. Vet. Med. Sci.* 80, 1540–1543. doi: 10.1292/jvms.18-0283

Cao, Y. P., Lin, Q. Q., He, W. Y., Wang, J., Yi, M. Y., and Lv, L. C., et al. (2020). Co-selection may explain the unexpectedly high prevalence of plasmid-mediated colistin resistance gene *mcr-1* in a chinese broiler farm. *Zool. Res.* 41, 569–575. doi: 10.24272/j.issn.2095-8137.2020.131

Carattoli, A., Villa, L., Fortini, D., and Garcia-Fernandez, A. (2021). Contemporary IncI1 plasmids involved in the transmission and spread of antimicrobial resistance in *Enterobacteriaceae*. *Plasmid* 118, 102392. doi: 10.1016/j.plasmid.2018.12.001

Chong, Y., Shimoda, S., and Shimono, N. (2018). Current epidemiology, genetic evolution and clinical impact of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*. *Infect. Genet. Evol.* 61, 185–188. doi: 10.1016/j.meegid.2018.04.005

Dong, H., Li, Y., Cheng, J., Xia, Z., Liu, W., and Yan, T., et al. (2022). Genomic epidemiology insights on ndm-producing pathogens revealed the pivotal role of plasmids on *bla*(NDM) transmission. *Microbiol. Spectr.* 10, e215621. doi: 10.1128/spectrum.02156-21

El-Gamal, M. I., Brahim, I., Hisham, N., Aladdin, R., Mohammed, H., and Bahaaeldin, A. (2017). Recent updates of carbapenem antibiotics. *Eur. J. Med. Chem.* 131, 185–195. doi: 10.1016/j.ejmech.2017.03.022

He, W., Gao, M., Lv, L., Wang, J., Cai, Z., and Bai, Y., et al. (2023). Persistence and molecular epidemiology of *bla*(NDM)-positive gram-negative bacteria in three broiler farms: a longitudinal study (2015-2021). *J. Hazard. Mater.* 446, 130725. doi: 10.1016/j.jhazmat.2023.130725

Hornsey, M., Phee, L., and Wareham, D. W. (2011). A novel variant, NDM-5, of the new delhi metallo-beta-lactamase in a multidrug-resistant *Escherichia coli* ST648 isolate recovered from a patient in the United Kingdom. *Antimicrob. Agents Chemother.* 55, 5952–5954. doi: 10.1128/AAC.05108-11

Huang, E., Yang, X., Leighton, E., and Li, X. (2023). Carbapenem resistance in the food supply chain. *J. Food Prot.* 86, 100108. doi: 10.1016/j.jfp.2023.100108

Iovleva, A., and Doi, Y. (2017). Carbapenem-resistant *Enterobacteriaceae*. *Clin. Lab. Med.* 37, 303–315. doi: 10.1016/j.cll.2017.01.005

Jean, S. S., Harnod, D., and Hsueh, P. R. (2022). Global threat of carbapenem-resistant gram-negative bacteria. *Front. Cell. Infect. Microbiol.* 12, 823684. doi: 10.3389/fcimb.2022.823684

Kanaan, M., Khalil, Z. K., Khashan, H. T., and Ghasemian, A. (2022). Occurrence of virulence factors and carbapenemase genes in *salmonella enterica* serovar enteritidis isolated from chicken meat and egg samples in Iraq. *BMC Microbiol.* 22, 279. doi: 10.1186/s12866-022-02696-7

Kapena, M. S., Muma, J. B., Mubita, C. M., and Munyeme, M. (2020). Antimicrobial resistance of *Escherichia coli* and *salmonella* in raw retail table eggs in Lusaka, Zambia. *Vet. World* 13, 2528–2533. doi: 10.14202/vetworld.2020.2528-2533

Li, C., Gu, X., Zhang, L., Liu, Y., Li, Y., and Zou, M., et al. (2022). The occurrence and genomic characteristics of *mcr-1*-harboring *salmonella* from retail meats and eggs in qingdao, China. *Foods* 11, 1–12. doi: 10.3390/foods11233854

Liu, Y., and Liu, J. H. (2018). Monitoring colistin resistance in food animals, an urgent threat. *Expert Rev. Anti Infect. Ther.* 16, 443–446. doi: 10.1080/14787210.2018.1481749

Liu, Y. Y., Chen, S., Burrus, V., and Liu, J. H. (2021). Editorial: globally or regionally spread of epidemic plasmids carrying clinically important resistance genes: epidemiology, molecular mechanism, and drivers. *Front. Microbiol.* 12, 822802. doi: 10.3389/fmicb.2021.822802

Lv, L. C., Lu, Y. Y., Gao, X., He, W. Y., Gao, M. Y., and Mo, K. B., et al. (2022). Characterization of ndm-5-producing *Enterobacteriaceae* isolates from retail grass carp (ctenopharyngodon idella) and evidence of *bla*(ndm-5)-bearing IncHI2 plasmid transfer between ducks and fish. *Zool. Res.* 43, 255–264. doi: 10.24272/j.issn.2095-8137.2021.426

Ma, J., Song, X., Li, M., Yu, Z., Cheng, W., and Yu, Z., et al. (2023). Global spread of carbapenem-resistant *Enterobacteriaceae*: epidemiological features, resistance mechanisms, detection and therapy. *Microbiol. Res.* 266, 127249. doi: 10.1016/j.micres.2022.127249

Ma, T., Fu, J., Xie, N., Ma, S., Lei, L., and Zhai, W., et al. (2020). Fitness cost of *bla*(NDM-5)-carrying p3r-IncX3 plasmids in wild-type ndm-free *Enterobacteriaceae*. *Microorganisms* 8, 1–11. doi: 10.3390/microorganisms8030377

Ma, Z., Zeng, Z., Liu, J., Liu, C., Pan, Y., and Zhang, Y., et al. (2021). Emergence of IncHI2 plasmid-harboring *bla*NDM-5 from porcine *Escherichia coli* isolates in guangdong, China. *Pathogens* 10, 1–7. doi: 10.3390/pathogens10080954

Mmatli, M., Mbelle, N. M., and Osei, S. J. (2022). Global epidemiology, genetic environment, risk factors and therapeutic prospects of *mcr* genes: a current and emerging update. *Front. Cell. Infect. Microbiol.* 12, 941358. doi: 10.3389/fcimb.2022.941358

Nordmann, P., and Poirel, L. (2019). Epidemiology and diagnostics of carbapenem resistance in gram-negative bacteria. *Clin. Infect. Dis.* 69, S521–S528. doi: 10.1093/cid/ciz824

Saravanan, B. S., Kumar, D. J. M., and Venkataramaniah, C. (2023). A review on molecular description of carbapenem resistant gram-negative *Bacilli*. *Clin. Med.* 10.

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Shen, Y., Hu, F., Wang, Y., Yin, D., Yang, L., and Chen, Y., et al. (2022). Transmission of carbapenem resistance between human and animal NDM-positive *Escherichia coli* Strains. *Engineering* 15, 24–33. doi: 10.1016/j.eng.2021.07.030

Sullivan, M. J., Petty, N. K., and Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics* 27, 1009–1010. doi: 10.1093/bioinformatics/btr039

Wang, J., Tang, B., Lin, R., Zheng, X., Ma, J., and Xiong, X., et al. (2022). Emergence of *mcr-1*- and *bla*(NDM-5)-harbouring IncHI2 plasmids in *Escherichia coli* strains isolated from meat in zhejiang, China. *J. Glob. Antimicrob. Resist.* 30, 103–106. doi: 10.1016/j.jgar.2022.06.002

Webb, H. E., Granier, S. A., Marault, M., Millemann, Y., den Bakker, H. C., and Nightingale, K. K., et al. (2016). Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect. Dis.* 16, 144–145. doi: 10.1016/S1473-3099(15)00538-1

Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* 13, e1005595. doi: 10.1371/journal.pcbi.1005595

Wu, R., Yi, L. X., Yu, L. F., Wang, J., Liu, Y., and Chen, X., et al. (2018). Fitness advantage of *mcr-1*-bearing IncI2 and IncX4 plasmids *in vitro*. *Front. Microbiol.* 9, 331. doi: 10.3389/fmicb.2018.00331

Wu, W., Feng, Y., Tang, G., Qiao, F., McNally, A., and Zong, Z. (2019). Ndm metallo-beta-lactamases and their bacterial producers in health care settings. *Clin. Microbiol. Rev.* 32, 2–30. doi: 10.1128/CMR.00115-18

Yang, X., Liu, W., Liu, Y., Wang, J., Lv, L., and Chen, X., et al. (2014). F33: A-: B-, IncHI2/ST3, and IncI1/ST71 plasmids drive the dissemination of *fosA3* and *bla* CTX-M-55/-14/-65 in *Escherichia coli* from chickens in China. *Front. Microbiol.* 5, 688. doi: 10.3389/fmicb.2014.00688

Zhai, R., Fu, B., Shi, X., Sun, C., Liu, Z., and Wang, S., et al. (2020). Contaminated in-house environment contributes to the persistence and transmission of ndm-producing bacteria in a Chinese poultry farm. *Environ. Int.* 139, 105715. doi: 10.1016/j.envint.2020.105715

Zhang, Q., Lv, L., Huang, X., Huang, Y., Zhuang, Z., and Lu, J., et al. (2019). Rapid increase in carbapenemase-producing *Enterobacteriaceae* in retail meat driven by the spread of the *bla*(NDM-5)-carrying IncX3 plasmid in China from 2016 to 2018. *Antimicrob. Agents Chemother.* 63, 1–5. doi: 10.1128/AAC.00573-19

Zhang, X., Chen, L., Zhang, X., Wang, Q., Quan, J., and He, J., et al. (2022a). Emergence of coexistence of a novel *bla*(NDM-5)-harbouring IncI1-i plasmid and an *mcr-1.1*-harbouring IncHI2 plasmid in a clinical *Escherichia coli* isolate in China. *J. Infect. Public Health* 15, 1363–1369. doi: 10.1016/j.jiph.2022.10.020

Zhang, X., Ma, M., Cheng, Y., Huang, Y., Tan, Y., and Yang, Y., et al. (2022b). Spread and molecular characteristics of *Enterobacteriaceae* carrying *fosA*-like genes from farms in China. *Microbiol. Spectr.* 10, e54522. doi: 10.1128/spectrum.00545-22

Zhang, Y., Liu, K., Zhang, Z., Tian, S., Liu, M., and Li, X., et al. (2021). A severe gastroenteritis outbreak of *Salmonella enterica* serovar enteritidis linked to contaminated egg fried rice, China, 2021. *Front. Microbiol.* 12, 779749. doi: 10.3389/fmicb.2021.779749

Zhao, Q., Berglund, B., Zou, H., Zhou, Z., Xia, H., and Zhao, L., et al. (2021). Dissemination of *bla*(NDM-5) via IncX3 plasmids in carbapenem-resistant *Enterobacteriaceae* among humans and in the environment in an intensive vegetable cultivation area in eastern China. *Environ. Pollut.* 273, 116370. doi: 10.1016/j.envpol.2020.116370

Zhao, Q. Y., Zhu, J. H., Cai, R. M., Zheng, X. R., Zhang, L. J., and Chang, M. X., et al. (2021). IS26 is responsible for the evolution and transmission of *bla*(NDM)-harboring plasmids in *Escherichia coli* of poultry origin in china. *Msystems* 6, e64621. doi: 10.1128/mSystems.00646-21

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Genomic characterization of *Salmonella* isolated from retail chicken and humans with diarrhea in Qingdao, China

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Salmonella, especially antimicrobial resistant strains, remains one of the leading causes of foodborne bacterial disease. Retail chicken is a major source of human salmonellosis. Here, we investigated the prevalence, antimicrobial resistance (AMR), and genomic characteristics of *Salmonella* in 88 out of 360 (24.4%) chilled chicken carcasses, together with 86 *Salmonella* from humans with diarrhea in Qingdao, China in 2020. The most common serotypes were Enteritidis and Typhimurium (including the serotype I 4,[5],12:i:-) among *Salmonella* from both chicken and humans. The sequence types were consistent with serotypes, with ST11, ST34 and ST19 the most dominantly identified. Resistance to nalidixic acid, ampicillin, tetracycline and chloramphenicol were the top four detected in *Salmonella* from both chicken and human sources. High multi-drug resistance (MDR) and resistance to third-generation cephalosporins resistance were found in *Salmonella* from chicken (53.4%) and humans (75.6%). In total, 149 of 174 (85.6%) *Salmonella* isolates could be categorized into 60 known SNP clusters, with 8 SNP clusters detected in both sources. Furthermore, high prevalence of plasmid replicons and prophages were observed among the studied isolates. A total of 79 antimicrobial resistant genes (ARGs) were found, with *aac(6')-Iaa*, bla_{TEM-1B}, *tet(A)*, *aph(6)-Id*, *aph(3")-Ib*, *sul2*, *floR* and *qnrS1* being the dominant ARGs. Moreover, nine CTX-M-type ESBL genes and the genes bla_{NMD-1}, *mcr-1.1*, and *mcr-9.1* were detected. The high incidence of MDR *Salmonella*, especially possessing lots of mobile genetic elements (MGEs) in this study posed a severe risk to food safety and public health, highlighting the importance of improving food hygiene measures to reduce the contamination and transmission of this bacterium. Overall, it is essential to continue monitoring the *Salmonella* serotypes, implement the necessary prevention and strategic control plans, and conduct an epidemiological surveillance system based on whole-genome sequencing.

KEYWORDS

***Salmonella*, retail chicken, genome sequencing, humans with diarrhea, antimicrobial resistance**

1 Introduction

Salmonella is one of the common pathogens causing sporadic cases or outbreaks of gastroenteritis (Jain et al., 2020). In 2010, there were an estimated 153 million cases of non-typhoidal *Salmonella* enteric infections worldwide, of which about 50% were foodborne (Kirk et al., 2015; WHO, 2022). In the United States, foodborne salmonellosis causes an estimated 212,500 infections and 90 deaths annually (CDC, 2019). In China, *Salmonella* is the second most common bacteria causing foodborne outbreaks (Sun et al., 2021). In fact, consuming contaminated foods, especially poultry meat, directly threatens human health, which appears to be one of the major sources of human infection (Heredia and García, 2018; Pan et al., 2019; Yang et al., 2020). Poultry meat in the food production supply chain have been frequently associated with human salmonellosis cases and are an important cause of *Salmonella* transmission between poultry farms and humans (Antunes et al., 2016; Rincón-Gamboa et al., 2021). Furthermore, *Salmonella* is also a significant repository of antimicrobial resistance genes, posing substantial challenges to public health and security (Jajere, 2019).

Over recent years, resistance to cephalosporins have been increasingly reported in humans and poultry industry and the contribution of poultry products to the dissemination of extended-spectrum β-lactamase (ESBL) producing *Salmonella* strains, and the associated dangers for human health, is well documented in certain countries (Antunes et al., 2016). Of concern is the increased incidence of infections caused by ESBL-producing organisms, including *Salmonella*, because they are resistant not only to most of the β-lactam antimicrobials but also to other antimicrobial classes, leaving few treatment options and the potential for clinical outcomes worse than those of infections caused by non-ESBL-producing strains (Wei et al., 2019; Wang et al., 2020a). Meanwhile, carbapenems and colistin are often used as the last-line treatment for infections caused by multidrug-resistant (MDR), especially ESBL-producing gram-negative bacteria. However, selective pressure from the overuse or misuse of both antimicrobials has resulted in the emergence of carbapenem- and colistin-resistant Enterobacteriaceae (CRE) (Nang et al., 2019; Octavia et al., 2020). Regarding *Salmonella* isolates, recent studies have demonstrated their resistance to these critical antimicrobial drugs (Wang et al., 2017; Fan et al., 2022; Zhang et al., 2022b).

The New Delhi metallo (NDM)-β-lactamases that can hydrolyze almost all β-lactam antibiotics, have become one of the most commonly reported carbapenemase resistance mechanisms worldwide (Poirel et al., 2010). According to the Comprehensive Antibiotic Resistance Database (CARD[1]), 43 variants of NDM genes have been identified in 2022, with NDM-1 and NDM-5 being particularly widespread among *Enterobacteriaceae* including *Salmonella* (Iovleva and Doi, 2017). Colistin resistance in bacteria has become another significant threat to food safety and public health, and its development was mainly attributed to the plasmid-mediated *mcr* genes. Currently, a variety of *mcr* genes, including *mcr-1* to *-10* have been reported with *mcr-9.1* being prevalent in *Salmonella* (Ling et al., 2020; Hussein et al., 2021). Hence, the increasing antimicrobial resistance in *Salmonella* needs to be monitored.

Our previous investigation has described the emergence of the $bla_{\text{NDM-1}}$ gene in *Salmonella* recovered from chicken carcass in slaughterhouse, while the *mcr* genes were also detected from living chicken in Qingdao (Wang et al., 2017; Zou et al., 2021). However, there is limited available information on the surveillance and genomics studies of *Salmonella* in retail chicken in Qingdao. In the current study, we therefore investigated the prevalence, AMR, and genomic characteristics of *Salmonella* from chilled chicken carcasses and humans with diarrhea in Qingdao. Specially, we identified four *Salmonella* isolates carrying the plasmid-borne *mcr-1.1*, *mcr-9.1*, and NDM-1 genes in this study.

2 Materials and methods

2.1 Bacterial isolation

A total of 360 chilled chicken carcasses were collected from local markets in Qingdao, China in 2020. All samples were subjected to qualitative analysis for *Salmonella* using an enrichment method described by the National Food Safety Standard of China-Food microbiological examination, *Salmonella* (GB 4789.4–2016). Finally, presumptive *Salmonella* was selected for biochemical confirmation using API 20E test identification test strips (bioMérieux, Marcy l′ Etoile, France), as well as for molecular identification using PCR assay targeting the *invA* gene (Ed-Dra et al., 2018). In addition, 86 *Salmonella* isolates from humans with diarrhea collected from enteric clinic settings in the study year in Qingdao, were also included in the study (Supplementary Table S1). The isolates were serotyped using O- and H-antigens by slide agglutination with hyperimmune sera, and the serotypes of *Salmonella* samples were identified following the Kauffmann-White scheme (Grimont and Weill, 2007). All confirmed *Salmonella* isolates were stored in brain heart infusion broth with 40% glycerol (Land Bridge, Beijing, China) at −80°C. One isolate was retained from each sample. All the procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee of China National Center of Food Safety Risk Assessment, Beijing, China (approval no. 2014003).

2.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) of the *Salmonella* isolates was evaluated using the broth dilution method by the Biofosun Gram-negative panels (Shanghai Biofosun Biotech, China) by the manufacturer's instructions. The MICs of 13 antimicrobial agents, including ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (CFX), imipenem (IPM), meropenem (MEM), trimethoprim-sulfamethoxazole (SXT), gentamicin (GEN), tetracycline (TET), ciprofloxacin (CIP), nalidixic acid (NAL), chloramphenicol (CHL), and polymyxin E (CT) were determined, and the results were interpreted using Clinical and Laboratory Standards Institute guidelines (CLSI,

1 https://card.mcmaster.ca/home

2017). *Escherichia coli* ATCC 25922 was used as quality control. Isolates showed resistance to all tested antimicrobials in this study were defined as pan-resistance, while isolates showed resistance to at least three classes of antimicrobials were defined as MDR.

2.3 Whole-genome sequencing and *in silico* analysis

Whole-genome sequencing of the *Salmonella* isolates from both chicken and humans was carried out using the Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd. Trimmomatic (Bolger et al., 2014), FastQC,[2] SPAdes v3.14 (Bankevich et al., 2012), and Prokka v1.14.5 (Seemann, 2014) were used for reads quality control, assembly, and annotation. MLST v2.19.0[3] was used for identifying the sequence type (ST). SeqSero2 v1.2.1[4] was used for identifying the *Salmonella* serotypes (Zhang et al., 2019). Antimicrobial resistance genes (ARGs) were identified using ResFinder v4.0 (Bortolaia et al., 2020). Virulence genes were identified by ABRicate v1.01[5] using the VFDB database (Liu et al., 2022) with 80% identity and 80% query coverage cutoffs. Plasmid replicon types were identified using PlasmidFinder v2.1 (Carattoli and Hasman, 2020). Prophage predictions were carried out using PHASTER to explore intact prophages (Arndt et al., 2016). Pan-genome analysis was performed using Roary v3.13.0 (Page et al., 2015). Core genome phylogeny was built using PhyML v3.3.20200621 (Guindon et al., 2010). The gene presence/absence of ARGs, virulence genes, plasmid replication types, and prophage types, along with the phylogenetic tree were visualized through R script (v3.6.2) with the package ggtree (Yu, 2020). The genomes were submitted to EnteroBase for cgMLST profiling (Zhou et al., 2020) and submitted to NCBI Pathogen Detection portal for SNP clustering (Timme et al., 2020).

2.4 Statistical analysis

The Chi-square test was performed to analyze differences between groups. Data analysis was performed using SPSS 20.0 (SPSS, Chicago, United States). All statistical tests were two-sided; $p < 0.05$ were considered statistically significant.

2.5 Data availability statement

The sequences obtained in this study have been deposited in the NGDC Genome Sequence Archive[6] under accession number CRA012442.

2 https://www.bioinformatics.babraham.ac.uk/projects/fastqc
3 https://github.com/tseemann/mlst
4 http://www.denglab.info/SeqSero2
5 https://github.com/tseemann/abricate
6 https://ngdc.cncb.ac.cn/gsa/

3 Results

3.1 Distribution of *Salmonella* serotypes and sequence types in retail chilled chicken carcasses and humans with diarrhea

A total of 88 (24.4%) *Salmonella* isolates were recovered from 360 retail chilled chicken carcasses in the local markets in Qingdao, China in 2020. Using both traditional and *in silico* serotyping methods, which yielded identical results, we determined that the 88 isolates represented 18 different serotypes (Figure 1, Supplementary Table S1). The serotypes of Enteritidis, Indiana, and Typhimurium were frequently detected (each was 17.0%, 15/88) among *Salmonella* from retail chilled chicken carcasses, followed by Derby (12.5%, 11/88), Agona (6.8%, 6/88), and Thompson (5.7%, 5/88) (Supplementary Table S2). The results also demonstrated that the 86 isolates collected from humans with diarrhea belonged to 15 different serotypes, with Enteritidis (23.3%, 20/86), Typhimurium (17.4%, 15/86), and I 4,[5],12:i:- (17.4%, 15/86) frequently detected, followed by Agona (8.1%, 7/86), London (7.0%, 6/86), and Derby (4.7%, 4/86) (Supplementary Table S2). There were six and three serotypes were isolated only in retail chilled chicken carcasses or humans with diarrhea, respectively.

The sequence types of all 174 studied *Salmonella* isolates showed strong clustering by serotypes (Figure 1, Supplementary Table S3). The most prevalent sequence type was ST11 (20.1%, 35/174, *S.* Enteritidis), followed by ST34 (13.2%, 23/174, *S.* Typhimurium/*S.* I 4,[5],12:i:-), ST19 (12.1%, 21/174, *S.* Typhimurium). The ST17 (8.6%, 15/174), ST40 (8.6%, 15/174), and ST13 (7.5%, 13/174) clones were detected in *S.* Indiana, *S.* Derby, and *S.* Agona, respectively. We also noticed that each serotype was associated with one ST, except for *S.* Typhimurium (ST19, ST34, ST36, ST99, and ST3557), *S.* Indiana (ST17 and ST3558), *S.* Rissen (ST469 and ST1836), and *S.* Schwarzengrund (ST96 and ST241).

3.2 Antimicrobial resistance phenotype

The results showed that 85.2% (75/88) of the *Salmonella* from retail chilled chicken carcasses were resistant to at least one antimicrobial agent, whereas 13 isolates (14.8%) were susceptible to all tested antimicrobials (Supplementary Table S1). In detail, the top four resistant antimicrobials were nalidixic acid (60.2%, 53/88), ampicillin (51.1%, 45/88), tetracycline (47.7%, 42/88) and chloramphenicol (40.9%, 36/88) (Figure 2A). Meanwhile, 47 isolates (53.4%) were MDR (i.e., could resist ≥3 antimicrobial classes) (Figure 2B). A total of 23 isolates (26.1%) were resistant to at least one of the third-generation cephalosporins (ceftazidime and cefotaxime), including 13 *S.* Indiana (86.7%, 13/15), 5 *S.* Thompson (5/5), 2 *S.* Typhimurium (13.3%, 2/15), 1 *S.* Agona (1/6), 1 *S.* Rissen (1/1), and 1 *S.* Enteritidis (6.7%, 1/15) (Supplementary Table S1). Most of these 23 isolates showed concurrent resistance to ciprofloxacin ($n = 17$). Notably, Polymyxin E resistance was observed in 16 isolates, including 8 *S.* Enteritidis, 2 *S.* Derby, and one each of *S.* Agona, *S.* I 4,[5],12:i:-, *S.* Indiana, *S.* Reading, *S.* Schwarzengrund, and *S.* Typhimurium.

Among the *Salmonella* isolates from humans with diarrhea, 93.0% (80/86) displayed resistance to at least one antimicrobial agent, while the remaining 6 isolates (7.0%) were susceptible to all tested antimicrobials (Supplementary Table S1). In detail, the top four resistant antimicrobials

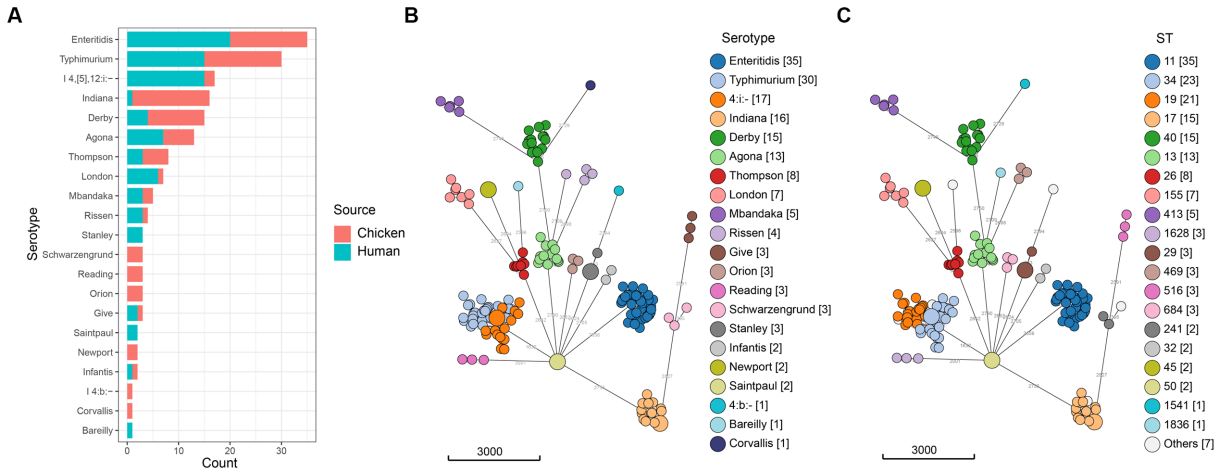

FIGURE 1
Distribution of *Salmonella* isolates. **(A)** Number of isolates from retail chilled chicken carcasses and humans with diarrhea. **(B)** Minimum spanning tree of the 174 *Salmonella* genomes colored by serotype. **(C)** Minimum spanning tree of the 174 *Salmonella* genomes colored by ST. The minimum spanning tree was built using cgMLST allelic profiles with the MSTreeV2 algorithm.

were also found to be ampicillin (84.9%, 73/86), tetracycline (72.1%, 62/86), nalidixic acid (68.6%, 59/86), and chloramphenicol (54.7%, 47/86), while 65 isolates (75.6%) were MDR (Figures 2A,B). A total of 34 isolates (39.5%) were resistant to at least one of the third-generation cephalosporins (ceftazidime and cefotaxime), including 9 *S.* Enteritidis (45%, 9/20), 8 *S.* Typhimurium (50%, 8/16), 7 *S.* I 4,[5],12:i:- (50%, 7/14), 2 *S.* Derby (2/4), 2 *S.* Saintpaul (2/2), 2 *S.* Stanley (2/3), one of each *S.* Agona (1/7), *S.* Bareilly (1/1), *S.* Infantis (1/1), *S.* Mbandaka (1/3), and *S.* Thompson (1/3), while 13 of these 34 isolates showed concurrent resistance to ciprofloxacin (Supplementary Table S1). Five isolates including 4 *S.* Enteritidis and 1 *S.* Agona showed resistant to polymyxin E. Moreover, two isolates (one of each *S.* Enteritidis and *S.* Infantis) were found to be resistant to carbapenems (imipenem and meropenem).

Furthermore, the *Salmonella* isolates from humans with diarrhea displayed higher resistance rates to several antimicrobials, including ampicillin, tetracycline, ceftazidime, and cefotaxime ($p < 0.01$), than the isolates from retail chilled chicken carcasses (Figure 2A). In addition, the human-associated *Salmonella* isolates showed higher MDR ($p < 0.05$) than those from retail chilled chicken carcasses (Figure 2B).

The AMR of the mainly detected *Salmonella* serotypes ($n > 10$) from retail chilled chicken carcasses and humans with diarrhea were estimated that the *S.* Indiana isolates exhibited the most pan-resistance (100%) and MDR (93.8%), followed by *S.* I 4,[5],12:i:- (pan-resistance, 100%; MDR, 87.5%), and *S.* Enteritidis (pan-resistance, 97.1%; MDR, 71.4%) (Table 1). Of each serotype, the human-associated isolates *S.* Agona (71.4%), *S.* I 4,[5],12:i:- (92.9%), and *S.* Typhimurium (81.3%) showed higher MDR than those of chicken-associated isolates (16.7, 50, and 40%, respectively).

3.3 Phylogenetic analysis revealed genomic diversity of *Salmonella*

All of the 174 isolates including 88 chicken-associated and 86 human-associated *Salmonella* isolates were subjected to whole genome sequencing (WGS). Based on data from the NCBI Pathogen Detection, 149 out of the 174 isolates (85.6%) could be categorized into known SNP clusters ($n = 60$). The distribution of SNP clusters differed between retail chicken and humans, with 27 unique SNP clusters found in chicken source and 25 unique cluster found in human source (Figure 3). Eight SNP clusters (PDS000078427.2, PDS000043691.33, PDS000096798.86, PDS000106143.144, PDS000144678.1, PDS000004748.67, PDS000101103.13, and PDS000026869.266) were detected in both sources, which belonged to six serotypes (*S.* London, *S.* Indiana, *S.* I 4,[5],12:i:-, *S.* Agona, *S.* Typhimurium, and *S.* Enteritidis) (Supplementary Table S4), revealing diverse origins of *Salmonella* isolated in this study.

Notably, the top six prevalent SNP clusters among the 174 isolates included PDS000026869.266, PDS000043691.33, PDS000004748.67, PDS000115660.28, PDS000101103.13, and PDS000106143.144. All of these, except PDS000115660.28, were detected in both chicken and humans. PDS000115660.28 only contained isolates from humans. The most prevalent SNP cluster PDS000026869.266 contained 10 isolates from humans and 7 isolates from retail chicken.

S. Typhimurium/*S.* I 4,[5],12:i:- displayed the highest diversity with 15 detected SNP clusters, followed by *S.* Derby with 8, *S.* Thompson with 6, and *S.* Enteritidis with 5 (Figure 3A). Among the designated SNP clusters ($n = 35$) of the chicken isolates, 21 of them had a minimum SNP distance within environmental source below 21 (Figure 3B), while 14 of them had a minimum SNP distance within human source below 21 (Figure 3C). In contrast, 24 of the designated SNP clusters ($n = 33$) of the human-associated isolates had a minimum SNP distance within human source below 21 (Figure 3B), while 15 clusters had a minimum SNP distance within environmental source below 21 (Figure 3C). These findings demonstrated that a large number of the designated SNP clusters contained isolates circulating in both human and environmental sources (Pightling et al., 2018). For the 25 isolates that did not fit into designated SNP clusters, 15 were from retail chicken and 10 were from humans. The most common serotypes among these were *S.* Typhimurium, *S.* Enteritidis, and *S.* Agona.

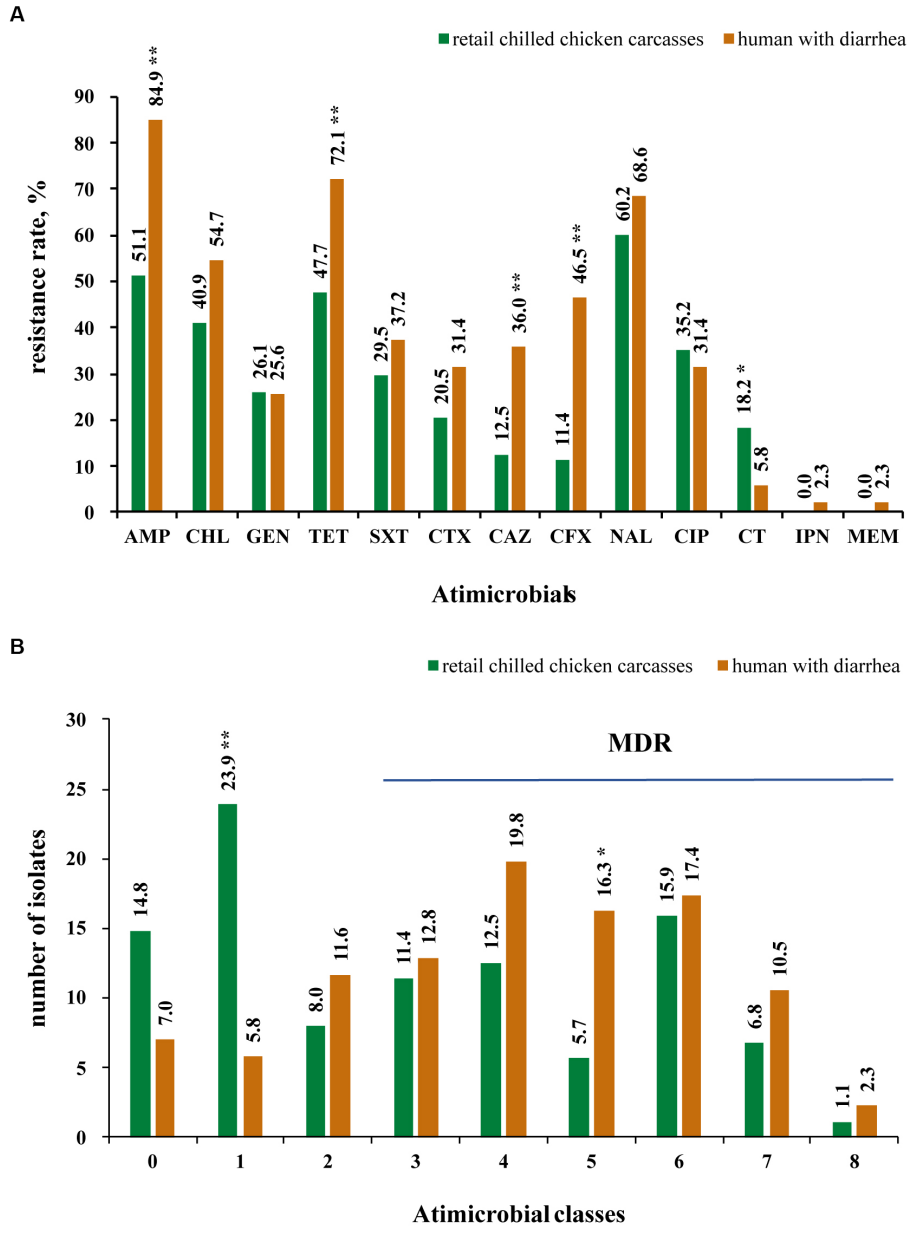

FIGURE 2
Antimicrobial resistance of 174 Salmonella isolates collected from retail chilled chicken carcasses and humans with diarrhea in this study. **(A)** Antimicrobial resistance rate of 174 Salmonella isolates to 13 antimicrobials. **(B)** Number of Salmonella isolates showing resistance to studied antimicrobials. * and ** mean significant difference with the *p* value less than 0.05 and 0.01, respectively.

TABLE 1 AMR of the mainly detected *Salmonella* serotypes, %(n/N).

Serotypes	Total		Retail chilled chicken carcasses		Humans with diarrhea	
	Pan-resistant	MDR	Pan-resistant	MDR	Pan-resistant	MDR
Agona	84.6 (11/13)	46.2 (6/13)	83.3 (5/6)	16.7 (1/6)	85.7 (6/7)	71.4 (5/7)*
Derby	93.3 (14/15)	60 (9/15)	90.9 (10/11)	45.5 (5/11)	100 (4/4)	100 (4/4)
Enteritidis	97.2 (33/35)	72.2 (25/35)	100 (15/15)	60 (9/15)	95 (19/20)	80 (16/20)
I 4,[5],12:i:-	100 (16/16)	87.5 (14/16)	100 (2/2)	50 (1/2)	100 (14/14)	92.9 (13/14)*
Indiana	100 (16/16)	93.8 (15/16)	100 (15/15)	93.3 (14/15)	100 (1/1)	100 (1/1)
Typhimurium	83.9 (26/31)	61.3 (19/31)	80 (12/15)	40 (6/15)	87.5 (14/16)	81.3 (13/16)*

* means significant difference with the *p* value less than 0.05.

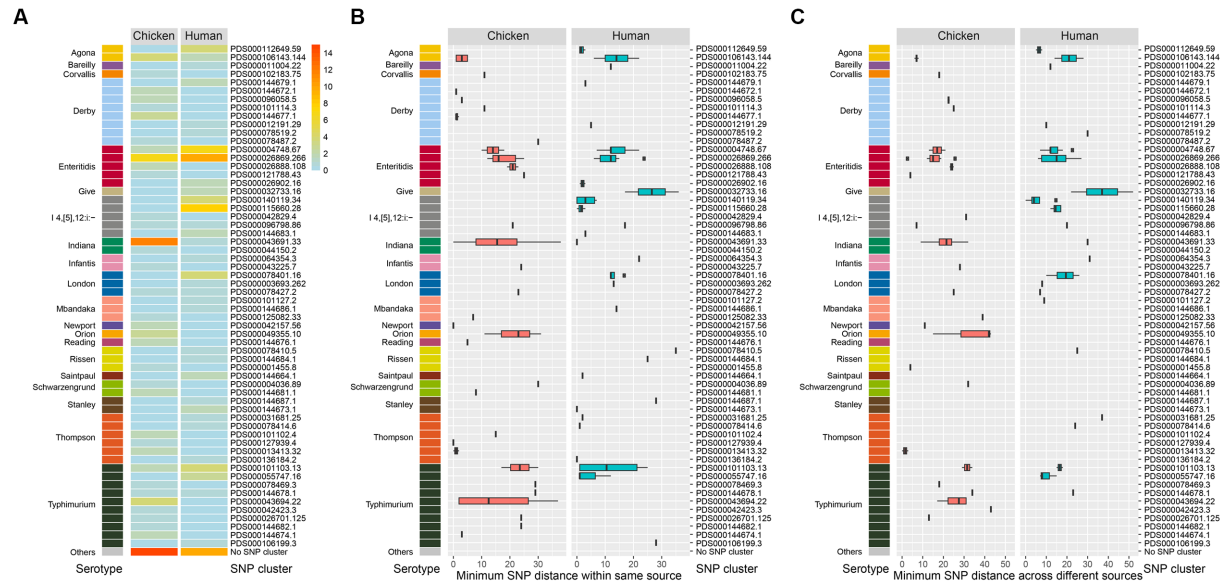

FIGURE 3
Genomic diversity of *Salmonella* isolates. **(A)** Heat map of serotype distribution and the designated SNP clusters from different sources. Color indicates the number of isolates. **(B)** Box plot of minimum SNP distance within same source of the designated SNP clusters of different serotypes. **(C)** Box plot of minimum SNP distance across different sources of the designated SNP clusters of different serotypes.

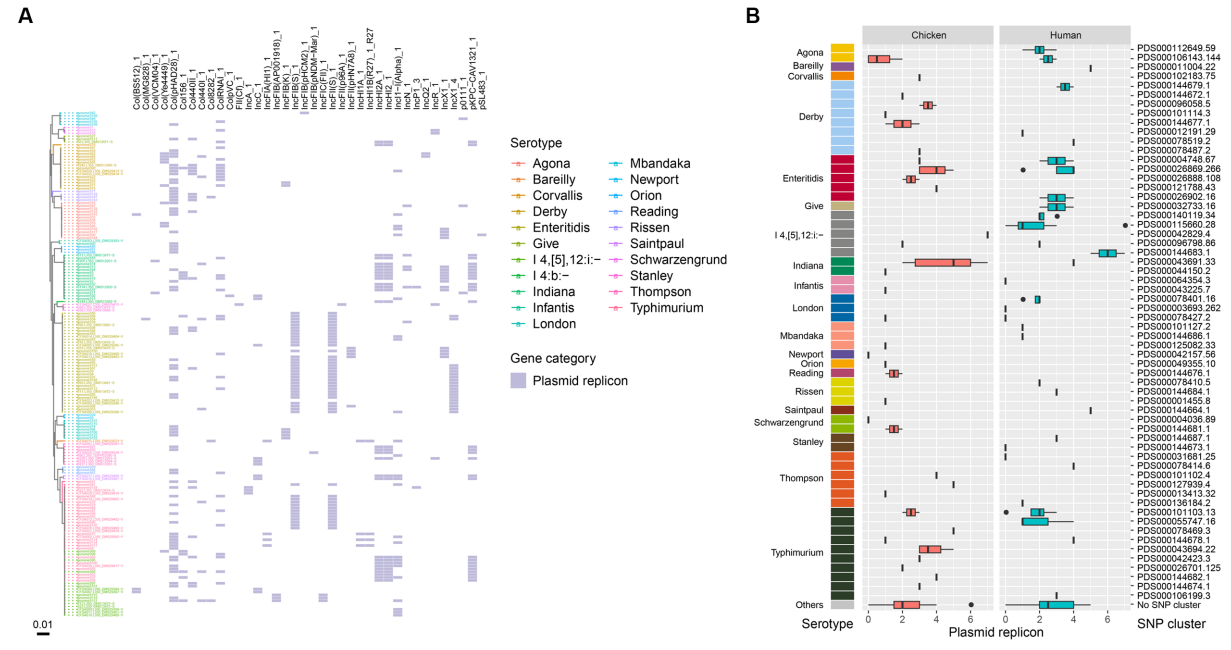

FIGURE 4
Plasmid replicons in the *Salmonella* genomes. **(A)** Core genome phylogeny of the *Salmonella* genomes and distribution of plasmid replicons. Color of the heatmap indicates the presence of plasmid replicons. **(B)** Box plot of plasmid replicon counts of the designated SNP clusters of different serotypes.

3.4 MGE profiles

Plasmid replicons were detected in a majority of the bacterial isolates from this study, with only 9.8% (17/174) showing no evidence of plasmids in their genomes. Ten of the human-associated isolates and seven of the chicken-associated isolates had no detected plasmid replicons. A total of 38 plasmid replicons were identified among the 174 isolates, with 5 isolates showing the highest number of 7 plasmid replicons (Figure 4A). The most frequently identified plasmid replicons were Col(pHAD28), IncFIB(S), and IncFII(S) (Figure 4A). Conversely, less common types such as Col(MG828), ColpVC, FII(*Cf*), IncFIB(pHCM2), IncFIB(pNDM-Mar), IncFII(p96A), and pSL483 were present in certain single isolate, respectively. Additionally, the prevalence of plasmid replicons exhibited variations

over isolates from retail chicken and humans. For instance, strains isolated in chicken showed a higher prevalence of IncHI2A, IncHI2 and pKPC-CAV1321, while those isolated in humans showed a higher prevalence of IncI1-I(Alpha) and Col440II. When considering different serotypes, *S.* Typhimurium and *S.* Indiana exhibited the largest number of plasmid types (16 in total, respectively), followed by *S.* I 4,[5],12:i:- (15 in total), and *S.* Enteritidis (14 in total) (Figure 4A). Notably, isolates from *S.* Enteritidis were more likely to carry plasmid replicons of IncFIB(S), IncFII(S), and IncX1, isolates from *S.* Typhimurium were more likely to carry Col(pHAD28), IncFIB(S), and IncFII(S), and isolates from *S.* Indiana were more likely to carry IncHI2A, IncHI2, and pKPC-CAV1321 (Figure 4A). Among the designated SNP clusters, PDS000043691.33 (*S.* Indiana), PDS000115660.28 (*S.* I 4,[5],12:i:-), PDS000042829.4 (*S.* I 4,[5],12:i:-), and PDS000144683.1 (*S.* I 4,[5],12:i:-) each harbored the highest number of plasmid replicon types, with seven types each (Figure 4B). Chicken-associated SNP clusters had a higher median number of plasmid replicons (3) compared to human-associated SNP clusters (2) (Figure 4B).

We also observed a high prevalence of intact prophages among the isolates, with only 17 lacking any. A total of 45 intact prophages were identified among the 174 isolates, with 31 isolates harboring only one prophage type (Figure 5A). Most isolates (72.4%, 126/174) contained at least two intact prophages. Seven isolates harbored more than 5 intact prophages, which belonged to serotypes such as Mbandaka, Typhimurium, Schwarzengrund, and Reading. Notably, 64 isolates, representing various serotypes, contained both Gifsy (Gifsy-1/Gifsy-2) and Salmon 118,970 sal3 prophages (Figure 5A). Among the various prophages identified, Gifsy-2 was more commonly present in isolates from humans (41/86) than retail chicken (26/88). When examining different serotypes, the Gifsy and Salmon 118,970 sal3 prophages were more likely to be carried by *S.* Enteritidis, *S.* Typhimurium, and *S.* I 4,[5],12:i:-, and were less frequently found in other serotypes. All the *S.* Enteritidis isolates carried Gifsy-2 and Salmon 118,970 sal3 prophages (Figure 5A). It is worth noting that all the isolates of *S.* I 4,[5],12:i:- carried Gifsy-2 other than Gifsy-1 prophage, while isolates of *S.* Typhimurium carried both types of Gifsy-1 and Gifsy-2 prophages. Most *S.* Indiana isolates (9/16) did not carry any intact prophages, while a small portion of *S.* Indiana isolates (6/16) carried only one intact prophage. The two *S.* Newport isolates were found to be free of intact prophages. In addition, SNP clusters that were associated a high number of intact prophage types (more than 5) contained PDS000144674.1 of *S.* Typhimurium (2/30), PDS000144676.1 of *S.* Reading (2/3), PDS000004036.89 of *S.* Schwarzengrund (1/3), and PDS000125082.33 of *S.* Mbandaka (1/5) (Figure 5B).

3.5 ARG profiles

To determine the presence of resistance genes, all 174 genome sequences were screened against the ResFinder database. A total of 79 different antibiotic resistance genes (ARGs) were identified in the isolates (Figure 6A). The majority of these ARGs belonged to beta-lactam resistance ($n = 19$) and aminoglycoside resistance ($n = 19$) categories. Among the CTX-M-type beta-lactamases, 9 different $bla_{\text{CTX-M}}$ genes were detected, with $bla_{\text{CTX-M-55}}$ ($n = 11$), $bla_{\text{CTX-M-65}}$ ($n = 10$), and $bla_{\text{CTX-M-14}}$ ($n = 6$) being the most prevalent (Figure 6A).

Notably, all isolates were positive for the aminoglycoside resistance gene *aac(6′)-Iaa*, and a large number of them carried the beta-lactam resistance gene $bla_{\text{TEM-1B}}$ ($n = 67$), the tetracycline resistance gene *tet(A)* ($n = 64$), aminoglycoside resistance genes *aph(6)-Id* ($n = 65$) and *aph(3″)-Ib* ($n = 60$), the sulfonamide resistance gene *sul2* ($n = 50$), and the amphenicol resistance gene *floR* ($n = 50$) (Figure 6A). Six types of

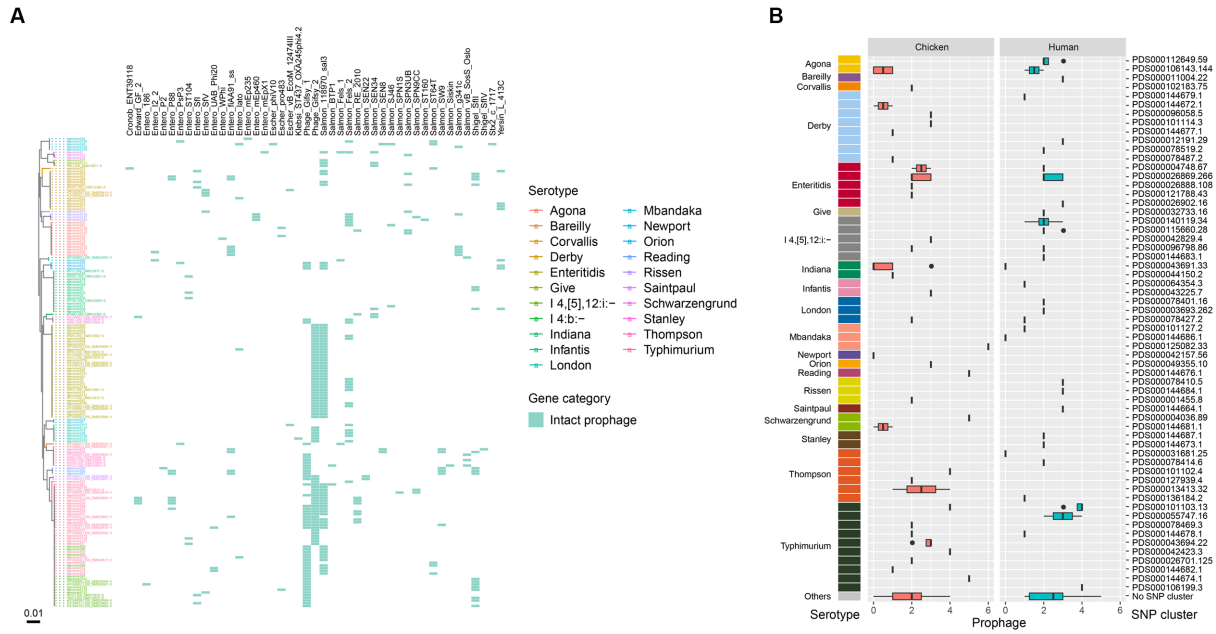

FIGURE 5
Prophages in the *Salmonella* genomes. **(A)** Core genome phylogeny of the *Salmonella* genomes and distribution of intact prophages. Color of the heatmap indicates the presence of prophages. **(B)** Box plot of prophage counts of the designated SNP clusters of different serotypes.

quinolone resistance genes were found, including *qnrS1* ($n = 23$), *qnrB4* ($n = 7$), *qnrB6* ($n = 6$), *qepA1* ($n = 3$), *qnrS2* ($n = 2$), and *qnrA7* ($n = 1$). Additionally, one *S.* Typhimurium isolate was positive for the colistin resistance gene *mcr-1.1*, and two *S.* Thompson isolates carried *mcr-9.1* (Figure 6A). All these *mcr*-positive isolates were from chicken. One *S.* Enteritidis isolate was positive for the $bla_{\text{NDM-1}}$ gene. Furthermore, the *mcr-1.1* gene was detected on an IncHI2 plasmid replicon, while the $bla_{\text{NDM-1}}$ gene was carried on an IncC plasmid. The plasmid carrying the *mcr-9.1* gene could not be identified as the gene was located on small contigs with a length of ~2,500 bp (data not shown).

Due to the high prevalence of genes related to aminoglycoside and beta-lactam resistance, all isolates contained at least one ARG in their genomes (Figure 6A). Three isolates, genomeS31, genomeS44, and genomeS52, harbored the largest number of ARGs ($n = 21$), which belonged to two serotypes, *S.* Indiana and *S.* Typhimurium. When considering different serotypes, *S.* Indiana exhibited the largest number of ARGs (14.5 in median), followed by *S.* Thompson (12.5 in median), *S.* I 4,[5],12:i:- (6 in median), and *S.* Typhimurium (5.5 in median). SNP clusters that were associated with higher number of ARGs included PDS000043691.33 (14 in median, *S.* Indiana), PDS000044150.2 (20, *S.* Indiana), PDS000055747.16 (16 in median, *S.* Typhimurium), PDS000043694.22 (17 in median, *S.* Typhimurium), PDS000144683.1 (15.5 in median, *S.* I 4,[5],12:i:-), PDS000127939.4 (19, *S.* Thompson), PDS000078401.16 (16 in median, *S.* London), PDS000144664.1 (16 in median, *S.* Saintpaul), and PDS000078519.2 (16, *S.* Derby) (Figure 6B).

It is worth noting that most of the ARGs were detected on plasmid sequences (Supplementary Figure S1A). The aminoglycoside resistance gene *aac(6′)-Iaa* presented in all the isolates was found located on chromosome sequences (Supplementary Figure S1B). Additionally, a total of 40 isolates were found carrying no ARGs other than the *aac(6′)-Iaa* gene, which belonged to a variety of serotypes such as *S.* Typhimurium ($n = 10$), *S.* Enteritidis ($n = 9$), *S.* Mbandaka ($n = 5$), etc.

4 Discussion

Salmonella is one of the most widespread pathogenic foodborne bacteria in Chinese food products and retail chicken have been recognized as one of the major sources of human salmonellosis (Paudyal et al., 2018). Moreover, there has been an increase in reports of the dissemination of resistant *Salmonella* from chicken to humans through the chicken production chain (Kongsanan et al., 2021; Manzari et al., 2022). In this study, we surveyed the prevalence, serovar distribution, MDR, and genomic characteristics of *Salmonella* in chilled chicken carcass and humans with diarrhea sources in Qingdao, China in 2020.

Overall, 24.4% of the retail chilled chicken carcass samples purchased in 2020 in local markets contained *Salmonella*. This prevalence is lower than what was found in other provinces (average, 39.3%) in China (Wang et al., 2020a), Brazil (31.5%) (Perin et al., 2020), but higher than those in Korea (14.1%) (La et al., 2021), the USA (17.9%) (Peng et al., 2016), and European Union (7.1%) (Gonçalves-Tenório et al., 2018). Thus, chicken meat in retail may be an important source of human salmonellosis in China. Besides, data reported that *Salmonella* prevalence in chilled poultry meat was statistically higher than that of frozen poultry meat and ambient poultry meat in China (Sun et al., 2021). In China, immersion chilling is employed more frequently and Chinese consumers have a preference for chilled meat (25% market share) over frozen meat (15% market share) (Liu et al., 2017). However, once a sample is contaminated with *Salmonella* during the immersion process, the contamination may spread among the whole batch of carcasses, leading to an increase in the prevalence of pathogens on finished products (Marmion et al., 2021). Therefore, in response to potential public health pressures, more effective intervention strategies during immersion chilling should be implemented to control the quality and safety of chicken products.

Serotype results suggested a wide range of *Salmonella* serotypes present in retail chilled chicken carcasses and humans with diarrhea. The dominance of *S.* Enteritidis and *S.* Typhimurium among the

FIGURE 6
ARGs in the *Salmonella* genomes. **(A)** Core genome phylogeny of the *Salmonella* genomes and distribution of ARGs. Color of the heatmap indicates the presence of ARGs. **(B)** Box plot of ARG counts of the designated SNP clusters of different serotypes.

studied *Salmonella* isolates, is consistent with the data obtained from chicken and human previously in China and other regions worldwide (EFSA and ECDC, 2018; Balasubramanian et al., 2019; Wang et al., 2020a). Besides, *S.* Indiana is another frequently detected serotype among chicken samples and is also detected from humans with diarrhea in this study. Accordingly, since 2009, the presence of *S.* Indiana has seen a remarkable increase, which has become one of the top three common serotypes in China (Zhang et al., 2022a). *S.* Indiana has been isolated from a wide and diverse variety of sample sources in 18 provinces throughout China and was most frequently recovered from food, followed by animals, the environment and then people, especially children under 6 years old with low immunity (Gong et al., 2017). What is more, *Salmonella* serovars Enteritidis, Typhimurium, and Indiana are also reported as the most common serotypes associated with human infections and outbreaks (Kuang et al., 2018b; Wu et al., 2018). Thus, the high prevalence of these *Salmonella* serotypes in chicken carcasses and humans with diarrhea indicates a significant risk to public health. The monitoring of the emergence and prevalence of these *Salmonella* serotypes is essential for the better control of salmonellosis.

The combination of MLST and serotype detection can facilitate research on the hereditary and evolutionary relationships of *Salmonella* (Xu et al., 2022). The most common *Salmonella* sequence types were found to be ST11, ST34, and ST19 in this study, where the corresponding serotypes were *S.* Enteritidis and *S.* Typhimurium (including *S.* I 4,[5],12:i:-). This is consistent with findings from previous reports in China (Wang et al., 2020b; Xu et al., 2022). Accordingly, among *S.* Enteritidis worldwide, ST11 is the predominant sequence type, accounting for 89% of the sequence types in the EnteroBase database (Ashton et al., 2016). However, a recent report from Malaysia showed that ST1925 was dominantly detected among chicken-associated *S.* Enteritidis strains (Zakaria et al., 2022). In America, the major prevalent STs are ST34, ST33, and ST11 (Pethplerdprao et al., 2017), which shows that STs may be associated with regions. Furthermore, the other mainly identified sequence types, ST13 (*S.* Agona), ST17 (*S.* Indiana), and ST40 (*S.* Derby), were also previously reported to be associated with salmonellosis in human (Luo et al., 2022; Sun et al., 2022; De Jesus Bertani et al., 2023). Observations from the present study therefore provided important evidence and confirmed further that these types of *Salmonella* can serve an important role in human diarrhea in Qingdao, China.

Herein, we found that 53.4 and 75.6% of retail chilled chicken carcass- and humans with diarrhea-associated *Salmonella* isolates were MDR, respectively. High percentages of MDR strains were also observed in *Salmonella* isolated from poultry (Li et al., 2022), food animals (Tang et al., 2023) and humans (Chen et al., 2022) in various regions of China. The surge in antimicrobial-resistant *Salmonella* isolates is recognized as a crucial public health issue (Eng et al., 2015). The major of *Salmonella* isolates among both sources were resistant to four antimicrobials of nalidixic acid, ampicillin, tetracycline and chloramphenicol, which are the first-line drugs used against bacterial infection in animal farms worldwide (Nhung et al., 2016; Lekagul et al., 2019). This finding is consistent with the literature from different countries, including China (Tamang et al., 2011; Cai et al., 2016; EFSA and ECDC, 2018). Our results also suggested that *Salmonella* isolates in this study showed high level resistance to third generation cephalosporins (ceftazidime and cefotaxime) and quinolone (ciprofloxacin), which are the first-line drugs used against bacterial infection in clinics (Tack et al., 2020). These findings are regarded as significant threats to public health, leading to limited drugs of choice for salmonellosis infection treatment in animals and humans. The high AMR rate of *S.* Enteritidis, *S.* Typhimurium (including *S.* I 4,[5],12:i:-) and *S.* Indiana, with a widespread AMR spectrum is of concern. Moreover, several *Salmonella* isolates showed resistance to colistin, which is considered as one of the last-resort therapeutic options for the defense of multidrug-resistant Enterobacteriaceae (Elbediwi et al., 2019). This finding of colistin resistance agreed with results reported by other recent studies in *Salmonella* isolates from poultry and humans (Fortini et al., 2022; Tang et al., 2022). Of note, two carbapenems (imipenem and meropenem) resistant *Salmonella* isolates were also found in this study. Therefore, the spread of MDR *Salmonella* isolates, especially resistant to these clinically important antimicrobials, including fluoroquinolone, third-generation cephalosporins, colistin and carbapenems, will result in challenges with public health.

Phylogenetic analysis revealed a high level of genomic diversity of the *Salmonella* isolates collected from Qingdao region in China. Eight SNP clusters containing both chicken and human isolated collected in this study were identified through NCBI Pathogen Detection. Six of these clusters (PDS000043691.33, PDS000096798.86, PDS000106143.144, PDS000004748.67, PDS000101103.13, and PDS000026869.266) were featured by involving a large number of strains from different countries, indicating these isolates had been widely spread around the world. In contrast, the other two clusters (PDS000078427.2 and PDS000144678.1) formed distinct, locally restricted clades circulating in a limited number of cities in China. Most of the chicken and human isolates shared no common SNP clusters, indicating multiple origins of these isolates. Interestingly, the major SNP clusters found in the present study comprised numerous strains from clinical infections in China and other countries. This highlights the importance of conducting additional research on the dissemination of strains associated with these SNP clusters.

Mobile genetic elements (MGEs), such as bacteriophages and plasmids, are DNA segments that carry genes involving with DNA movement within genomes (Frost et al., 2005). The presence of intact Salmon 118,970 sal3, Gifsy-2, and Gifsy-1 was found at a higher frequency in the genomes of *S.* Typhimurium, *S.* I 4,[5],12:i:-, and *S.* Enteritidis, whereas such prophage types were seldom observed in other prevalent serotypes such as *S.* Indiana, *S.* Agona, and *S.* Derby. These findings indicate the presence of prophage types is associated with serotypes and may be related to the evolutionary history of different serotypes. Recently, a study of a large-scale investigation on *S.* Typhimurium has reported the common presence of the above-mentioned prophages in *S.* Typhimurium isolated in China (Wang et al., 2023). Furthermore, the specific phage, Salmon 118,970 sal3 was not only found in *Salmonella*, but also found in *E. coli* (Li et al., 2020) and *Morganella morganii* (Minnullina et al., 2019), suggesting that this phage has a relatively wide host range. The presence of Gifsy-1 and Gifsy-2 and their contribution to virulence in *S.* Typhimurium has been widely reported (Ho and Slauch, 2001).

Plasmids play a crucial role as the primary vehicles for horizontal gene transfer (HGT) in bacteria (De La Cruz and Davies, 2000). Among the isolates, the Col(pHAD28) plasmid replicon was the most common, and the IncF plasmid replicon was the second most common. Interestingly, the IncF is also one of the most prevalent plasmid replicons in *Salmonella* isolated from food animals in the

United States (Mcmillan et al., 2019). The presence of Col(pHAD28) plasmid replicon was observed in a total of 14 serotypes identified in this study, indicating its high occurrence in *Salmonella* isolated from Qingdao region in China. Continuous monitoring of the spread of this plasmid is essential, as several studies have reported the presence of PMQR genes within this plasmid (Li et al., 2021; Hurtado et al., 2022).

Genomic analysis showed that the *Salmonella* isolates harbored different antimicrobial resistant genes. All of the studied *Salmonella* isolates harbored the *aac(6′)-Iaa* gene. However, *aac(6′)-Iaa* gene and similar genes usually are transcriptionally silent and rarely become transcriptionally active. AAC(6′)-Iaa is a typical member of the [A] family in that it acetylates tobramycin, kanamycin, and amikacin effectively but acetylates gentamicin ineffectively (Salipante and Hall, 2003). The mere presence of this gene does not confer aminoglycoside resistance in *Salmonella* (Magnet et al., 1999; Neuert et al., 2018). Besides, 81.4% (96/118) of the isolates resistant to the aminoglycoside class harbored different aminoglycoside resistance genes in addition to the *aac(6′)-Iaa* gene. Among diverse mechanisms of aminoglycoside resistance, enzymatic modification is the most prevalent mechanism in pathogenic bacteria, including *Salmonella* (Ramirez and Tolmasky, 2010; Biswas et al., 2019). Moreover, our study revealed 52/57 (91.2%) of the isolates resistant to the third-generation cephalosporins harbored different beta-lactam resistance genes, which were common detected in *S.* Enteritidis, *S.* Typhimurium, *S.* I 4,[5],12:i:-, and *S.* Indiana. The *bla* genes control the resistance to beta-lactam antimicrobials by hydrolyzing the beta-lactam ring, leading to antibiotic inactivation (Jacoby, 2009; Eguale et al., 2017). In this study, the frequently detected $bla_{\text{TEM-1B}}$ gene (37.9%, 67/174) conferring resistance to ampicillin, is the dominant beta-lactam in most *Salmonella* serotypes worldwide (Eguale et al., 2017). Meanwhile, nine different CTX-M-type ESBL-producing were found in this study. During the last decade, the most frequently encountered (particularly in areas of Europe and Asia) ESBL genes were those encoding the CTX-M enzyme family, primarily carried by transferable plasmids and transposons (Cantón et al., 2012). The emergence of CTX-M-type ESBL-producing *Salmonella* has been reported in clinical cases, animals, and food samples worldwide, including China (Brown et al., 2018; Wang et al., 2020a). Five different *tet* resistance genes were found in 88/174 (50.6%) of the studied isolates, 84 of which were resistant to tetracyclines. Additionally, six plasmid-mediated quinolone resistance (PMQR) genes, with *qnrS1* prevalently detected, were identified in among the examined isolates belonging to different serotypes. These critical genes in *Salmonella* isolates from both chicken and human related samples present a tremendous public health concern. It is essential that the existence of the acquired antimicrobial resistant genes in bacterial genomes does not inevitably confer phenotypic resistance and vice versa.

Through NCBI's Pathogen Detection platform, a total of 60 SNP clusters was categorized using isolates collected in this study (Supplementary Table S4). A SNP cluster is a cluster of isolates where each isolate is less than or equal to 50 SNPs distant from others, which offers higher resolution than serotype and MLST for outbreak surveillance purpose. In the designated SNP clusters, we identified several SNP clusters carrying PMQR genes. Some of these SNP clusters contained a large number of isolates from various isolation locations harboring specific PMQR genes, for example, *qnrS1* in PDS000112649.59 of *S.* Agona, *qnrB6* in PDS000102183.75 of *S.* Corvallis, and *qnrS1* in PDS000115660.28 of *S.* 4,[5],12:i:-; *qnr*-carrying strains with the same sequence types have been reported to be circulating globally with a high rate of quinolone resistance (Fernández et al., 2018; Zhang et al., 2020; Cadel-Six et al., 2021; Lee et al., 2021; Chen et al., 2023).

On the other hand, there are *qnr*-carrying isolates collected uniquely or mostly from China in SNP clusters with small numbers of matched isolates such as PDS000078519.2 of *S.* Derby, PDS000013413.32 of *S.* Thompson, and PDS000055747.16 of *S.* Typhimurium, indicating that these strains were circulating locally in specific regions in China and tend to be emerging threat to public health. Consistently, in a recent study investigating ciprofloxacin resistance in *Salmonella* from humans, food and animals collected in Shanghai, China, *qnr*-positive isolates were mostly *S.* Thompson, *S.* Derby and *S.* Typhimurium (Kuang et al., 2018a).

Apart from *qnr*-carrying isolates, this study also detected three *mcr*-carrying isolates. The isolate harboring *mcr-1.1* was found clustered in the SNP cluster PDS000042829.4 of *S.* I 4,[5],12:i:-, which contained a total of four *mcr-1.1*-carrying isolates uniquely from China, with one isolated from a diarrheal patient (Sun et al., 2023) and another one isolated from ready-to-eat pork (Wang et al., 2018). The two *mcr-9.1*-carrying isolates were designated to the SNP cluster PDS000101102.4 of *S.* Thompson. This SNP cluster had a total of 14 isolates collected from China, of which 11 were *mcr-9.1* positive and 9 were *qnrB4* positive. Interestingly, there are 8 isolates carrying both *mcr-9.1* and *qnrB4*. A recent study also reported a *S.* I 4,[5],12:i:- isolate carrying both *mcr-9.1* and *qnrA1* and it was found that these clinically relevant resistance genes were driven by IncHI2-ST1 plasmids (Vázquez et al., 2023).

Additionally, the $bla_{\text{NDM-1}}$-carrying *S.* Enteritidis isolate was associated with the SNP cluster PDS000004748.67, which had a total of 258 matched isolates, but no other isolates within this cluster were $bla_{\text{NDM-1}}$ positive, suggesting the $bla_{\text{NDM-1}}$ positive isolate has recently acquired plasmid carrying $bla_{\text{NDM-1}}$ gene. It should be paid great attention to as the NDM-1-harboring plasmids confer resistance to all β-lactams (Huang et al., 2017).

In summary, the frequently detection of *Salmonella* from retail chilled chicken carcasses in Qingdao, China, highlighted the importance of improving food hygiene measures to reduce the contamination and transmission of this bacterium. We found a considerable diversity of *Salmonella* serotypes and sequence types among both chicken and human, with MDR, posing a severe risk to food safety and public health. The finding of eight worldwide spread SNP clusters in this study emphasized additional research on the dissemination of strains associated with these SNP clusters. The MGE results showed high prevalence of plasmid replicons and prophages among *Salmonella* from both sources, and the presence of these MGEs associated with specific serotypes indicated different evolutionary history of these serotypes. Given the high prevalence of CTX-M type ESBL-producing and PMQR genes identified in this study, we strongly recommend that both clinical and veterinary sectors routinely test for AMR when resistance to these antimicrobials is detected in *Salmonella*. This will enhance monitoring and guide the selection of effective treatments. Overall, it is essential to continue monitoring the *Salmonella* serotypes, implement the necessary prevention and strategic control plans, and conduct an epidemiological surveillance system based on whole-genome sequencing.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

WW: Writing – original draft, Writing – review & editing. JC: Writing – original draft, Writing – review & editing. FLiu: Writing – review & editing. YH: Writing – review & editing. FLi: Writing – review & editing. XD: Writing – review & editing. YD: Writing – review & editing. SL: Writing – original draft, Writing – review & editing. JX: Writing – review & editing. ZZ: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1295769/full#supplementary-material

References

Antunes, P., Mourão, J., Campos, J., and Peixe, L. (2016). Salmonellosis: the role of poultry meat. *Clin. Microbiol. Infect.* 22, 110–121. doi: 10.1016/j.cmi.2015.12.004

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., et al. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* 44, W16–W21. doi: 10.1093/nar/gkw387

Ashton, P. M., Nair, S., Peters, T. M., Bale, J. A., Powell, D. G., Painset, A., et al. (2016). Identification of Salmonella for public health surveillance using whole genome sequencing. *PeerJ* 4:e1752. doi: 10.7717/peerj.1752

Balasubramanian, R., Im, J., Lee, J. S., Jeon, H. J., Mogeni, O. D., Kim, J. H., et al. (2019). The global burden and epidemiology of invasive non-typhoidal Salmonella infections. *Hum. Vaccin. Immunother.* 15, 1421–1426. doi: 10.1080/21645515.2018.1504717

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Biswas, S., Li, Y., Elbediwi, M., and Yue, M. (2019). Emergence and dissemination of mcr-carrying clinically relevant *Salmonella Typhimurium* monophasic clone ST34. *Microorganisms* 7:298. doi: 10.3390/microorganisms7090298

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., et al. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother.* 75, 3491–3500. doi: 10.1093/jac/dkaa345

Brown, A. C., Chen, J. C., Watkins, L. K. F., Campbell, D., Folster, J. P., Tate, H., et al. (2018). Ctx-m-65 extended-spectrum β-lactamase-producing *Salmonella enterica* serotype Infantis, United States. *Emerg. Infect. Dis.* 24, 2284–2291. doi: 10.3201/eid2412.180500

Cadel-Six, S., Cherchame, E., Douarre, P.-E., Tang, Y., Felten, A., Barbet, P., et al. (2021). The spatiotemporal dynamics and microevolution events that favored the success of the highly clonal multidrug-resistant monophasic *Salmonella Typhimurium* circulating in Europe. *Front. Microbiol.* 12:651124. doi: 10.3389/fmicb.2021.651124

Cai, Y., Tao, J., Jiao, Y., Fei, X., Zhou, L., Wang, Y., et al. (2016). Phenotypic characteristics and genotypic correlation between Salmonella isolates from a slaughterhouse and retail markets in Yangzhou, China. *Int. J. Food Microbiol.* 222, 56–64. doi: 10.1016/j.ijfoodmicro.2016.01.020

Cantón, R., González-Alba, J. M., and Galán, J. C. (2012). CTX-M enzymes: origin and diffusion. *Front. Microbiol.* 3:110. doi: 10.3389/fmicb.2012.00110

Carattoli, A., and Hasman, H. (2020). Plasmidfinder and *in silico* pMLST: identification and typing of plasmid replicons in whole-genome sequencing (WGS). *Methods Mol. Biol.* 2075, 285–294. doi: 10.1007/978-1-4939-9877-7_20

CDC (2019). Antibiotic resistance threats in the United States. Available at: https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf. (Accessed October 2022).

Chen, J., Ed-Dra, A., Zhou, H., Wu, B., Zhang, Y., and Yue, M. (2022). Antimicrobial resistance and genomic investigation of non-typhoidal Salmonella isolated from outpatients in Shaoxing city, China. *Front. Public Health* 10:988317. doi: 10.3389/fpubh.2022.988317

Chen, K., Xie, M., Wang, H., Chan, E. W.-C., and Chen, S. (2023). Intercontinental spread and clonal expansion of ColRNA1 plasmid-bearing Salmonella Corvallis ST1541 strains: a genomic epidemiological study. *One Health Adv.* 1, 1–15. doi: 10.1186/s44280-023-00017-9

CLSI (2017). *Performance standards for antimicrobial susceptibility testing*. Clinical and Laboratory Standards Institute, Wayne, PA.

De Jesus Bertani, A. M., Vieira, T., Reis, A. D., Dos Santos, C. A., De Almeida, E. A., Camargo, C. H., et al. (2023). Whole genome sequence analysis of the first reported isolate of Salmonella Agona carrying blaCTX-M-55 gene in Brazil. *Sci. Rep.* 13:2299. doi: 10.1038/s41598-023-29599-5

De La Cruz, F., and Davies, J. (2000). Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends Microbiol.* 8, 128–133. doi: 10.1016/S0966-842X(00)01703-0

Ed-Dra, A., Karraouan, B., Allaoui, A. E., Khayatti, M., Ossmani, H. E., Filali, F. R., et al. (2018). Antimicrobial resistance and genetic diversity of Salmonella Infantis isolated from foods and human samples in Morocco. *J. Glob. Antimicrob. Resist.* 14, 297–301. doi: 10.1016/j.jgar.2018.05.019

EFSA and ECDC (2018). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. *EFSA J.* 16:e05500. doi: 10.2903/j.efsa.2018.5500

Eguale, T., Birungi, J., Asrat, D., Njahira, M. N., Njuguna, J., Gebreyes, W. A., et al. (2017). Genetic markers associated with resistance to beta-lactam and quinolone antimicrobials in non-typhoidal Salmonella isolates from humans and animals in Central Ethiopia. *Antimicrob. Resist. Infect. Control* 6:13. doi: 10.1186/s13756-017-0171-6

Elbediwi, M., Li, Y., Paudyal, N., Pan, H., Li, X., Xie, S., et al. (2019). Global burden of colistin-resistant bacteria: mobilized colistin resistance genes study (1980-2018). *Microorganisms* 7:461. doi: 10.3390/microorganisms7100461

Eng, S.-K., Pusparajah, P., Ab Mutalib, N.-S., Ser, H.-L., Chan, K.-G., and Lee, L.-H. (2015). Salmonella: a review on pathogenesis, epidemiology and antibiotic resistance. *Front. Life Sci.* 8, 284–293. doi: 10.1080/21553769.2015.1051243

Fan, J., Cai, H., Fang, Y., He, J., Zhang, L., Xu, Q., et al. (2022). Molecular genetic characteristics of plasmid-borne mcr-9 in *Salmonella enterica* serotype typhimurium and Thompson in Zhejiang, China. *Front. Microbiol.* 13:852434. doi: 10.3389/fmicb.2022.852434

Fernández, J., Guerra, B., and Rodicio, M. R. (2018). Resistance to carbapenems in non-typhoidal *Salmonella enterica* serovars from humans, animals and food. *Vet. Sci.* 5:40. doi: 10.3390/vetsci5020040

Fortini, D., Owczarek, S., Dionisi, A. M., Lucarelli, C., Arena, S., Carattoli, A., et al. (2022). Colistin resistance mechanisms in human *Salmonella enterica* strains isolated by the National Surveillance Enter-net Italia (2016-2018). *Antibiotics (Basel)* 11:102. doi: 10.3390/antibiotics11010102

Frost, L. S., Leplae, R., Summers, A. O., and Toussaint, A. (2005). Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* 3, 722–732. doi: 10.1038/nrmicro1235

Gonçalves-Tenório, A., Silva, B. N., Rodrigues, V., Cadavez, V., and Gonzales-Barron, U. (2018). Prevalence of pathogens in poultry meat: a meta-analysis of European published surveys. *Foods* 7:69. doi: 10.3390/foods7050069

Gong, J., Kelly, P., and Wang, C. (2017). Prevalence and antimicrobial resistance of *Salmonella enterica* serovar Indiana in China (1984-2016). *Zoonoses Public Health* 64, 239–251. doi: 10.1111/zph.12328

Grimont, P. A. D., and Weill, F.-X. (2007). "Antigenic formulae of the *Salmonella* serovars" in *WHO collaborating centre for reference and research on Salmonella*, vol. 9, 1–166.

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321. doi: 10.1093/sysbio/syq010

Heredia, N., and García, S. (2018). Animals as sources of food-borne pathogens: a review. *Anim. Nutr.* 4, 250–255. doi: 10.1016/j.aninu.2018.04.006

Ho, T. D., and Slauch, J. M. (2001). OmpC is the receptor for Gifsy-1 and Gifsy-2 bacteriophages of Salmonella. *J. Bacteriol.* 183, 1495–1498. doi: 10.1128/JB.183.4.1495-1498.2001

Huang, J., Deng, S., Ren, J., Tu, J., Ye, M., and Wang, M. (2017). Characterization of a blaNDM-1-harboring plasmid from a *Salmonella enterica* clinical isolate in China. *Mol. Med. Rep.* 16, 1087–1092. doi: 10.3892/mmr.2017.6733

Hurtado, R., Barh, D., Weimer, B. C., Viana, M. V. C., Profeta, R., Sousa, T. J., et al. (2022). WGS-based lineage and antimicrobial resistance pattern of *Salmonella Typhimurium* isolated during 2000-2017 in Peru. *Antibiotics (Basel)* 11:1170. doi: 10.3390/antibiotics11091170

Hussein, N. H., Al-Kadmy, I. M. S., Taha, B. M., and Hussein, J. D. (2021). Mobilized colistin resistance (mcr) genes from 1 to 10: a comprehensive review. *Mol. Biol. Rep.* 48, 2897–2907. doi: 10.1007/s11033-021-06307-y

Iovleva, A., and Doi, Y. (2017). Carbapenem-resistant Enterobacteriaceae. *Clin. Lab. Med.* 37, 303–315. doi: 10.1016/j.cll.2017.01.005

Jacoby, G. A. (2009). AmpC beta-lactamases. *Clin. Microbiol. Rev.* 22, 161–182. doi: 10.1128/CMR.00036-08

Jain, P., Chowdhury, G., Samajpati, S., Basak, S., Ganai, A., Samanta, S., et al. (2020). Characterization of non-typhoidal Salmonella isolates from children with acute gastroenteritis, Kolkata, India, during 2000-2016. *Braz. J. Microbiol.* 51, 613–627. doi: 10.1007/s42770-019-00213-z

Jajere, S. M. (2019). A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Vet. World* 12, 504–521. doi: 10.14202/vetworld.2019.504-521

Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Devleesschauwer, B., et al. (2015). World health organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. *PLoS Med.* 12:e1001921. doi: 10.1371/journal.pmed.1001921

Kongsanan, P., Angkititrakul, S., Kiddee, A., and Tribuddharat, C. (2021). Spread of antimicrobial-resistant Salmonella from poultry to humans in Thailand. *Jpn. J. Infect. Dis.* 74, 220–227. doi: 10.7883/yoken.JJID.2020.548

Kuang, D., Zhang, J., Xu, X., Shi, W., Chen, S., Yang, X., et al. (2018a). Emerging high-level ciprofloxacin resistance and molecular basis of resistance in *Salmonella enterica* from humans, food and animals. *Int. J. Food Microbiol.* 280, 1–9. doi: 10.1016/j.ijfoodmicro.2018.05.001

Kuang, D., Zhang, J., Xu, X., Shi, W., Yang, X., Su, X., et al. (2018b). Increase in ceftriaxone resistance and widespread extended-spectrum β-lactamases genes among *Salmonella enterica* from human and nonhuman sources. *Foodborne Pathog. Dis.* 15, 770–775. doi: 10.1089/fpd.2018.2468

La, T. M., Kim, T., Lee, H. J., Lee, J. B., Park, S. Y., Choi, I. S., et al. (2021). Whole-genome analysis of multidrug-resistant *Salmonella Enteritidis* strains isolated from poultry sources in Korea. *Pathogens* 10:1615. doi: 10.3390/pathogens10121615

Lee, S., Park, N., Yun, S., Hur, E., Song, J., Lee, H., et al. (2021). Presence of plasmid-mediated quinolone resistance (PMQR) genes in non-typhoidal Salmonella strains with reduced susceptibility to fluoroquinolones isolated from human salmonellosis in Gyeonggi-do, South Korea from 2016 to 2019. *Gut Pathog.* 13:35. doi: 10.1186/s13099-021-00431-7

Lekagul, A., Tangcharoensathien, V., and Yeung, S. (2019). Patterns of antibiotic use in global pig production: a systematic review. *Vet. Anim. Sci.* 7:100058. doi: 10.1016/j.vas.2019.100058

Li, Y., Kang, X., Ed-Dra, A., Zhou, X., Jia, C., Müller, A., et al. (2022). Genome-based assessment of antimicrobial resistance and virulence potential of isolates of non-Pullorum/Gallinarum Salmonella serovars recovered from dead poultry in China. *Microbiol. Spectr.* 10:e0096522. doi: 10.1128/spectrum.00965-22

Li, Y., Ma, X., Li, C., Dai, X., and Zhang, L. (2020). Occurrence and genomic characterization of ESBL-producing *Escherichia coli* ST29 strains from swine with abundant virulence genes. *Microb. Pathog.* 148:104483. doi: 10.1016/j.micpath.2020.104483

Li, L., Olsen, R. H., Wang, C., Song, A., Xiao, J., Meng, H., et al. (2021). First report of two foodborne *Salmonella enterica* subsp. enterica serovar Bovismorbificans isolates carrying a novel mega-plasmid harboring Bla(DHA-1) and qnrB4 genes. *Int. J. Food Microbiol.* 360:109439. doi: 10.1016/j.ijfoodmicro.2021.109439

Ling, Z., Yin, W., Shen, Z., Wang, Y., Shen, J., and Walsh, T. R. (2020). Epidemiology of mobile colistin resistance genes mcr-1 to mcr-9. *J. Antimicrob. Chemother.* 75, 3087–3095. doi: 10.1093/jac/dkaa205

Liu, R., Xing, L., Zhou, G., and Zhang, W. (2017). What is meat in China? *Anim. Front.* 7, 53–56. doi: 10.2527/af.2017.0445

Liu, B., Zheng, D., Zhou, S., Chen, L., and Yang, J. (2022). VFDB 2022: a general classification scheme for bacterial virulence factors. *Nucleic Acids Res.* 50, D912–d917. doi: 10.1093/nar/gkab1107

Luo, M., She, Y., Jiang, Y., Xie, L., Yang, C., Qiu, Y., et al. (2022). Population dynamics and antimicrobial resistance of Salmonella derby ST40 from Shenzhen, China. *Front. Microbiol.* 13:1065672. doi: 10.3389/fmicb.2022.1065672

Magnet, S., Courvalin, P., and Lambert, T. (1999). Activation of the cryptic aac(6′)-Iy aminoglycoside resistance gene of Salmonella by a chromosomal deletion generating a transcriptional fusion. *J. Bacteriol.* 181, 6650–6655. doi: 10.1128/JB.181.21.6650-6655.1999

Manzari, M., Fani, F., Alebouyeh, M., Moaddeli, A., Rahnamaye Farzami, M., Amin Shahidi, M., et al. (2022). Multidrug-resistant Salmonella strains from food animals as a potential source for human infection in Iran. *Comp. Immunol. Microbiol. Infect. Dis.* 90-91:101898. doi: 10.1016/j.cimid.2022.101898

Marmion, M., Ferone, M. T., Whyte, P., and Scannell, A. G. M. (2021). The changing microbiome of poultry meat; from farm to fridge. *Food Microbiol.* 99:103823. doi: 10.1016/j.fm.2021.103823

Mcmillan, E. A., Gupta, S. K., Williams, L. E., Jové, T., Hiott, L. M., Woodley, T. A., et al. (2019). Antimicrobial resistance genes, cassettes, and plasmids present in *Salmonella enterica* associated with United States food animals. *Front. Microbiol.* 10:832. doi: 10.3389/fmicb.2019.00832

Minnullina, L., Pudova, D., Shagimardanova, E., Shigapova, L., Sharipova, M., and Mardanova, A. (2019). Comparative genome analysis of uropathogenic *Morganella morganii* strains. *Front. Cell. Infect. Microbiol.* 9:167. doi: 10.3389/fcimb.2019.00167

Nang, S. C., Li, J., and Velkov, T. (2019). The rise and spread of mcr plasmid-mediated polymyxin resistance. *Crit. Rev. Microbiol.* 45, 131–161. doi: 10.1080/1040841X.2018.1492902

Neuert, S., Nair, S., Day, M. R., Doumith, M., Ashton, P. M., Mellor, K. C., et al. (2018). Prediction of phenotypic antimicrobial resistance profiles from whole genome sequences of non-typhoidal *Salmonella enterica*. *Front. Microbiol.* 9:592. doi: 10.3389/fmicb.2018.00592

Nhung, N. T., Cuong, N. V., Thwaites, G., and Carrique-Mas, J. (2016). Antimicrobial usage and antimicrobial resistance in animal production in Southeast Asia: a review. *Antibiotics (Basel)* 5:37. doi: 10.3390/antibiotics5040037

Octavia, S., Chew, K. L., Chew, K. L., Lin, R. T. P., and Teo, J. W. P. (2020). Multidrug-resistant *Salmonella enterica* serovar London carrying Bla(NDM-1) encoding plasmid from Singapore. *Clin. Microbiol. Infect.* 26, 963–966. doi: 10.1016/j.cmi.2020.01.033

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Pan, H., Zhou, X., Chai, W., Paudyal, N., Li, S., Zhou, X., et al. (2019). Diversified sources for human infections by *Salmonella enterica* serovar Newport. *Transbound. Emerg. Dis.* 66, 1044–1048. doi: 10.1111/tbed.13099

Paudyal, N., Pan, H., Liao, X., Zhang, X., Li, X., Fang, W., et al. (2018). A meta-analysis of major foodborne pathogens in Chinese food commodities between 2006 and 2016. *Foodborne Pathog. Dis.* 15, 187–197. doi: 10.1089/fpd.2017.2417

Peng, M., Salaheen, S., Almario, J. A., Tesfaye, B., Buchanan, R., and Biswas, D. (2016). Prevalence and antibiotic resistance pattern of Salmonella serovars in integrated crop-livestock farms and their products sold in local markets. *Environ. Microbiol.* 18, 1654–1665. doi: 10.1111/1462-2920.13265

Perin, A. P., Martins, B. T. F., Barreiros, M. A. B., Yamatogi, R. S., Nero, L. A., and dos Santos Bersot, L. (2020). Occurrence, quantification, pulse types, and antimicrobial susceptibility of Salmonella sp. isolated from chicken meat in the state of Paraná, Brazil. *Braz. J. Microbiol.* 51, 335–345. doi: 10.1007/s42770-019-00188-x

Pethplerdprao, P., Supa-Amornkul, S., Panvisavas, N., and Chaturongakul, S. (2017). *Salmonella enterica* multilocus sequence typing and its correlation with serotypes. *Food Biotechnol.* 31, 73–79. doi: 10.1080/08905436.2017.1301820

Pightling, A. W., Pettengill, J. B., Luo, Y., Baugher, J. D., Rand, H., and Strain, E. (2018). Interpreting whole-genome sequence analyses of foodborne bacteria for regulatory applications and outbreak investigations. *Front. Microbiol.* 9:1482. doi: 10.3389/fmicb.2018.01482

Poirel, L., Lagrutta, E., Taylor, P., Pham, J., and Nordmann, P. (2010). Emergence of metallo-β-lactamase NDM-1-producing multidrug-resistant *Escherichia coli* in Australia. *Antimicrob. Agents Chemother.* 54, 4914–4916. doi: 10.1128/AAC.00878-10

Ramirez, M. S., and Tolmasky, M. E. (2010). Aminoglycoside modifying enzymes. *Drug Resist. Updat.* 13, 151–171. doi: 10.1016/j.drup.2010.08.003

Rincón-Gamboa, S. M., Poutou-Piñales, R. A., and Carrascal-Camacho, A. K. (2021). Analysis of the assessment of antimicrobial susceptibility. Non-typhoid Salmonella in meat and meat products as model (systematic review). *BMC Microbiol.* 21:223. doi: 10.1186/s12866-021-02268-1

Salipante, S. J., and Hall, B. G. (2003). Determining the limits of the evolutionary potential of an antibiotic resistance gene. *Mol. Biol. Evol.* 20, 653–659. doi: 10.1093/molbev/msg074

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Sun, R. Y., Fang, L. X., Ke, B. X., Sun, J., Wu, Z. W., Feng, Y. J., et al. (2023). Carriage and transmission ofmcr-1in Salmonella typhimurium and its monophasic 1,4,[5],12:i:-variants from diarrheal outpatients: a 10-year genomic epidemiology in Guangdong, southern China. *Microbiol. Spectr.* 11:e0311922. doi: 10.1128/spectrum.03119-22

Sun, R. Y., Guo, W. Y., Zhang, J. X., Wang, M. G., Wang, L. L., Lian, X. L., et al. (2022). Phylogenomic analysis of Salmonella Indiana ST17, an emerging MDR clonal group in China. *J. Antimicrob. Chemother.* 77, 2937–2945. doi: 10.1093/jac/dkac243

Sun, T., Liu, Y., Qin, X., Aspridou, Z., Zheng, J., Wang, X., et al. (2021). The prevalence and epidemiology of salmonella in retail raw poultry meat in China: a systematic review and meta-analysis. *Foods* 10:2757. doi: 10.3390/foods10112757

Sun, L., Zhang, H., Chen, J., Chen, L., Qi, X., and Zhang, R. (2021). Epidemiology of foodborne disease outbreaks caused by nontyphoidal Salmonella in Zhejiang province, China, 2010–2019. *Foodborne Pathogens and Disease*, 18, 880–886. doi: 10.1089/fpd.2021.0006

Tack, B., Vanaenrode, J., Verbakel, J. Y., Toelen, J., and Jacobs, J. (2020). Invasive non-typhoidal Salmonella infections in sub-Saharan Africa: a systematic review on antimicrobial resistance and treatment. *BMC Med.* 18:212. doi: 10.1186/s12916-020-01652-4

Tamang, M. D., Nam, H. M., Kim, T. S., Jang, G. C., Jung, S. C., and Lim, S. K. (2011). Emergence of extended-spectrum beta-lactamase (CTX-M-15 and CTX-M-14)-producing nontyphoid Salmonella with reduced susceptibility to ciprofloxacin among food animals and humans in Korea. *J. Clin. Microbiol.* 49, 2671–2675. doi: 10.1128/JCM.00754-11

Tang, B., Elbediwi, M., Nambiar, R. B., Yang, H., Lin, J., and Yue, M. (2022). Genomic characterization of antimicrobial-resistant *Salmonella enterica* in duck, chicken, and pig farms and retail markets in eastern China. *Microbiol. Spectr.* 10:e0125722. doi: 10.1128/spectrum.01257-22

Tang, B., Siddique, A., Jia, C., Ed-Dra, A., Wu, J., Lin, H., et al. (2023). Genome-based risk assessment for foodborne *Salmonella enterica* from food animals in China: a one health perspective. *Int. J. Food Microbiol.* 390:110120. doi: 10.1016/j.ijfoodmicro.2023.110120

Timme, R. E., Wolfgang, W. J., Balkey, M., Venkata, S. L. G., Randolph, R., Allard, M., et al. (2020). Optimizing open data to support one health: best practices to ensure interoperability of genomic data from bacterial pathogens. *One Health Outlook* 2, 1–11. doi: 10.1186/s42522-020-00026-3

Vázquez, X., Fernández, J., Alkorta, M., De Toro, M., Rodicio, M. R., and Rodicio, R. (2023). Spread of Bla(ctx-m-9) and other clinically relevant resistance genes, such as mcr-9 and qnra1, driven by inchi2-st1 plasmids in clinical isolates of monophasic *Salmonella enterica* serovar typhimurium ST34. *Antibiotics (Basel)* 12:547. doi: 10.3390/antibiotics12030547

Wang, W., Baloch, Z., Zou, M., Dong, Y., Peng, Z., Hu, Y., et al. (2018). Complete genomic analysis of a *Salmonella enterica* serovar typhimurium isolate cultured from ready-to-eat pork in China carrying one large plasmid containing mcr-1. *Front. Microbiol.* 9:616. doi: 10.3389/fmicb.2018.00616

Wang, Y., Liu, Y., Lyu, N., Li, Z., Ma, S., Cao, D., et al. (2023). The temporal dynamics of antimicrobial-resistant Salmonella enterica and predominant serovars in China. *Natl. Sci. Rev.* 10:nwac269. doi: 10.1093/nsr/nwac269

Wang, W., Peng, Z., Baloch, Z., Hu, Y., Xu, J., Zhang, W., et al. (2017). Genomic characterization of an extensively-drug resistance *Salmonella enterica* serotype Indiana strain harboring Bla(NDM-1) gene isolated from a chicken carcass in China. *Microbiol. Res.* 204, 48–54. doi: 10.1016/j.micres.2017.07.006

Wang, X., Wang, H., Li, T., Liu, F., Cheng, Y., Guo, X., et al. (2020b). Characterization of Salmonella spp. isolated from chickens in Central China. *BMC Vet. Res.* 16:299. doi: 10.1186/s12917-020-02513-1

Wang, W., Zhao, L., Hu, Y., Dottorini, T., Fanning, S., Xu, J., et al. (2020a). Epidemiological study on prevalence, serovar diversity, multidrug resistance, and ctx-m-type extended-spectrum β-lactamases of Salmonella spp. from patients with diarrhea, food of animal origin, and pets in several provinces of China. *Antimicrob. Agents Chemother.* 64:aac, 00092–00020. doi: 10.1128/AAC.00092-20

Wei, Z., Xu, X., Yan, M., Chang, H., Li, Y., Kan, B., et al. (2019). Salmonella typhimurium and *Salmonella Enteritidis* infections in sporadic diarrhea in children: source tracing and resistance to third-generation cephalosporins and ciprofloxacin. *Foodborne Pathog. Dis.* 16, 244–255. doi: 10.1089/fpd.2018.2557

WHO (2022). Food safety. Available at: https://www.who.int/en/news-room/fact-sheets/detail/food-safety (Accessed October 2022).

Wu, C., Yan, M., Liu, L., Lai, J., Chan, E. W., and Chen, S. (2018). Comparative characterization of nontyphoidal Salmonella isolated from humans and food animals in China, 2003-2011. *Heliyon* 4:e00613. doi: 10.1016/j.heliyon.2018.e00613

Xu, L., He, Q., Tang, Y., Wen, W., Chen, L., Li, Y., et al. (2022). Multi-locus sequence and drug resistance analysis of Salmonella infection in children with diarrhea in Guangdong to identify the dominant ST and cause of antibiotic-resistance. *Exp. Ther. Med.* 24:678. doi: 10.3892/etm.2022.11614

Yang, X., Huang, J., Zhang, Y., Liu, S., Chen, L., Xiao, C., et al. (2020). Prevalence, abundance, serovars and antimicrobial resistance of Salmonella isolated from retail raw poultry meat in China. *Sci. Total Environ.* 713:136385. doi: 10.1016/j.scitotenv.2019.136385

Yu, G. (2020). Using ggtree to visualize data on tree-like structures. *Curr. Protoc. Bioinformatics* 69:e96. doi: 10.1002/cpbi.96

Zakaria, Z., Hassan, L., Sharif, Z., Ahmad, N., Mohd Ali, R., Amir Husin, S., et al. (2022). Virulence gene profile, antimicrobial resistance and multilocus sequence typing of *Salmonella enterica* subsp. enterica serovar Enteritidis from chickens and chicken products. *Animals (Basel)* 12:97. doi: 10.3390/ani12010097

Zhang, S., Den Bakker, H. C., Li, S., Chen, J., Dinsmore, B. A., Lane, C., et al. (2019). SeqSero2: rapid and improved Salmonella serotype determination using whole-genome sequencing data. *Appl. Environ. Microbiol.* 85, e01746–e01719. doi: 10.1128/AEM.01746-19

Zhang, Z., He, S., Xu, X., Chang, J., Zhan, Z., Cui, Y., et al. (2022a). Antimicrobial susceptibility and molecular characterization of *Salmonella enterica* serovar Indiana from foods, patients, and environments in China during 2007–2016. *Food Control* 131:108427. doi: 10.1016/j.foodcont.2021.108427

Zhang, Z., Tian, X., and Shi, C. (2022b). Global spread of mcr-producing *Salmonella enterica* isolates. *Antibiotics (Basel)* 11:998. doi: 10.3390/antibiotics11080998

Zhang, C. Z., Zhang, Y., Ding, X. M., Lin, X. L., Lian, X. L., Trampari, E., et al. (2020). Emergence of ciprofloxacin heteroresistance in foodborne *Salmonella enterica* serovar Agona. *J. Antimicrob. Chemother.* 75, 2773–2779. doi: 10.1093/jac/dkaa288

Zhou, Z., Alikhan, N. F., Mohamed, K., Fan, Y., and Achtman, M. (2020). The EnteroBase user's guide, with case studies on Salmonella transmissions, *Yersinia pestis* phylogeny, and Escherichia core genomic diversity. *Genome Res.* 30, 138–152. doi: 10.1101/gr.251678.119

Zou, M., Ma, P. P., Liu, W. S., Liang, X., Li, X. Y., Li, Y. Z., et al. (2021). Prevalence and antibiotic resistance characteristics of extraintestinal pathogenic *escherichia coli* among healthy chickens from farms and live poultry markets in China. *Animals (Basel)* 11:1112. doi: 10.3390/ani11041112

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Genomic characterization of *Bacillus cereus* isolated from food poisoning cases revealed the mechanism of toxin production

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Introduction: *Bacillus cereus* is a ubiquitous opportunistic human pathogen that causes food intoxications worldwide. However, the genomic characteristics and pathogenic mechanisms of *B. cereus* are still unclear.

Methods: Here, we isolated and purified nine strains of *B. cereus* (*LY01-LY09*) that caused vomiting, diarrhea and other symptoms from four foodborne outbreaks happened in Guizhou Province in southwest China from June to September 2021. After colony observation, Gram staining, microscopic examination and biochemical test, they were identified as *B. cereus*. The genomic characteristics, phylogenetic relationships and virulence factors of the isolated strains were analyzed at the genome level. Genome sequencing, comparative genomic analysis, secondary metabolite analysis and quantitative PCR were utilized to give a thorough exploration of the strains.

Results: We obtained the genome maps of *LY01-LY09* and found that *LY01-LY09* had a complex interspecific relationship with B. anthracis and *B. thuringiensis*. We also observed a contraction of gene families in *LY01-LY09*, and the contracted families were mainly associated with prophage, which contributed to the species diversity of *B. cereus*. The *Hsp20* gene family underwent a rapid evolution in *LY01-LY09*, which facilitated the adaptation of the strains to adverse environmental conditions. Moreover, the *LY01-LY09* strains exhibited a higher copy number in the non-ribosomal polypeptide synthetase (NRPS) genes and carried the complete cereulide synthetase (*ces*) gene cluster sequences. Considering that the *NRPS* system is a classical regulatory mechanism for emetic toxin synthesis, we hypothesized that *LY01-LY09* could synthesize emetic toxins through the regulation of *ces* gene clusters by the *NRPS* system.

Discussion: These findings are important for further investigation into the evolutionary relationship between *B. cereus* and their related species, as well as the underlying mechanisms governing the synthesis and secretion of bacterial toxins.

KEYWORDS

***Bacillus cereus*, comparative genomic analysis, virulence factor, metabolic pathway, foodborne outbreak**

1 Introduction

Foodborne pathogens frequently cause public health emergencies (Josephs-Spaulding et al., 2016; Antunes et al., 2020) and have become a critical concern in food safety in China (He and Shi, 2021). Pathogenic microorganisms can lead to both food poisoning and contamination. Microbial contamination poses a significant threat to food safety and the issue of foodborne illnesses resulting from such contamination is also highly critical (Tran et al., 2011; Pires et al., 2021). *B. cereus* is a frequently encountered foodborne pathogen, and *B. cereus*-related food poisoning primarily manifests as acute gastroenteritis characterized by symptoms such as vomiting, fatigue, nausea, and diarrhea (Kotiranta et al., 2000). *B. cereus* causes two distinct types of food poisoning based on symptoms: food intoxication (vomiting type) and food toxico-infection (diarrhea type). According to relevant statistics, the vomiting type accounts for 75.9% of *B. cereus* poisoning cases, while the diarrheal type 11.4%, thus making vomiting the predominant manifestation of *B. cereus* foodborne illness (Zhou et al., 2008). The diarrheal type is mainly attributed to the *B. cereus* production of enterotoxins, including hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), and cytotoxin K (CytK) (Dietrich et al., 2021). In contrast, the vomiting type is caused by cereulide, an emetic toxin produced by *B. cereus* through a non-ribosomal polypeptide synthetase (NRPS), which is encoded by the cereulide synthetase (*ces*) gene cluster (Marxen et al., 2015; Carroll and Wiedmann, 2020). The *ces* gene cluster, about 24 kb long, comprises seven genes, and is typically situated on a megaplasmid of *B. cereus* (Ehling-Schulz et al., 2006). The *B. cereus*-caused vomiting type of food poisoning is often mild, although severe and even lethal conditions might happen in rare cases (Stenfors Arnesen et al., 2008).

Genome sequencing technology allows for a comprehensive analysis of the molecular biological characteristics of pathogenic bacteria at the genetic level, and is widely used in epidemiological investigations of foodborne disease outbreaks (Li et al., 2021). This technology provides a crucial foundation for molecular epidemiological investigations of foodborne disease outbreaks caused by *B. cereus*, enabling not only pathogen identification, but also the detection and classification of virulence and drug resistance genes, and further the analysis of pathogenicity (Balloux et al., 2018). A previous study detected hemolytic (*hblA*, *hblC,* and *hblD*) and non-hemolytic (*nheA*, *nheB,* and *nheC*) enterotoxin genes in two *B. cereus* strains isolated from indoor air, suggesting that airborne isolates may cause diarrhea rather than vomiting (Premkrishnan et al., 2021). Toxigenic heterogeneity was found to be related to toxin genes such as *nheABC*, *hblCDAB*, *cytK2*, *entFM,* and *CesB* in the genomes of *B. cereus sensu lato* (s.l.) isolated from ready-to-eat foods and powdered milk (Sánchez Chica et al., 2020). The characterization of the genomes of *B. cereus* and the identification of virulence-related genes are of great significance for determining the causes of foodborne poisoning. However, few studies have dealt with the genomic profiling of *B. cereus* isolated from food sources that induces symptoms such as vomiting and diarrhea.

In this study, we performed genome sequencing and comparative genetic analysis on nine strains of *B. cereus* (*LY01-LY09*) isolated from contaminated food linked to foodborne outbreaks in Guizhou Province, China to analyze the evolution of these strains at the genome level, explore their virulence factors, and evaluate their pathogenic potential. By analyzing the genomic information of *LY01-LY09*, our objective was to establish a fundamental basis for research on the pathogenic mechanisms of *B. cereus*, as well as for the development of antibiotics, disease prevention and control. This study is of great significance in comprehending the evolutionary traits and patterns of *B. cereus*, and may provide a rapid method for the detection of *B. cereus* strains.

2 Materials and methods

2.1 Outbreak investigation

From June to September 2021, four foodborne outbreaks had occurred in Ziyun County and Tongren City, Guizhou Province. Persons affected reported symptoms of gastroenteritis. Afterward, technicians from Guizhou Provincial Centre for Disease Control and Prevention carried out epidemiological and environmental investigations. Food samples were collected for laboratory test.

In the 23 cases, more than 86% experienced nausea or vomiting (**Supplementary Table 1**). Few reported abdominal pain, diarrhea, dizziness, fatigue, etc. In the three outbreaks happened in Ziyun County, all persons had eaten rice noodle (a popular local food) bought from the same vendors or stores. The Tongren outbreak occurred in a nursery, and all affected children had eaten meals served by the nursery kitchen (**Supplementary Table 1**). By laboratory test, *B. cereus* colonies were isolated from food samples. In addition, two out of four vomit samples from children of the Tongren outbreak yielded toxin-producing *B. cereus*. All those affected were managed symptomatically, except for six were hospitalized and given intravenous rehydration.

2.2 Isolation, culture, and identification of bacterial strains

Food samples were approximately diluted, and the diluted solution was inoculated on Mannitol-Egg Yolk-Polymyxin (MYP) medium (Tryptone 10 g/L, Mannitol 10 g/L, Meat extract 1 g/L, NaCl 10 g/L and Phenol red 0.025 g/L) and Nutrient Agar (NA) medium. Number of colonies was enumerated after culturing at 37°C for 24 h (Anon, 1995). Five bacterial colonies from each plate were selected and inoculated on an NA inclined surface for pure culture at 37°C for 24 h. After being confirmed as Gram-positive, the morphology of the bacteria was observed under microscopic examination. Finally, the EasylD Biochemical Identification Kit for *B. cereus* (HuanKai microbial, China) was utilized to identify strains presenting in contaminated food.

2.3 Sequencing and assembling of *B. cereus* strain genomes

The genomic DNA of *B. cereus* strains was extracted using SDS, and total DNA was fragmented using the Covaris ultrasonic

breaker (Covaris, East Sussex, UK). The library was constructed by the NEBNext®Ultra™ DNA Library Prep Kit (NEB, USA) in accordance with the manufacturer's protocol. After a thorough quality inspection, the library was sequenced by Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) to obtain raw data, which were then filtered to acquire clean data. SOAP *de novo* (v2.04), SPAdes (v 3.15.5), and ABySS (v 2.0) assembly software were utilized for *de novo* assembly of high-quality sequencing data obtained from the *B. cereus* strains, while Contig Integrator for Sequence Assembly (CISA) software (Lin and Liao, 2013) was employed to obtain preliminary assembly results. The assembly output was optimized using GapClose (v1.12), which was employed to fill gaps and improve genome quality.

2.4 Genome annotation and collinearity analysis

GeneMarkS (Version 4.17) software was utilized to predict coding genes in the *B. cereus* strains, which were mapped to functional databases (E-value $\leq 1e^{-5}$) in Kyoto Encyclopedia of Genes and Genomes (KEGG),[1] Transporter Classification Database (TCDB),[2] pathogen–host interactions database (PHI),[3] and carbohydrate active enzyme (CAZy)[4] to obtain functional annotation of the protein-coding genes. In addition, repeated and tandem repeats were identified using RepeatMasker (Version open-4.0.5) and TRF (Tandem Repeats Finder, Version 4.07b), respectively. To search for homology, BLASTP software (Version 2.12.0+) was utilized to compare the protein-coding genes among the *LY01-LY09* strains. The GENESPACE package of R language was employed for the visualization of genome collinearity.

2.5 Gene family clustering and phylogenetic tree construction

Furthermore, the complete genome sequences of 26 *Bacillus* strains were downloaded from the NCBI GenBank database, including four *B. anthracis*, two *B. amyloliquefaciens*, four *B. thuringiensis*, one *B. pumilusstrain*, ten *B. cereus*, two *B. velezensis*, and three *B. subtilis*. Strain names and accession numbers of the reference sequences are available in **Figure 1**. Altogether, 1269 single-copy orthologs were obtained from the whole-genome sequences of the 35 *Bacillus* strains (9 isolated and 26 downloaded) using OrthoFinder (v.2.5.4) (Emms and Kelly, 2019). Multiple alignments of amino acid sequences of orthologs were carried out by MUSCLE (v.3.8.1551) (Edgar, 2004). Conserved blocks from multiple alignments of protein sequences were obtained by Gblocks (Castresana, 2000). Maximum likelihood (ML) tree was constructed using RAxML v.8.2.12 software (Stamatakis, 2014) with 1000 bootstrap replicates by PROTGAMMAILGF model, which was built using ProtTest (v.3.4.2) software (Darriba et al., 2011). Tree visualization was completed by FigTree (v.1.4.4) software. The expansion and contraction analysis of gene family was performed by CAFE (v.4.2.1) software, afterward representative species on the branch were selected.

2.6 Virulence factor prediction of the *B. cereus* strains

Protein sequences of the nine *B. cereus* strains were subjected to BLAST with an E-value threshold $\leq 1e^{-5}$ against the virulence factor database (VFDB)[5] (Liu et al., 2022). The resulting data were then integrated with corresponding functional annotations for each identified virulence factor and its target species, obtaining comprehensive annotation results for the virulence factors of the nine *B. cereus* strains. *NRPS* gene sequences were identified by BLAST and motif prediction were performed using the MEME website.[6] The phylogenetic relationship of *NRPS* gene family was constructed based on IQ-TREE (v. 2.1.4-beta) according ML method.

2.7 Verification of virulence factors by q-PCR

The expressions of *hblC, nheB* and *cesB* of the nine *B. cereus* strains were detected by quantitative polymerase chain reaction (q-PCR). Specifically, primers and probes were designed based on *B. cereus* virulence factor genes (*hblC, nheB, cesB*). During PCR amplification, TaqMan probes were hydrolyzed by the action of Taq DNA polymerase to generate fluorescence signals. Cycle threshold (Ct) values ≤ 30 were regarded as positive. Amplification curves were obtained for the detection of *B. cereus* virulence factors genes.

2.8 Comparative genome analysis

The fastANI program (Jain et al., 2018) was used to calculate the average nucleotide identity (ANI) between the whole genome sequences of two *B. cereus* strains. In addition, MUMmer (v. 4.0.0beta2) (Kurtz et al., 2004) was used to analyze the single nucleotide polymorphism (SNP) and InDels among strains based on the whole genome sequence, with the genome of LY03 as a reference. CIRCOS (v.0.69-9) software (Krzywinski et al., 2009) was used to visualize SNP.

3 Results

3.1 Identification and characterization of isolates

A total of nine strains of bacteria were isolated and purified from food samples. The selective medium MYP was used to

1 https://www.genome.jp/kegg
2 http://www.tcdb.org
3 www.phi-base.org
4 http://www.cazy.org/
5 http://www.mgc.ac.cn/VFs/
6 https://meme-suite.org/meme/

FIGURE 1
Phylogenetic analysis of the 9 *Bacillus* isolates and 26 *Bacillus* strains downloaded from databases based on whole genome sequence. Strain names and accessions of the downloaded strains are available in the figure. Bootstrap frequencies were obtained with 1,000 replicates.

screen and identify the strains. White colonies surrounded by pink areas were observed on the MYP medium, while colonies formed rough white surface with irregular edges on the NA medium (Supplementary Figure 1A). This was consistent with the typical characteristics of *B. cereus*. The nine strains were stained with Gram's stain and identified as Gram-positive (Supplementary Figure 1B). Finally, the strains were tested for starch hydrolysis, glucose fermentation, Voges-Proskauer (V-P) reaction, etc., using the identification kit. A diffuse growth out into the medium along and away from the stab was observed in the motility test, thus excluding the possibility of *B. mycoides* strains (Supplementary Table 2). Based on the aforementioned testing results, the strains isolated and purified in this study were identified as *B. cereus* according to the National Standard of the People's Republic of China GB 4789.14 (2014).

3.2 Genome structure and linkage map of the *LY01-LY09* strains

The nine strains of *B. cereus* had similar genome sizes, with all but the *LY08* strain (5.68 Mb) possessing a genome size of 5.64 Mb. The GC content ranges from 35.23 to 35.25%, exhibiting a small variation as well. The lengths of their coding genes ranged from 4.75 Mb to 4.79 Mb, accounting for approximately 84.3–84.4% of their respective genome sizes (Supplementary Table 3). The proportions of repeat sequences (LINE, RC, SINE, and LTR) in each genome were similar among the strains (Supplementary Figure 2A). The number of transfer RNAs in each strain ranged from 92 to 93, with a length of 7,000–7,200 bp. Meanwhile, the number of small RNAs varied from 5 to 6, with lengths ranging from 400 to 550 nucleotides (Supplementary Figure 2B). CRISPR (clustered regularly interspaced short palindromic repeat sequences) elements counts ranged from 10 to 29, with lengths varying between 2,476 and 6,175 bp. Furthermore, the nine strains had similar numbers of CRISPR, genomic island (Gis) and prophage (Supplementary Figure 2C). Based on the KEGG database, the metabolic pathways of the *LY01-LY09* cellular gene products and compounds were assigned to six categories: metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, and human diseases. At level B, a total of 41 metabolic pathways were involved, with a primary focus on amino acid metabolism, carbohydrate metabolism,

membrane transport, caffeine and vitamin metabolism, as well as energy metabolism (Supplementary Figure 3A). Functional analysis of the transport proteins in the nine isolates was performed using the TCDB database. We found that the transport proteins were mainly primary active transporters and electrochemical potential-driven transporters (Supplementary Figure 3B). Based on the CAZy database, glycosyltransferase (GTs), Glycoside hydrolases (GHs), carbohydrate esterases (CEs) and auxiliary activities (AAs) were detected as the four enzyme classifications (Supplementary Figure 3C). In addition, we noticed that reduced virulence and unaffected pathogenicity were the phenotypes with the most matched genes (Supplementary Figure 3D).

3.3 Comparative genome analysis of the *LY01-LY09* strains

Pairwise macrosynteny analysis revealed a high collinearity among the nine strains, with high conservation at the genome level, except for contig rearrangements on contig3 of *LY02*, and contig3 and contig6 of *LY01* (Supplementary Figure 4). A further analysis detected a fission phenomenon on contig7 and contig9 only, compared with a high collinearity among the isolated strains and the reference *B. cereus* strain. The genomic characteristics of the nine strains of *B. cereus* were compared with each other, and the results showed that pairwise ANI values were greater than 99.98% (Supplementary Table 4). This may indicate that these nine strains were the same strain, which was consistent with the results of collinear analysis. In addition, the genomic sequences of the other eight strains were compared with *LY03* to obtain mutation information (SNPs and InDels). We found few mutation sites between the genomes of the nine strains, indicating their low level of heterogeneity (Supplementary Figure 5).

3.4 Phylogenetic analysis based on whole genome sequencing

The evolutionary relationships between the nine isolated strains with other related *Bacillus* strains were analyzed using whole-genome ML phylogenetic tree (Figure 1). The *LY01-LY09* strains showed 100 bootstrap frequency rooted at the branch. The *LY01-LY09* strains exhibited a close genetic relationship with *B. anthracis* and *B. thuringiensis*, which formed sister groups with *B. anthracis* (Figure 1). In addition, *B. thuringiensis*, *B. cereus* and *B. anthracis* were in the same branch, the boundaries between some *B. cereus* and *B. anthracis* strains were not clear. The results showed that the genomes of the three *Bacillus* species were highly similar.

3.5 Regulatory networks of the *LY01-LY09* strains and identification of key genes

To understand genetic alterations of the *LY01-LY09* strains and evolutionary forces driving these changes, we examined the interactions among the gene families that have expanded, contracted, and rapidly evolved (Supplementary Figure 6), and identified key genes in gene co-expression networks. The findings indicate that *BA_0477, BA_3774,* and *BA_3775*, which were closely associated with prophage (Supplementary Table 5), were the key genes within the contracted gene families of the *LY01-LY09* strains (Supplementary Figure 7A). The genes belonging to the contracted gene families were primarily associated with phage terminase, phage portal protein, DNA binding, and extracellular region (Supplementary Figure 7B). Similarly, GO enrichment analysis were performed on the expanded gene families, which showed close relationships with DNA recombination, resolvase, N-terminal domain, Tn3 transposase DDE domain, recombinase conserved site and other related factors (Supplementary Figure 7C). To better understand species evolution, we investigated the complete genome sequences of *B. anthracis*, *B. amyloliquefaciens*, *B. thuringiensis, B. velezensis, B. cereus* and *Clostridium botulinum*, together with those of the nine *Bacillus* isolates. By doing so, we intended to explore the interactions among rapidly evolving gene families of the 15 strains. It was discovered that the key genes of these families were the same as those of the contracted gene families in the *LY01-LY09* strains, and all of them were closely associated with prophage (Figure 2A; Supplementary Table 5). The Heat-shock protein 20 (*Hsp20*) family changed significantly in gene number among the rapidly evolving gene families. Structural analysis revealed that the *Hsp20* families of LY09Scaffold63 and LY07LY09Scaffold61 were the most similar in terms of gene number and structure. We thus speculate that they performed similar functions as well (Figure 2B). The protein sequences of the *Hsp20* families in *LY01-LY09*, *B. thuringiensis*, and *B. velezensis* exhibited significant variations due to point mutations and fragment insertions (Figure 2C).

3.6 Virulence factors of the *LY01-LY09* strains causing vomiting

We also observed a similar number of identical gene clusters across the nine *B. cereus* strains. Moreover, these gene clusters mainly focused on *NRPS*, leucine aminopeptidase (*LAP*), bacteriocin, and terpene (Figure 3A), among which the number of the *NRPS* genes was the highest. A further analysis of the *NRPS* genes revealed that Motif-1, Motif-2 and Motif-3 were the shared motifs among the genes (Figure 3B). In addition, the motif sequences were 2-3-1 and 3-2-1, indicating that the *NRPS* gene family in the *LY01-LY09* strains was highly conservative. According to the maximum likelihood-based phylogenetic tree of NRPS proteins, all but one of the 27 NRPS proteins could be assigned to three groups (Figure 3C). Group 1 comprised of eight protein-coding genes, group 2 nine genes, and group 3 also nine genes.

The consistency of virulence factors among strains *LY01-LY09* was further confirmed by analyzing the VFDB database (Table 1). The polysaccharide capsule involved the most virulence genes, totaling 16 in quantity. Capsular polysaccharide is a major virulence determinant for numerous bacteria, as it has the ability to evade host phagocytosis and immune responses, resulting in immunodeficiency and bacterial resistance against host defenses (Wang et al., 2023). Cereulide ranked second in virulence gene number (7 genes). It is an important toxin produced by pathogenic

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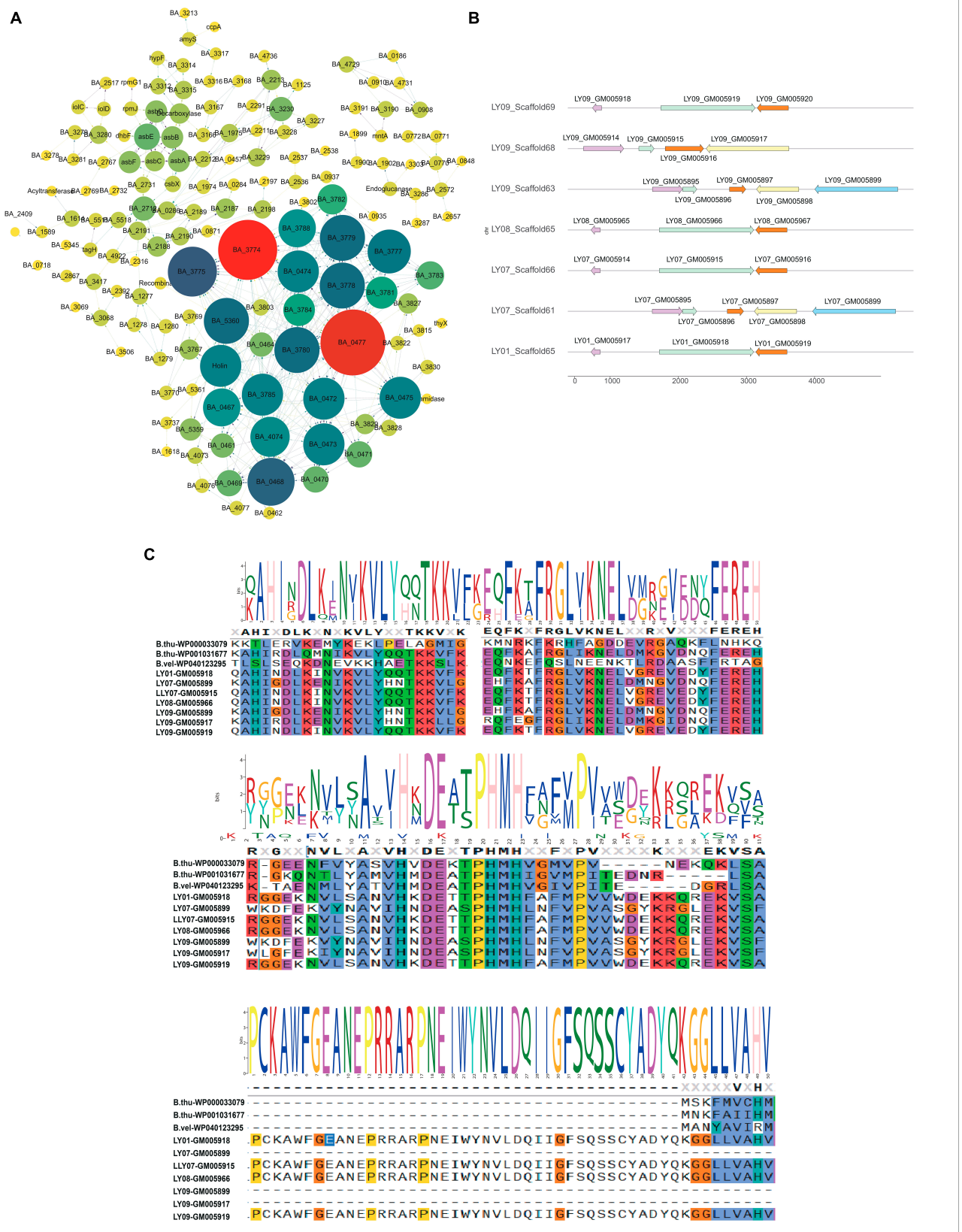

FIGURE 2
Functions of fast evolving genes and structure of heat-shock protein 20 (*HSP20*) *B. anthracis*, *B. amyloliquefaciens*, *B. thuringiensis*, *B. velezensis*, *B. cereus* and *Clostridium botulinum* downloaded from database and the nine *Bacillus* isolates. **(A)** Functions of fast-evolving genes in the 15 strains; **(B)** *HSP20* gene structure of *LY01-LY09*; **(C)** Conserved motifs and loci of *HSP20*.

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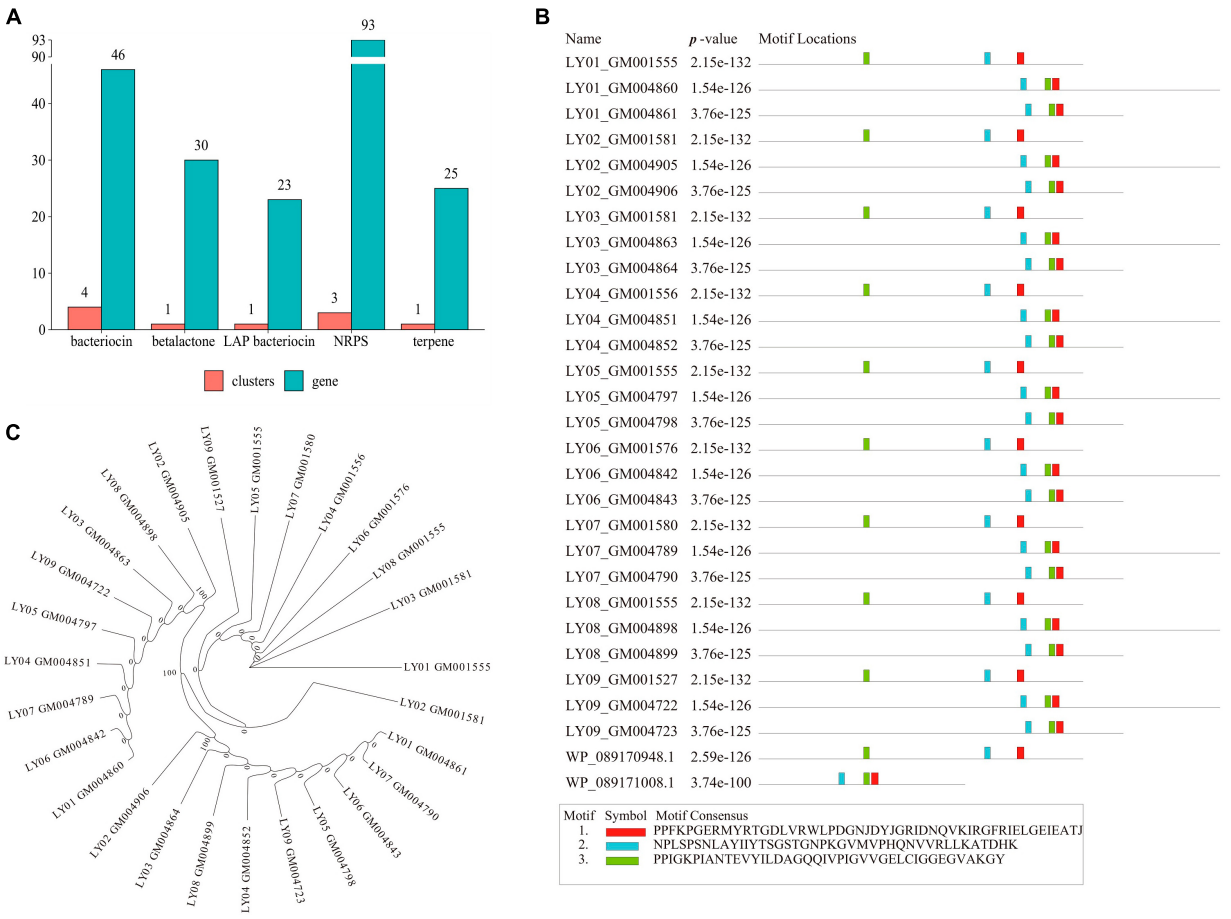

FIGURE 3
Gene clusters and *NRPS* gene analysis of *B. cereus*. **(A)** Gene cluster analysis of *B. cereus*; **(B)** Motif analysis of the *NRPS* genes; **(C)** Phylogenetic analysis of the *NRPS* genes.

TABLE 1 **Analysis of virulence factors.**

Classification	Virulence factor	Related gene	Gene number
Enzymes	Sphingomyelinase (SMase)	*sph*	1
Immune escape	*B. cereus* exo-polysaccharide (BPS)	*bpsC*	1
	Polysaccharide capsule	Undetermined	16
Iron to obtain	Bacillibactin	*dhbA, dhbB, dhbC, dhbE, dhbF*	4
	Hal	*hal*	1
	IlsA	*ilsA*	1
Regulation	PagR-XO2	*pagR-XO2*	1
	PlcR-PapR quorum sensing	*papR*	1
		plcR	1
	CheA/CheY (Listeria)	*cheA*	1
Toxin	Cereulide	*cesA, cesB, cesC, cesD, cesH, cesP, cesT*	7
	Non-hemolytic enterotoxin (Nhe)	*nheC*	1
Others	O-antigen (Yersinia)	*ddhA*	1

B. cereus, and is encoded by the *ces* gene cluster and synthesized by the regulation of RRPS (Kalbhenn et al., 2022). By q-PCR, *hblC, nheB,* and *cesB* were detected but only *cesB* was positive (**Supplementary Table 6**). In addition, the hemolysin BL gene (*hblA*, *hblC*, *hblD*) and non-hemolytic enterotoxin Nhe gene (*nheC*) associated with diarrhea toxins were discovered in the nine *B. cereus* strains (**Table 1**). The results showed that *cesB* had a high copy number, but the *hblC* gene was not detected in *B. cereus*.

4 Discussion

According to annotation analysis of public databases, the metabolic pathways of the nine *B. cereus* isolates were mainly focused on amino acid metabolism, carbohydrate metabolism, membrane transport, caffeine, vitamin metabolism, and energy metabolism. Amino acid metabolism in organisms mainly includes the synthesis of proteins, peptides and other nitrogen-containing substances. Amino acids can also be broken down into α-keto acids, amines and carbon dioxide through deamination, transamination, combined deamination or decarboxylation. The synthesis of *B. cereus* toxins is catalyzed by multiple enzymes. Therefore, the metabolism of amino acids in *B. cereus* has potential functions in regulating the synthesis and expression of virulence factors (Tran et al., 2011; Yu et al., 2011). Carbohydrates function primarily as the energy source for organisms through glycolysis, tricarboxylic acid cycle, pentose phosphate pathway, gluconeogenesis, gluconeogenesis, etc. (Li et al., 2020). The functions of carbohydrates in *LY01-LY09* fell into four categories according to the CAZy database, whose number of genes from high to low were glycosyltransferases (GTs), glycoside hydrolases (GHs), carbohydrate esterolytic enzymes (CEs), and oxidoreductase (AAs). GTs can catalyze the formation of glycosidic bonds between specific sugars and receptors. Their substrates encompass a range of biomolecules, including sugars, proteins, lipids, and other small molecules that are essential for the synthesis of biological compounds (Hsieh et al., 2014). The analysis of the metabolic pathways of *B. cereus* increases our understanding of the mechanism of synthesis and secretion of bacterial toxins.

By comparative genomics, we found more than 99.98% ANI between each pair of the nine *B. cereus* genomes. At the same time, the results of collinearity and SNP analyses also showed high similarity between strains. Therefore, we speculate these nine strains to be the same one, which caused the foodborne outbreaks. The results of phylogenetic and virulence factor analyses were consistent with the above conclusion. Interestingly, phylogenetic analysis revealed that the *LY01-LY09* strains identified in this study was closely related to *B. cereus* and *B. anthracis* (**Figure 1**). *B. cereus* and *B. anthracis,* which are closely related, are members of the *B. cereus* s.l. group, as evidenced by the high collinearity and similarity in their chromosomes and proteins (Pilo and Frey, 2011). Therefore, it is difficult to discriminate *B. cereus* from *B. anthracis*, particularly for isolates exhibiting unusual biochemical or physiological characteristics. It is usually not enough when relying solely on genome-wide information to differentiate between the two species. Phenotype and plasmid content are also needed to take into consideration (Ehling-Schulz et al., 2019; Lin et al., 2022; Trunet et al., 2023). Phylogenetic analysis revealed that *B. cereus* and *B. thuringiensis* were clustered within the same clade, indicating a close evolutionary relationship. Previous research has indicated that *B. cereus* and *B. thuringiensis* are highly similar in genome. The only difference between the two strains is that *B. thuringiensis* can produce parasporal crystals in the outer spore membrane during spore formation, which is encoded by the insecticidal plasmids. The removal of this plasmid can result in the conversion of *B. thuringiensis* to *B. cereus* (Shu-sen et al., 2007; Venkateswaran et al., 2017). These results further confirm the high genetic similarity and close relationship between diarrheal toxin-producing *B. cereus* and *B. thuringiensis*.

By exploring the alterations in gene families across species, it was found that *B. cereus*, *B. thuringiensis* and *B. anthracis* have expanded significantly. Conversely, the gene families of the isolated *B. cereus* strains have contracted. We speculate that variations in the number of gene families may be responsible for the phenotypic differences of the three *Bacillus* species. This reflects genomic variations among different strains of the same species. To further investigate the relationship among the *LY01-LY09* strains, we constructed an evolutionary tree based on single-copy genes. The analysis revealed that *B. thuringiensis* strains, *B. anthracis* strains, *LY01* and *LY09* formed a closely related clade, which is consistent with conclusions of previous studies. Previous research has shown that *B. cereus* s.l. can be classified into three separate clades. Most *B. cereus* can be classified into two clades: clade A, which is capable of producing emetic toxin, and clade B, which cannot. *B. thuringiensis*, an insect pathogen, mainly belongs to clade B, whereas *B. anthracis* was relatively conservative and restricted to clade A (Ehling-Schulz et al., 2019; Biggel et al., 2022; Carroll et al., 2022). As illustrated in **Figure 1**, the close clustering pattern between *LY01-LY09* and *B. anthracis* suggests that *LY01-LY09* may be pathogens that are harmful to humans as well. Previous studies have demonstrated that *HSP20* exhibits chaperone activity, which can enhance cellular tolerance to damage and maintain normal cellular metabolism under various stresses, thus improving cell survival rates (Kranzler et al., 2016; Ling et al., 2022; Niu et al., 2022). *LY07* and *LY09* had more than one *Hsp20* genes, some of which undergone point mutations and loss of long amino acids. Therefore, we speculated that the *LY07* and *LY09* strains may exhibit lower susceptibility to environmental stressors such as temperature, demonstrating greater adaptation to adverse conditions in comparison to the *LY01* and *LY08* strains.

Non-ribosomal polypeptide synthetase are the major systemic enzymes that regulate the synthesis of emetic toxins (Dommel et al., 2010). This study identified many *NRPS* genes in the *LY01-LY09* strains, which may suggest that the *LY01-LY09* strains can induce the vomiting-type food poisoning by synthesizing the emetic toxin cereulide regulated by *NRPS*. The *NRPS* biosynthetic machinery is a vast modular multi-enzyme complex that participates in the production of diverse natural products, including toxins, antibiotics, and surfactants. The emetic toxin cereulide is a typical product among these compounds (Bozhüyük et al., 2019). In a previous study, PCR was employed to explore the *NRPS* genes of an emetic strain of *B. cereus*, which confirmed that cereulide was produced via NRPS in *B. cereus* (Toh et al., 2004). *NRPS* plays a key role in the biosynthesis of emetic toxins (cereulide). The gene locus encoding Cereulide synthetase shows the typical structure of the *NRPS* genes clusters, which plays an important role in the synthetic expression of *B. cereus* cereulide (Magarvey et al., 2006; Makarasen et al., 2009). Meanwhile, we have identified seven genes (*cesA, cesB, cesC, cesD, cesH, cesP,* and *cesT*) within the emetic toxin synthetase gene cluster (*ces* gene cluster) in the *LY01-LY09* strains. *CesPTABCD* can form a ces-operon in the megaplasmid pBCE4810, which is transcribed into a 23 kb polycistronic mRNA chain driven by the main promoter p1. Adjacent to *CesPTABCD*, cesH is transcribed as a single cistron by its own promoter PH and encodes a 31 kDa hydrolase (Ehling-Schulz et al., 2015; Lücking et al., 2015). *CesT* is involved in

the synthesis of phosphopantetheinyl transferase encoded by *ces*P, which plays a crucial role in initiating non-ribosomal synthesis. In contrast, *cesA* and *cesB* are important in peptide assembly due to their high affinity for Nicotinamide adenine dinucleotide phosphate (NADPH) (Alonzo et al., 2015), while *cesC* and *cesD* are involved in encoding ATP-binding cassette (ABC) transporters (Dommel et al., 2010). The *B. cereus* strains isolated in this study carried a complete *ces* gene-cluster sequence and the diarrheal toxin *nheC*, as well as a substantial number of *NRPS* genes. We speculate that the isolated *B. cereus* regulated the *ces* gene cluster to synthesize emetic toxins mainly via the NRPS system, thereby inducing foodborne illness associated with vomiting (Ehling-Schulz et al., 2006).

5 Conclusion

In this study, nine strains of *B. cereus* (*LY01-LY09*) were successfully isolated from food samples of foodborne outbreaks. Their whole genome sequences were obtained by high-throughput sequencing. Based on this, the genetic relationship and gene family of *LY01-LY09* and their related strains were studied via comparative genomics. The results showed that the nine strains belonged to *B. cereus* s.l and they were the same strain. Their genome has unique characteristics compared with *B. thuringiensis* and *B. anthracis*. According to the analysis of virulence factors of *LY01-LY09*, it was found that they all contained emetic toxin and *NRPS* gene clusters. Genomic characteristics of the strains were explained. These results provide insights for further study of the evolutionary relationship of *B. cereus* and the mechanism of toxin synthesis and secretion. This study provides a scientific basis for the prevention and rapid diagnosis of foodborne diseases at the genome level.

Data availability statement

The original contributions presented in this study are included in this article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

LZ designed the experiments and applied for fund. SZ, SL, JH, and YW went to the incidence sites to collect food samples and conducted the tests to identify the *B. cereus* strains. YL performed the q-PCR and prepared data. QZ, GL, YC, and JX have worked together to analyze data and wrote the manuscript, in which QZ drafted the Results and Discussion section. GL conducted comparative genome analysis. All authors participated in the rounds of manuscript revision, contributed to the manuscript, and approved its submission and publication.

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Conflict of interest

YS was employed by Yunnan Pulis Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1238799/full#supplementary-material

References

Alonzo, D., Magarvey, N., and Schmeing, T. (2015). Characterization of cereulide synthetase, a toxin-producing macromolecular machine. *PLoS One* 10:e0128569. doi: 10.1371/journal.pone.0128569

Anon (1995). *Bacillus cereus in foods. Enumeration and Confirmation. Microbiological Methods*. Rockville: AOAC International.

Antunes, P., Novais, C., and Peixe, L. (2020). Food-to-humans bacterial transmission. *Microbiol Spectr.* 8, 161–193. doi: 10.1128/microbiolspec.MTBP-0019-2016

Balloux, F., Brønstad Brynildsrud, O., van Dorp, L., Shaw, L., Chen, H., Harris, K., et al. (2018). From theory to practice: Translating whole-genome sequencing (WGS) into the clinic. *Trends Microbiol.* 26, 1035–1048. doi: 10.1016/j.tim.2018.08.004

Biggel, M., Jessberger, N., Kovac, J., and Johler, S. (2022). Recent paradigm shifts in the perception of the role of *Bacillus thuringiensis* in foodborne disease. *Food Microbiol.* 105:104025. doi: 10.1016/j.fm.2022.104025

Bozhüyük, K., Micklefield, J., and Wilkinson, B. (2019). Engineering enzymatic assembly lines to produce new antibiotics. *Curr. Opin. Microbiol.* 51, 88–96. doi: 10.1016/j.mib.2019.10.007

Carroll, L., Cheng, R., Wiedmann, M., and Kovac, J. (2022). Keeping up with the *Bacillus cereus* group: Taxonomy through the genomics era and beyond. *Crit Rev Food Sci Nutr.* 62, 7677–7702. doi: 10.1080/10408398.2021.1916735

Carroll, L., and Wiedmann, M. (2020). Cereulide synthetase acquisition and loss events within the evolutionary history of group III *Bacillus cereus* sensu lato facilitate

the transition between emetic and diarrheal foodborne pathogens. *mBio* 11:e01263-20. doi: 10.1128/mBio.01263-20

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540–552. doi: 10.1093/oxfordjournals.molbev.a026334

Darriba, D., Taboada, G., Doallo, R., and Posada, D. (2011). ProtTest 3: Fast selection of best-fit models of protein evolution. *Bioinformatics* 27, 1164–1165. doi: 10.1093/bioinformatics/btr088

Dietrich, R., Jessberger, N., Ehling-Schulz, M., Märtlbauer, E., and Granum, P. (2021). The food poisoning toxins of *Bacillus cereus*. *Toxins* 13:98. doi: 10.3390/toxins13020098

Dommel, M., Frenzel, E., Strasser, B., Blöchinger, C., Scherer, S., and Ehling-Schulz, M. (2010). Identification of the main promoter directing cereulide biosynthesis in emetic *Bacillus cereus* and its application for real-time monitoring of ces gene expression in foods. *Appl. Environ. Microbiol.* 76, 1232–1240. doi: 10.1128/AEM.02317-09

Edgar, R. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 32, 1792–1797. doi: 10.1093/nar/gkh340

Ehling-Schulz, M., Frenzel, E., and Gohar, M. (2015). Food-bacteria interplay: Pathometabolism of emetic *Bacillus cereus*. *Front. Microbiol.* 6:704. doi: 10.3389/fmicb.2015.00704

Ehling-Schulz, M., Fricker, M., Grallert, H., Rieck, P., Wagner, M., and Scherer, S. (2006). Cereulide synthetase gene cluster from emetic *Bacillus cereus*: Structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1. *BMC Microbiol.* 6:20. doi: 10.1186/1471-2180-6-20

Ehling-Schulz, M., Lereclus, D., and Koehler, T. (2019). The *Bacillus cereus* group: *Bacillus species* with pathogenic potential. *Microbiol. Spectr.* 7.

Emms, D., and Kelly, S. (2019). OrthoFinder: Phylogenetic orthology inference for comparative genomics. *Genome Biol*. 20:238. doi: 10.1186/s13059-019-1832-y

He, S., and Shi, X. (2021). Microbial food safety in China: Past, present, and future. *Foodborne Pathog. Dis.* 18, 510–518. doi: 10.1089/fpd.2021.0009

Hsieh, Y., Chiu, H., Huang, Y., Fun, H., Lu, C., Li, Y., et al. (2014). Purification, crystallization and preliminary X-ray crystallographic analysis of glycosyltransferase-1 from *Bacillus cereus*. *Acta Crystallogr. F Struct. Biol. Commun*. 70(Pt 9), 1228–1231. doi: 10.1107/S2053230X14014629

Jain, C., Rodriguez-R, L., Phillippy, A., Konstantinidis, K., and Aluru, S. (2018). High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* 9:5114. doi: 10.1038/s41467-018-07641-9

Josephs-Spaulding, J., Beeler, E., and Singh, O. (2016). Human microbiome versus food-borne pathogens: Friend or foe. *Appl. Microbiol. Biotechnol.* 100, 4845–4863. doi: 10.1007/s00253-016-7523-7

Kalbhenn, E., Kranzler, M., Gacek-Matthews, A., Grass, G., Stark, T., Frenzel, E., et al. (2022). Impact of a novel pagr-like transcriptional regulator on cereulide toxin synthesis in emetic *Bacillus cereus*. *Int. J. Mol. Sci*. 23:11479. doi: 10.3390/ijms231911479

Kotiranta, A., Lounatmaa, K., and Haapasalo, M. (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infect*. 2, 189–198. doi: 10.1016/s1286-4579(00)00269-0

Kranzler, M., Stollewerk, K., Rouzeau-Szynalski, K., Blayo, L., Sulyok, M., and Ehling-Schulz, M. (2016). Temperature exerts control of *Bacillus cereus* emetic toxin production on post-transcriptional levels. *Front. Microbiol.* 7:1640. doi: 10.3389/fmicb.2016.01640

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., et al. (2009). Circos: An information aesthetic for comparative genomics. *Genome Res.* 19, 1639–1645. doi: 10.1101/gr.092759.109

Kurtz, S., Phillippy, A., Delcher, A., Smoot, M., Shumway, M., Antonescu, C., et al. (2004). Versatile and open software for comparing large genomes. *Genome Biol*. 5:R12. doi: 10.1186/gb-2004-5-2-r12

Li, W., Cui, Q., Bai, L., Fu, P., Han, H., Liu, J., et al. (2021). Application of whole-genome sequencing in the national molecular tracing network for foodborne disease surveillance in China. *Foodborne Pathog. Dis.* 18, 538–546. doi: 10.1089/fpd.2020.2908

Li, Y., Zhao, M., Chen, W., Du, H., Xie, X., Wang, D., et al. (2020). Comparative transcriptomic analysis reveals that multiple hormone signal transduction and carbohydrate metabolic pathways are affected by *Bacillus cereus* in *Nicotiana tabacum*. *Genomics* 112, 4254–4267. doi: 10.1016/j.ygeno.2020.07.022

Lin, S., and Liao, Y. C. (2013). CISA: Contig integrator for sequence assembly of bacterial genomes. *PLoS One* 8:e60843. doi: 10.1371/journal.pone.0060843

Lin, Y., Briandet, R., and Kovács, Á (2022). *Bacillus cereus* sensu lato biofilm formation and its ecological importance. *Biofilm* 4:100070. doi: 10.1016/j.bioflm.2022.100070

Ling, Y., Ling, Z., and Zhao, R. (2022). Construction of a heat-resistant strain of Lentinus edodes by fungal Hsp20 protein overexpression and genetic transformation. *Front. Microbiol.* 13:1009885. doi: 10.3389/fmicb.2022.1009885

Liu, B., Zheng, D., Zhou, S., Chen, L., and Yang, J. (2022). VFDB 2022: A general classification scheme for bacterial virulence factors. *Nucleic Acids Res*. 50, D912–D917. doi: 10.1093/nar/gkab1107

Lücking, G., Frenzel, E., Rütschle, A., Marxen, S., Stark, T., Hofmann, T., et al. (2015). Ces locus embedded proteins control the non-ribosomal synthesis of the cereulide toxin in emetic *Bacillus cereus* on multiple levels. *Front. Microbiol.* 6:1101. doi: 10.3389/fmicb.2015.01101

Magarvey, N., Ehling-Schulz, M., and Walsh, C. (2006). Characterization of the cereulide NRPS alpha-hydroxy acid specifying modules: Activation of alpha-keto acids and chiral reduction on the assembly line. *J. Am. Chem. Soc.* 128, 10698–10699. doi: 10.1021/ja0640187

Makarasen, A., Yoza, K., and Isobe, M. (2009). Higher structure of cereulide, an emetic toxin from *Bacillus cereus*, and special comparison with valinomycin, an antibiotic from *Streptomyces fulvissimus*. *Chem. Asian J.* 4, 688–698. doi: 10.1002/asia.200900011

Marxen, S., Stark, T., Rütschle, A., Lücking, G., Frenzel, E., Scherer, S., et al. (2015). Depsipeptide intermediates interrogate proposed biosynthesis of cereulide, the emetic toxin of *Bacillus cereus*. *Sci. Rep.* 5:10637. doi: 10.1038/srep10637

Niu, H., Yang, M., Qi, Y., Liu, Y., Wang, X., and Dong, Q. (2022). Heat shock in *Cronobacter sakazakii* induces direct protection and cross-protection against simulated gastric fluid stress. *Food Microbiol.* 103:103948. doi: 10.1016/j.fm.2021.103948

Pilo, P., and Frey, J. (2011). *Bacillus anthracis*: Molecular taxonomy, population genetics, phylogeny and patho-evolution. *Infect. Genet. Evol.* 11, 1218–1224. doi: 10.1016/j.meegid.2011.05.013

Pires, S., Desta, B., Mughini-Gras, L., Mmbaga, B., Fayemi, O., Salvador, E., et al. (2021). Burden of foodborne diseases: Think global, act local. *Curr. Opin. Food Sci.* 39, 152–159. doi: 10.1016/j.cofs.2021.01.006

Premkrishnan, B., Heinle, C., Uchida, A., Purbojati, R., Kushwaha, K., Putra, A., et al. (2021). The genomic characterisation and comparison of *Bacillus cereus* strains isolated from indoor air. *Gut Pathog.* 13:6. doi: 10.1186/s13099-021-00399-4

Sánchez Chica, J., Correa, M., Aceves-Diez, A., Rasschaert, G., Heyndrickx, M., and Castañeda-Sandoval, L. (2020). Genomic and toxigenic heterogeneity of bacillus cereus sensu lato isolated from ready-to-eat foods and powdered milk in day care centers in Colombia. *Foodborne Pathog. Dis.* 17, 340–347. doi: 10.1089/fpd.2019.2709

Shu-sen, S., Li-li, J., and Fang, W. (2007). Study on genetic relationship between *Bacillus thuringiensis* and *Bacillus cereus*. *Chin. J. Public Health* 23, 321–323.

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Stenfors Arnesen, L., Fagerlund, A., and Granum, P. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev*. 32, 579–606. doi: 10.1111/j.1574-6976.2008.00112.x

Toh, M., Moffitt, M., Henrichsen, L., Raftery, M., Barrow, K., Cox, J., et al. (2004). Cereulide, the emetic toxin of *Bacillus cereus*, is putatively a product of nonribosomal peptide synthesis. *J Appl Microbiol.* 97, 992–1000. doi: 10.1111/j.1365-2672.2004.02381.x

Tran, S., Guillemet, E., Ngo-Camus, M., Clybouw, C., Puhar, A., Moris, A., et al. (2011). Haemolysin II is a *Bacillus cereus* virulence factor that induces apoptosis of macrophages. *Cell Microbiol.* 13, 92–108. doi: 10.1111/j.1462-5822.2010.01522.x

Trunet, C., Cauquil, A., Hymery, N., Koullen, L., Postollec, F., and Coroller, L. (2023). Are *Bacillus thuringiensis* strains like any other *Bacillus cereus* strains? Phenotypic-based tools to locate *Bacillus thuringiensis* in the diversity of the *Bacillus cereus* sensu lato group. *Res. Microbiol.* 174:104077. doi: 10.1016/j.resmic.2023.104077

Venkateswaran, K., Singh, N., Checinska Sielaff, A., Pope, R., Bergman, N., van Tongeren, S., et al. (2017). Non-toxin-producing *Bacillus cereus* strains belonging to the B. Anthracis clade isolated from the International Space Station. *mSystems* 2:e00021-17. doi: 10.1128/mSystems.00021-17.

Wang, Y. J., Li, L., Yu, J., Hu, H. Y., Liu, Z. X., Jiang, W. J., et al. (2023). Imaging of *Escherichia coli* K5 and glycosaminoglycan precursors via targeted metabolic labeling of capsular polysaccharides in bacteria. *Sci. Adv.* 9:eade4770. doi: 10.1126/sciadv.ade4770

Yu, C., Wang, Y., Xu, C., He, J., Zhang, Q., and Yu, Z. (2011). [Analyze and compare metabolic pathways of *Bacillus cereus* group]. *Yi Chuan* 33, 1057–1066. doi: 10.3724/sp.j.1005.2011.01057

Zhou, G., Liu, H., He, J., Yuan, Y., and Yuan, Z. (2008). The occurrence of *Bacillus cereus*, *B. thuringiensis* and *B. mycoides* in Chinese pasteurized full fat milk. *Int J Food Microbiol.* 121, 195–200. doi: 10.1016/j.ijfoodmicro.2007.11.028

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Genomic analysis of *Salmonella enterica* from Metropolitan Manila abattoirs and markets reveals insights into circulating virulence and antimicrobial resistance genotypes

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The integration of next-generation sequencing into the identification and characterization of resistant and virulent strains as well as the routine surveillance of foodborne pathogens such as *Salmonella enterica* have not yet been accomplished in the Philippines. This study investigated the antimicrobial profiles, virulence, and susceptibility of the 105 *S. enterica* isolates from swine and chicken samples obtained from slaughterhouses and public wet markets in Metropolitan Manila using whole-genome sequence analysis. Four predominant serovars were identified in genotypic serotyping, namely, Infantis (26.7%), Anatum (19.1%), Rissen (18.1%), and London (13.3%). Phenotypic antimicrobial resistance (AMR) profiling revealed that 65% of the isolates were resistant to at least one antibiotic, 37% were multidrug resistant (MDR), and 57% were extended-spectrum β-lactamase producers. Bioinformatic analysis revealed that isolates had resistance genes and plasmids belonging to the *Col* and *Inc* plasmid families that confer resistance against tetracycline (64%), sulfonamide (56%), and streptomycin (56%). Further analyses revealed the presence of 155 virulence genes, 42 of which were serovar-specific. The virulence genes primarily code for host immune system modulators, iron acquisition enzyme complexes, host cell invasion proteins, as well as proteins that allow intracellular and intramacrophage survival. This study showed that virulent MDR *S. enterica* and several phenotypic and genotypic AMR patterns were present in the food chain. It serves as a foundation to understand the current AMR status in the Philippines food chain and to prompt the creation of preventative measures and efficient treatments against foodborne pathogens.

KEYWORDS

***Salmonella enterica*, whole-genome sequencing, antimicrobial resistance genes, plasmids, virulence, food chains**

1 Introduction

Salmonellosis, caused by *Salmonella* spp., is among the most frequently reported foodborne diseases worldwide and has a high health and economic burden. *Salmonella enterica*, with its six distinct subspecies (I, II, IIIa, IIIb, IV, and VI), is a leading cause of global human diarrheal cases and outbreaks annually, including in the Philippines. While there are numerous potential means and sources of transmission, chicken and pig meat, along with other farm animals and products, have been identified as the dominant food vehicles for *S. enterica* due to their wide distribution and natural, chronic carriage among livestock (World Health Organization, 2018; Ferrari et al., 2019; Edrington and Brown, 2022). The predicted increase in consumption of swine and poultry products in the Philippines in the next ten years is over 3 million metric tons *per annum* (Organisation for Economic Cooperation and Development/Food and Agriculture Organization, 2023), which increases the potential for exposure to the pathogen. In 2021, the agricultural sector contributed to 9.6% of the Philippines' national gross domestic product (GDP) (Philippine Statistics Authority, 2022). However, due to several outbreak events in the country such as African swine fever (Cooper et al., 2022) and COVID-19 pandemic (Espino et al., 2021), a significant decline in hog and poultry production output was reported. Approximately 16% (53 K metric tons) increase in imported frozen chicken meat was reported from 2020 to 2021 (Philippine Statistics Authority, 2022). In 2022 alone, pork (52%; 710 M metric tons) and chicken (30%; 411 M metric tons) topped the total meat importation to address the local supply shortage due to production loss and growing meat consumption (Bureau of Animal Industry, 2022).

Studies on raw and processed meats from abattoirs and wet markets in Metro Manila, Philippines, have revealed a high prevalence of *S. enterica* (>30%) and the additional high frequency of samples positive for the *spvC* virulence gene, which is strongly associated with strains that cause non-typhoidal bacteremia (Calayag et al., 2017; Santos et al., 2020). Subsequent work in Metro Manila has revealed highly frequent virulence genes (e.g., SPIs 1–5 genes), some co-occurring, and others linked to location and animal source (Pavon et al., 2022). In this setting, *S. enterica* has been identified primarily from chicken samples and ground pork (both >65%), and with multiple and mixed serogroups E1, C1, C2, B, and D being abundant (all >6%). *S. enterica* types vary significantly in their host range and their degree of host adaptation. Other studies set in Metro Manila in swine have found similarly high *S. enterica* bacterium prevalence across accredited and locally registered abattoirs (~50%) but with most bacteria under serogroup O (Ng and Rivera, 2015; Calayag et al., 2017). Collectively, these studies point to *S. enterica* circulating in the poultry and swine food chains in Metro Manila, with virulence genes, and thereby a likely major and increasing cause of gastroenteritis and enteric fever.

In 2015, a study in slaughtered swine in Metro Manila, Philippines detected five serotypes, namely, *S. enterica* Typhimurium, Agona, Heidelberg, Choleraesuis, and Weltevreden from tonsils and jejuna of freshly slaughtered swine (Ng and Rivera, 2015). In a separate work, serotypes Anatum, Kentucky, and Saintpaul have been found in bovine, porcine, and poultry meat from wet markets (Santos et al., 2020). The presence of these serotypes in meat samples can be correlated to disease. In a 15-year report of *Salmonella* serotype distribution in the Philippines by the Antimicrobial Resistance Surveillance Reference Laboratory of the Research Institute for Tropical Medicine (RITM), *S. enterica* Enteritidis, Typhimurium, Weltevreden, Stanley, and Anatum were found to be the five most prevalent non-typhoidal serotypes in clinical samples. The other serotypes found in meat samples such as Agona, Heidelberg, Choleraesuis, and Kentucky were also detected from clinical samples, although not as prevalent (Sia et al., 2020). This gives us a picture of how parallel the prevalent serotypes in meat samples and clinical samples are, and how tracking the source of these pathogens is paramount to reducing risk, designing mitigation strategies, and predicting future outbreaks.

Treatment options for salmonellosis are decreasing as the underlying bacteria continue to show antimicrobial resistance (AMR). Due to frequent antibiotic exposure, swine and poultry are now recognized as potential risks in disseminating drug-resistant *S. enterica*, with multidrug resistant (MDR) strains now being present in the Philippines (Calayag et al., 2017). As defined by Magiorakos et al. (2012), MDR organisms have non-susceptibility to at least one antimicrobial agent from three or more antimicrobial classes. Studies of *S. enterica* from slaughtered swine in Metro Manilla revealed high rates of resistance to ampicillin, trimethoprim/sulfamethoxazole, and MDR (all >67%) (Calayag et al., 2017). These forms of resistance can be detected by polymerase chain reaction (PCR)-based approaches that target known regions (e.g., on plasmids) or, phenotypically, using systems such as the VITEK® 2 Compact 60 ID/AST System (bioMérieux, 2005) for antimicrobial susceptibility testing. However, in other infection settings, e.g., tuberculosis (Phelan et al., 2019), *Klebsiella pneumoniae* (Spadar et al., 2023), next-generation sequencing (NGS) has gained traction for fast and affordable AMR profiling (genotyping). Whole-genome sequencing (WGS) analysis can be a rapid and cost-effective approach to define resistance genotypes, predict resistance phenotypes, and identify identical isolate genomes that are part of transmission chains (Sobkowiak et al., 2020; Napier et al., 2022).

To demonstrate the utility of sequencing, we have performed WGS on 105 *S. enterica* isolates across live animal and processed meat domains in the poultry and swine food chains in Metro Manila between 2018 and 2022. This study is part of a bigger project that aimed to recover 2,500 *S. enterica* isolates from abattoirs and wet markets in eight cities in Metro Manila, Philippines. The 105 isolates in this study have been obtained from six out of the eight cities, and were the first batch subjected to whole genome sequencing. The resulting genomic variation is used to understand circulating AMR and virulence gene repertoires. Our work provides a baseline set of genomic data and a snapshot of diversity, which can be used in emerging applications of NGS for the routine monitoring of meat product safety in the Philippines.

2 Materials and methods

2.1 Sample collection and processing

A collection of *S. enterica* strains isolated from swine and poultry meat was established to understand the AMR profiles and determine the virulence genes associated with swine and poultry food chains in Metro Manila, Philippines. The meat samples came from public wet markets and accredited as well as locally registered abattoirs in the

four Metro Manila districts, namely, Capital, Eastern, Northern, and Southern. Sample collection from freshly slaughtered swine in slaughterhouses includes the 15-cm segment of the jejunum (Calayag et al., 2017). All sample collection was performed between 2018 and 2022. Different parts of swine and chicken meat were sampled from public wet markets. Raw meat samples include ground or cut-up meat, while processed meat samples include marinated, salted, cured, and pre-cooked products. The samples were transported to the laboratory in sterile plastic bags and kept cold in a cooler. Upon arrival at the laboratory, 25 g of meat sample was weighed and pre-enriched with 225 mL of sterile buffered peptone water (BD Difco, NJ, USA) in a sterile Rollbag® (Interscience, France), homogenized with BagMixer® 400 (Interscience, France) for 1 min, and incubated for 24 h at 37°C. For the single-enrichment broth culture method, 100-μL of the pre-enriched culture was transferred into Rappaport-Vassiliadis (RV) broth (10 mL; Difco, BD, Sparks, MD) and incubated at 42°C for 24 h (Ng and Rivera, 2015). A loopful of incubated RV broth was streak plated onto xylose lysine deoxycholate (XLD) agar (BD Diagnostics System, NJ, USA) plates for isolation and purification, and incubated at 37°C for 18–24 h. Typical *Salmonella* colonies, i.e., colonies with black centers and clear or transparent halo, were then subcultured on nutrient agar (NA) (BD Diagnostics System, NJ, USA) for further confirmation analysis (Pavon et al., 2022).

2.2 DNA extraction, molecular detection, and sequencing

Presumptive colonies of *S. enterica* were subjected to DNA extraction using a DNA purification kit (Monarch®, New England BioLabs, MA, USA) and stored in a −20°C freezer (Schnee Irish, Vienna, Austria). PCR-based identification was conducted using the species-specific *invA* gene to confirm the identity of the isolates (Ng and Rivera, 2015). The amplicons were subjected to agarose gel electrophoresis using 1.5% agarose stained with 10,000 × GelRed® in water (Biotium, CA, USA). Electrophoresis runs were made to proceed under 280 V for 35 min. The gels were visualized using a UV gel documentation system (Vilber Lourmat, France) (Pavon and Rivera, 2021). Amplicon size was estimated using 100-bp HyperLadder™ (Bioline, Meridian Bioscience, London, UK) as the molecular weight marker. Amplicons with the approximate size of ~244 bp were considered positive for the *invA* gene, and thus *Salmonella*-positive. The concentration and purity of the *invA*-positive DNA extracts were measured using a microplate spectrophotometer (Multiskan SkyHigh, Thermo Scientific™) before submission to the sequencing facilities. From a collection of 2,500 *S. enterica* isolates, a subset of 105 isolates was randomly selected for Illumina TruSeq library construction and sequencing undertaken at the DNA Sequencing Core Facility of the Philippine Genome Center and through the Applied Genome Centre at London School of Hygiene and Tropical Medicine (LSHTM).

2.3 AMR genotyping and phenotyping and virulence gene profiling

ABRicate software (v1.0.1; minimum %ID = 80, min % coverage = 80) (Seemann, 2020) was applied to find the presence of resistance genes (NCBI database, as of March 27, 2021) and plasmids (PlasmidFinder database; as of April 4, 2023) (Carattoli et al., 2014). The VITEK® 2 Compact 60 ID/AST System (AST-GN70 card panel, bioMérieux, Marcy-l'Étoile, France) was used to test the sensitivity of 105 isolates to 15 antimicrobial agents, including ampicillin, sulbactam, tazobactam, ceftriaxone, cefepime, aztreonam, ertapenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, tigecycline, nitrofurantoin, and trimethoprim-sulfamethoxazole. Interpretive criteria and breakpoints from the Clinical and Laboratory Standards Institute (CLSI) (2022) 32nd Edition were used in the analysis. *S. enterica* subsp. *enterica* Le Minor and Popoff serovar Typhimurium ATCC 14028™, *Escherichia coli* ATCC 25922™, and *K. pneumoniae* ATCC 600703™ were used as quality control (QC) reference strains. ABRicate software (v1.0.1) (Seemann, 2020) with the same parameters was also applied to find the presence of virulence genes (VFDB database, as of April 4, 2023) (Chen et al., 2016).

2.4 Bioinformatics, sample genotyping, and phylogeny

Kraken2 software (v2.1.2) was used with the standard database to scan for any possible contamination (Wood et al., 2019). Genomes were assembled with Shovill software (v1.1.0) (Seemann, 2019) and were used by the SISTR tool (v1.1.1) (Yoshida et al., 2016) to predict serovars and cgMLST sequence types (STs), and to perform basic quality control checks. QUAST software (v5.2.0) was used to check the quality of the assemblies with all genomes passing a minimum of 95% of BUSCO genes found (Mikheenko et al., 2018). ParSNP software (v1.7.4) was used to perform a core genome analysis to identify genomic regions present in most strains. These regions were identified and aligned by ParSNP and this was used as input to build a phylogeny using the RAxML tool (v8.2.12; parameters: -m GTRGAMMA -N 100 -k -f a) (Stamatakis, 2014) for all isolates together, as well as for the four main individual serovar clades separately. The sequence data were scanned for 155 known virulence genes. The phylogenetic trees were used to identify potential transmission chains, through the application of established methods (Sobkowiak et al., 2020; Napier et al., 2022). The isolates have been run through Snippy (v4.6.0)[1] (Seemann, 2015) to find single nucleotide polymorphisms (SNPs) and determine genetic similarities within serovars and across sampling sites (slaughterhouses and markets), cities, and matrices (swine or chicken). Genetically similar isolates (i.e., less than 20 SNPs) have been plotted in single-linkage clusters using transmission graph viewer.[2]

2.5 Ethical approval and consent to participate

Ethical review and approval were waived for this study due to informed consent obtained from the National Meat Inspection Service of the Philippine Department of Agriculture. Animal slaughter and evisceration were performed according to Philippine national

1 https://github.com/tseemann/snippy
2 https://jodyphelan.github.io/tgv/

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regulations. Informed consent was also obtained from veterinarians in charge of the abattoirs, and farm owners for sample collection.

3 Results

3.1 Characteristics of the *Salmonella* isolates

In total, 105 isolates for the study were sourced from six different locations around Metro Manila (Supplementary Figure S1; Supplementary Table S1). These were collected from both markets (74.3%) and abattoirs (25.7%), and from both chicken (26.7%) and swine (73.3%) (Table 1). Phylogenetic reconstruction using a core genome alignment revealed distinct clades which correlated perfectly with the serovars (N = 18) derived from the sequence data (Figure 1). Most of the isolates belonged to the Infantis ($n = 28$, 26.7%), Anatum (20, 19.1%), Rissen (19, 18.1%), and London (14, 13.3%) serovar classes (Table 2).

Phenotypic analysis of susceptibility to 15 antimicrobial drugs using VITEK® 2 Compact 60 ID/AST System found that 65% of isolates were resistant to at least one drug, with 57% categorized as extended-spectrum β-lactamases (ESBL) and 37% categorized as MDR. Out of the total 105 strains tested, 63% (66/105) were resistant to ampicillin, 78% (82/105) had intermediate to ciprofloxacin, a quinolone, while 23% (24/105) were resistant. No intermediate resistance to any cephalosporin antimicrobials was detected. Resistance was only observed in cefazolin and ceftriaxone, with 24% (25/105) resistant isolates. For the remaining antibiotics, the resistance percentages were as follows: 31% (33/105) nitrofurantoin, 27% (28/105) gentamicin, 26% (27/105) tobramycin, and 23% (24/105) sulbactam. The isolates were all susceptible to aztreonam, carbapenem, amikacin, and tigecycline (Table 3). The sequences were scanned for AMR loci, revealing the presence of 35 genes and 12 plasmids conferring resistance to 14 different drugs. Most of the isolates (67/105; 64%) exhibited AMR genes against tetracycline (*tetABDM*), followed by 56% of isolates with sulfonamide (*sul1, sul2, sul3*) and streptomycin (*aadA1*, *aadA2*, *aph(6)-Id*, and *aph(3′)-Ib*) AMR genes. In contrast with the phenotypic susceptibility test, there are 56 isolates with trimethoprim-sulfamethoxazole genes (*sul1, sul2, sul3, dfrA1, dfrA12*, and *dfrA14*) but only 9.5% (10/105) of samples exhibited resistance to the antimicrobials. Consistent with the high resistance observed against ampicillin and ciprofloxacin, several isolates have AMR genes and plasmids promoting β-lactam ($bla_{\text{EC-18}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{TEM-1}}$, $bla_{\text{TEM-150}}$, and $bla_{\text{TEM-176}}$) and quinolone resistance (*qnrS1* and *qnrB2*) (Table 3).

TABLE 1 Sample characteristics.

Characteristic	*N*	%
Year of collection		
2018	33	31.4
2019	44	41.9
2022	28	26.7
Location*		
Valenzuela	33	31.4
San Juan	25	23.8
Caloocan	20	19.1
Muntinlupa	15	14.3
Quezon	10	9.5
Pasay	2	1.9
Source		
Market	78	74.3
Abattoir	27	25.7
Food chain		
Swine	77	73.3
Chicken	28	26.7
Meat cuts		
Chicken		
Breast	6	5.7
Drumstick	6	5.7
Thigh	12	11.4
Wing	4	3.8
Swine		
Tongue	2	1.9
Meatloaf	1	1.0
Ground	14	13.3
Jejunum	21	20.0
Shoulder	9	8.6
Chorizo	7	6.7
Cubed	1	1.0
Chop	12	11.4
Neck	1	1.0
Cured	3	2.9
Tonsils	6	5.7

*All markets, except from abattoirs in Valenzuela ($N = 7$) and Caloocan ($N = 20$).

3.2 *Salmonella* serovars and AMR

Genomic diversity existed within the isolates, but a clonal group is evident from both the phylogenetic tree and the presence of multiple isolates from the same cgMLST ST, all sourced from the same location and meat type. Resistance was common with isolate genomes containing genes that confer resistance to 9 drug types, including some isolates potentially simultaneously resistant to 8 different drugs. Plasmids were also found in all isolates, with two out of six being present across all (Figure 2A).

The Infantis serovar were all sourced from markets, and almost exclusively found in chicken (26/28). Some isolates sourced from different locations had the same cgMLST ST but significant genomic diversity existed preventing close clusters on the phylogenetic tree. AMR was also common in this serovar with genes conferring resistance to 13 different drug groups being found with some isolates presenting resistance to 11 different drugs. Six different plasmids were found with the IncFIB(K) type being present in all samples except one (Figure 2B).

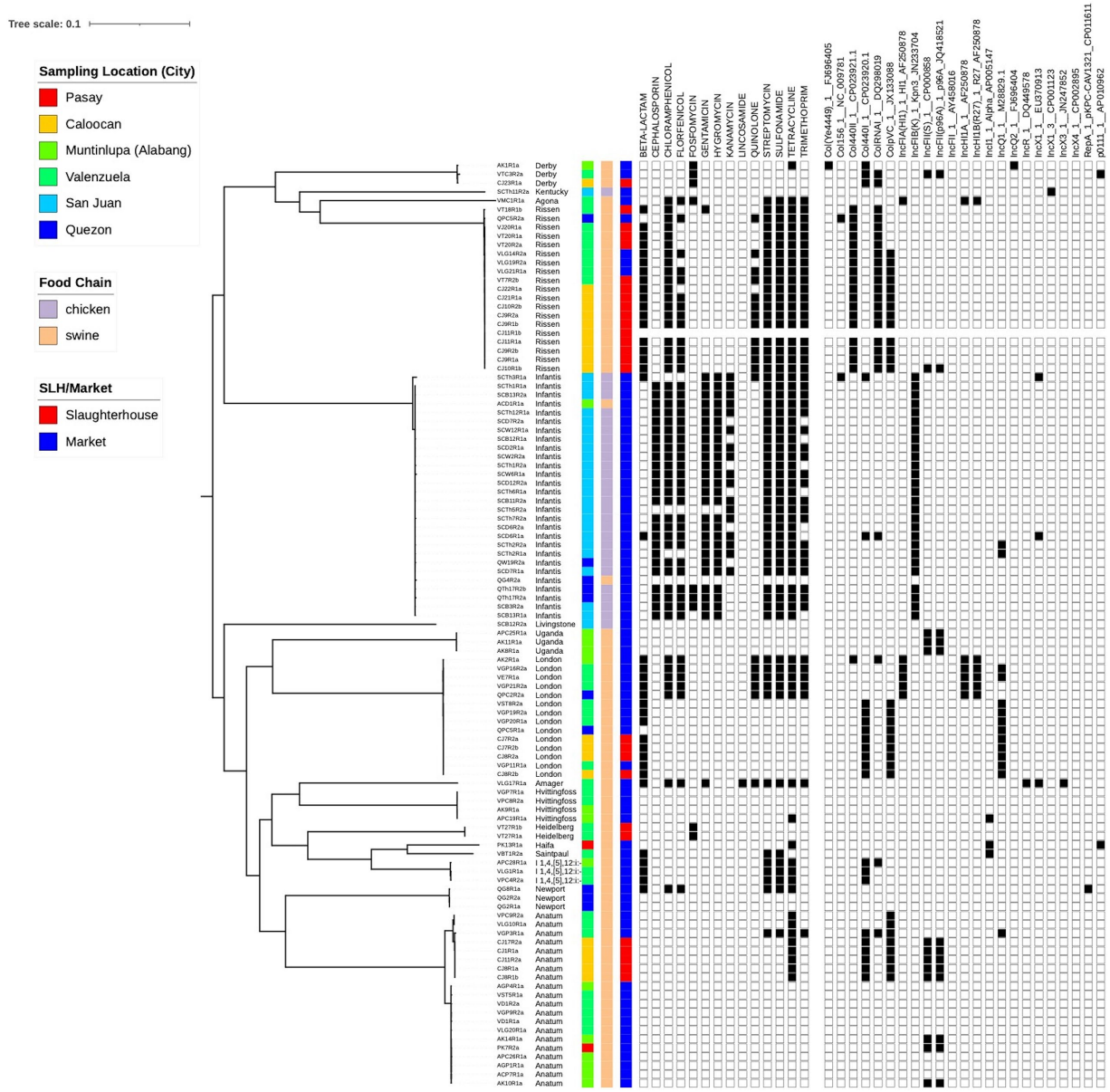

FIGURE 1
Phylogenetic tree of study isolates ($N = 105$). A core genome analysis was performed to identify genomic regions common to all strains. This was used as input to RaxML to build a maximum likelihood tree. Sampling location, food chain and source type (slaughterhouse/market) are visualized together with phenotypic resistance and the presence of plasmids.

The London serovar was found only in swine and was sourced from both markets and slaughterhouses in four different locations. While some isolates clustered closely on the phylogenetic tree, every isolate had a unique cgMLST ST indicating a significant amount of diversity. Genes conferring resistance to 8 different drugs were found with one clade containing all 8 AMR genes, while the other clade displayed very little resistance with all isolates possessing, at most, one resistance gene. This may be reflective of the different plasmid content between the clades with 8 different plasmids found in total (Figure 2C).

The Anatum serovar was sourced in four different locations and exclusively found in swine from both markets and abattoirs. The phylogenetic tree formed two main clades, and all isolates had different cgMLST STs. AMR loci linked to four different drugs were found, with one isolate presenting all resistance genes. However, one large clade did not present any resistance genes. Six different plasmids were found, including in the clade without resistance genes (Figure 2D). There was evidence of high similarity of samples across different swine markets (Valenzuela, Muntinlupa, Pasay) suggestive of transmission within abattoirs or farms.

3.3 Virulence genes

The presence of 155 known virulence genes was detected across the 105 isolates (Supplementary Table S1). More than half of the genes (87/155; 56.12%) were found in all isolates; while a minority (24/155;

TABLE 2 Serovars identified.

Serovar	Chicken *N* = 28	Swine *N* = 75	Total *N* = 105	%
Infantis	26	2	28	26.7
Anatum	0	20	20	19.1
Rissen	0	19	19	18.1
London	0	14	14	13.3
Hvittingfoss	0	4	4	3.8
Derby	0	3	3	2.9
I 1,4,[5],12:i:-	0	3	3	2.9
Newport	0	3	3	2.9
Uganda	0	3	3	2.9
Heidelberg	0	2	2	1.9
Agona	0	1	1	<1.0
Amager	0	1	1	<1.0
Haifa	0	0	1	<1.0
Kentucky	1	0	1	<1.0
Livingstone	1	1	1	<1.0
Saintpaul	0	1	1	<1.0

15.48%) were absent in all isolates. For the other (non-fixed) 42 virulence genes, some were serovar-specific (Figure 3). The most frequent serovar, Infantis, had the highest number of virulence genes (median number: Infantis 116; vs. other serovars 102; Wilcoxon $p < 10^{-13}$). Excluding one outlier, 11 virulence genes were found exclusively in the Infantis serovar (*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU*, and *ybtX*). Similarly, the aerobactin biosynthesis *iucA, iucB, iucC, iucD*, and *iutA* virulence genes were exclusive to the single Kentucky serovar. The fatty acyl-CoA dehydrogenase (*faeD* and *faeE*) genes were found exclusively in 5 serovars–Anatum (20), Haifa (1), Infantis (27), Kentucky (1), Saintpaul (1).

3.4 Genetic similarities within serovars

For the four most isolated serovars, isolates with less than 20 SNPs have been plotted in single-linkage clusters reflecting sampling sites, cities, and matrices in Figure 4.

For the Rissen serovar, isolates sourced from Caloocan slaughterhouse congregated in one cluster, while isolates from Valenzuela slaughterhouse and markets form multiple clusters (Figure 4A). For Anatum serovar, clustering can be observed per sampling site and city, apart from market isolates from Pasay and one isolate from Muntinlupa which have clustered together. Valenzuela Anatum isolates, on the other hand, formed multiple clusters (Figure 4B). Isolates under the London serovar from one slaughterhouse and three markets, all from three cities, are found to congregate in one major cluster. Meanwhile, Valenzuela and Quezon isolates form one minor cluster (Figure 4C). Finally, for the Infantis serovar, the isolates which were mostly sourced from chicken market samples and a few swine samples formed one major cluster which branches out into smaller clusters. Several San Juan isolates link to a few Quezon isolates, forming three minor clusters (Figure 4D).

4 Discussion

Due to its emergence, transmission, and persistence, AMR continues to be a serious global health problem. This issue is greatly exacerbated internationally through the use of antimicrobials in various food chains, notably in the production of livestock and poultry. It adversely affects food security in low- and middle-income countries such as the Philippines which is a large consumer of hog and poultry products. This study is the first to report on AMR and transmission of *S. enterica* using a WGS of isolates from swine and poultry in the Philippines.

4.1 Serovars

The four most dominant serovars found in this study, Infantis, Anatum, Rissen, and London are consistently detected in several institutional and independent studies in different countries and territories such as the European Union [European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2022], the United States (Centers for Disease Control and Prevention (CDC), 2013; Bearson, 2021), and China (Liu et al., 2020; Tang et al., 2022, 2023), to mention a few. In this study, the most dominant is *S.* Infantis which is one of the increasingly emerging and spreading serovars globally (Alvarez et al., 2023) and is widely linked to human salmonellosis (Montone et al., 2023). In our study, this serovar was associated with raw chicken meat, consistent with epidemiological data from the EU [European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2022] and the USA [Centers for Disease Control and Prevention (CDC), 2013]. Due to outbreaks in the EU, it has been recommended that this serovar (among others) be included in surveillance schemes (Ferrari et al., 2019).

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TABLE 3 AMR and linked genes and plasmids.

Drug	*N* (%) phenotypic resistant*	Genes	Plasmids
AMP	63.0	bla_{EC-18} $bla_{CTX-M-65}$ bla_{TEM-1} $bla_{TEM-150}$ $bla_{TEM-176}$	*Col156_1* *Col440I_1* *Col440II_1* *IncFIA(HI1)_1_HI1* *IncFIB(K)_1_Kpn3* *IncFII(p96A)_1_p96A* *IncFII(S)_1* *IncFII_1* *IncFII_1_pSFO* *IncHI1A_1* *IncHI1B(R27)_1_R27* *IncQ1_1* *IncQ2_1* *IncI1_1_Alpha* *IncR_1* *IncX1_1* *IncX1_3* *IncX3_1* *IncX4_1* *p0111_1*
AMS	23.0		
TZP	0		
CZN	24.0	bla_{EC-18} $bla_{CTX-M-65}$ bla_{TEM-1} $bla_{TEM-150}$ $bla_{TEM-176}$	*IncFIA(HI1)_1_HI1* *IncFIB(K)_1_Kpn3* *IncHI1A_1* *IncHI1B(R27)_1_R27* *IncQ1_1* *IncQ2_1* *IncI1_1_Alpha* *IncR_1* *IncX1_1* *IncX1_3* *IncX3_1* *IncX4_1* *p0111_1* *RepA_1_pKPC-CAV1321*
CTR	24.0		
CEF	0		
NFN	31.0	-	-
GEN	27.0	*aadA1* *aph(4)-Ia* *aac(3)-Iva* *aph(3')-Ia* *aadA2* *aph(6)-Id* *aph(3")-Ib* *aac(3)-Iid* *aac(3)-Iie*	*IncFIA(HI1)_1_HI1* *IncFIB(K)_1_Kpn3* *IncHI1A_1* *IncHI1B(R27)_1_R27* *IncQ1_1* *IncQ2_1* *IncI1_1_Alpha* *IncR_1* *IncX1_1* *IncX1_3* *IncX3_1* *IncX4_1* *p0111_1*
SXT	27.0	*sul1* *dfrA14* *dfrA12* *sul2* *sul3* *dfrA1*	-

(Continued)

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TABLE 3 (Continued)

Drug	*N* (%) phenotypic resistant*	Genes	Plasmids
TOB	26.0	*aadA1* *aph(4)-Ia* *aac(3)-Iva* *aph(3′)-Ia* *aadA2* *aph(6)-Id* *aph(3″)-Ib* *aac(3)-Iid* *aac(3)-Iie*	*IncFIA(HI1)_1_HI1* *IncFIB(K)_1_Kpn3* *IncHI1A_1* *IncHI1B(R27)_1_R27* *IncQ1_1* *IncQ2_1* *IncI1_1_Alpha* *IncR_1* *IncX1_1* *IncX1_3* *IncX3_1* *IncX4_1* *p0111_1*
CIP	23.0	*qnrS1* *qnrB2*	*IncFIB(K)_1_Kpn3* *IncQ1_1* *IncQ2_1* *IncI1_1_Alpha* *IncR_1* *IncX1_1* *IncX1_3* *IncX3_1* *IncX4_1*
AZT	0	$bla_{\text{EC-18}}$ $bla_{\text{CTX-M-65}}$ $bla_{\text{TEM-1}}$ $bla_{\text{TEM-150}}$ $bla_{\text{TEM-176}}$	*Col156_1* *Col440I_1* *Col440II_1* *IncFIA(HI1)_1_HI1* *IncFIB(K)_1_Kpn3* *IncFII(p96A)_1_p96A* *IncFII(S)_1* *IncFII_1* *IncFII_1_pSFO* *IncHI1A_1* *IncHI1B(R27)_1_R27* *IncQ1_1* *IncQ2_1* *IncI1_1_Alpha* *IncR_1* *IncX1_1* *IncX1_3* *IncX3_1* *IncX4_1* *p0111_1*
ETP	0	-	*RepA_1_pKPC-CAV1321*
MEM	0	-	

(Continued)

TABLE 3 (Continued)

Drug	*N* (%) phenotypic resistant*	Genes	Plasmids
AMK	0	*aadA1* *aph(4)-Ia* *aac(3)-Iva* *aph(3′)-Ia* *aadA2* *aph(6)-Id* *aph(3″)-Ib* *aac(3)-Iid* *aac(3)-Iie*	*IncFIA(HI1)_1_HI1* *IncFIB(K)_1_Kpn3* *IncHI1A_1* *IncHI1B(R27)_1_R27* *IncQ1_1* *IncQ2_1* *IncI1_1_Alpha* *IncR_1* *IncX1_1* *IncX1_3* *IncX3_1* *IncX4_1* *p0111_1* *RepA_1_pKPC-CAV1321*
TGC	0	*tet(A)* *tet(M)* *tet(B)* *tet(D)* *tet(J)*	-

VITEK; AMP, ampicillin; NFN, nitrofurantoin; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; TOB, tobramycin; CZN, cefazolin; CTR, ceftriaxone; CIP, ciprofloxacin; AMS, ampicillin/sulbactam; TZP, piperacillin/tazobactam; CEF, cefepime; AZT, aztreonam; ETP, ertapenem; MEM, meropenem; AMK, amikacin; TGC, tigecycline.

From the four most dominant serovars, the most AMR genes have been detected in *S.* Infantis. Likewise, *S.* Infantis harbored the most number of virulence genes. These AMR and virulence determinants would have likely propelled the increased emergence and rapid spread of *S.* Infantis. In a large-scale review by Alvarez et al. (2023) which included 3,725 independent studies on *S.* Infantis in different countries, *S.* Infantis isolates collectively exhibited resistance to all antibiotic classes examined, including aminoglycosides, amphenicols, β-lactams, quinolones, sulfonamides, and tetracyclines, among others. They have also exhibited the presence of resistance genes attributed to specific antimicrobials such as *aad, aac, aph, sul, bla, qnr*, and *tet* which are consistent with the results of this study.

The second most dominant serovar was Anatum, which was exclusively isolated from swine samples, consistent with settings such as Latin America (Ferrari et al., 2019). However, previous reviews have revealed that it is associated with beef and seafood, globally, which were not sources considered in our work (Ferrari et al., 2019). The London serovar has been associated with various infections in humans (Yong et al., 2005), animals, and food products (Meunsene et al., 2021). Although it is uncommonly detected and studied among *Salmonella* serovars, London isolates have exhibited potential zoonotic transmission and increasing resistance to antibiotics (Fang et al., 2022). In this study, London isolates were exclusively found in swine samples and only conferred resistance to limited antimicrobial classes. Lastly, the Rissen serovar is currently one of the emerging serovars in various countries worldwide. In a large-scale global review by Elbediwi et al. (2021), it was revealed that the bulk of Rissen isolates (~2/3) were obtained from human samples, more than half of which were asymptomatic individuals. While most non-clinical Rissen isolates in global studies were associated with poultry and porcine samples, several studies have also found the serovar in seafood (Atwill and Jeamsripong, 2021; Lozano-León et al., 2022), and other sources (Prasertsee et al., 2019; Silveira et al., 2019; Nguyen et al., 2021; Sanguankiat et al., 2023).

In the Philippines, *Salmonella* detection studies in non-clinical samples would often be limited to presence-absence tests. Only a few studies would have data on *Salmonella* serovars. One study on retail meats sampled from wet markets in Metro Manila revealed that bovine meat mostly harbored Anatum and Saintpaul, porcine meat mostly harbored Anatum, and poultry meat mostly harbored Kentucky (Santos et al., 2020). However, these identities were only obtained using H typing, and thus are not confirmed. *Salmonella* studies with confirmed serovar identification are limited to clinical samples from the Philippine Department of Health – Antimicrobial Resistance Surveillance Program (DOH-ARSP) of the RITM. In a surveillance study covering blood and stool samples obtained from 17 sentinel sites all over the country from 2013 to 2014, *in silico* genotyping using WGS revealed that the most dominant serovars were *S.* Typhi as well as *S.* Enteritidis and ST34 (I 4, [5],12: i: -) for the non-typhoidal types (Lagrada et al., 2022).

The genetic similarity of the isolates that clustered together can possibly be traced to having the same slaughterhouse or farm source(s), thus harboring the same strains. This can also indicate transmission, as clones of the same strain can be circulating in one sampling site. It is important to note that local farms supply livestock to different slaughterhouses, and these slaughterhouses do not necessarily supply markets within the same city. This means that a farm can send livestock for slaughter to one slaughterhouse, and the slaughtered meat can be supplied to different markets in different cities. Genetically distinct isolates, or those that form multiple clusters, could likely be traced from various slaughterhouses and/or farm sources. Cross-contamination, as apparent in Infantis isolates, could likely be traced to large-scale commercial and backyard farms that rear mixed livestock animals and/or market stalls that sell both pig and chicken meat.

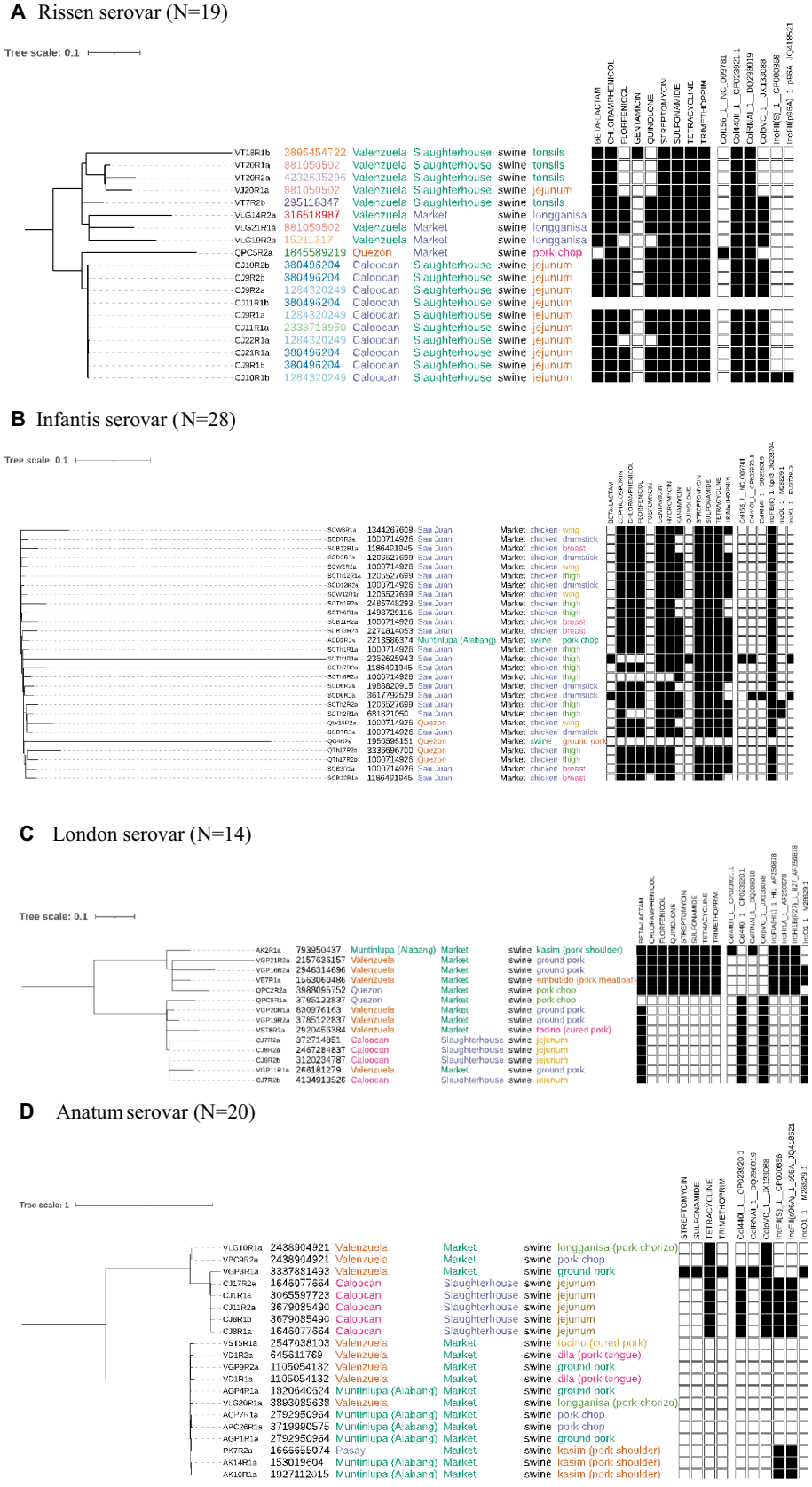

FIGURE 2
Phylogenetic trees built using on the core genome of isolates across different serovars. Annotated are the sequence IDs, cgMLST ST, source location, type, animal and meat cut. Presence (black square) or absence (white square) of genes conferring resistance to drugs and presence of plasmids are also indicated. **(A)** Rissen serovar (*N* = 19). **(B)** Infantis serovar (*N* = 28). **(C)** London serovar (*N* = 14). **(D)** Anatum serovar (*N* = 20).

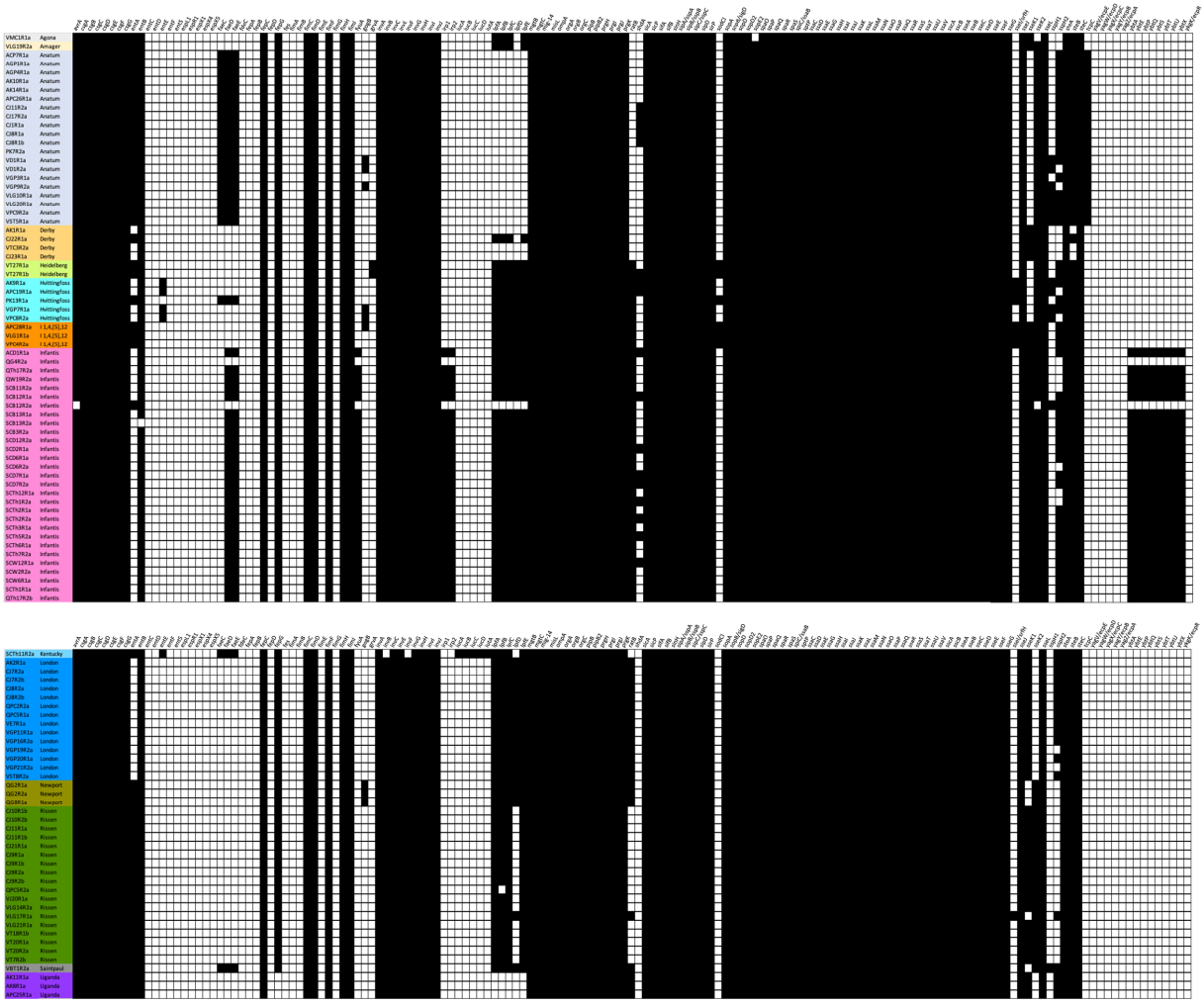

FIGURE 3
Presence (black square) or absence (white square) of genes conferring virulence characteristics of the isolates. Virulence genes were mined using ABRicate software (v1.0.1; minimum %ID = 80, min % coverage = 80; VFDB database, as of April 4, 2023).

4.2 Correlation between AMR and antimicrobial use

Salmonella has been found to have intrinsic resistance to β-lactams, macrolides (except azithromycin), lincosamides, glycopeptides, and fusidane (Stock and Wiedemann, 2000). The genetic determinants of these resistances are usually chromosome-encoded or because of functional and/or structural characteristics as opposed to plasmid-borne resistances that can be acquired via horizontal gene transfer. For example, macrolides, a family of antimicrobials that have a characteristic macrocyclic lactone ring structure, have difficulty traversing the Gram-negative cell wall, specifically through the polar and negatively charged outer membrane, consequent to their affinity to efflux pumps that actively transport them out of the cell (McDermott et al., 2003; Myers and Clark, 2021). Another example of this would be their intrinsic resistance to β-lactam antibiotics, such as penicillin which is the highest conferred phenotypic resistance (60/105 isolates) in this study. β-lactam antibiotics, as aptly named, would have a characteristic β-lactam ring, which, similar to macrolides, would have restricted access to the outer membrane. In addition, *S. enterica* would also have a chromosomally-encoded or plasmid-borne *ampC* gene that codes for β-lactamases–enzymes that can hydrolyze β-lactams (Narendrakumar et al., 2023).

The intrinsic resistance of *Salmonella* complicates treatment regimens and raises the need for other treatment options. As this is the case, other drugs of choice are usually prescribed for both typhoidal and non-typhoidal infections such as fluoroquinolones or cephalosporins (Shane et al., 2017; Tack et al., 2020). While these drugs still exhibit efficacy against *Salmonella*, there is a marked increase in the occurrence of intermediate and resistant phenotypes, as exhibited in this study. Alarmingly, there have also been many reports of emerging fluoroquinolone and cephalosporin resistance from various continents. In Russia, fluoroquinolone- and cephalosporin-resistant *Salmonella* strains have been isolated from raw poultry products as well as ready-to-eat chicken products (Egorova et al., 2021). A study in Ghana by Dekker et al. (2018) reported 63% fluoroquinolone resistance in *S. enterica* isolated from local and imported meat. In addition, this resistance is mostly conferred by *qnrB2* resistance plasmids, which means that these resistance determinants can be transferable.

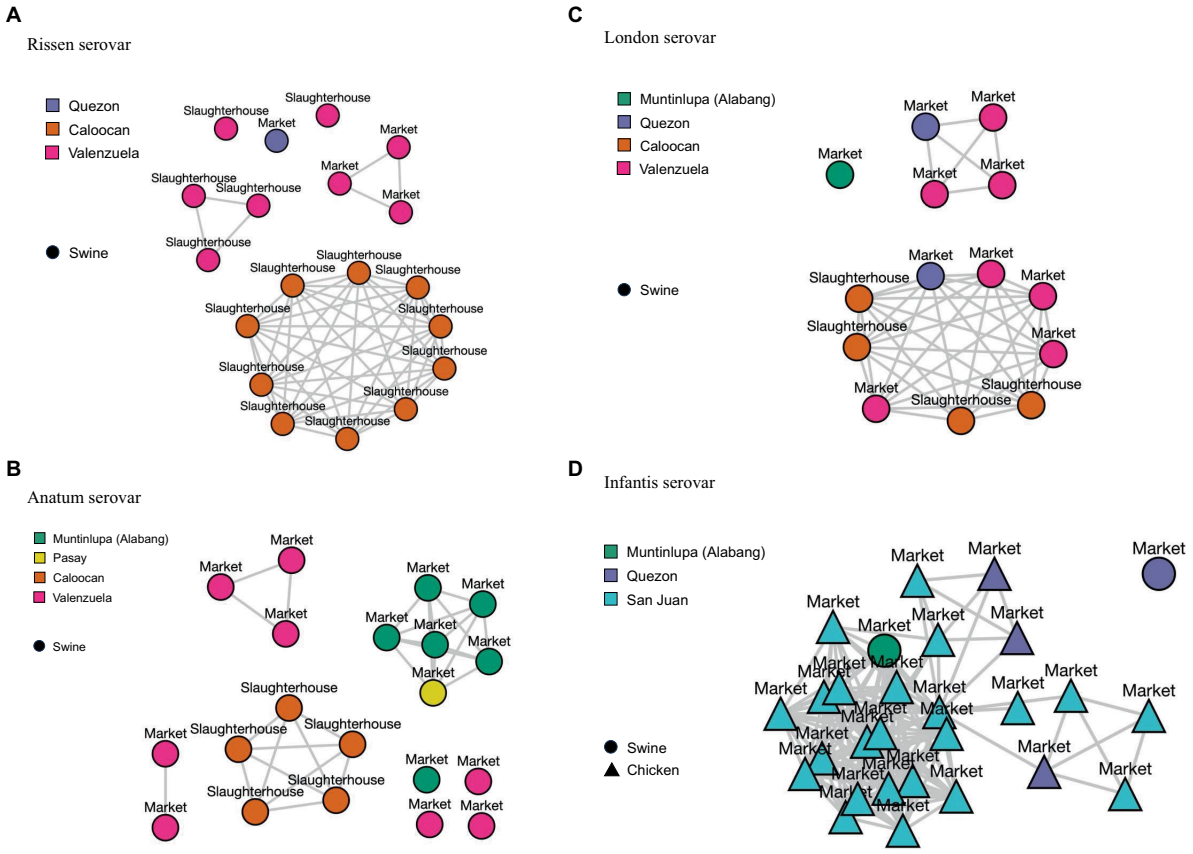

FIGURE 4
Single-linkage clusters reflecting single-nucleotide polymorphisms (SNPs) at (distance: < 20) within serovars. Sampling sites (market or slaughterhouse), sampling locations (cities), and sample matrices (swine or chicken) are also indicated. **(A)** Rissen serovar. **(B)** Anatum serovar. **(C)** London serovar. **(D)** Infantis serovar.

In this study, a significant number of isolates had transferable resistance plasmids such as the *Col* plasmid family that facilitate resistance to glycopeptides (vancomycin), polymyxin (colistin), β-lactam antibiotics (e.g., penicillin and ampicillin), as well as quinolones (ciprofloxacin) (McMillan et al., 2020). The isolates also harbor *Inc* plasmids that confer resistance against aminoglycosides, β-lactams, and fluoroquinolones (Hiley et al., 2021). The modes of action of these resistance genes are primarily related to the production of efflux pumps that actively expel antimicrobial agents from the cell. In addition, several genes code for enzymes that promote the inactivation of antimicrobial agents, either by hydrolysis or through the production of inactivated forms of the drug (Helinski, 2022).

The increasing trend of resistance to fluoroquinolones can be traced to the growth of drug sales, especially for use in livestock settings. A study by Yin et al. (2022) was able to establish a link between a 41.67% increase in fluoroquinolone sales and a 5% increase in the prevalence of quinolone-resistant *Salmonella* in retail meat from 2013 to 2018. In 2020, the estimated use of antimicrobials for the rearing of cattle, sheep, poultry, and swine worldwide was 99,502 tons, with the highest usage recorded in Asia at 67% (66,666 tons). This is particularly important since the volume of imported meat and meat products in the Philippines has ballooned to 16% in 2022, with imports coming in from Brazil, Australia, Germany, India, Italy, and the USA, among others (Bureau of Animal Industry, 2022). Incidentally, these countries are considered antimicrobial use hotspots in 2020 and are projected to remain so come 2050 (Mulchandani et al., 2023). The high consumption of antimicrobials in these hotspot countries could be likely linked to their intensive farming practices to meet local and export demands.

The same literature estimated the global use of tetracycline to be 33,305 tons, making it the most consumed antimicrobial in the world, with the second highest antimicrobial being penicillin, at around 15,000 tons–only half as much as tetracycline. This trend could be correlated with the detection of tetracycline resistance genes in 64% of the isolates. Sulfonamides and aminoglycosides have ranked fourth and sixth, respectively, in global consumption. Resistance genes against these antimicrobials were also detected in 56% of isolates.

4.3 Virulence determinants

A total of 155 virulence genes were detected in 105 isolates, 42 of which are serovar-specific. The virulence-associated determinants are grouped into six categories: fimbriae adherence determinants, element-uptake determinants, secretion system, protein synthesis, colonization, and survival against the host immune system.

Fimbriae function as an adhesion factor necessary for bacterial colonization and infection. The prevalence of *csg* and *fim* genes are conserved adhesion and infection factors in *Enterobacteriaceae*. Two *csg* operons, *csg*BAC, and *csg*DEFG, encode for curli fimbriae and mediate binding to tissue matrices, while *fim* genes that encode hair-like appendages called type 1 fimbriae (Römling et al., 1998; Zeiner et al., 2012). These two gene clusters were present in all 105 isolates in this study as they facilitate adhesion and binding to eukaryotic blood and tissue matrices. While *lpf*ACE and *fae*DE were serovar-specific genes determined in the sequence analysis, both were present in Haifa, Kentucky, Saintpaul, and Infantis serovars. These genes are unique for long polar fimbriae and fimbrial adhesin production, respectively. The homologous interchange of horizontally transmitted segments may have allowed the deletion of serovar-specific genes to spread to other *Salmonella* serovars. In particular, the *lpf* operon was initially present in a lineage ancestor of *Salmonella* and has been found to be deleted in several lineages of the genus, contributing to its diverse phylogenetic distribution (Bäumler et al., 1997). Interestingly, most jejunum samples in this study acquired these *lpf* genes since *lpf*-mediated adhesion targeted the alimentary tract such as animal ileum (Bäumler and Heffron, 1995), induced biofilm formation (Ledeboer et al., 2006) and promoted long-term intestinal persistence (Weening et al., 2005), indicating a function-mediated gene deletion and acquisition.

Genes associated with type III secretion system (T3SS) encoded by *Salmonella* pathogenicity islands (SPI) are also predominantly present among all the isolates. Present genes associated with SPI-1 include the *inv*/*spa* and *prg* genes that aid in the invasion and infection of *Salmonella*. The genes encoding for SP 1–2 regulation, and production of its chaperone proteins, effector proteins, and T3SS2 apparatus were also detected. However, the T3SS *esp* genes involved in the regulation and transport of proteins in the host cells during the invasion were absent in all isolates. The lack of genes and operons for the synthesis and regulation of extracellular proteins was also evident.

Other functional sets of genes that are present were responsible for the uptake and transport of organic compounds such as magnesium and iron. All 105 isolates have genes for the uptake of magnesium from the environment. Interestingly, only two serovars possess genes for iron transport and synthesis and encode for a different type of siderophore. The single Kentucky serovar isolate from a chicken sample had genes that encode for aerophore, while most Infantis isolates (27/28) possess genes associated with the uptake, transport, and biosynthesis of yersiniabactin. Iron uptake is important for host-pathogen interactions that are often overlooked in non-typhoidal *Salmonella*. The presence of *Yersinia* high pathogenicity island (HPI) in the Infantis isolates may also promote infection (Fetherston et al., 1996; Oelschlaeger et al., 2003), suppress host immune response (Autenrieth et al., 1991; Gehring et al., 1998) and increase the fitness and persistence in the environment (Oelschlaeger et al., 2003).

The expression of genes associated with pathogenicity islands 1 and 2 as well as fimbriae production among all isolates suggest the potential harm and disease development in humans. The genome sequences in this study served as a basis for traceback investigations to animal and food products as the possible cause of human salmonellosis. A comparative analysis of the SPIs, adhesin molecules, secretion systems, virulence plasmid, and epidemiological characteristics could elucidate the role of food-borne infections in humans.

5 Conclusion

Through the application of next-generation sequencing, we found resistance and virulence determinants that contribute to the persistence of *S. enterica* in the poultry and swine food chains in the Philippines. Our study of 105 *S. enterica* isolates provides proof of principle that WGS approaches can decipher the complex AMR and virulence patterns and shows that sequencing should be implemented by meat inspection networks to augment the existing presence–absence detection tests as acceptability, safety, and quality criteria. In addition, WGS gave insights into strain clustering and evidence of infection cross-contamination. Indeed, there are large-scale follow-up studies to assess AMR and virulence diversity and capture transmission, and the current work provides a baseline set of data. Ultimately, the genetic insights from this study and similar works, especially with additional number of samples and isolates could lead to enhanced diagnostics for disease management and control across the entire food chain, including farm, abattoir, and market settings. New genetic markers revealed should be integrated into current testing routines and may replace those currently used to give a more accurate picture of the strains and their AMR profiles. More isolates must be obtained in order to more clearly and accurately visualize the current and possibly predict future trends relating to *S. enterica* transmission, AMR, and virulence. The identification of AMR and transmission chains from farm to fork will assist surveillance and clinical decision-making, thereby improving the food security and health of both humans and animals.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary materials, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by National Meat Inspection Service, Philippines. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JM: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. VM: Data curation, Formal analysis, Investigation, Writing – original draft. AC: Data curation, Investigation, Writing – original draft. SC: Conceptualization, Writing – review & editing. JH: Conceptualization, Methodology, Writing – review & editing. MH: Conceptualization, Methodology, Writing – original draft. JP: Data curation, Formal analysis, Methodology, Software, Writing – original draft. TC: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. WR: Conceptualization, Funding acquisition,

Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Alvarez, D. M., Barrón-Montenegro, R., Conejeros, J., Rivera, D., Undurraga, E. A., and Moreno-Switt, A. I. (2023). A review of the global emergence of multidrug-resistant *Salmonella enterica* subsp. *enterica* serovar Infantis. *Int. J. Food Microbiol.* 403:110297. doi: 10.1016/j.ijfoodmicro.2023.110297

Atwill, E. R., and Jeamsripong, S. (2021). Bacterial diversity and potential risk factors associated with *Salmonella* contamination of seafood products sold in retail markets in Bangkok, Thailand. *PeerJ* 9:e12694. doi: 10.7717/peerj.12694

Autenrieth, I., Hantke, K., and Heesemann, J. (1991). Immunosuppression of the host and delivery of iron to the pathogen: a possible dual role of siderophores in the pathogenesis of microbial infections? *Med. Microbiol. Immunol.* 180, 135–141. doi: 10.1007/BF00206117

Bäumler, A. J., Gilde, A. J., Tsolis, R. M., Van Der Velden, A. W., Ahmer, B. M., and Heffron, F. (1997). Contribution of horizontal gene transfer and deletion events to development of distinctive patterns of fimbrial operons during evolution of *Salmonella* serotypes. *J. Bacteriol.* 179, 317–322. doi: 10.1128/jb.179.2.317-322.1997

Bäumler, A. J., and Heffron, F. (1995). Identification and sequence analysis of lpfABCDE, a putative fimbrial operon of *Salmonella typhimurium*. *J. Bacteriol.* 177, 2087–2097. doi: 10.1128/jb.177.8.2087-2097.1995

Bearson, S. M. D. (2021). *Salmonella* in swine: Prevalence, multidrug resistance, and vaccination strategies. *Annu. Rev. Anim. Biosci.* 10:1. doi: 10.1146/annurev-animal-013120

bioMérieux (2005). VITEK®2 compact 60 microbial identification and antimicrobial susceptibility testing system [Equipment and Software]. Available at: https://www.biomerieux-usa.com/clinical/vitek-2-healthcare (Accessed September 20, 2023).

Bureau of Animal Industry. (2022). Volume (kgs) of meat and meat products importation arrivals per month. Available at: https://www.bai.gov.ph/stakeholders?Rep=Importation%20Data (Accessed September 16, 2023).

Calayag, A. M. B., Paclibare, P. A. P., Santos, P. D. M., Bautista, C. A. C., and Rivera, W. L. (2017). Molecular characterization and antimicrobial resistance of *Salmonella enterica* from swine slaughtered in two different types of Philippine abattoir. *Food Microbiol.* 65, 51–56. doi: 10.1016/j.fm.2017.01.016

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., et al. (2014). *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 58, 3895–3903. doi: 10.1128/AAC.02412-14

Centers for Disease Control and Prevention (CDC). (2013). *An atlas of Salmonella in the United States, 1968–2011: Laboratory-based enteric disease surveillance*. Atlanta, Georgia: US Department of Health and Human Services, CDC.

Chen, L., Zheng, D., Liu, B., Yang, J., and Jin, Q. (2016). VFDB 2016: hierarchical and refined dataset for big data analysis – 10 years on. *Nucleic Acids Res.* 44, D694–D697. doi: 10.1093/nar/gkv1239

Clinical and Laboratory Standards Institute. (2022). *M100: Performance standards for antimicrobial susceptibility testing. 32nd Edn*. Berwyn, PA: Clinical and Laboratory Standards Institute.

Cooper, T. L., Smith, D., Gonzales, M. J. C., Maghanay, M. T., Sanderson, S., Cornejo, M. R. J. C., et al. (2022). Beyond numbers: determining the socioeconomic and livelihood impacts of African swine fever and its control in the Philippines. *Front. Vet. Sci.* 8:734236. doi: 10.3389/fvets.2021.734236

Dekker, D., Krumkamp, R., Eibach, D., Sarpong, N., Boahen, K. G., Frimpong, M., et al. (2018). Characterization of *Salmonella enterica* from invasive bloodstream infections and water sources in rural Ghana. *BMC Infect. Dis.* 18:47. doi: 10.1186/s12879-018-2957-4

Edrington, T. S., and Brown, T. R. (2022). A commentary on *Salmonella* from a pre-harvest perspective. *Front. Anim. Sci.* 3:877392. doi: 10.3389/fanim.2022.877392

Egorova, A., Mikhaylova, Y., Saenko, S., Tyumentseva, M., Tyumentsev, A., Karbyshev, K., et al. (2021). Comparative whole-genome analysis of Russian foodborne multidrug-resistant *Salmonella* Infantis isolates. *Microorganisms* 10:89. doi: 10.3390/microorganisms10010089

Elbediwi, M., Shi, D., Biswas, S., Xu, X., and Yue, M. (2021). Changing patterns of *Salmonella enterica* serovar Rissen from humans, food animals, and animal-derived foods in China, 1995–2019. *Front. Microbiol.* 12:702909. doi: 10.3389/fmicb.2021.702909

Espino, A., Itliong, K., Ruba, C. D., Thy, O., Barbon, W. J., Monville-Oro, E., et al. (2021). COVID-19 impact on local Agri-food system in Cambodia, Myanmar, and the Philippines: Findings from a rapid assessment. CGIAR Research Program on Climate Change, Agriculture and Food Security. Available at: https://cgspace.cgiar.org/handle/10568/113843 (Accessed April 11, 2023)

European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC) (2022). The European Union one health 2021 zoonoses report. *EFSA J.* 20:e07666. doi: 10.2903/j.efsa.2022.7666

Fang, L., Lin, G., Li, Y., Lin, Q., Lou, H., Lin, M., et al. (2022). Genomic characterization of *Salmonella enterica* serovar Kentucky and London recovered from food and human salmonellosis in Zhejiang Province, China (2016–2021). *Front. Microbiol.* 13:961739. doi: 10.3389/fmicb.2022.961739

Ferrari, R. G., Rosario, D. K. A., Cunha-Neto, A., Mano, S. B., Figueiredo, E. E. S., and Conte-Junior, C. A. (2019). Worldwide epidemiology of *Salmonella* serovars

in animal-based foods: a meta-analysis. *Appl. Environ. Microbiol.* 85, e00591–e00519. doi: 10.1128/AEM.00591-19

Fetherston, J. D., Bearden, S. W., and Perry, R. D. (1996). YbtA, an AraC-type regulator of the *Yersinia pestis* pesticin/yersiniabactin receptor. *Mol. Microbiol.* 22, 315–325. doi: 10.1046/j.1365-2958.1996.00118.x

Gehring, A. M., DeMoll, E., Fetherston, J. D., Mori, I., Mayhew, G. F., Blattner, F. R., et al. (1998). Iron acquisition in plague: modular logic in enzymatic biogenesis of yersiniabactin by *Yersinia pestis*. *Chem. Biol.* 5, 573–586. doi: 10.1016/S1074-5521(98)90115-6

Helinski, D. R. (2022). A brief history of plasmids. *EcoSal Plus* 10:eESP00282021. doi: 10.1128/ecosalplus.esp-0028-2021

Hiley, L., Graham, R. M. A., and Jennison, A. V. (2021). Characterisation of IncI1 plasmids associated with change of phage type in isolates of *Salmonella enterica* serovar typhimurium. *BMC Microbiol.* 21:2151. doi: 10.1186/s12866-021-02151-z

Lagrada, M. L., Argimón, S., Borlasa, J. B., Abad, J. P., Gayeta, J. M., Masim, M. L., et al. (2022). Genomic surveillance of *Salmonella* spp. in the Philippines during 2013–2014. *Trans. R. Soc. Trop. Med. Hyg.* 116, 1202–1213. doi: 10.1093/trstmh/trac080

Ledeboer, N. A., Frye, J. G., McClelland, M., and Jones, B. D. (2006). *Salmonella enterica* serovar typhimurium requires the Lpf, Pef, and Tafi fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. *Infect. Immun.* 74, 3156–3169. doi: 10.1128/IAI.01428-05

Liu, Q., Chen, W., Elbediwi, M., Pan, H., Wang, L., Zhou, C., et al. (2020). Characterization of *Salmonella* resistome and plasmidome in pork production system in Jiangsu, China. *Front. Vet. Sci.* 7:617. doi: 10.3389/fvets.2020.00617

Lozano-León, A., García-Omil, C., Rodríguez-Souto, R. R., Lamas, A., and Garrido-Maestu, A. (2022). An evaluation of the pathogenic potential and the antimicrobial resistance of *Salmonella* strains isolated from mussels. *Microorganisms* 10:126. doi: 10.3390/microorganisms10010126

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

McDermott, P. F., Walker, R. D., and White, D. G. (2003). Antimicrobials: modes of action and mechanisms of resistance. *Int. J. Toxicol.* 22, 135–143. doi: 10.1080/10915810305089

McMillan, E. A., Jackson, C. R., and Frye, J. G. (2020). Transferable plasmids of *Salmonella enterica* associated with antibiotic resistance genes. *Front. Microbiol.* 11:562181. doi: 10.3389/fmicb.2020.562181

Meunsene, D., Eiamsam-ang, T., Patchanee, P., Pascoe, B., Tadee, P., and Tadee, P. (2021). Molecular evidence for cross boundary spread of *Salmonella* spp. in meat sold at retail markets in the middle Mekong basin area. *PeerJ* 9:e11255. doi: 10.7717/peerj.11255

Mikheenko, A., Prjibelski, A., Saveliev, V., Antipov, D., and Gurevich, A. (2018). Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics* 34, i142–i150. doi: 10.1093/bioinformatics/bty266

Montone, A. M. I., Cutarelli, A., Peruzy, M. F., La Tela, I., Brunetti, R., Pirofalo, M. G., et al. (2023). Antimicrobial resistance and genomic characterization of *Salmonella* Infantis from different sources. *Int. J. Mol. Sci.* 24:5492. doi: 10.3390/ijms24065492

Mulchandani, R., Wang, Y., Gilbert, M., and Van Boeckel, T. P. (2023). Global trends in antimicrobial use in food-producing animals: 2020 to 2030. *PLoS Glob. Public Health* 3:e0001305. doi: 10.1371/journal.pgph.0001305

Myers, A. G., and Clark, R. B. (2021). Discovery of macrolide antibiotics effective against multi-drug resistant gram-negative pathogens. *Acc. Chem. Res.* 54, 1635–1645. doi: 10.1021/acs.accounts.1c00020

Napier, G., Khan, A. S., Jabbar, A., Khan, M. T., Ali, S., Qasim, M., et al. (2022). Characterisation of drug-resistant *Mycobacterium tuberculosis* mutations and transmission in Pakistan. *Sci. Rep.* 12:7703. doi: 10.1038/s41598-022-11795-4

Narendrakumar, L., Chakraborty, M., Kumari, S., Paul, D., and Das, B. (2023). β-Lactam potentiators to re-sensitize resistant pathogens: discovery, development, clinical use and the way forward. *Front. Microbiol.* 13:1092556. doi: 10.3389/fmicb.2022.1092556

Ng, K. C. S., and Rivera, W. L. (2015). Multiplex PCR–based serogrouping and serotyping of *Salmonella enterica* from tonsil and jejunum with jejunal lymph nodes of slaughtered swine in Metro Manila, Philippines. *J. Food Prot.* 78, 873–880. doi: 10.4315/0362-028X.JFP-14-342

Nguyen, T. K., Nguyen, L. T., Chau, T. T. H., Nguyen, T. T., Tran, B. N., Taniguchi, T., et al. (2021). Prevalence and antibiotic resistance of *Salmonella* isolated from poultry and its environment in the Mekong Delta, Vietnam. *Vet. World.* 14, 3216–3223. doi: 10.14202/vetworld.2021.3216-3223

Oelschlaeger, T. A., Zhang, D., Schubert, S., Carniel, E., Rabsch, W., Karch, H., et al. (2003). The high-pathogenicity island is absent in human pathogens of *Salmonella enterica* subspecies I but present in isolates of subspecies III and VI. *J. Bacteriol.* 185, 1107–1111. doi: 10.1128/JB.185.3.1107-1111.2003

Organisation for Economic Cooperation and Development/Food and Agriculture Organization (2023). *OECD-FAO Agricultural Outlook 2023-2032*. Paris: OECD Publishing.

Pavon, R. D. N., Mendoza, P. D. G., Flores, C. A. R., Calayag, A. M. B., and Rivera, W. L. (2022). Genotypic virulence profiles and associations in *Salmonella* isolated from meat samples in wet markets and abattoirs of Metro Manila, Philippines. *BMC Microbiol.* 22:292. doi: 10.1186/s12866-022-02697-6

Pavon, R. D. N., and Rivera, W. L. (2021). Molecular serotyping by phylogenetic analyses of a 1498bp segment of the *invA* gene of *Salmonella*. *ASM Sci. J.* 14, 1–14. doi: 10.32802/asmscj.2020.602

Phelan, J. E., O'Sullivan, D. M., Machado, D., Ramos, J., Oppong, Y. E. A., Campino, S., et al. (2019). Integrating informatics tools and portable sequencing technology for rapid detection of resistance to anti-tuberculous drugs. *Genome Med.* 11:41. doi: 10.1186/s13073-019-0650-x

Philippine Statistics Authority. (2022). 2017–2021 Livestock and Poultry Statistics of the Philippines. Available at: https://psa.gov.ph/sites/default/files/L%26P%20Stat%20of%20the%20Phil_2017-2021-signed%20Copy.pdf (Accessed April 11, 2023)

Prasertsee, T., Chokesajjawatee, N., Santiyanont, P., Chuammitri, P., Deeudom, M., Tadee, P., et al. (2019). Quantification and rep-PCR characterization of *Salmonella* spp. in retail meats and hospital patients in northern Thailand. *Zoonoses Public Health* 66, 301–309. doi: 10.1111/zph.12565

Römling, U., Bian, Z., Hammar, M., Sierralta, W. D., and Normark, S. (1998). Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation. *J. Bacteriol.* 180, 722–731. doi: 10.1128/JB.180.3.722-731.1998

Sanguankiat, A., Pinniam, N., and Tulayakul, P. (2023). Surveillance of antimicrobial resistance, phenotypic, and genotypic patterns of *Salmonella enterica* isolated from animal feedstuffs: annual study. *Vet. World.* 16, 939–945. doi: 10.14202/vetworld.2023.939-945

Santos, P. D. M., Widmer, K. W., and Rivera, W. L. (2020). PCR-based detection and serovar identification of *Salmonella* in retail meat collected from wet markets in Metro Manila, Philippines. *PLoS One* 15:e0239457. doi: 10.1371/journal.pone.0239457

Seemann, T. (2015). Snippy: Fast bacterial variant calling from NGS reads. Available at: https://github.com/tseemann/snippy (Accessed September 14, 2023).

Seemann, T. (2019). Shovill. Available at: https://github.com/tseemann/shovill (Accessed September 14, 2023).

Seemann, T. (2020). ABRicate. Available at: https://github.com/tseemann/abricate (Accessed September 14, 2023).

Shane, A. L., Mody, R. K., Crump, J. A., Tarr, P. I., Steiner, T. S., Kotloff, K., et al. (2017). 2017 Infectious Diseases Society of America clinical practice guidelines for the diagnosis and Management of Infectious Diarrhea. *Clin. Infect. Dis.* 65, e45–e80. doi: 10.1093/cid/cix669

Sia, S., Olorsoa, A., Limas, M., Jamoralin Jr, M., Macaranas, P. K., Espiritu, H. G., et al. (2020). A fifteen-year report of serotype distribution and antimicrobial resistance of *Salmonella* in the Philippines. *PJP* 5, 19–29. doi: 10.21141/PJP.2020.04

Silveira, L., Pinto, M., Isidro, J., Pista, Â., Themudo, P., Vieira, L., et al. (2019). Multidrug-resistant *Salmonella enterica* serovar Rissen clusters detected in Azores archipelago, Portugal. *Int. J. Genomics* 2019:1860275. doi: 10.1155/2019/1860275

Sobkowiak, B., Banda, L., Mzembe, T., Crampin, A. C., Glynn, J. R., and Clark, T. G. (2020). Bayesian reconstruction of *Mycobacterium tuberculosis* transmission networks in a high incidence area over two decades in Malawi reveals associated risk factors and genomic variants. *Microb. Genomics.* 6:e000361. doi: 10.1099/mgen.0.000361

Spadar, A., Perdigão, J., Campino, S., and Clark, T. (2023). Large-scale genomic analysis of global *Klebsiella pneumoniae* plasmids reveals multiple simultaneous clusters of carbapenem-resistant hypervirulent strains. *Genome Med.* 15:3. doi: 10.1186/s13073-023-01153-y

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Stock, I., and Wiedemann, B. (2000). Natural antibiotic susceptibility of *Salmonella enterica* strains. *Int. J. Antimicrob. Agents* 16, 211–217. doi: 10.1016/S0924-8579(00)00204-1

Tack, B., Vanaenrode, J., Verbakel, J. Y., Toelen, J., and Jacobs, J. (2020). Invasive non-typhoidal *Salmonella infections* in sub-Saharan Africa: a systematic review on antimicrobial resistance and treatment. *BMC Med.* 18:212. doi: 10.1186/s12916-020-01652-4

Tang, B., Elbediwi, M., Nambiar, R. B., Yang, H., Lin, J., and Yue, M. (2022). Genomic characterization of antimicrobial-resistant *Salmonella enterica* in duck, chicken, and pig farms and retail markets in eastern China. *Microbiol. Spectrum* 10:e0125722. doi: 10.1128/spectrum.01257-22

Tang, B., Siddique, A., Jia, C., Ed-Dra, A., Wu, J., Lin, H., et al. (2023). Genome-based risk assessment for foodborne *Salmonella enterica* from food animals in China: a one health perspective. *Int. J. Food Microbiol.* 390:110120. doi: 10.1016/j.ijfoodmicro.2023.110120

Weening, E. H., Barker, J. D., Laarakker, M. C., Humphries, A. D., Tsolis, R. M., and Bäumler, A. J. (2005). The *Salmonella enterica* serotype typhimurium *lpf, bcf, stb, stc, std,* and *sth* fimbrial operons are required for intestinal persistence in mice. *Infect. Immun.* 73, 3358–3366. doi: 10.1128/IAI.73.6.3358-3366.2005

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with kraken 2. *Genome Biol.* 20:257. doi: 10.1186/s13059-019-1891-0

World Health Organization. (2018). *Salmonella* (non-typhoidal). Available at: https://www.who.int/news-room/fact-sheets/detail/salmonella-(non-typhoidal) (Accessed September 18, 2023).

Yin, X., Dudley, E. G., Pinto, C. N., and M'ikanatha, N. M. (2022). Fluoroquinolone sales in food animals and quinolone resistance in non-typhoidal *Salmonella* from retail meats: United States, 2009-2018. *J. Glob. Antimicrob. Resist.* 29, 163–167. doi: 10.1016/j.jgar.2022.03.005

Yong, D., Lim, Y. S., Yum, J. H., Lee, H., Lee, K., Kim, E. C., et al. (2005). Nosocomial outbreak of pediatric gastroenteritis caused by CTX-M-14-type extended-spectrum beta-lactamase-producing strains of *Salmonella enterica* serovar London. *J. Clin. Microbiol.* 43, 3519–3521. doi: 10.1128/JCM.43.7.3519-3521.2005

Yoshida, C. E., Kruczkiewicz, P., Laing, C. R., Lingohr, E. J., Gannon, V. P. J., Nash, J. H. E., et al. (2016). The *Salmonella in silico* typing resource (SISTR): an open web-accessible tool for rapidly typing and subtyping draft *Salmonella* genome assemblies. *PLoS One* 11:e0147101. doi: 10.1371/journal.pone.0147101

Zeiner, S. A., Dwyer, B. E., and Clegg, S. (2012). FimA, FimF, and FimH are necessary for assembly of type 1 fimbriae on *Salmonella enterica* serovar typhimurium. *Infect. Immun.* 80, 3289–3296. doi: 10.1128/IAI.00331-12

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Prevalence and antimicrobial susceptibility profile of bacteria isolated from the hands of housemaids in Jimma City, Ethiopia

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Introduction: Bacterial pathogens continue to be a major cause of foodborne gastroenteritis in humans and remain a public health problem. Housemaids operating inside a kitchen could be the source of infection and may transmit disease-inflicting pathogens through contaminated hands.

Objective: This study aimed to assess the prevalence and antimicrobial susceptibility profile of bacteria isolated from the hands of housemaids in Jimma City, Ethiopia.

Methods: A laboratory-based cross-sectional study was employed among 234 housemaids. Hand swab samples from the dominant hand of the study participants were collected under sterile conditions following standard operating procedures. Then, in the laboratory, the swabs were inoculated aseptically using streak-plating methods on the growth media, such as mannitol salt agar [*Staphylococcus aureus* and coagulase-negative staphylococci], MacConkey agar [*Klebsiella* species and *Proteus* species], salmonella-shigella agar [*Salmonella* species and *Shigella* species], and eosin methylene blue agar [*Escherichia coli* (*E. coli*)]. In addition, a set of biochemical tests was applied to examine bacterial species. Data were double-entered into EpiData version 3.1 and then exported to the Statistical Package for Social Science (SPSS) version 26 for further analysis. Descriptive analyses were summarized using frequency and percentage.

Results: The proportion of housemaids' hands containing one or more positive bacterial isolates was 72% (95% CI: 66.2, 77.8). The dominant bacterial isolates were *Staphylococcus aureus* (31.6%), *Escherichia coli* (21.3%), *Salmonella* species (1.3%), *Shigella* species (6.7%), *Klebsiella* species (23.1%) and *Proteus* species (14.7%). Fingernail status (AOR =15.31, 95% CI: 10.372, 22.595) and the removal of a watch, ring, and bracelet during hand washing (AOR = 20.844, 95% CI: 2.190, 9.842) were significantly associated with the prevalence of bacterial isolation. Most *Staphylococcus aureus* isolates were susceptible to chloramphenicol (98.6%). *Escherichia coli* isolates were susceptible to tetracycline (75%), ceftriaxone (79.2%), chloramphenicol (87.5%), and ceftazidime (77.1%). Eighty percent of isolated *Shigella* species were susceptible to chloramphenicol and gentamicin respectively. In addition, *Klebsiella* and *Proteus species* exhibited high susceptibility to chloramphenicol. However, their isolates showed resistance against a number of the tested antimicrobials. *Staphylococcus aureus* isolates (28.2%) were resistance to tetracycline. Moreover, One-quarter of *Escherichia coli* isolates were resistance to tetracycline, ceftriaxone, chloramphenicol,

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and ceftazidime. Whereas 46.7% and 48.5% of isolated *Shigella* species and Proteus species were resistance to tetracycline and ceftriaxone.

Conclusion: The hands of housemaids are important potential sources of pathogenic bacteria that would result in the potential risk of foodborne diseases. Most bacteria isolates were resistant to tetracycline, ceftriaxone, and ceftazidime. Therefore, practicing good hand hygiene helps to prevent and control the spread of antimicrobial-resistant microbes.

KEYWORDS

bacterial isolate, antimicrobial resistance, housemaids, Jimma City, Ethiopia

1 Introduction

Enteric bacterial pathogens are a major cause of foodborne gastroenteritis in humans and remain a public health problem worldwide (1). They are common foodborne disease agents and persist as a major public health threat. A study revealed that food commodities were contaminated by food handlers or housemaids (2). Moreover, the majority of foodborne outbreak causative agents enter the body through the ingestion of contaminated food (3, 4). Banik et al. state that foodborne illnesses occurred after the entrance of those disease-causing microbes into the food supply chain (5). In 2020, the World Health Organization reported approximately 600 million cases and 420,000 deaths related to contaminated food around the world (3). According to the Centers for Disease Control and Prevention estimates, approximately 1 in 6 Americans (48 million people) get sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases each year (6). However, the problem is severe in developing countries, including Ethiopia. Furthermore, it was estimated that approximately 700,000 deaths per year in Africa are caused by foodborne diseases (7). The summary report of the Federal Ministry of Health revealed that the annual incidences of foodborne illnesses ranged from 3.4 to 9.3% in Ethiopia (8). In addition, several studies have been performed to assess and estimate pathogenic bacteria and related rates of infection in Ethiopia (1, 7, 9–20).

The fecal–oral route of pathogen transmission is the most common among the other methods of infection transmission for heterogeneous pathogens (21, 22). In this sense, varieties of bacterial isolates, particularly *Staphylococcus aureus*, *Klebsiella* species, *Proteus* species, *E. coli*, *Shigella* species, *Salmonella* species, *Campylobacter*, *Vibrio cholerae*, and *Streptococcus pneumonia*, might be ingested that results from the hand contact with feces (5, 12–14, 17, 22–26). Furthermore, studies revealed that bacterial pathogens were the most extensively identified infectious agent for the majority of foodborne outbreak types (8, 11). Housemaids perform various daily activities and work at home; their hands quickly become contaminated with different kinds of microbes and therefore, become asymptomatic carriers of pathogens.

The public health importance of bacterial infection continues to pose a challenge to community health systems worldwide (27–29). Food poisoning (gastroenteritis), skin, ear, or sinus infections, sexually transmitted infections, bacterial pneumonia, and urinary tract infections are typical bacterial infections (6, 30). For instance, *S. aureus* produces toxins that cause staphylococcal food poisoning and gastroenteritis with emesis and with or without diarrhea (23, 31). Infections associated with *Salmonella* species and *Shigella* species are among the major public health problems in many countries, including Ethiopia (7, 9). For instance, *Salmonella* species is the most common cause of foodborne illnesses (23). *Shigella* causes a foodborne illness with common symptoms of diarrhea, fever, and stomach cramps (7). The estimated annual incidences of *Shigella* species and *Salmonella* species are 165 and 25 million, respectively (9). Moreover, a study disclosed that *Klebsiella* species cause infections at multiple sites in the bodies of people with preexisting health conditions (32).

Due to the high prevalence of antimicrobial-resistant (AMR) pathogens, advances in infection control have not completely eradicated the problem (13). In addition, the constant increase in AMR bacterial strains has become an important clinical problem (33, 34). Those AMR bacterial strains include members of Enterobacteriaceae and continue the increasing concern, which could lead to the narrowing of available therapeutic options (34, 35). Antimicrobial resistance could be determined by many contributing factors. The microbial evolution and transmission of genetic determinants of resistance between microbes enable the spread of pathogenic bacteria (34–38). In addition, the widespread and prolonged use of antibiotics leads to the emergence of resistant bacterial pathogens (13, 18, 38). Moreover, the problems of infectious diseases were worsened by the improper use of antibiotics by humans and animals, which contributed to the rise of AMR globally (13, 33–41). In sum, AMR is an emerging global challenge that results in the spread of infectious diseases that affect human populations (38, 39).

Therefore, pathogenic microbes continue to challenge the healthcare systems in developing countries, including Ethiopia (40). Evidence from studies revealed an increasing incidence of multidrug resistance in foodborne pathogens, particularly to the commonly used antimicrobial agents (40, 41). Most studies in Ethiopia have been conducted in institutions such as hospitals, mass food processing, and catering establishments (11, 13, 14, 18). There is a paucity of data showing profiles of bacteria isolated from the hands of housemaids and their antimicrobial susceptibility profile in dwellings, particularly in the study area. Therefore, the present study aimed to assess the prevalence and antimicrobial susceptibility profile of bacteria isolated from the hands of housemaids in Jimma City, Ethiopia.

Abbreviations: AMR, Antimicrobial resistance; EMB, Eosin methylene blue; KIA, Kliger iron agar; MSA, Mannitol salt agar; CLSI, Clinical Laboratory Standards Institute; SCA, Simon citrate agar; SIM, Sulfide indole motility; SSA, *Salmonella*–Shigella agar.

2 Materials and methods

2.1 Study area, design, and period

The current laboratory-based cross-sectional survey was conducted in residential settings in Jimma City, Southwest Ethiopia, from April to June 2022. Jimma City is located 352 km southwest of Addis Ababa. The physical location of Jimma City lies between latitude 7°41′ N and longitude 36°50′ E. It has an average altitude of 1,780 m above sea level. It receives a mean annual rainfall of approximately 1,530 mm. The mean annual minimum and maximum temperatures of Jimma City are 14.4 and 26.7°C, respectively (42).

2.2 Source and study population

All housemaids who have been engaged in work in Jimma City were the source population. Those housemaids who could fulfill the eligibility criteria and were available during the data collection period were considered the study population.

2.3 Eligibility criteria

Housemaids who reported having respiratory infections such as the common cold or fecal–oral diseases such as diarrhea, as well as those who had pores, skin irritation, inflammation, eczema, or scars on their palms during the data collection period, were not included in the analysis.

2.4 Sample size determination and sampling technique

The sample size was determined by applying Yamane's simplified formula for proportions. A 95% confidence interval (CI) and 0.5 or 50% proportion were assumed

$$n = \frac{N}{1 + Ne^2}$$

$$n = \frac{455}{1 + 455(0.05)^2} = 213$$

where n is the sample size, N is the population size, and e is the level of precision (5%). After considering a 10% sample size (21 study subjects) with a non-response rate, the final sample size was 213 + 21 = 234.

All residential settings in Jimma City were included in the study. An inventory assessment was conducted to gather information about housemaids employed in residential settings, and data about the total number of housemaids were obtained from households and local administrations [Kebele]. In sum, 455 housemaids were engaged in residential settings in Jimma City (43). Those housemaids who were available during the data collection period were included until the sample size (234) was fulfilled. To minimize sampling bias, after convincing the heads of households, their house number was used for identification.

2.5 Data collection techniques

Data collection tools for sociodemographic data and other relevant data related to hand hygiene practices among housemaids were adapted from the World Health Organization and published articles (44–46). Whereas, hand swab sample collection procedures followed the guidelines of the Clinical and Laboratory Standards Institute (CLSI), the American Type Culture Collection, and others (47–49).

Data were collected by the data collectors after obtaining written informed consent using a pre-tested semi-structured questionnaire and observation designed to obtain sociodemographic data such as sex, age and educational status, and other relevant data related to housemaids' hand hygiene practices such as fingernail status, frequent handwashing, handwashing method, use of soap and water for frequent handwashing, following the five steps to washing hands in the right way, removal of watch, ring, and bracelets during handwashing, and time in second to wash hands. The data were collected from the study participants after receiving their written informed consent and after receiving the ethical approval for the study from the Institutional Review Board of the Institute of Health, Jimma University.

Three data collectors with prior experience in the field and who were fluent in speaking and reading in the local language and English were hired. The data collection survey underwent 5-day training sessions on informed consent and data collection procedures from 20 to 25 March 2022. The data collectors were two individuals with Bachelor of Science degrees in medical laboratory technology and one individual with a Bachelor of Science degree in environmental health science.

2.6 Laboratory data, analysis, and interpretation

For the laboratory investigation of the commensal microbes from the hands of housemaids, a swab sample was collected following standard operating procedures of CLSI, American-type culture collection, and other guidelines (47–49). In advance, hand swab samples were collected following sterile conditions for the segregation of commensal microbes.

2.6.1 Sample collection and transport

The samples were collected and transported using sterile cotton swabs and 10 mL saline-filled sterile test containers. Following handwashing, the participant's hands were sampled for the hand swabs by rubbing the entire surface with sterile, moistened cotton-tipped swabs. The sample was then placed or soaked in a labeled 0.85% saline solution containing sterile test tubes for microbial culturing. Nevertheless, no prior notice was given, and extra hand cleanliness was not practiced when collecting samples (18, 50). Three well-trained laboratory personnel gathered swab samples using

accepted aseptic methods. Samples were transferred to Jimma University's Department of Medical Microbiology Laboratory soon after collection. Next, in the laboratory, the samples were enhanced in a nutrient broth for a whole day to promote bacterial recovery, as handwashing has an impact on the survival of the bacteria that were collected.

2.6.2 Sample culturing and identification

The most popular techniques for identifying bacteria include the use of differential media, which makes it simpler to separate colonies of desired microorganisms from other colonies growing on the same plate, or selective media, which can prevent or reduce the growth of undesirable commensal microbes (47, 49). By providing the growth media, it is essential to grow and maintain them in carefully regulated laboratory settings. The preparation of each culture medium utilized in this investigation was performed in accordance with the manufacturer's instructions, and aseptic culturing techniques were employed. A loop full of each hand swab sample enriched on the nutrient broth in the laboratory soon after collection was inoculated aseptically using streak-plating methods on the selective and differential such as mannitol salt agar (MSA) (*S. aureus* and Coagulase-negative staphylococci), MacConkey agar (MCA) (*Klebsiella* species and *Proteus* species), salmonella-shigella agar (SSA) (*Salmonella* species and *Shigella* species), and eosin methylene blue agar (EMBA) (*E. coli*). Then, it was incubated at 37°C for 24 h. Following an incubation period, the culture plates were inspected to see whether there was any suspected bacterial growth present (positive) or absent (negative). Biochemical and morphological testing were used to corroborate the positive laboratory results.

2.6.3 Biochemical tests

The single colony of bacteria grown on selective and differential media was then subcultured into nutrient agar to determine growth patterns and for further biochemical tests. Then, after obtaining pure colonies, identification of bacteria isolates was performed by using standard microbiology techniques such as the morphology of its colonies and a battery (set) of biochemical tests such as a response on catalase, coagulase, oxidase, Simon citrate agar (SCA), urease, sulfide indole motility (SIM), Kliger's Iron Agar (KIA), and gas and hydrogen sulfide (H_2S) generation (17). The isolation and identification of bacteria from the hand swabs from the hands of housemaids are shown (Figure 1).

2.6.4 Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed on Muller Hinton Agar (HIMEDIA, TITAN BIOTECH LTD, Rajasthan, India) by the disk diffusion method. The following antimicrobial drugs were used to test susceptibility: tetracycline (30 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), and ceftazidime (30 μg). The selection of drugs was based on availability and pieces of literature (17, 48). The selections of drugs for antimicrobial sensitivity tests were

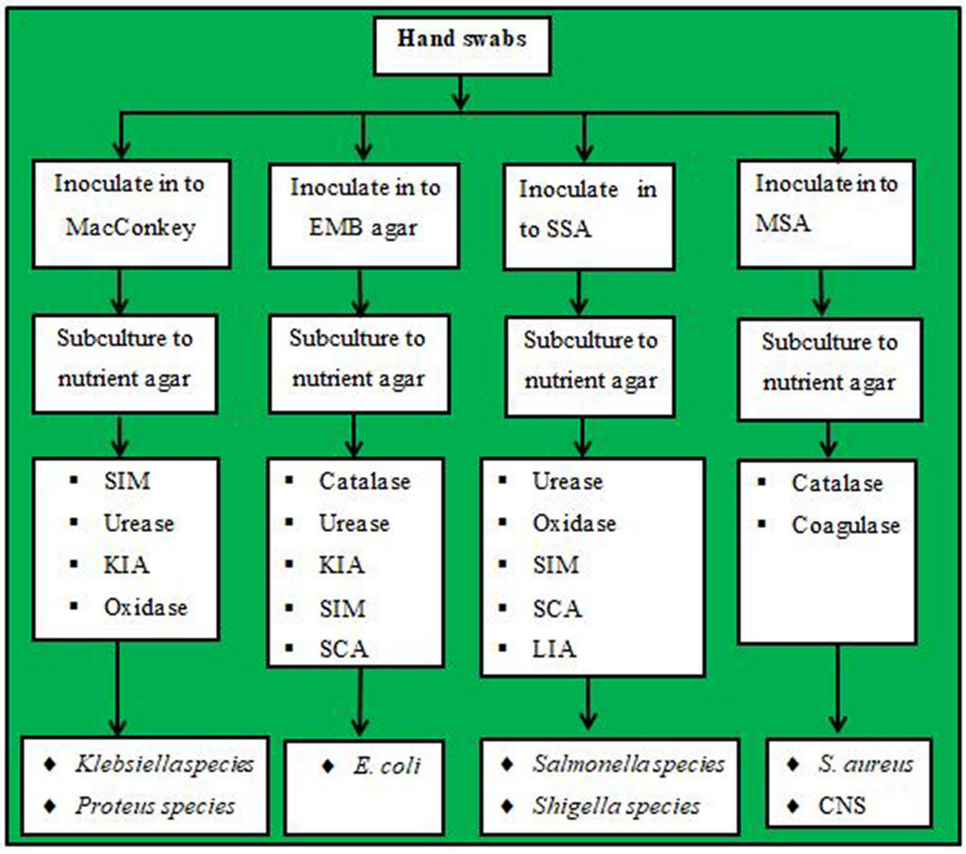

FIGURE 1
Laboratory flowchart showing bacteria isolations from hand swab samples.

based on the standards for antimicrobial susceptibility guidelines (48), pieces of literature (13, 14, 18), and the current availability of drugs in the market that the community is accessing. The sensitivity, intermediate, and resistance of the bacterial isolates were interpreted according to CLSI guidelines (48).

All culture media used for antimicrobial susceptibility tests were prepared according to the manufacturer's instructions, and culturing procedures were carried out aseptically. Each batch of the prepared media was checked for sterility by incubating the sample medium at 37°C for a day (14). *Staphylococcus aureus* ATCC25923 and *E. coli* ATCC25922, sensitive to all antimicrobial agents, were used as control strains (48). The zone diameter interpretive standards for the determination of antimicrobial agents are shown below (Table 1).

2.7 Quality control

The data collection questionnaire was designed, modified, and contextualized after reviewing related pieces of literature (44–46). Before data collection, a pre-test was conducted among 10% of the total sample size of the study subjects in Mizan-Aman town to determine whether any corrections were made or not.

To manage the quality of work, standard operating procedures have been strictly adhered to in laboratory tests for the investigation of commensal microbes (49). In addition, the proper functioning of the instruments utilized was checked before processing samples, and the known strains of selected organisms (*S. aureus* ATCC25923 and *E. coli* ATCC25922) were used for comparison purposes while distinguishing quality. The hand swab samples were collected aseptically, and the temperature range until the final laboratory analysis was checked. Then, the bacterial enrichment broth and growth media sterility were ensured using an autoclave and sample media storage in an incubator overnight, respectively. Interpretation of laboratory findings was confirmed by using updated microbiology guidelines such as CLSI (48).

2.8 Data processing and analysis

Data were edited, cleaned, and double-entered into EpiData version 3.1 and then exported to the statistical package for social science statistics version 26 for further analysis. Descriptive analyses were summarized using frequency and percentage and presented in texts, tables, and figures. Binary logistic regression was analyzed to assess associated factors with the prevalence of bacteria isolated from the hands of housemaids. The variables with a value of $p \leq 0.25$ were fitted into the multivariable analysis. A Hosmer and Lemeshow statistical test was carried out to check the goodness of fitness. Variables were selected through a backward, stepwise selection technique. The odds ratio with a respective 95% confidence interval was used to measure the strength of the association. A p value of <0.05 was considered statistically significant.

2.9 Ethical consideration

The Institute of Health Sciences at Jimma University's IRB granted ethical clearance for the study which was carried out under reference number IHRPGS/437/22. Every respondent was asked for their informed and oral consent. Codes were used to maintain the complete confidentiality of the information collected from study participants, including their privacy. Concerning parties, such as research participants and households, would be connected to the atypical clinical result. When gathering data, personal safety measures were taken to prevent the spread of COVID-19 from the data collector to study participants and vice versa. These measures included wearing a mask, and gloves, wiping hands with sanitizer or alcohol, and washing hands with detergent.

3 Results

3.1 Sociodemographic characteristics

Two hundred and twenty-five study subjects participated in this study, with a response rate of 96.2%. All the respondents were women. The age of study participants ranged from 18 to 36, with a mean age of 21.41 ± SD of (3.961). The majority, 182(81%) and 34(15%) of the study participants were between the age categories of 18–30 and 18–24 years, respectively (Figure 2). More than half (53%) of study participants attended primary school, while 7(3%) of respondents could not read or write (Figure 3).

TABLE 1 Zone diameter interpretive standards for the determination of antimicrobial agent sensitivity and resistance tested by disk diffusion method.

Bacteria category	Antimicrobial agent	Disk content	Interpretive categories and zone diameter breakpoints nearest whole mm		
			Sensitive	Intermediate	Resistant
Gram-positive bacteria [*S. aureus* and Coagulase-negative staphylococci]	Tetracycline	30 µg	≥19	15–18	≤14
	Chloramphenicol	30 µg	≥18	13–17	≤12
	Gentamicin	10 µg	≥15	13–14	≤12
Enterobacteriaceae [*Escherichia coli, Salmonella* species, *Shigella* species, *Klebsiella* species, *and Proteus* species]	Tetracycline	30 µg	≥15	12–14	≤11
	Ceftriaxone	30 µg	≥23	20–22	≤19
	Chloramphenicol	30 µg	≥18	13–17	≤12
	Gentamicin	10 µg	≥15	13–14	≤12
	Ceftazidime	30 µg	≥21	18–20	≤17

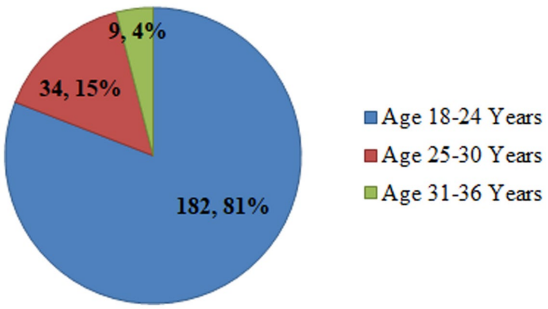

FIGURE 2
Age category of housemaids ($n = 225$) working in Jimma City, Southwest Ethiopia, 2022.

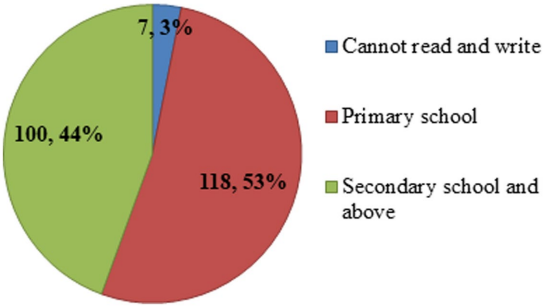

FIGURE 3
Educational status of housemaids ($n = 225$) working in Jimma City, Southwest Ethiopia, 2022.

3.2 Prevalence of bacterial isolates

The overall prevalence of one or more bacteria isolated from the hands of housemaids was 72% (95% CI: 66.2–77.8). The total number of bacteria isolated from hand swab samples was 224. *Staphylococcus aureus* 71(31.6%) was the predominant bacterial species, followed by *Klebsiella* species 52(23.1%) and *E. coli* 48(21.3%), whereas the least isolated bacteria was *Coagulase-negative staphylococci* (0.90%). However, bacteria were not isolated from 63(28%) of the study participants' hands (Table 2).

3.2.1 Factors associated with bacterial isolation

In bivariable logistic regression, variables including fingernail status, frequent handwashing, how to wash hands, washing hands frequently with soap/other detergents, following five steps to wash hands the right way, removing a watch, ring, and bracelet during handwashing, and washing hands for 20 s were significantly associated with bacterial isolates from hands. In multivariable logistic analysis, all variables with a value of $p \leq 0.25$ in bivariable analyses were included. Fingernail status and removing a watch, ring, and bracelet during handwashing were found to be significantly associated with bacterial isolates from the hands of housemaids, with a value of $p < 0.05$ (Table 3).

This study suggested that housemaids who did not trim their fingernails were 15.31 times more likely to have tested positive for bacteria cultures from the hand swabs compared to housemaids who trimmed their fingernails (adjusted odd ratio = 15.31, 95% confidence interval: 10.372, 22.595). Housemaids who did not experience the removal of a watch, ring, or bracelet during handwashing had 20.844 times higher odds of positive bacteria cultures than their counterparts (adjusted odd ratio = 20.844, 95% confidence interval: 2.190, 9.842; Table 3).

3.3 Antimicrobial susceptibility pattern of bacteria isolates

The majority of *Staphylococcus aureus* were susceptible to chloramphenicol ($n = 70$; 98.6%) followed by 46 (64.8%) and 42 (59.2%) sensitive to gentamicin and tetracycline, respectively. *Coagulase-negative staphylococci* were sensitive to all tested antibiotics (Table 4).

TABLE 2 Types of bacterial isolates from the hands of housemaids ($n = 225$) in Jimma City, Southwest Ethiopia, 2022.

Bacteria isolates	Frequency	Percent
Staphylococcus aureus	71	31.6
Coagulase-negative staphylococci	2	0.9
Escherichia coli	48	21.3
Salmonella species	3	1.3
Shigella species	15	6.7
Klebsiella species	52	23.1
Proteus species	33	14.7

However, approximately 20 (28.2%) of isolated *S. aureus* were resistant to tetracycline. In addition, no resistance was reported regarding *Coagulase-negative staphylococci*. Regarding *S. aureus* isolates, no resistance was reported to chloramphenicol or gentamicin (Table 4).

For *E. coli* ($n = 48$), approximately 36 (75%), 38 (79.2%), 42 (87.5%), and 37 (77.1%) isolates were sensitive to tetracycline, ceftriaxone, chloramphenicol, and ceftazidime. For *Salmonella* species, all three isolates and two of them were sensitive to chloramphenicol, ceftriaxone, and ceftazidime. Approximately 80% of the *Shigella* isolate was sensitive to chloramphenicol and gentamicin, respectively. For *Klebsiella* isolates ($n = 52$), 46 (88.5%), 31 (59.6%), and 28 (53.8%) were sensitive to chloramphenicol, gentamicin, ceftriaxone, and ceftazidime. Moreover, 100, 81.8, and 69.7% of *Proteus* species were sensitive to chloramphenicol, gentamicin, and tetracycline, respectively (Table 4).

One-quarter of *E. coli* isolate was resistant to tetracycline, ceftriaxone, chloramphenicol, and ceftazidime, while a single isolate of *Salmonella* was resistant to tetracycline, ceftriaxone, and gentamicin. Most *Shigella* isolates were resistant to tetracycline (46.7%), ceftriaxone (26.7%), and ceftazidime (26.7%). Moreover, 16 (48.5%), 9 (27.3%), and 8 (24.2%) of isolated *Proteus* species

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TABLE 3 Factors associated with the bacterial isolation from the hands of housemaids ($n = 225$) in Jimma City, Ethiopia, 2022.

Study variables	Category	Bacterial culture results from hands		Chi-square and p value	COR (95%CI)	Sig.	AOR (95% CI)
		Negative	Positive				
Age of housemaids	18–24 years	49	133	$\chi^2_{(df=2)} = 3.534$	1.24 (0.571, 2.839)		
	25–30 years	9	25	$p = 0.1710$	5.02 (0.029, 1.951)		
	≥31 years	5	4		1	1	1
Educational status	Cannot read and write	1	6	$\chi^2_{(df=2)} = 1.835$	9 (3.012, 12.002)		
	Primary school	30	88	$p = 0.3990$	0.84 (0.192, 11.219)		
	Secondary and above	32	68		1	1	1
Fingernail status	Not trimmed	10	39	$\chi^2_{(df=1)} = 1.791$	6.54 (1.424, 9.375)	0.000	15.31 (10.372,22.595)**
	Trimmed	53	123	$p = 0.1810$	1	1	1
Frequently washing hands	No	2	16	$\chi^2_{(df=1)} = 2.768$	5.65 (2.196, 14.532)		
	Yes	61	146	$p = 0.0960$	1	1	1
How to wash hands	Water only	22	8	$\chi^2_{(df=1)} = 0.133$	6.96 (2.958, 16.369)		
	Water and soap	53	124	$p = 0.7160$	1	1	1
Frequent HW with soap or other detergents	No	3	2	$\chi^2_{(df=1)} = 2.216$	6.00 (2.713,13.270)		
	Yes	50	122	$p = 0.1370$	1	1	1
Follow five steps to wash hands the right way	No	36	100	$\chi^2_{(df=1)} = 1.715$	10.71 (2.500,5.861)		
	Yes	25	46	$p = 0.1900$	1	1	1
Removing watch, ring, and bracelet during HW	No	33	128	$\chi^2_{(df=1)} = 5.517$	1.97 (0.820, 4.743)	0.008	20.844 (2.190,9.842)*
	Yes	30	34	$p = 0.0190$	1	1	1
Washing hands for 20 s	No	28	89	$\chi^2_{(df=1)} = 3.283$	3.02 (1.388, 6.572)		
	Yes	35	73	$p = 0.070$	1	1	1

CI, Confidence interval; 1: for reference category; COR, Crude odd ratio; AOR, Adjusted odd ratio; HW, Handwashing. *p value < 0.05. **p value < 0.001.

were resistant to ceftriaxone, tetracycline, and ceftazidime, respectively (Table 4).

However, no resistance was reported to chloramphenicol regarding *Salmonella*, *Shigella*, *Klebsiella,* and *Proteus* species. In addition, no resistance was reported regarding gentamicin on isolates of *E. coli* and *Proteus* (Table 4).

From total bacteria isolates, no resistant chloramphenicol was recorded among six (85.7%), namely *S. aureus*, *Coagulase-negative staphylococci*, *Salmonella*, *Shigella*, *Klebsiella* species, and *Klebsiella* species Similarly, no gentamicin resistance was recorded among four (57.1%), namely *S. aureus*, *Coagulase-negative staphylococci*, *E.coli*, and *Klebsiella* species (Table 4).

4 Discussion

Housemaids with poor hand hygiene could be potential sources of infection due to pathogenic bacteria, which can cause food contamination and, consequently, foodborne diseases that pose a potential risk to public health (18, 25). Due to the scarcity of published information, the bacterial contamination level among housemaids in Ethiopia is underexplored. Therefore, the present study was undertaken to assess the prevalence and antibiotic susceptibility profile of bacteria isolated from the hands of housemaids in Jimma City, Ethiopia.

The prevalence of bacterial isolation from the hands of housemaids was 72%. This result is comparable with a study conducted in Tripoli, Libya with the prevalence of bacterial growth (71.41%) (51). The bacteria isolation from hands could be due to poor hand hygiene practices such as untrimmed fingernails and not removing the watch, rings, and bracelets during handwashing. Thus, jewelry could lead to bacteria colonizing the hands (52). In addition, bacterial isolation from the hands of housemaids illustrates the concept of fecal contamination (53). The other reason might be the quality of the handwashing water. Pieces of evidence revealed that bacterial contamination of hands is significantly affected by handwashing water (54, 55).

However, the result is higher than the reported prevalence by a previous study performed in Iran with a bacteria isolation rate of 62.2% (25), Egypt with a positive culture for one or more microbial contaminants (60%) (56), Sudan with a carrier of pathogenic bacteria (23.2%) (24), and in different parts of Ethiopia: Jimma (49.6,

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TABLE 4 Antimicrobial susceptibility pattern of bacteria isolates from the hands of housemaids in Jimma City, Southwest Ethiopia, 2022.

Bacteria isolate	Total	SP	TE [*n* (%)]	CRO [*n* (%)]	C [*n* (%)]	GEN [*n* (%)]	CAZ [*n* (%)]
Staphylococcus aureus	71	Sensitivity	42 [59.2]	NA	70 [98.6]	46 [64.8]	NA
		Intermediate	9 [12.7]		1 [1.4]	25 [35.2]	
		Resistant	20 [28.2]		0 [0.0]	0 [0.0]	
Coagulase-negative staphylococci	*2*	Sensitivity	2		2	2	
		Intermediate	0		0	0	
		Resistant	0		0	0	
E.coli	48	Sensitivity	36 [75.0]	38 [79.2]	42 [87.5]	30 [62.5]	37 [77.1]
		Intermediate	0 [0.0]	4 [8.3]	0 [0.0]	18 [37.5]	5 [10.4]
		Resistant	12 [25.0]	6 [12.5]	6 [12.5]	0 [0.0]	6 [12.5]
Salmonella species	3	Sensitivity	1	2	3	1	2
		Intermediate	1	0	0	1	1
		Resistant	1	1	0	1	0
Shigella species	15	Sensitivity	3 [20.0]	8 [53.3]	12 [80.0]	12 [80.0]	8 [53.3]
		Intermediate	5 [33.3]	3 [20.0]	3 [20.0]	0 [0.0]	3 [20.0]
		Resistant	7 [46.7]	4 [26.7]	0 [0.0]	3 [20.0]	4 [26.7]
Klebsiella species	52	Sensitivity	25 [48.1]	28 [53.8]	46 [88.5]	31 [59.6]	28 [53.8]
		Intermediate	15 [28.8]	14 [26.9]	6 [11.5]	21 [40.4]	7 [13.5]
		Resistant	12 [23.1]	10 [19.2]	0 [0.0]	0 [0.00]	17 [32.7]
Proteus species	33	Sensitivity	23 [69.7]	9 [27.3]	33 [100.0]	27 [81.8]	5 [15.2]
		Intermediate	1 [3.0]	8 [24.2]	0 [0.0]	5 [15.2]	20 [60.6]
		Resistant	9 [27.3]	16 [48.5]	0 [0.0]	1 [3.0]	8 [24.2]

SP, Sensitivity pattern; TE, Tetracycline; CRO, Ceftriaxone; C, Chloramphenicol; GEN, Gentamycin; CAZ, Ceftazidime; *n*, number; %, percent; NA, not applicable.

6.9, and 19.0%) (11, 15, 20), Gondar town (13. 2%) (1), Debre Markos (29.5 and 46.7%) (16, 17), and Dessie town (59.4%) (12). On the other hand, the result is lower than the study conducted in Mauritius, in which the prevalence of bacteria growth from hands was 91.0% (26), and in Ethiopia, at the University of Gondar Referral Hospital (UoGRH), the prevalence of bacterial isolation was 83.9% (13). The observed discrepancy in the bacteria isolation rate might be due to the differences in the study settings and periods, study participants, hygiene practices, and referent pressure for hygienic conditions.

In this study, *S. aureus* was the predominant bacterial species in the hands of housemaids, with an isolation rate of 31.6%. This result is comparable to a study conducted in the ICUs of United States medical centers (30.0%) (57) and UoGRH, Ethiopia, where the prevalence of *S. aureus* was 34% (13). The isolation of *S. aureus* could be because it is a pathogenic bacterium that is part of the normal flora of the skin and other body parts.

However, the result is lower than the study performed in Sudan (71.8%) (24), Iran (46%) (25), Nigeria (68.9%) (58), Eritrea (63.1%) (59), and Ethiopia; Addis Ababa Regional Laboratory (50.0%) (60) and Ethiopia's Debre Markos Comprehensive Specialized Hospital (DMCSH) (46.2%) (19), but higher than the study conducted in Alexandria, Egypt (22%) (56), Eastern India (3.44%) (5), and Ethiopia; University of Gondar (16%) (14); Debre Markos (5%) (17); Gondar Town (16.5%) (61), and Jimma University main campus (23.5%) (15). The discrepancy in the isolation rate of *S. aureus* might be due to differences in the sociodemographic characteristics, the study periods, the study settings and participants, the working environment, and the sample size.

The majority of *S. aureus* was sensitive to chloramphenicol (98.6%), followed by gentamycin (64.8%) and tetracycline (59.2%). This result is higher compared to the results reported in the UoGRH with sensitivity to chloramphenicol (76.9%) (13), the Addis Ababa regional laboratory with sensitivity to chloramphenicol (53.7%) (60), and DMCSH with sensitivity to chloramphenicol (66.7%) (19). Regarding resistance, isolates of *S. aureus* were resistant to tetracycline (28.2%). The result is lower than the study performed by Addis Ababa Regional Laboratory (74.30%) (60), Debre Markos (54.5%) (17), and UoGRH (64.1%) (13), but the result is higher than the study conducted at the University of Gondar (21.9%) (14). Different mechanisms play a pivotal role in how *S. aureus* became resistant to antimicrobials. The antimicrobial resistance of *S. aureus* to tetracycline might be due to increased efflux, the production of β-lactamase to β-lactam-sensitive antibiotics, the presence of acetyltransferase, a decrease in accumulation of macrolide antibiotics, the expression of the mec gene, and the formation of alternative pathways for sulphonamides (62). The reason for the antimicrobial resistance could be the dissemination of the strain (31). Moreover, the antimicrobial resistance of *S. aureus* could be determined by a lack of access to appropriate antimicrobial susceptibility tests and bacteriological diagnosis that could lead to the misuse of antimicrobials by patients (16).

In the present study, the isolation rate of *E. coli* was 21.3%, which is comparable with a study conducted in India (20.68%) (5). The result is lower than the study performed in Iran (29.2%) (51), Nigeria

(25.0%) (58), and Jimma University Specialized Hospital (JUSH) (25.4%) (20). However, the result is higher compared to previous studies conducted in Mauritius (0.5%) (26), in the ICUs of United States medical centers (7.1%) (57), and in the different parts of Ethiopia: Jimma University (10.9%) (15), Gondar Town (3.1 and 1.9%) (1, 61), Debre Markos (2.7%) (17), UoGRH (5.9%) (13), University of Gondar (2.67%) (14), DMCSH (3.9%) (19), and Addis Ababa Regional Laboratory (3.6%) (60). The isolation of *E. coli* illustrates the concept of fecal contamination in the hands of housemaids. In addition, De Alwis et al. (53) revealed that contaminated surfaces such as toilets and washrooms could be the sources of contamination of the hands when a person comes into contact.

The majority of *E. coli* isolates (87.5%) were sensitive to chloramphenicol. This result is lower than a study conducted in Gondar town (100%) (1); however, the result is higher compared to other studies with a sensitivity of 50% (11, 14, 17, 60), 75% (13), (66.7%) (19) and 56% to chloramphenicol (16). Similarly, approximately 79.2 and 75% of *E. coli* isolates were sensitive to ceftriaxone and tetracycline which is higher than the results reported in previous studies (11, 16, 17, 19, 60). However, resistance to tetracycline, ceftriaxone, chloramphenicol, and ceftazidime was reported for *E. coli* isolates (12.5%). It is lower than the results reported in other studies (11, 14, 16, 17, 60). A study conducted in the JUSH showed that *E. coli* isolates were resistant to ceftriaxone (73%) and ceftazidime (65%) (20). Another study carried out in DMCSH revealed that *E. coli* was resistant to tetracycline (44.4%), chloramphenicol (22.2%), and ceftazidime (33.3) (19). *Escherichia coli* were sensitive to chloramphenicol, which might be due to decreased levels of acetyl coenzyme A incat-expressing CM2555 cells in the presence of chloramphenicol (63). The antimicrobial resistance of *E. coli* to antimicrobial drugs could be due to its outer membrane and the expression of numerous efflux pumps (64). In addition, the antimicrobial resistance of *E. coli* might be due to the transmission of resistance genes and the unrestricted use of antimicrobials that perpetuate antimicrobial-resistant plasmids (65). Furthermore, other contributors to antimicrobial resistance are the spread of *E. coli*-resistant strains, overuse or inappropriate prescribing, use of antibiotics in livestock, hygiene/fecal colonization, and antibiotic resistance mechanisms (β-lactams) (64).

In the current study, the isolation rate of *Shigella* species was 6.70%, which is comparable to the result reported in Debre Markos University, northwest Ethiopia (5.9%) (18). The isolation of *Shigella* species from the hands of housemaids illustrates poor hand hygiene practices due to fecal contamination. The other reason might be due to cross-contamination of hands with surfaces such as toilets and washrooms (53). The result is lower than a study performed in Gondar town with a prevalence of 10.1% (1), but higher compared to previous studies conducted in Jimma town (0.2%) (11), the University of Gondar (2.7%) (14), and Debre Markos Referral Hospital (5.4%) (16). The variation might be due to differences in the demographic characteristics, study settings and period, study design and sampling techniques, and geographical variation of study areas.

Concerning the antimicrobial resistance profile of isolates, approximately 80% of isolated *Shigella* species were sensitive to chloramphenicol and gentamicin, respectively. This is comparable to a study performed in Gondar town (1) and Debre Markos University (18), but the result is higher than previous studies performed at the Debre Markos Referral Hospital (16) and the University of Gondar (14). On the other hand, approximately 46.7 and 26.7% of isolated *Shigella* species were resistant to tetracycline, ceftriaxone, and ceftazidime. This is consistent with reported results in Gondar town (1) and Debre Markos University (18). The result is lower than a previous study performed in Debre Markos Referral Hospital (16), Gondar town (1), and Jimma town (11), but higher than a previous study in Gondar town (1) and Jimma town (11). The resistance of *Shigella* species to tested antibiotics might be due to high descriptions from clinics available in the locality as well as self-medication. In addition, it might be due to genetic diversity (66).

In the present study, the isolation rate of *Klebsiella* species was 23.1%, which is higher than the previous study conducted in different parts of Ethiopia: Debre Markos Referral Hospital (4.3%) (16), Gondar town (1.67%) (14), Debre Markos University (2.7%) (17), UoGRH (12.5%), Gondar town (5.5%) (61), and ICUs of US medical centers (11.8%) (57). Regarding the antimicrobial susceptibility pattern of *Klebsiella* species, the majority of *Klebsiella* isolates (88.5%) were sensitive to chloramphenicol. The result is in line with a previous study conducted in Ethiopia (17). While antibiotic resistance to tetracycline, ceftriaxone, and ceftazidime among *Klebsiella* isolates was recorded, a study performed in different parts of Ethiopia showed antibiotic resistance of *Klebsiella* species regarding chloramphenicol, tetracycline, ceftriaxone, gentamicin, and ceftazidime (13, 14, 16, 17, 20). The antimicrobial resistance of *Klebsiella* species might be due to its strains having a *β*-lactam ring provided with a Zwitterionic structure (20). Another aggravating factor for antimicrobial resistance of *Klebsiella* species might be the self-prescribing of antibiotics by a few people due to the availability of antibiotics on the market in the study area and inappropriate use of antibiotics (60).

The isolation of *Proteus* species is 14.7% in the present study, which is inconsistent with Debre Markos University (1.4%) (17), JUSH (2.0%) (20), Jimma University (2.2%) (15), and UoGRH (9.6%) (13). Moreover, we observed antimicrobial resistance of *Proteus* species to ceftriaxone, tetracycline, and ceftazidime in the current study. It is consistent with a previous study that showed antimicrobial resistance recorded for tested drugs such as ceftriaxone, tetracycline, and ceftazidime (13, 17, 20).

In sum, sensitivity to chloramphenicol on most bacteria isolates was observed, but resistance to tetracycline, ceftriaxone, and ceftazidime among isolates *E. coli*, *Salmonella*, *Shigella*, *Klebsiella* species, and *Proteus* species was observed in the present study. At present, antimicrobial resistance is an emerging global challenge that results in the spread of infectious diseases that could affect human populations (38, 39). This might be due to a complex set of causes such as biological processes, human behaviors, and social factors that support the microbes to multiply, carry on, and produce harm (37). In addition, antimicrobial resistance could be due to the evolutionary processes or natural phenomena to which microbes tend to adapt (36, 37). The other reason for AMR could be the inappropriate use of drugs by the community, the use of antibiotics in animals, or the external environment (18, 38, 39). Moreover, the global connection of a large human population allows microbes to move from place to place and spread, allowing them to easily enter the environment (39).

In comparison to housemaids who clipped their fingernails, housemaids who neglected to do so were 15.31 times more likely to have positive bacteria cultures (AOR = 15.31, 95% CI: 10.372, 22.595). This result is in line with the result reported in Poland (OR: 7.1; 95% CI:1.83, 27.39) (67). The result is also supported by a study performed by Mengist et al. (17). In addition to limiting the use of proper hand hygiene, long or sharp fingernails might promote bacteria development. The likelihood of

positive bacterial isolation was 20.844 times higher in housemaids who did not remove their accessories before washing their hands than their counterparts (AOR = 20.844, 95% CI: 2.190, 9.842). During handwashing, accessories must be taken off to prevent the growth and spread of harmful microorganisms. They should be well rinsed unless doing so would cause bacterial growth (53).

4.1 Limitations

This study did not identify important microbial hand contaminants such as *V. cholerae*, *Helicobacter, and Campylobacter* due to constraints on resources. In addition, some antimicrobial susceptibility tests were performed in this study due to the lack of antimicrobial disks. Furthermore, a multidrug resistance test was not conducted for bacteria isolated from the hands of housemaids.

5 Conclusion

Housemaids' hands are very important potential sources of disease-causing bacterial pathogens that would result in the potential risk of gastrointestinal tract infections. Fingernail status and the removal of accessories during handwashing were found to be significantly associated with the prevalence of bacterial isolation from the hands of housemaids. Moreover, most of the bacterial isolates were sensitive to chloramphenicol and gentamycin, while the majority of them were resistant to tetracycline, vancomycin, and ceftazidime.

Therefore, keeping fingernail status short, removing accessories during handwashing, and practicing good hand hygiene are crucial to preventing and controlling antimicrobial-resistant microbes. In addition, any community health worker, regional, national, and other stakeholders who are engaged in community health should create awareness about regular handwashing and its relevance in the prevention and reduction of pathogenic microorganisms from hands in the wider community.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The study was conducted after obtaining an ethical clearance with reference number IHRPGS/437/22 approved by the Institutional Review Board of the Institute of Health Sciences, Jimma University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

TA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. NG: Visualization, Writing – original draft, Writing – review & editing. GZ: Visualization, Writing – original draft, Writing – review & editing. TT: Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. TG: Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Getie M, Abebe W, Tessema B. Prevalence of enteric bacteria and their antimicrobial susceptibility patterns among food handlers in Gondar town, Northwest Ethiopia. *Antimicrob Resist Infect Control*. (2019) 8:1–6. doi: 10.1186/s13756-019-0566-7

2. Grace D. Food safety in low and middle-income countries. *Int J Environ Res Public Health*. (2015) 12:10490–507. doi: 10.3390/ijerph120910490

3. World Health Organization (2020). *Food safety*. 1–9.

4. Grace D. (2015). Food safety in developing countries: An overview. A learning resource for Department for International Development (DFID) livelihoods advisers.

5. Banik S, Chakrabarty S, Das N. Hand hygiene in food handlers working in canteens of an educational institution in eastern India. *Int J Commun Med Public Heal*. (2020) 7:2602–6. doi: 10.18203/2394-6040.ijcmph20202983

6. Thobaben M. Causes and prevention of foodborne illness. *Home Health Care Manag Pract*. (2010) 22:533–5. doi: 10.1177/1084822310376611

7. Tadesse G, Mitiku H, Teklemariam Z, Marami D. Salmonella and Shigella among asymptomatic street food vendors in the Dire Dawa city, eastern Ethiopia: prevalence, antimicrobial susceptibility pattern, and associated factors. *Environ Health Insights*. (2019) 13:117863021985358–8. doi: 10.1177/1178630219853581

8. Kalekidan T, Behailu K, Rediet H. The Ethiopian perception of the food safety system. *Adv Food Sci Technol*. (2014) 2:260–8.

9. Mardu F, Negash H, Legese H, Berhe B, Tesfay K, Haileslasie H, et al. Assessment of knowledge, practice, and status of food handlers toward Salmonella, Shigella, and intestinal parasites: a cross-sectional study in Tigrai prison centers, Ethiopia. *PLoS One*. (2020) 15:1–13. doi: 10.1371/journal.pone.0241145

10. Mama M, Alemu G. Prevalence, antimicrobial susceptibility patterns and associated risk factors of Shigella and Salmonella among food handlers in Arba Minch University, South Ethiopia. *BMC Infect Dis*. (2016) 16:1–7. doi: 10.1186/s12879-016-2035-8

11. Worku T, Jejaw A, Kannan S, Wondafrash B. Isolation and antimicrobial sensitivity patterns of enteric bacterial pathogens from asymptomatic food handlers, Jimma, Ethiopia. *Am J Heal Res*. (2015) 3:399–406. doi: 10.11648/j.ajhr.20150306.24

12. Shiferaw B, Gelaw B, Assefa A, Assefa Y, Addis Z. Bacterial isolates and their antimicrobial susceptibility pattern among patients with external ocular infections at Borumeda hospital, Northeast Ethiopia. *BMC Ophthalmol*. (2015) 15:1–8. doi: 10.1186/s12886-015-0078-z

13. Mohammed A, Seid ME, Gebrecherkos T, Tiruneh M, Moges F. Bacterial isolates and their antimicrobial susceptibility patterns of wound infections among inpatients and outpatients attending the University of Gondar Referral Hospital, Northwest Ethiopia. *Int J Microbiol*. (2017) 2017:8953829. doi: 10.1155/2017/8953829

14. Dagnew M, Tiruneh M, Moges F, Gizachew M. Bacterial profile and antimicrobial susceptibility pattern among food handlers at Gondar University cafeteria, Northwest Ethiopia. *J Infect Dis Ther*. (2013) 1:105. doi: 10.4172/2332-0877.1000105

15. Assefa T, Tasew H, Wondafrash B, Beker J. Contamination of Bacteria and associated factors among food handlers working in the student cafeterias of Jimma University-Main campus, Jimma, south West Ethiopia. *Altern Integr Med*. (2015) 4:185. doi: 10.4172/2327-5162.1000185

16. Abebe M, Tadesse S, Meseret G, Derbie A. Type of bacterial isolates and antimicrobial resistance profile from different clinical samples at a referral hospital, Northwest Ethiopia: five years data analysis. *BMC Res Notes*. (2019) 12:568. doi: 10.1186/s13104-019-4604-6

17. Mengist A, Aschale Y, Reta A. Bacterial and parasitic assessment from fingernails in Debre Markos, Northwest Ethiopia. *Can J Infect Dis Med Microbiol*. (2018) 2018:6532014. doi: 10.1155/2018/6532014

18. Mengist A, Mengistu G, Reta A. Prevalence and antimicrobial susceptibility pattern of Salmonella and Shigella among food handlers in catering establishments at Debre Markos university, Northwest Ethiopia. *Int J Infect Dis*. (2018) 75:74–9. doi: 10.1016/j.ijid.2018.08.008

19. Haile Z, Mengist HM, Dilnessa T. Bacterial isolates, their antimicrobial susceptibility pattern, and associated factors of external ocular infections among patients attending eye clinic at Debre Markos comprehensive specialized hospital, Northwest Ethiopia. *PLoS One*. (2022) 17:e0277230. doi: 10.1371/journal.pone.0277230

20. Gashe F, Mulisa E, Mekonnen M, Zeleke G. Antimicrobial resistance profile of different clinical isolates against third-generation Cephalosporins. *Aust J Pharm*. (2018) 2018:1–7. doi: 10.1155/2018/5070742

21. Mekonnen M, Aga F, Kinati T, Shifera D. Assessment of hand washing practice and associated factors among primary school children in Sebeta town Oromia regional state, Ethiopia. *Health Sci J*. (2018) 12:1–6. doi: 10.21767/1791-809X.1000605

22. Pickering AJ, Boehm AB, Mwanjali M, Davis J. Efficacy of waterless hand hygiene compared with handwashing with soap: a field study in Dar Es Salaam, Tanzania. *Am J Trop Med Hyg*. (2010) 82:270–8. doi: 10.4269/ajtmh.2010.09-0220

23. World Health Organization (2016). Burden of Foodborne Diseases in the South-East Asia Region. New Delhi, India.

24. Ahmed H, Hassan H. Bacteriological and parasitological assessment of food handlers in the Omdurman area of Sudan. *J Microbiol Immunol Infect*. (2010) 43:70–3. doi: 10.1016/S1684-1182(10)60010-2

25. Nasrolahei M, Mirshafiee S, Kholdi S, Salehian M, Nasrolahei M. Bacterial assessment of food handlers in Sari City, Mazandaran Province, north of Iran. *J Infect Public Health*. (2016) 10:1–6. doi: 10.1016/j.jiph.2016.03.006

26. Padaruth SK, Biranjia-Hurdoyal SD. Hygiene practices and fecal contamination of the hands of children attending primary school in Mauritius. *Int Health*. (2015) 7:280–4. doi: 10.1093/inthealth/ihu080

27. Doron S, Gorbach SL. Bacterial infections: overview. *Int Encycl Public Heal*. (2008) 3:273–82. doi: 10.1016/B978-012373960-5.00596-7

28. Ks I, Lr S, Gr A, F S, T M, Ap G, et al. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the global burden of disease study 2019. *Lancet*. (2022) 400:2221–48. doi: 10.1016/S0140-6736(22)02185-7

29. Mahtab T, Jyoti A, Khusro A, Redwan BM, Zidan M, Mitra S, et al. Antibiotic resistance in microbes: history, mechanisms, therapeutic strategies, and prospects. *J Infect Public Health*. (2021) 14:1750–66. doi: 10.1016/j.jiph.2021.10.020

30. Cleveland Clinic (2023). Bacterial infection 1–10. Available at: https://my.clevelandclinic.org/health/diseases/24189-bacterial-infection

31. Castro A, Santos C, Meireles H, Silva J, Teixeira P. Food handlers as potential sources of dissemination of virulent strains of *Staphylococcus aureus* in the community. *J Infect Public Health*. (2016) 9:153–60. doi: 10.1016/j.jiph.2015.08.001

32. Chang D, Sharma L, Dela Cruz CS, Zhang D. Clinical epidemiology, risk factors, and control strategies of *Klebsiella pneumoniae* infection. *Front Microbiol*. (2021) 12:750662. doi: 10.3389/fmicb.2021.750662

33. Adamus-Bialek W, Zajac E, Parniewski P, Kaca W. Comparison of antibiotic resistance patterns in collections of Escherichia coli and *Proteus mirabilis* uropathogenic strains. *Mol Biol Rep*. (2013) 40:3429–35. doi: 10.1007/s11033-012-2420-3

34. Kamil TD, Jarjes SF. Isolation, identification, and antibiotics susceptibility determination of Proteus species obtained from various clinical specimens in Erbil City. *Polytech J*. (2019) 9:86–92. doi: 10.25156/ptj.v9n2y2019.pp86-92

35. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis*. (2009) 48:1–12. doi: 10.1086/595011

36. Giubilini A, Savulescu J. Moral responsibility and the justification of policies to preserve antimicrobial effectiveness In: E Jamrozik and M Selgelid, editors. *Ethics and Drug Resistance: Collective Responsibility for Global Public Health*. Cham, Switzerland: Springer (2020). 141–54.

37. Cheah PY, Parker M, Day NPJ. Ethics and antimalarial drug resistance In: E Jamrozik and M Selgelid, editors. *Ethics and Drug Resistance: Collective Responsibility for Global Public Health*. Cham, Switzerland: Springer (2020). 55–73.

38. Nijsingh N, Larsson DGJ, de Fine LK, Munthe C. Justifying antibiotic resistance interventions: uncertainty, precaution and ethics In: E Jamrozik and M Selgelid, editors. *Ethics and Drug Resistance: Collective Responsibility for Global Public Health*. Cham, Switzerland: Springer (2020). 357–75.

39. Michael CA, Dominey-Howes D, Labbate M. The antimicrobial resistance crisis: causes, consequences, and management. *Front Public Health*. (2014) 2:1–8. doi: 10.3389/fpubh.2014.00145

40. Okeke IN, Laxminarayan R, Bhutta ZA, Duse AG, Jenkins P, Brien TFO, et al. Antimicrobial resistance in developing countries. Part I: recent trends and current status. *Lancet Infect Dis*. (2005) 5:481–93. doi: 10.1016/S1473-3099(05)70189-4

41. Woodward DL, Rodgers FG. Surveillance of antimicrobial resistance in salmonella, shigella and *Vibrio cholerae* in Latin America and the Caribbean: a collaborative project. *Can J Infect Dis Med Microbiol*. (2000) 11:181–6. doi: 10.1155/2000/743969

42. Tadesse T, Deneke Y, Deresa B. Seroprevalence of bovine viral diarrhea virus and its potential risk factors in dairy cattle of Jimma town, southwestern Ethiopia. *J Dairy, Vet Anim Res*. (2019) 8:11–7. doi: 10.15406/jdvar.2019.08.00235

43. Jimma city municipality office (2022). Population profiles of Jimma city administration, Jimma zone, Oromia.

44. Bekele D, Tolossa T, Tsegaye R, Teshome W. The knowledge and practice towards COVID-19 pandemic prevention among residents of Ethiopia. An online cross-sectional study. *PLoS One*. (2021) 16:e0234585. doi: 10.1371/journal.pone.0234585

45. Engdaw GT, Gebrehiwot M, Andualem Z. Hand hygiene compliance and associated factors among health care providers in Central Gondar zone public primary hospitals, Northwest Ethiopia. *Antimicrob Resist Infect Control*. (2019) 8:190. doi: 10.1186/s13756-019-0634-z

46. WHO (2009). Hand Hygiene Knowledge Questionnaire for Health-Care Workers.

47. American Type Culture Collection (2022). *Introduction to microbiology*. Manassas, Virginia 20110-2209.

48. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing. 30th edition. CLSI supplement M100*. Wayne, PA: Clinical and Laboratory Standards Institute (2020).

49. Cheesbrough M. *District Laboratory in Tropical Practice Countries: Part 2. 2nd ed*. Cambridge, UK: Cambridge University Press (2006).

50. Bargellini A, Ferranti G, Santangelo M, Venturelli F. Hand hygiene knowledge, hand contamination and practice of Italian nursing and medical students. *Epidemiol Biostat Public Health*. (2014) 11:e9971. doi: 10.2427/9971

51. Alsagher MR, Soudah SA, Khsheba AE, Fadel SM, Dadiesh MA, Houme MA, et al. Hand washing before and after applying different hand hygiene techniques in places of public concern in Tripoli-Libya. *Open Microbiol J*. (2018) 12:364–75. doi: 10.2174/1874285801812010364

52. Kampf G, Löffler H. Hand disinfection in hospitals-benefits and risks. *JDDG*. (2010) 8:978–83. doi: 10.1111/j.1610-0387.2010.07501.x

53. De Alwis WR, Pakirisamy P, Wai San L, Xiaofen EC. A study on hand contamination and hand washing practices among medical students. *ISRN Public Health*. (2012) 2012:1–5. doi: 10.5402/2012/251483

54. Berhanu L, Mereta ST, Gume B, Kassa T, Berihun G, Dadi LS, et al. Effect of microbial quality of washing water on hand hygiene status of food handlers in Jimma

town: implication for food hygiene and safety. *J Multidiscip Healthc*. (2021) 14:1129–34. doi: 10.2147/JMDH.S306359

55. Burton M, Cobb E, Donachie P, Judah G, Curtis V, Schmidt WP. The effect of handwashing with water or soap on bacterial contamination of hands. *Int J Environ Res Public Health*. (2011) 8:97–104. doi: 10.3390/ijerph8010097

56. El-Kady H. Efficacy of different alcohol-based hand disinfectants in reduction of hand contamination among food handlers in Alexandria, Eqypt. *JAMB*. (2018) 13:1–13. doi: 10.9734/JAMB/2018/45345

57. Sader HS, Castanheira M, Mendes RE, Flamm RK. Frequency and antimicrobial susceptibility of gram-negative bacteria isolated from patients with pneumonia hospitalized in ICUs of US medical centres (2015-17). *J Antimicrob Chemother*. (2018) 73:3053–9. doi: 10.1093/jac/dky279

58. Ogba OM, Asukwo PE, Otu-Bassey IB. Assessment of bacterial carriage on the hands of primary school children in Calabar municipality, Nigeria. *Biomed dermatol*. (2018) 2:1–7. doi: 10.1186/s41702-017-0017-0

59. Garoy EY, Gebreab YB, Achila OO, Tekeste DG, Kesete R, Ghirmay R, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA): prevalence and antimicrobial sensitivity pattern among patients-a multicenter study in Asmara, Eritrea. *Can J Infect Dis Med Microbiol*. (2019):8321834. doi: 10.1155/2019/8321834

60. Kitila KT, Taddese BD, Hailu TK, Sori LM, Geleto SE, Mengistu GZ, et al. Assessment of bacterial profile and antimicrobial resistance pattern of bacterial isolates from blood culture in Addis Ababa regional laboratory, Addis Ababa, Ethiopia. *Clin Microbiol*. (2018) 7:312. doi: 10.4172/2327-5073.1000312

61. Gashaw A, Afework K, Feleke M, Moges T, Kahsay H. Prevalence of bacteria and intestinal parasites among food-handlers in Gondar town, Northwest Ethiopia. *J Health Popul Nutr*. (2008) 26:451–5. doi: 10.3329/jhpn.v26i4.1887

62. Deyno S, Fekadu S, Astatkie A. Resistance of *Staphylococcus aureus* to antimicrobial agents in Ethiopia: a meta-analysis. *Antimicrob Resist Infect Control*. (2017) 6:1–15. doi: 10.1186/s13756-017-0243-7

63. Potrykus J, Wegrzyn G. Chloramphenicol-sensitive *Escherichia coli* strain expressing the chloramphenicol acetyltransferase (cat) gene. *Antimicrob Agents Chemother*. (2001) 45:3610–2. doi: 10.1128/AAC.45.12.3610-3612.2001

64. Galindo-Méndez M. Antimicrobial resistance in *Escherichia coli E. coli* infections—importance of early diagnosis and efficient treatment. *Dermatol Int*. (2020):1–20. doi: 10.5772/intechopen.93115

65. Uma B, Prabhakar K, Rajendran S, Kavitha K, Sarayu Y. Antibiotic sensitivity and plasmid profiles of *Escherichia coli* isolated from pediatric diarrhea. *J Global Infect Dis*. (2009) 1:107. doi: 10.4103/0974-777X.56255

66. Pakbin B, Amani Z, Allahyari S, Mousavi S, Mahmoudi R, Brück WM, et al. Genetic diversity and antibiotic resistance of Shigella spp. isolates from food products. *Food Sci Nutr*. (2021) 9:6362–71. doi: 10.1002/fsn3.2603

67. Wałaszek MZ, Kołpa M, Różańska A, Jagiencarz- B. Effectiveness of hand hygiene and the condition of fingernails. A qualitative evaluation of nail microbial colonization following hand disinfection, a pilot study. *J Hosp Infect*. (2018) 100:207–10. doi: 10.1016/j.jhin.2018.06.023

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Differences in molecular characteristics and expression of virulence genes in carbapenem-resistant and sensitive *Klebsiella pneumoniae* isolates in Ningbo, China

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Background: In recent years, *Klebsiella pneumoniae* has attracted attention because of its increasing drug resistance. At the same time, the migration and pathogenicity caused by its virulence genes also bring many difficulties to the diagnosis and treatment of clinical infections. However, it is currently unclear whether there are differences in virulence and pathogenicity with changes in drug resistance.

Objective: To understand the differences in molecular characteristics and expression of virulence genes in carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and carbapenem-sensitive *Klebsiella pneumoniae* (CSKP).

Methods: Using polymerase chain reaction (PCR), we examined capsule polysaccharide-related genes and virulence genes in 150 clinical isolates of CRKP and 213 isolates of CSKP from the local area in Ningbo, China. Multilocus sequence typing (MLST) was used to analyze the phylogenetic relationships of clinical *Klebsiella pneumoniae* isolates. Furthermore, real-time quantitative PCR (RT-qPCR) was used to analyze the expression differences of common virulence genes in CSKP and CRKP, and the virulence was further verified by the larval model of *Galleria mellonella*.

Results: The study found that the detection rates of genes *rmpA*, *iroB*, *peg-344*, *magA*, *aerobactin*, *alls*, *kfu*, and *entB* were significantly higher in CSKP compared to CRKP. The capsule gene types K1 and K2 were more common in CSKP, while K5 was more common in CRKP. Hypervirulent *Klebsiella pneumoniae* (hvKP) was predominantly from CSKP. CRKP strains exhibited noticeable homogeneity, with ST11 being the predominant sequence type among the strains. CSKP strains showed greater diversity in ST types, but ST23 was still the predominant sequence type. Carbapenem-sensitive hypervirulent *Klebsiella pneumoniae* (CS-hvKP) had higher expression of *rmpA* and *rmpA2* genes compared to carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP). In the

wax moth virulence model, the survival rate of CS-hvKP was significantly lower than that of CR-hvKP.

Conclusion: There is a significant difference in the distribution of virulence genes between CSKP and CRKP, with CSKP carrying a significantly greater number of virulence genes. Furthermore, compared to CSKP, CRKP strains exhibit noticeable homogeneity, with ST11 being the predominant sequence type among the strains. Additionally, in terms of virulence gene expression efficiency and virulence, CSKP is significantly higher than CRKP.

KEYWORDS

***Klebsiella pneumoniae*, carbapenem-resistant, hvKp, virulence, MLST**

1 Introduction

Klebsiella pneumoniae (KP) is a Gram-negative rod-shaped bacterium belonging to the *Enterobacteriaceae* family. It is an opportunistic pathogen that is widely present in the human intestinal and respiratory tracts. KP can cause primary pneumonia or extrapulmonary infections when the host's immune system is compromised, invasive procedures are performed, or antibiotics are used improperly, including bloodstream infections, urinary tract infections, meningitis, enteritis, liver abscesses, etc. (Choby et al., 2020; Wang et al., 2020; Zhang H. et al., 2021; Kuo et al., 2022). KP can be categorized into two types based on pathogenicity: classical *Klebsiella pneumoniae* (cKP) and hypervirulent *Klebsiella pneumoniae* (hvKP). Compared to cKP, infections caused by hvKP are more severe and widespread (Russo and Marr, 2019; Zhu et al., 2021a). HvKP exhibits stronger tissue invasiveness and cell migration, often resulting in concurrent infections in the bloodstream, eyes, liver, lungs, and central nervous system (Choby et al., 2020; Chang et al., 2021; Dai and Hu, 2022). The enhanced virulence of hvKP is mainly associated with factors such as capsular polysaccharides, mucoid phenotype regulatory genes, iron carriers, fimbriae, lipopolysaccharides, mobile genetic elements, etc. Most hvKP strains have a hypermucoviscosity phenotype (usually identified in string test), but it has been found that cKP can also exhibit hypermucoviscosity while string test of hvKP could be negative (Catalán-Nájera et al., 2017; Russo et al., 2018; Yang et al., 2020). In order to detect hvKP more accurately, in-depth studies have been conducted at the molecular level, using the five genes "*rmpA*, *rmpA2*, *peg-344*, *iucA*, *iroB*" for the diagnosis of hvKP with an accuracy of over 95% (Bulger et al., 2017; Russo and Marr, 2019). Capsular genes K1, K2, K5, K20, K54, and K57 have been shown to be highly associated with hvKP (Zhan et al., 2017; Russo et al., 2018; Yuan et al., 2019; Choby et al., 2020). With the horizontal transfer of virulence plasmids and carbapenemase-encoding plasmids (Gu et al., 2018; Zhang et al., 2020; Zhou et al., 2020), carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP) has been increasingly detected and even cause outbreaks in hospitals (Gu et al., 2018). In China, CR-hvKP has been detected in Zhejiang, Jiangsu, Beijing, Shanghai, Henan, Shandong, Hebei, and Inner Mongolia (Li et al., 2023; Pu et al., 2023; Yin et al., 2023). With the exponential increase in the detection of CR-hvKP, it poses a serious threat to public health. Due to the different selection of strains, the detection rate of virulence genes varied greatly in different selection studies. For example, the detection rate of *rmpA* gene can range from 2.2 to 87.2% (Lin et al., 2020; Li et al., 2023; Yin et al., 2023). Therefore, the differences in distribution and pathogenicity of virulence genes in CRKP and CSKP remain to be studied. Therefore, we used the five genes "*rmpA*, *rmpA2*, *peg-344*, *iucA*, *iroB*" as markers for hvKP and investigated the distribution and molecular epidemiological characteristics of virulence factors in CSKP and CRKP, as well as the differences in the expression levels of virulence genes and their pathogenicity in animal models.

2 Materials and methods

2.1 Bacterial strains and specimen source

In this study, a total of 363 strains of *Klebsiella pneumoniae* (KP) were selected from multiple hospitals located in Ningbo, Zhejiang Province, China, from January 2019 to December 2021. These included 150 strains of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and 213 strains of carbapenem-sensitive *Klebsiella pneumoniae* (CSKP). This study was approved by the Ethics Committee of Ningbo Medical Center LiHuiLi Hospital, Ningbo University (KY2023SL347-01). The specimens were obtained from various sources, including sputum, throat swabs, wound secretions, blood, and urine.

2.2 Identification of bacterial strains and drug susceptibility test

We used the VITEK 2 Compact automated system (bioMérieux, France) for bacterial strain identification and antimicrobial susceptibility testing. Data on drug susceptibility can be found in **Supplementary Table 1**. Carbapenem resistance was determined using the broth microdilution method. The quality control strains used for antimicrobial susceptibility testing were *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (purchased from the National Center for Clinical Laboratories, Ministry of Health). The definition of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) in this study was based

on the MIC breakpoints specified by the Clinical and Laboratory Standards Institute (CLSI) in 2023, which indicate intermediate or resistant levels to one or more carbapenem drugs.

2.3 Detection of virulence genes and capsule typing genes

Genomic DNA was extracted from the samples using a boiling method (Liao et al., 2020b). Subsequently, polymerase chain reaction (PCR) was employed to amplify various genes related to capsule polysaccharide-related genes (*rmpA*, *rmpA2*, *magA*), iron carrier-associated virulence genes (*iucA*, *iutA*, *entB*, *aerobactin*, *iroB*, *ybts*, *kfu*), fimbriae-related genes (*fimH*, *markD*, *alls*), lipopolysaccharide-related genes (*wabG*), as well as inner membrane transport protein (*peg-344*) and capsular typing genes (K1, K2, K5, K20, K54, K57). The primer sequences and annealing temperatures for each gene were provided in **Supplementary Table 2**. The PCR reaction consisted of 12.5 μL of 2X Taq MasterMix, 1.0 μL of forward and reverse primers each, 2.0 μL of DNA template, and 8.5 μL of ddH_2O, with a total volume of 25 μL. The PCR reaction conditions were as follows: initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at the respective temperatures for 30 s, extension at 72°C for 1 min, with a total of 30 cycles, and a final extension at 72°C for 10 min. The PCR products were subjected to agarose gel electrophoresis on a 1.0% agarose gel at a voltage of 110 V for 30 min. Gel imaging and analysis were performed using the GELDOC XR gel imaging analysis system (Bio-Rad, USA), and the presence or absence of the target genes was determined based on the position of the marker bands. For positive bands, sequencing analysis was performed after gel purification.

2.4 Detection of hypermucoviscosity phenotype

The strains were inoculated onto Columbia blood agar plates using the streaking method in three zones. The petri dishes were then placed in a constant temperature incubator and cultured at 37°C with 5% CO_2 for 18–24 h. A sterile disposable inoculation loop was used to pick up bacteria from a single colony and streaked upward. If the length of the mucoviscosity string observed during streaking was greater than 5 mm, and this phenomenon could be observed repeatedly more than 2 times, the string test result was considered positive, indicating that the strain was hypermucoviscosity *Klebsiella pneumoniae* (hmKP). Conversely, if the string test result was negative, the strain was considered non-hypermucoviscosity *Klebsiella pneumoniae* (n-hmKP).

2.5 Multilocus sequence typing (MLST)

Genomic DNA of *Klebsiella pneumoniae* was first extracted using a boiling method. PCR was then used to amplify 7 housekeeping gene fragments (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, *tonB*). The primer sequences and annealing temperatures for each gene can be found in **Supplementary Table 1**. The PCR reaction system consisted of 12.5 μL of 2X Taq MasterMix, 1.0 μL of forward and reverse primers each, 2.0 μL of DNA template, and 8.5 μL of ddH_2O, with a total volume of 25 μL. The PCR reaction conditions were as follows: initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at the respective temperatures for 45 s, extension at 72°C for 45 s, with a total of 35 cycles, and a final extension at 72°C for 5 min. The amplification products were sent to Shanghai Biotechnologies Corporation for sequencing. The sequencing results were uploaded to http://bigsdb.pasteur.fr/klebsiella/ and then compared with the database on the website to obtain the allele number and MLST type.

2.6 Relative expression levels of virulence genes

In this study, strains carrying the same virulence genes were selected from CSKP and CRKP, respectively, and their relative expression differences were analyzed using RT-qPCR. Details of the strains are shown in **Supplementary Table 3**. First, the Column Bacterial Total RNA Extraction Purification Kit (Bioteke, Shanghai) was used to extract RNA from the tested strains. Then, the One Step RT-qPCR Kit (Dye method) (Sangon Biotech, Shanghai) was used for RT-qPCR. The primer sequences can be found in **Supplementary Table 1**. Each gene was replicated three times. For data analysis, the 16S rRNA gene was used as the internal reference, and the $2^{-\Delta\Delta CT}$ method was used for calculation. Finally, GraphPad Prism 9.5 software (GraphPad Software, San Diego, CA, USA) was used for data visualization and analysis of relative gene expression levels.

2.7 Preparation of the infectious model using the *galleria mellonella* larvae

We used *Galleria mellonella* larvae as an infection model (Ménard et al., 2021; Asai et al., 2023). The strains were derived from RT-qPCR consistently, as detailed in **Supplementary Table 3**. *Galleria mellonella* larvae from Huiyude (Tianjin) weighing 250–350 milligrams, pale yellowish white in color, and exhibiting good activity and responsive behavior were selected as experimental subjects. Firstly, pilot experiments were conducted using control strains with pre-set concentrations of 10^7 CFU/mL, 10^6 CFU/mL, 10^5 CFU/mL of injection solutions to determine the optimal concentration by observing larval mortality. The criteria for determining larval mortality were blackening of the body and no response to touch (**Figure 7A** shows survival and **Figure 7B** shows death). Ten larvae were injected for each strain, with the injection site being the rear lateral side of the second-last abdominal proleg, and care was taken to prevent any obvious leakage during the injection process. The injected larvae were placed in sterile disposable culture dishes and incubated in a lightproof incubator (Shanghai Yiheng) at 37°C. The survival of larvae was recorded every 6 h, and a survival curve was plotted. A saline control group was set up (10 larvae injected with 10 μL of physiological saline). The negative control strain was *Klebsiella quasipneumoniae* ATCC 700603, and the positive control strain was NTUH-K2044

(hvKP) (purchased from the National Center for Clinical Medicine Examination, Ministry of Health).

2.8 Statistical analysis

In this study, we retrospectively analyzed the data of clinically isolated bacteria using WHONET 5.6 software. For data processing, we used SPSS 25.0 software (IBM, USA). The comparison of sample rates was analyzed using the Pearson's chi-square 2 × 2 (2-sided) test or Fisher's exact test (2-sided). Survival analysis was performed using the Log Rank (Mantel-Cox) test, with a *P*-value < 0.05 considered statistically significant. For the evaluation of virulence gene correlation, calculations, and graphical analyses were conducted using GraphPad Prism 9.5 software (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Strain source and specimen distribution

In this study, a total of 150 carbapenem-susceptible *Klebsiella pneumoniae* (CSKP) strains and 213 carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains were collected. The specimens comprised urine, sputum, bile, drainage fluid, puncture fluid, and blood. We found significant differences in the types of specimens between the two groups (detailed in **Figure 1**).

3.2 Detection of virulence genes and capsule genes

A total of 363 KP strains were examined for virulence genes. The positive rates of the 5 virulence genes (*rmpA*, *rmpA2*, *peg-344*, *iucA*, and *iroB*) in KP were 30.9, 34.2, 32.0, 41.9, and 27.3%, respectively. Further comparing the detection rates of virulence genes in carbapenem-susceptible KP (CSKP) and carbapenem-resistant KP (CRKP), we found that CSKP had significantly higher positive rates of genes *rmpA*, *peg-344*, and *iroB* compared to CRKP ($P < 0.01$), at rates of 44.1, 42.7, and 44.6%, respectively. However, there was no significant difference in the detection rates of genes *rmpA2* and *iucA* between the two groups ($P > 0.05$). Additionally, the detection rate of gene *ybts* in CSKP was 47.9%, significantly lower than that in CRKP ($P < 0.01$). Furthermore, CSKP and CRKP also showed significant differences in the detection rates of other virulence genes including *magA*, *aerobactin*, *alls*, *kfu*, and *entB* ($P < 0.01$) (**Table 1**).

For capsule genotypes, we found the number of K1, K2, K5, K20, K54, and K57 types were (35)9.6%, (21) 5.8%, (27)7.4%, (8) 2.2%, (8) 2.2%, and (12)3.3%, respectively. Furthermore, comparing the capsule genotypes between CSKP and CRKP, we found the detection rates of K1, K2, and K54 types in CSKP were significantly higher than in CRKP (**Table 1**).

We analyzed the distribution and correlation of virulence genes (**Figure 2**) and found that in CSKP, the genes *rmpA*, *rmpA2*, *iucA*, *iroB*, *peg-344*, and *aerobactin* tended to coexist. Additionally, the *iutA* gene showed a highly positive correlation with *rmpA*, *rmpA2*, *iucA*, *iroB*, and *aerobactin*. The *entB* gene showed a positive correlation with the *wabG* gene, and the K1 gene showed a positive correlation with the *magA* and *allS* genes. However, the K non-typeable gene showed a negative correlation with the *rmpA*, *iroB*, and *aerobactin* genes. In CRKP, the correlations among the genes were significantly reduced, the positive correlation was only found between the *iutA* and *iucA* genes, the *fimH* and *rmkD*, *wabG* genes, and between the *entB* and *wabG* genes.

3.3 Mucoid phenotype and virulent *Klebsiella pneumoniae*

Among the 363 strains of KP, 99 strains (27.3%) were identified as hypermucoviscous positive (hmKP). Among them, 90 strains (42.3%) in the CSKP group exhibited a high mucoviscosity

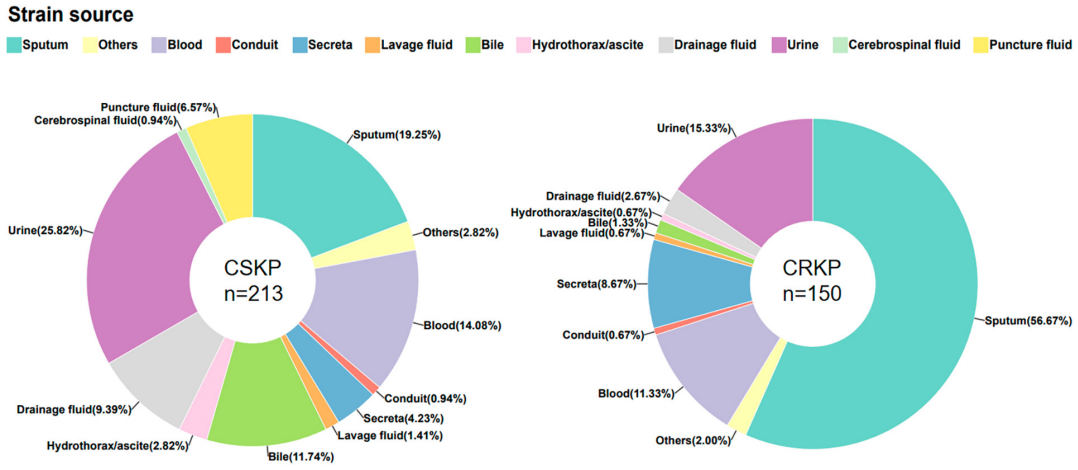

FIGURE 1
Source of CSKP and CRKP strains.

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TABLE 1 Detection rate of virulence and capsule genes.

Gene	CSKP [*n* (%)]	CRKP [*n* (%)]	*P*
rmpA	94 (44.1)	18 (12.0)	<0.001
rmpA2	74 (34.7)	50 (33.3)	0.781
iucA	95 (44.6)	57 (38.0)	0.209
iroB	97 (45.5)	2 (1.3)	<0.001
peg-344	91 (42.7)	25 (16.7)	<0.001
iutA	86 (40.4)	74 (49.3)	0.091
magA	32 (15.0)	0 (0)	<0.001
aerobactin	96 (45.1)	1 (0.7)	<0.001
ybts	102 (47.9)	113 (75.3)	<0.001
alls	44 (20.7)	2 (1.3)	<0.001
rmkD	185 (86.9)	137 (91.3)	0.184
fimH	200 (93.9)	138 (92.0)	0.482
kfu	81 (38.0)	27 (18.0)	<0.001
entB	206 (96.7)	134 (89.3)	0.004
wabG	203 (95.3)	140 (93.3)	0.417
K1	35 (16.4)	0 (0.0)	<0.001
K2	20 (9.4)	1 (0.7)	<0.001
K5	19 (8.9)	8 (5.3)	0.200
K20	7 (3.3)	1 (0.7)	0.147*
K54	8 (3.8)	0 (0.0)	0.023*
K57	10 (4.7)	2 (1.3)	0.133*
K non-typeable	114 (53.5)	138 (92.0)	<0.001

*Represents: the Fisher's exact test (2-sided) is used.

phenotype, while only 9 strains (6.0%) in the CRKP group showed this phenotype, indicating a significant difference between the two groups ($P < 0.01$). We defined hvKP based on the detection of the *rmpA*, *rmpA2*, *peg-344*, *iucA*, and *iroB* genes. A total of 65 strains (17.9%) were determined as hvKP. Among them, the detection rate of CS-hvKP (30.0%) was significantly higher than that of CR-hvKP (0.7%) (Figure 3). At the same time, we divided the strains into sputum, blood, urine, and others sources (mostly sterile body fluids). The positive rate of hmKP was 22.2% in sputum, 36.2% in blood, 17.9% in urine and 35.7% in others isolates. The positive rate of blood isolates was similar to others isolates and higher than that of sputum and urine isolates. The detection of hvKP in each group also showed acacia results. The positive rate of hvKP was 13.5% in sputum, 21.3% in blood, 12.8% in urine and 25.0% in others isolates. The positive rate of blood isolates was similar to others isolates and higher than that of sputum and urine isolates (Figure 3).

3.4 MLST typing and correlation of virulence

Among the 363 strains of KP, the sequence type (ST) was successfully determined for 358. A total of 71 different ST types were detected. For CSKP, 58 different ST types were identified, with ST23 being the most common sequence type ($n = 27$, 12.7%), followed by ST65 ($n = 11$, 5.2%), ST268 ($n = 10$, 4.7%), and ST1764 ($n = 10$, 4.7%). On the other hand, for CRKP, 19 different ST types were identified, with ST11 being the most common sequence type ($n = 75$, 50.0%), followed by ST437 ($n = 19$, 15.2%) and ST268 ($n = 10$, 4.7%) (Figure 4).

Through examination of virulence characteristics and the sequence type (Figure 5), we observed that 98.5% of hvKP strains in this study were CSKP, while 1.5% were CRKP. Among hvKP strains, 65.65% were identified as hmKP. Additionally, the positive rate of capsule genes in hvKP was 95.38%, Among them, the detection rates of K1, K2, K5, K20, K54, and K57 were 47.69, 16.92, 6.15, 6.15, 4.62, and 13.85%, respectively.

Among hmKp isolates, the most common ST type was ST23 ($n = 26$, 26.3%), followed by ST412 ($n = 7$, 7.1%) and ST65 ($n = 5$, 5.1%). Among hvKP isolates, the most common ST type was also ST23 ($n = 27$, 41.5%), followed by ST412 ($n = 7$, 10.8%) and ST65 ($n = 5$, 7.7%). The ST types of these two groups showed a high degree of similarity. According to the classification of capsule serotypes, there were differences in the distribution of ST types among different capsule types. ST23 was primarily associated with K1 type (77.1%), while K2 type showed diversity, with ST65 being the predominant type (23.8%). K20 type was mainly associated with ST268 (62.5%) and ST368 (25.0%), while K54 type was mainly associated with ST29 (50.0%), and K57 type was primarily associated with ST412 (83.3%). More details can be found in Figure 5.

3.5 Relative expression levels of virulence genes

Based on the screening of virulence genes in this study, we selected genes *rmpA*, *rmpA2*, and *iutA*, which showed high positive rate in both CSKP and CRKP for RT-qPCR experiments (for detailed information on experimental strains, please refer to Supplementary Table 3). The results (Figure 6) showed significant differences in the expression of the same virulence genes among different strains. In CSKP, the expression level of the *rmpA* gene was significantly higher than that in CRKP ($P < 0.01$), followed by *rmpA2* ($P = 0.015$), while there was no significant difference in the expression of *iutA* ($P = 0.200$).

3.6 Pathogenicity test of KP strains using animal model

We studied the impact of different KP strains on larval survival rates in the model of *Galleria mellonella* larvae. Control strains included the negative control strain ATCC 700603, the positive control strain NTUH-K2044 (representing hvKP), and larvae injected with physiological saline. The survival rates of abovementioned controls were 90.0, 50.0, and 100% respectively. We also selected strains positive or negative for genes *rmpA*, *rmpA2*, and *iutA* for injection experiments (Supplementary Table 3). Compared to the control groups, we found significant differences in larval survival rates after injection

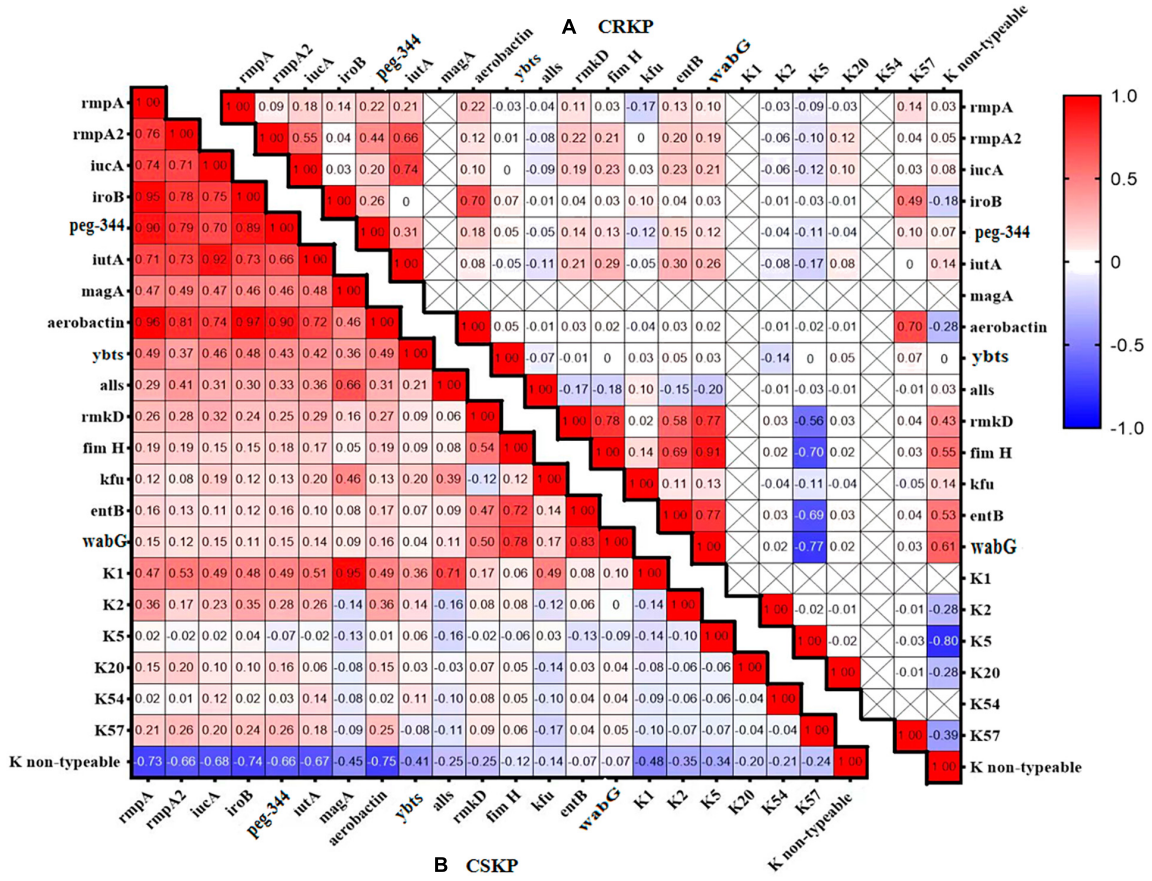

FIGURE 2
Heatmap of the distribution correlation of virulence genes. Panel **(A)** shows the correlation of virulence genes and capsule genes in CSKP, while Panel **(B)** shows the correlation in CRKP. The values in each square represent the correlation between the genes on the horizontal and vertical axes. Values closer to 1 indicate a stronger positive correlation, values closer to −1 indicate a stronger negative correlation, and values closer to 0 indicate no correlation. " × " indicates genes that were not detected and for which correlation cannot be calculated.

with different strains expressing virulence genes. Particularly, the larval survival rate after injection with *rmpA*-positive strain was 66.7%, significantly lower than *rmpA*-negative strain (90.0%) ($P = 0.005$) (Figure 7C). The larval survival rate after injection with *rmpA2*-positive strain was 55.8%, significantly lower than *rmpA2*-negative strain (89.0%) ($P < 0.001$) (Figure 7D). The larval survival rate after injection with *iutA*-positive strain was 62.5%, significantly lower than *iutA*-negative strain (93.3%) ($P < 0.001$) (Figure 7E). In hvKP strains, the larval survival rate after injection with CS-hvKP strain (13.3%) was significantly lower than CR-hvKP strain (70.0%) ($P = 0.003$) (Figure 7F).

4 Discussion

Klebsiella pneumoniae can cause infections of the respiratory tract, bloodstream, urinary tract, gastrointestinal tract, liver and gallbladder, and central nervous system. The site of infection may vary depending on the virulence and migratory ability of the strain. In this study, we found that CSKP was mainly isolated from urine, while CRKP was mainly isolated from sputum samples. Additionally, there were statistically significant differences in the percentage of the two strains among other samples, indicating that these two types of strains may differ in terms of virulence and pathogenicity.

To date, a gold standard for defining hvKP is still lacking. HvKP has been previously determined based on the hypermucoviscous phenotype of colonies (Li et al., 2014; Zhan et al., 2017; Russo and Marr, 2019) due to its association with the synthesis of capsular polysaccharides. Capsular polysaccharides can enhance the survival and migratory ability of KP by inhibiting the host's inflammatory response, leading to invasive infections. Our results showed that the detection rate of hypermucoviscous colonies in CSKP was 42.3%, while it was only 6.0% in CRKP. The positive rate of hvKP in blood (21.3%) was higher than that in sputum (13.5%) and urine (12.8%), indicating that blood isolates were more virulent than sputum and urine isolates. Soares de Moraes et al. (2022) proposed that *Klebsiella pneumoniae* of bloodstream infection was more virulent, and Li et al. (2023) found that the detection rate of virulence gene from blood isolates was higher than that of urine. These conclusions were consistent with our experimental results. It has been found that the virulence of KP is not solely dependent on its hypermucoviscosity (Zhang et al., 2015; Calfee, 2017). Our study showed that using hypermucoviscous as the criteria for hvKP resulted in a true positive rate of only 65.65%.

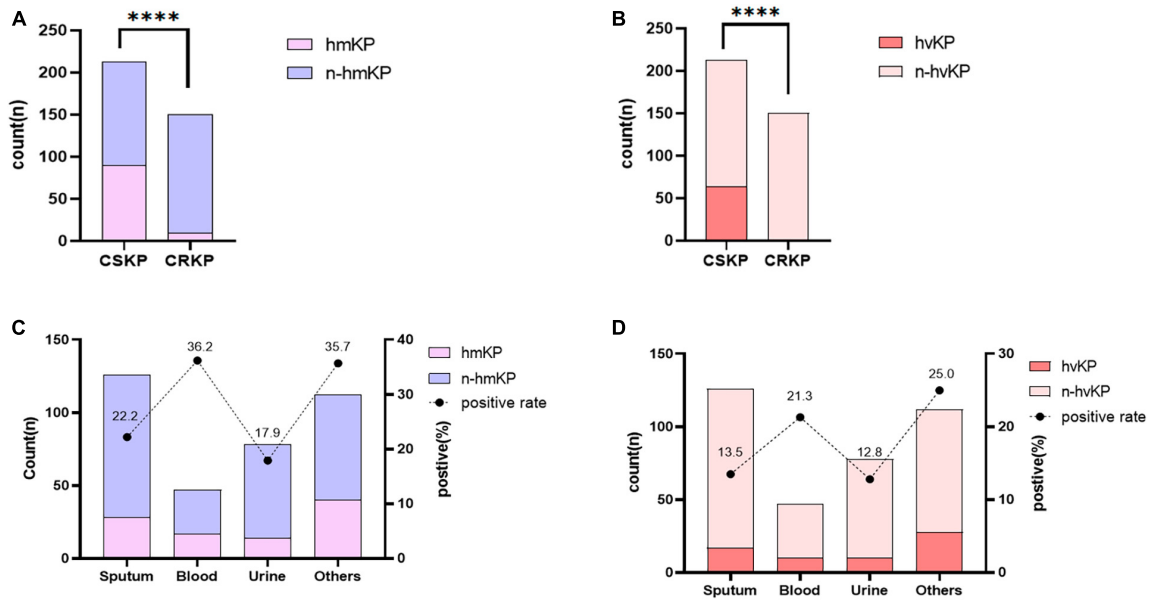

FIGURE 3
Differential distribution of high mucoid phenotype and high-virulence genes in different groups. Panel **(A)** shows the comparison of hmKP detection rates between CSKP and CRKP, while Panel **(B)** shows the comparison of hvKP detection rates. ****$P < 0.0001$. Panel **(C)** shows the comparison of hmKP detection rates between different sources, while Panel **(D)** shows the comparison of hvKP detection rates. The number indicates the positive rate of each group.

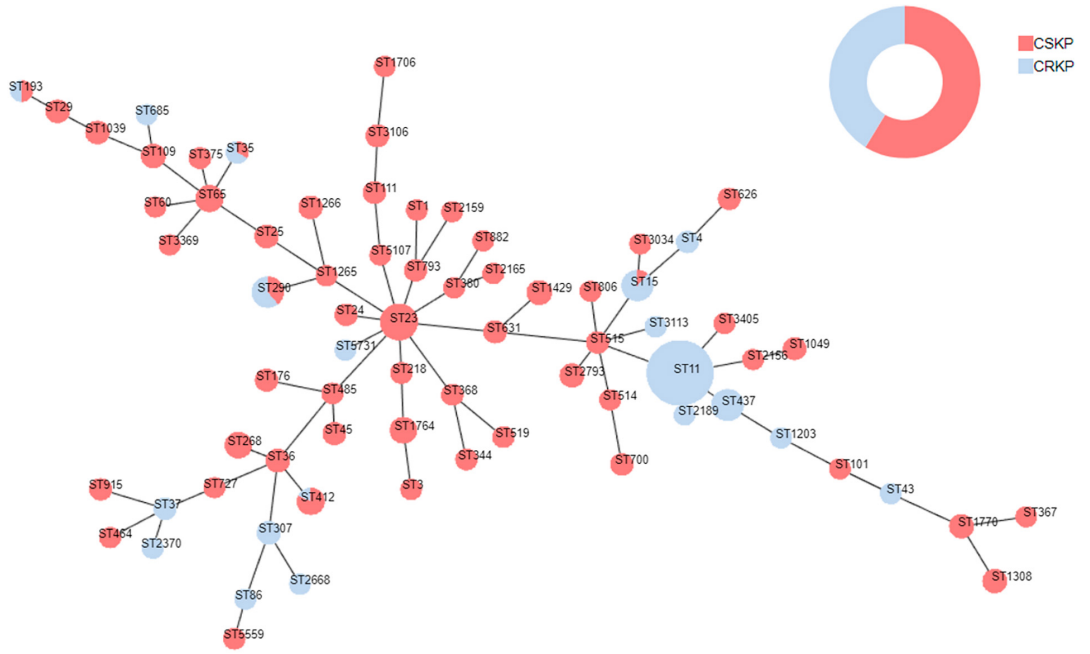

FIGURE 4
Minimum spanning tree of *Klebsiella pneumoniae*. Red represents CSKP, and blue represents CRKP. The minimum spanning tree is constructed using seven allelic genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, *tonB*) of *Klebsiella pneumoniae*. The size of the nodes is proportional to the number of isolates, and the length of the lines between nodes is proportional to the number of different alleles.

Previous work (Gu et al., 2018) indicated that pLVPK was an important virulence plasmid in hvKP, carrying various virulence genes. The absence of this plasmid reduces the virulence of hvKP strains. Therefore, researchers (Bialek-Davenet et al., 2014) have used genomic sequencing of the pLVPK plasmid to identify *rmpA*, *rmpA2*, *peg-344*, *iucA*, and *iroB* genes as molecular markers for hvKP, achieving an accuracy rate of over 95% (Russo and Marr, 2019). Among them, *rmpA* and *rmpA2* (Lin et al., 2020; Chen and Chen, 2021) are genes related to the regulation of mucoid phenotype and can regulate the synthesis and transcription of

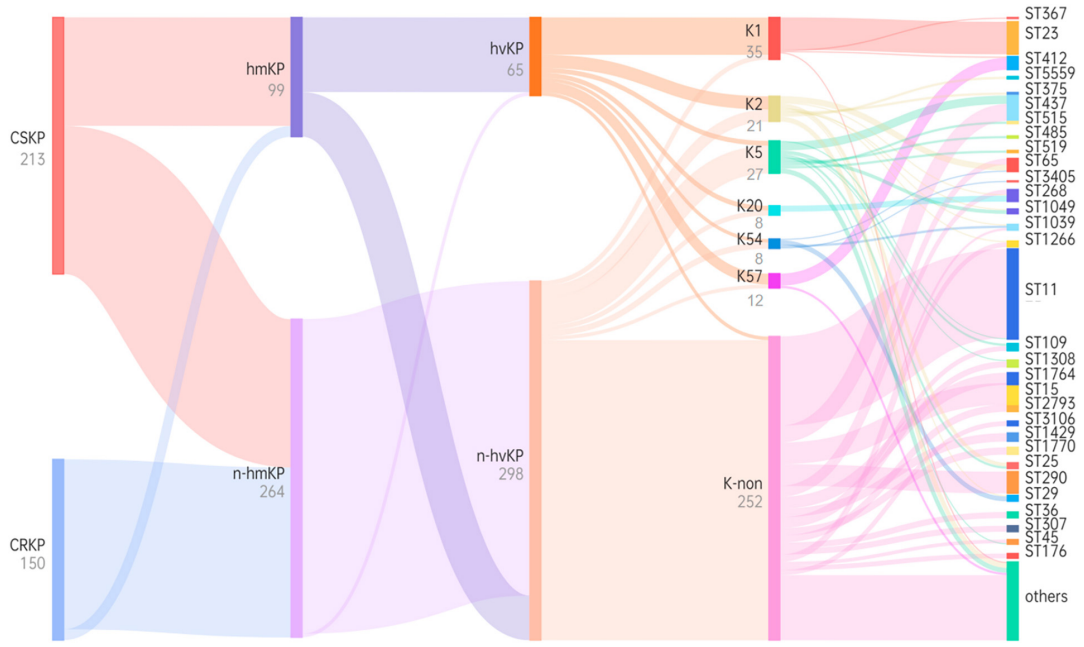

FIGURE 5
Phylogenetic tree based on virulence characteristics and ST typing. Black characters represent classifications, while gray characters represent quantities. The flow of bacterial strains is represented from **left** to **right**, and the thickness of the lines between nodes is proportional to the number of bacterial strains.

capsular polysaccharides, thickening the capsule and enhancing the virulence of the strain. *Peg-344* (Ye et al., 2016; Bulger et al., 2017) is a novel virulence factor that encodes an inner membrane transporter protein, the product of which is a permease in the superfamily of metabolite transporters. The *iucABCD* gene cluster encodes the aerobactin synthase, and the membrane protein receptor is encoded by the *iutA* gene. The *iroA* (*iroBCDN* gene cluster) encodes salmochelin (Paczosa and Mecsas, 2016; Palmer and Skaar, 2016; Nicolò et al., 2022), allowing bacteria to uptake sufficient iron for growth and reproduction. The results of this study showed that the positive rates of the *rmpA*, *peg-344*, and *iroB* genes in CSKP were 44.1, 42.7, and 45.5%, respectively, significantly higher than in CRKP, while there were no significant differences in the positive rates of the *rmpA2* and *iucA* genes. Moreover, the detection rates of genes such as *magA*, *aerobactin*, *alls*, *kfu*, and *entB* in CSKP were also higher than in CRKP. Capsule genes highly associated with hvKP included K1, K2, K5, K20, K54, and K57, and possess *rmpA*/*rmpA2* genes (Zhan et al., 2017; Russo et al., 2018; Yuan et al., 2019; Choby et al., 2020). This study found that in CSKP, the most common capsule types were K1, K2, and K5, while in CRKP, K5 was the main type, with significantly higher detection rates of K1, K2, and K54 in CSKP compared to CRKP.

By analyzing the correlation of virulence genes, we found that in CSKP, *rmpA*, *rmpA2*, *iucA*, *iroB*, *peg-344*, and *aerobactin* generally appeared together, and there was a high correlation among these virulence genes. However, in CRKP, the correlation between these genes was significantly reduced. This may be because these virulence genes participate in the expression of virulence and the pathogenic process, with the majority located on the same plasmids. Therefore, it can be speculated that the carriage of virulence plasmids may be more common in CSKP.

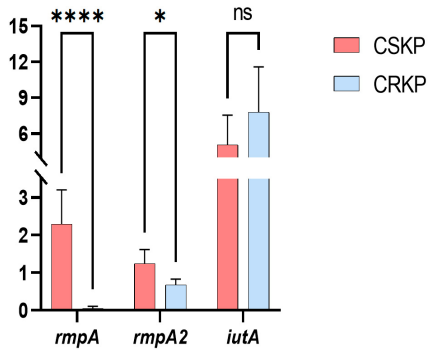

FIGURE 6
Differential gene expression efficiency of *rmpA*, *rmpA2*, and *iutA* in CSKP and CRKP. Using CSKP as a control, the differences in gene expression efficiency of CRKP in various genes are compared. ****$P < 0.0001$, *$P < 0.05$, and "ns" represents no statistically significant difference between the two groups.

To further clarify the homogeneity and spread of CSKP and CRKP in the local area, MLST typing was performed. In CRKP strains, the globally epidemic ST types are ST258 and ST11 (Liao et al., 2020a; Su et al., 2020; Wei et al., 2022), but their spread is regional (Han et al., 2022). In this study, 19 ST types were detected in CRKP strains, with the most common ST type being ST11 (75, 50.0%), but ST258 was not detected. Different strains are restricted to their distinct clonal lineages (Yin et al., 2023). In China, ST11 is the most common clone of CRKP, with a rate of 80.7% (Pu et al., 2023), and a previous study revealed that the majority of CR-hvKP strains belonged to the ST11 type (Zhang et al., 2020), which was consistented with our results. In CSKP, a total of 58

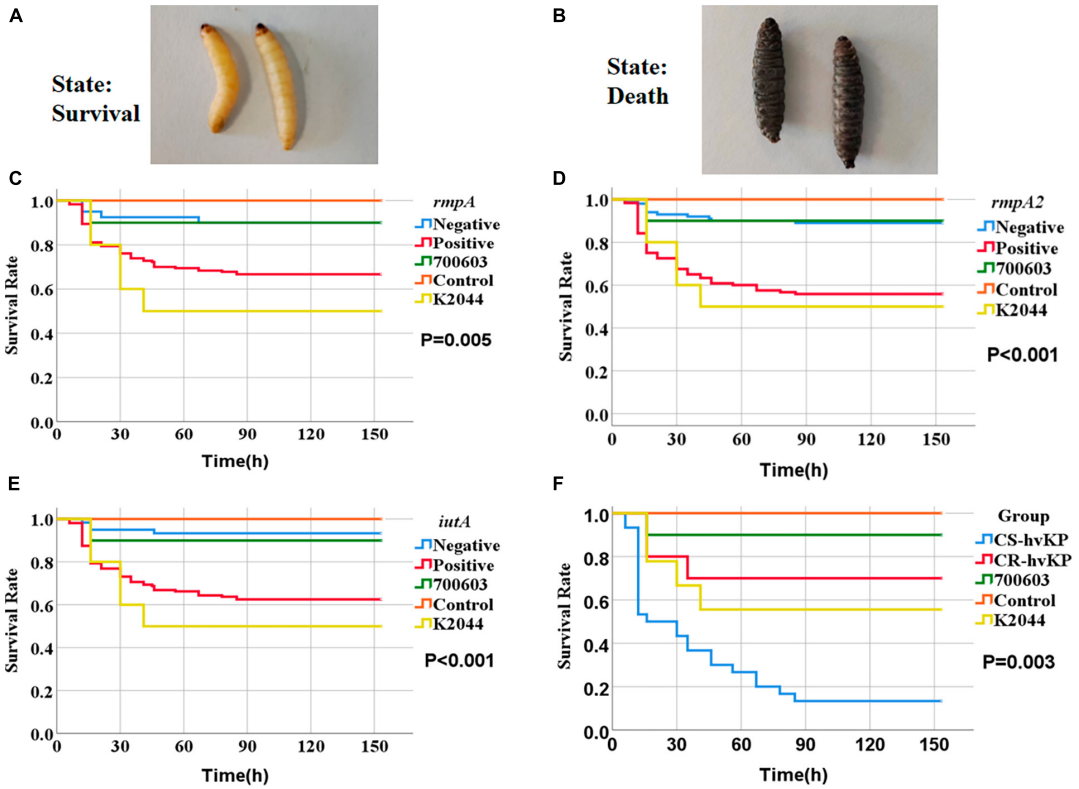

FIGURE 7
Survival curves of *Galleria mellonella* larvae in the infection model. The strains were derived from RT-qPCR consistently, as detailed in **Supplementary Table 3**. The criteria for determining larval mortality were blackening of the body and no response to touch. Panel **(A)** represents the living state of survival and panel **(B)** represents death. ATCC 700603 (shown as 700603), NTUH-K2044 (shown as K2044), and physiological saline control (shown as Control) in Panels **(C–E)** represent the survival rates of *Galleria mellonella* larvae at different time points with *rmpA*, *rmpA2*, and *iutA* gene-positive and gene-negative strains, respectively. The *P*-values indicate the significance between gene-positive and gene-negative strains in each case. Panel **(F)** shows the survival rates of *Galleria mellonella* larvae infected with CS-hvKP and CR-hvKP at different time points, with the *P*-value indicating the significance between the two groups.

ST types were detected, indicating significant diversity, but some epidemic strains were still observed. The most common ST type was ST23 (27, 12.7%). This is also the most common ST type in hvKP (Kocsis, 2023). Among the six common capsular serotype groups, we found that ST23 was mainly associated with K1 type (77.1%), while K2 type showed diversity and was mainly associated with ST65 (23.8%), K20 type was mainly associated with ST268 (62.5%) and ST368 (25.0%), K54 type was mainly associated with ST29 (50.0%), and K57 type was mainly associated with ST412 (83.3%), indicating clear clonal dissemination. This is consistent with the views of Kocsis (2023) and Li et al. (2023). Additionally, it was found that ST15, ST35, ST193, ST290, and ST412 types were detected in both CSKP and CRKP, which may be because the resistance of CRKP is mainly mediated by resistant plasmids, and as plasmids spread, it leads to differences in resistance among homologous strains. Similarly, transfer of virulence plasmids can occur between cKP and hvKP (Dong et al., 2019; Lam et al., 2019; Liao et al., 2020a; Han et al., 2022).

To further validate the virulence of CSKP and CRKP, we conducted RT-qPCR and a *Galleria mellonella* larvae infection model. Through RT-qPCR, we found that the relative expression level of the same virulence genes varied among different strains, with the most significant difference observed in *rmpA* gene between CSKP and CRKP ($P < 0.001$), followed by *rmpA2* ($P = 0.015$). In addition, Zhu et al. (2021b) constructed *blaKPC-2* transformants by expressing *blaKPC-2* in hvKP through *in vitro* transformation. In the *blaKPC-2* transformants, the relative expression levels of *rmpA* and *rmpA2* genes were approximately 50% of those in the wild-type strain, indicating that the expression level of virulence genes decreases after acquiring resistance. This may be related to the energy burden of bacteria, since the expression of resistant genes consumes energy, resulting in a reduction in the expression of virulence genes (Drancourt et al., 2001). Fursova et al. (2021) studied the bacterial resistance gene expression of multidrug-resistant KP (MDR-KP) and multidrug-resistant hvKP (MDR-hvKP) and found that the β-lactamase and carbapenemase genes of the former had higher expression levels, which also indicated that MDR-hvKP had a higher metabolic load due to multiple resistance and virulence factors (Mendes et al., 2023). Zhang Y. et al. (2021) studied the suitability of MDR-hvKPstrains after resistance plasmid and resistant plasmid, and found that the stability of resistant plasmid was higher. The results of the *Galleria mellonella* larvae infection model also supported this notion. We found that the survival rate of larvae infected with CS-hvKP strains was lower than that of larvae infected with CR-hvKP strains. Furthermore, the experimental results of the *Galleria mellonella* larvae infection model also revealed that the survival rate of larvae infected with strains positive for *rmpA*, *rmpA2*, and *iutA* genes was significantly

lower than that of larvae infected with strains negative for these genes. This further indicates that these genes play an important role in the expression of virulence of *Klebsiella pneumoniae*.

This study did not carry out drug resistance gene detection, could not combine carbapenem resistance phenotype with genes, and could not reflect the correlation between virulence genes and drug resistance genes, which has certain limitations. Further research will be carried out in the future.

5 Conclusion

In general, among clinically isolated *Klebsiella pneumoniae*, hmKP is more manifested as hvKP, which needs more attention. CSKP carries more virulence genes than CRKP, and the distribution of these genes in CSKP is relatively balanced. Most hvKP strains belong to CSKP, while there are fewer in CRKP. CRKP strains show significant homogeneity, with the predominant sequence type being ST11; whereas CSKP strains exhibit relative diversity, but there is still some evidence of clonal dissemination, with ST23 being the common type. There may be differences in virulence gene expression between hv-CSKP and hv-CRKP, and in the *Galleria mellonella* larvae model, the survival rate of hv-CSKP-infected larvae is lower than that of hv-CRKP-infected larvae.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Ethics statement

The manuscript presents research on animals that do not require Ethical approval for their study.

Author contributions

MJ: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. XQ: Investigation, Project administration, Supervision, Writing – original draft. SS: Conceptualization, Data curation, Investigation, Writing – original draft. RZ: Methodology, Writing – original draft. WL: Data curation, Investigation, Writing – original draft. CL: Writing – review and editing. YT: Funding acquisition, Resources, Writing – review and editing. YW: Funding acquisition, Resources, Writing – review and editing. QL: Methodology, Supervision, Writing – review and editing. QW: Funding acquisition, Resources, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1356229/full#supplementary-material

References

Asai, M., Li, Y., Newton, S. M., Robertson, B. D., and Langford, P. R. (2023). Galleria mellonella-intracellular bacteria pathogen infection models: The ins and outs. *FEMS Microbiol. Rev.* 47:fuad011. doi: 10.1093/femsre/fuad011

Bialek-Davenet, S., Criscuolo, A., Ailloud, F., Passet, V., Jones, L., Delannoy-Vieillard, A. S., et al. (2014). Genomic definition of hypervirulent and multidrug-resistant *Klebsiella pneumoniae* clonal groups. *Emerg. Infect. Dis.* 20, 1812–1820. doi: 10.3201/eid2011.140206

Bulger, J., Macdonald, U., Olson, R., Beanan, J., and Russo, T. A. (2017). Metabolite transporter PEG344 is required for full virulence of Hypervirulent *Klebsiella pneumoniae* Strain hvKP1 after pulmonary but not subcutaneous challenge. *Infect. Immun.* 85:e00093-17. doi: 10.1128/iai.00093-17

Calfee, D. P. (2017). Recent advances in the understanding and management of *Klebsiella pneumoniae*. *F1000Res* 6:1760. doi: 10.12688/f1000research.11532.1

Catalán-Nájera, J. C., Garza-Ramos, U., and Barrios-Camacho, H. (2017). Hypervirulence and hypermucoviscosity: Two different but complementary *Klebsiella spp. phenotypes*? *Virulence* 8, 1111–1123. doi: 10.1080/21505594.2017.1317412

Chang, D., Sharma, L., Dela Cruz, C. S., and Zhang, D. (2021). Clinical epidemiology, risk factors, and control strategies of *Klebsiella pneumoniae* infection. *Front. Microbiol.* 12:750662. doi: 10.3389/fmicb.2021.750662

Chen, Y., and Chen, Y. (2021). Clinical challenges with hypervirulent *Klebsiella Pneumoniae* (hvKP) in China. *J. Transl. Int. Med.* 9, 71–75. doi: 10.2478/jtim-2021-0004

Choby, J. E., Howard-Anderson, J., and Weiss, D. S. (2020). Hypervirulent *Klebsiella pneumoniae* - clinical and molecular perspectives. *J. Intern. Med.* 287, 283–300. doi: 10.1111/joim.13007

Dai, P., and Hu, D. (2022). The making of hypervirulent *Klebsiella pneumoniae*. *J. Clin. Lab. Anal.* 36:e24743. doi: 10.1002/jcla.24743

Dong, N., Sun, Q., Huang, Y., Shu, L., Ye, L., Zhang, R., et al. (2019). Evolution of carbapenem-resistant serotype K1 hypervirulent *Klebsiella pneumoniae* by acquisition of bla(VIM-1)-bearing plasmid. *Antimicrob. Agents Chemother.* 63:e01056-19. doi: 10.1128/aac.01056-19

Drancourt, M., Bollet, C., Carta, A., and Rousselier, P. (2001). Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. *Int. J. Syst. Evol. Microbiol.* 51, 925–932. doi: 10.1099/00207713-51-3-925

Fursova, A. D., Fursov, M. V., Astashkin, E. I., Novikova, T. S., Fedyukina, G. N., Kislichkina, A. A., et al. (2021). Early response of antimicrobial resistance and virulence genes expression in classical, hypervirulent, and hybrid hvKp-MDR *Klebsiella pneumoniae* on antimicrobial stress. *Antibiotics* 11:7. doi: 10.3390/antibiotics11010007

Gu, D., Dong, N., Zheng, Z., Lin, D., Huang, M., Wang, L., et al. (2018). A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital: A molecular epidemiological study. *Lancet Infect. Dis.* 18, 37–46. doi: 10.1016/s1473-3099(17)30489-9

Han, Y. L., Wen, X. H., Zhao, W., Cao, X. S., Wen, J. X., Wang, J. R., et al. (2022). Epidemiological characteristics and molecular evolution mechanisms of carbapenem-resistant hypervirulent *Klebsiella pneumoniae*. *Front. Microbiol.* 13:1003783. doi: 10.3389/fmicb.2022.1003783

Kocsis, B. (2023). Hypervirulent *Klebsiella pneumoniae*: An update on epidemiology, detection and antibiotic resistance. *Acta Microbiol. Immunol. Hung.* 70, 278–287. doi: 10.1556/030.2023.02186

Kuo, G., Yen, C. L., Lu, Y. A., Chen, C. Y., Sun, M. H., Lin, Y., et al. (2022). Clinical and visual outcomes following endogenous endophthalmitis: 175 consecutive cases from a tertiary referral center in Taiwan. *J. Microbiol. Immunol. Infect.* 55, 114–122. doi: 10.1016/j.jmii.2021.01.010

Lam, M. M. C., Wyres, K. L., Wick, R. R., Judd, L. M., Fostervold, A., Holt, K. E., et al. (2019). Convergence of virulence and MDR in a single plasmid vector in MDR *Klebsiella pneumoniae* ST15. *J. Antimicrob Chemother.* 74, 1218–1222. doi: 10.1093/jac/dkz028

Li, H. F., Zhang, L. X., Zhang, W. L., Li, J., Li, Y. Q., and Hu, T. P. (2023). Study on virulence genes, drug resistance and molecular epidemiology of *Klebsiella pneumoniae* with high virulence in inner Mongolia, China. *Infect. Drug Resist.* 16, 1133–1144. doi: 10.2147/idr.S391468

Li, W., Sun, G., Yu, Y., Li, N., Chen, M., Jin, R., et al. (2014). Increasing occurrence of antimicrobial-resistant hypervirulent (hypermucoviscous) *Klebsiella pneumoniae* isolates in China. *Clin. Infect. Dis.* 58, 225–232. doi: 10.1093/cid/cit675

Liao, W., Liu, Y., and Zhang, W. (2020a). Virulence evolution, molecular mechanisms of resistance and prevalence of ST11 carbapenem-resistant *Klebsiella pneumoniae* in China: A review over the last 10 years. *J. Glob. Antimicrob Resist.* 23, 174–180. doi: 10.1016/j.jgar.2020.09.004

Liao, W., Long, D., Huang, Q., Wei, D., Liu, X., Wan, L., et al. (2020b). Rapid detection to differentiate hypervirulent *Klebsiella pneumoniae* (hvKp) from classical *K. pneumoniae* by Identifying peg-344 With Loop-Mediated Isothermal Amplication (LAMP). *Front. Microbiol.* 11:1189. doi: 10.3389/fmicb.2020.01189

Lin, Z. W., Zheng, J. X., Bai, B., Xu, G. J., Lin, F. J., Chen, Z., et al. (2020). Characteristics of hypervirulent *Klebsiella pneumoniae*: Does low expression of rmpA contribute to the absence of hypervirulence? *Front. Microbiol.* 11:436. doi: 10.3389/fmicb.2020.00436

Ménard, G., Rouillon, A., Cattoir, V., and Donnio, P. Y. (2021). Galleria mellonella as a suitable model of bacterial infection: Past, present and future. *Front. Cell Infect. Microbiol.* 11:782733. doi: 10.3389/fcimb.2021.782733

Mendes, G., Santos, M. L., Ramalho, J. F., Duarte, A., and Caneiras, C. (2023). Virulence factors in carbapenem-resistant hypervirulent *Klebsiella pneumoniae*. *Front. Microbiol.* 14:1325077. doi: 10.3389/fmicb.2023.1325077

Nicolò, S., Mattiuz, G., Antonelli, A., Arena, F., Di Pilato, V., Giani, T., et al. (2022). Hypervirulent *Klebsiella pneumoniae* strains modulate human dendritic cell functions and affect T(H)1/T(H)17 response. *Microorganisms* 10:384. doi: 10.3390/microorganisms10020384

Paczosa, M. K., and Mecsas, J. (2016). *Klebsiella pneumoniae*: Going on the offense with a strong defense. *Microbiol. Mol. Biol. Rev.* 80, 629–661. doi: 10.1128/mmbr.00078-15

Palmer, L. D., and Skaar, E. P. (2016). Transition metals and virulence in bacteria. *Annu. Rev. Genet.* 50, 67–91. doi: 10.1146/annurev-genet-120215-035146

Pu, D., Zhao, J., Chang, K., Zhuo, X., and Cao, B. (2023). "Superbugs" with hypervirulence and carbapenem resistance in *Klebsiella pneumoniae*: The rise of such emerging nosocomial pathogens in China. *Sci. Bull.* 68, 2658–2670. doi: 10.1016/j.scib.2023.09.040

Russo, T. A., and Marr, C. M. (2019). Hypervirulent *Klebsiella pneumoniae*. *Clin Microbiol Rev* 32:e00001-19. doi: 10.1128/cmr.00001-19

Russo, T. A., Olson, R., Fang, C. T., Stoesser, N., Miller, M., Macdonald, U., et al. (2018). Identification of biomarkers for differentiation of hypervirulent *Klebsiella pneumoniae* from classical *K. pneumoniae*. *J. Clin. Microbiol.* 56:e00776-18. doi: 10.1128/jcm.00776-18

Soares de Moraes, L., Gomes Magalhaes, G. L., Material Soncini, J. G., Pelisson, M., Eches Perugini, M. R., and Vespero, E. C. (2022). High mortality from carbapenem-resistant *Klebsiella pneumoniae* bloodstream infection. *Microb. Pathog.* 167:105519. doi: 10.1016/j.micpath.2022.105519

Su, S., Zhang, J., Zhao, Y., Yu, L., Wang, Y., Wang, Y., et al. (2020). Outbreak of KPC-2 Carbapenem-resistant *Klebsiella pneumoniae* ST76 and Carbapenem-resistant K2 Hypervirulent *Klebsiella pneumoniae* ST375 strains in Northeast China: Molecular and virulent characteristics. *BMC Infect. Dis.* 20:472. doi: 10.1186/s12879-020-05143-y

Wang, G., Zhao, G., Chao, X., Xie, L., and Wang, H. (2020). The characteristic of virulence, biofilm and antibiotic resistance of *Klebsiella pneumoniae*. *Int. J. Environ. Res. Public Health* 17:6278. doi: 10.3390/ijerph17176278

Wei, T., Zou, C., Qin, J., Tao, J., Yan, L., Wang, J., et al. (2022). Emergence of hypervirulent ST11-K64 *Klebsiella pneumoniae* poses a serious clinical threat in older patients. *Front. Public Health* 10:765624. doi: 10.3389/fpubh.2022.765624

Yang, Q., Jia, X., Zhou, M., Zhang, H., Yang, W., Kudinha, T., et al. (2020). Emergence of ST11-K47 and ST11-K64 hypervirulent carbapenem-resistant *Klebsiella pneumoniae* in bacterial liver abscesses from China: A molecular, biological, and epidemiological study. *Emerg. Microbes Infect.* 9, 320–331. doi: 10.1080/22221751.2020.1721334

Ye, M., Tu, J., Jiang, J., Bi, Y., You, W., Zhang, Y., et al. (2016). Clinical and genomic analysis of liver abscess-causing *Klebsiella pneumoniae* identifies new liver abscess-associated virulence genes. *Front. Cell Infect. Microbiol.* 6:165. doi: 10.3389/fcimb.2016.00165

Yin, L., Yan, G., Lu, L., He, L., Wu, N., Cao, Y., et al. (2023). Molecular characteristics and virulence factors of carbapenem-resistant *Klebsiella pneumoniae* among pediatric patients in Shanghai, China. *Infect. Genet. Evol.* 112:105451. doi: 10.1016/j.meegid.2023.105451

Yuan, Y., Li, Y., Wang, G., Li, C., Chang, Y. F., Chen, W., et al. (2019). bla(NDM-5) carried by a hypervirulent *Klebsiella pneumoniae* with sequence type 29. *Antimicrob Resist. Infect. Control* 8:140. doi: 10.1186/s13756-019-0596-1

Zhan, L., Wang, S., Guo, Y., Jin, Y., Duan, J., Hao, Z., et al. (2017). Outbreak by hypermucoviscous *Klebsiella pneumoniae* ST11 Isolates with carbapenem resistance in a Tertiary Hospital in China. *Front. Cell Infect. Microbiol.* 7:182. doi: 10.3389/fcimb.2017.00182

Zhang, H., Zhang, G., Yang, Y., Zhang, J., Li, D., Duan, S., et al. (2021). Antimicrobial resistance comparison of *Klebsiella pneumoniae* pathogens isolated from intra-abdominal and urinary tract infections in different organs, hospital departments and regions of China between 2014 and 2017. *J Microbiol. Immunol. Infect.* 54, 639–648. doi: 10.1016/j.jmii.2020.03.009

Zhang, Y., Wang, X., Wang, Q., Chen, H., Li, H., Wang, S., et al. (2021). Emergence of tigecycline nonsusceptible and IMP-4 carbapenemase-producing K2-ST65 hypervirulent *Klebsiella pneumoniae* in China. *Microbiol. Spectr.* 9:e0130521. doi: 10.1128/Spectrum.01305-21

Zhang, Y., Jin, L., Ouyang, P., Wang, Q., Wang, R., Wang, J., et al. (2020). Evolution of hypervirulence in carbapenem-resistant *Klebsiella pneumoniae* in China: A multicentre, molecular epidemiological analysis. *J. Antimicrob. Chemother.* 75, 327–336. doi: 10.1093/jac/dkz446

Zhang, Y., Zeng, J., Liu, W., Zhao, F., Hu, Z., Zhao, C., et al. (2015). Emergence of a hypervirulent carbapenem-resistant *Klebsiella pneumoniae* isolate from clinical infections in China. *J. Infect.* 71, 553–560. doi: 10.1016/j.jinf.2015.07.010

Zhou, K., Xiao, T., David, S., Wang, Q., Zhou, Y., Guo, L., et al. (2020). Novel subclone of carbapenem-resistant *Klebsiella pneumoniae* sequence type 11 with enhanced virulence and transmissibility, China. *Emerg. Infect. Dis.* 26, 289–297. doi: 10.3201/eid2602.190594

Zhu, J., Wang, T., Chen, L., and Du, H. (2021a). Virulence factors in hypervirulent *Klebsiella pneumoniae*. *Front. Microbiol.* 12:642484. doi: 10.3389/fmicb.2021.642484

Zhu, J., Wei, Q., Shen, Z., Yuan, T., and Li, M. (2021b). Effect of KPC-2 type carbapenemase on the virulence of hypervirulent *Klebsiella pneumoniae*. *Chin. J. Infect. Chemother.* 21, 697–702. doi: 10.16718/j.1009-7708.2021.06.011

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Transcriptomic analysis using RNA sequencing and phenotypic analysis of *Salmonella enterica* after acid exposure for different time durations using adaptive laboratory evolution

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Introduction: This study is the final part of a two-part series that delves into the molecular mechanisms driving adaptive laboratory evolution (ALE) of *Salmonella enterica* in acid stress. The phenotypic and transcriptomic alterations in the acid-evolved lineages (EL) of *Salmonella enterica* serovar Enteritidis after 70 days of acid stress exposure were analyzed.

Materials and methods: The stability of phenotypic changes observed after 70 days in acetic acid was explored after stress removal using a newly developed evolutionary lineage EL5. Additionally, the impact of short-term acid stress on the previously adapted lineage EL4 was also examined.

Results: The results indicate that the elevated antibiotic minimum inhibitory concentration (MIC) observed after exposure to acetic acid for 70 days was lost when acid stress was removed. This phenomenon was observed against human antibiotics such as meropenem, ciprofloxacin, gentamicin, and streptomycin. The MIC of meropenem in EL4 on day 70 was 0.094 mM, which dropped to 0.032 mM when removed from acetic acid stress after day 70. However, after stress reintroduction, the MIC swiftly elevated, and within 4 days, it returned to 0.094 mM. After 20 more days of adaptation in acetic acid, the meropenem MIC increased to 0.125 mM. The other human antibiotics that were tested exhibited a similar trend. The MIC of acetic acid in EL4 on day 70 was observed to be 35 mM, which remained constant even after the removal of acetic acid stress. Readaptation of EL4 in acetic acid for 20 more days caused the acetic acid MIC to increase to 37 mM. Bacterial whole genome sequencing of EL5 revealed base substitutions in several genes involved in pathogenesis, such as the *phoQ* and *wzc* genes. Transcriptomic analysis of EL5 revealed upregulation of virulence, drug resistance, toxin-antitoxin, and iron metabolism genes. Unstable *Salmonella* small colony variants (SSCV) of *S.* Enteritidis were also observed in EL5 as compared to the wild-type unevolved *S.* Enteritidis.

Discussion: This study presents a comprehensive understanding of the evolution of the phenotypic, genomic, and transcriptomic changes in *S.* Enteritidis due to prolonged acid exposure through ALE.

KEYWORDS

***Salmonella enterica* serovar Enteritidis, acetic acid stress, adaptive laboratory evolution (ALE), minimum inhibitory concentration (MIC), human antibiotics, mutations, genome sequencing, RNA sequencing**

1 Introduction

Understanding how microorganisms adapt to harsh environmental conditions is paramount, particularly for food-borne pathogens such as *Salmonella enterica,* which pose significant threats to public health worldwide (Brown et al., 2021). Acid stress represents one such critical environmental challenge for enteric pathogens such as *Salmonella* Enteritidis, which encounter highly acidic conditions in external environments and within the host gastrointestinal tract (He et al., 2018; Kenney, 2019). During food processing, *S.* Enteritidis is exposed to acidic conditions commonly used for food preservation, flavoring, and fermentation (He et al., 2018). Additionally, *S.* Enteritidis present on food contact surfaces is often exposed to acidic cleaning agents such as organic acids (Aljumaah et al., 2020).

Analyzing how *S.* Enteritidis navigates these acidic environments is pivotal for safety and control strategies. As part of an ongoing investigation into the adaptive responses of *S.* Enteritidis to acid stress, this study constitutes the second phase of our two-part series, focusing on the comprehensive analysis of phenotypic and transcriptomic changes following adaptive laboratory evolution (ALE) of *S.* Enteritidis in acid stress. ALE entails the iterative cultivation of microbial populations under controlled stress conditions, thereby simulating natural selection in a laboratory setting (Sandberg et al., 2019). By subjecting *S.* Enteritidis to prolonged acid stress using ALE, we aim to unearth the intricate dynamics of its adaptive strategies.

In Part 1 of this series, we established the groundwork by introducing ALE as a powerful tool for investigating the adaptation of *S.* Enteritidis to acid stress. We delved into genotypic and phenotypic transformations of *S.* Enteritidis during prolonged exposure to acetic acid. ALE led to the generation of four acid-evolved lineages (EL) in our previous study, which were named EL1-EL4 (Ghoshal et al., 2023). We studied the changes in the growth rates of the ELs after prolonged and continuous exposure to acetic acid. We analyzed the changes in MIC against acetic acid and the concomitant changes in the MIC of human antibiotics in the ELs. Additionally, the presence of mutations in the form of base substitutions, insertions, and deletions was also explored, providing insights into the genetic basis of acid stress adaptation for 70 days using ALE. Building upon these findings, Part 2 of this series delves deeper into the molecular mechanisms that underpin the adaptive responses in *S.* Enteritidis through an integrated study of phenotypic transformations and transcriptomic alterations during ALE. The phenotypic changes serve as tangible manifestations of the bacterium's altered genome and physiology, while the transcriptomic shifts provide a window into the underlying gene expression patterns that drive these adaptations.

This study applies a multi-pronged approach, where we analyzed the stability of the phenotypic changes observed in the previous study (Ghoshal et al., 2023) after the removal and subsequent reintroduction of the acid stress. The ALE process was continued in EL4, and we worked on the development of another evolutionary lineage, EL5. We studied the transcriptomic changes in ELs after long-term continuous adaptation as well as the effects of short-term acid stress on the previously acid-adapted ELs.

Our goal is to present a holistic narrative of the journey of *S.* Enteritidis through adaptive evolution under acid-stress conditions, using the combined efforts of Part 1 and Part 2 of this series. By exploring both the phenotypic and molecular facets of adaptation (genetic and transcriptomic), we aspire to deepen our understanding of strategies employed by *S.* Enteritidis for survival and uncover novel perspectives for managing and combating its pathogenic effects. The culmination of this two-part series aims to provide a comprehensive resource for researchers and scientists delving into bacterial stress response and bacterial evolution under stress.

2 Materials and methods

2.1 Bacterial isolates and growth media

For this study, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC BAA 1045, phage type 30) was used. Frozen stocks of the bacteria were stored at −80°C in trypticase soy broth (TSB; Sigma-Aldrich) supplemented with 15% glycerol. To revive the frozen *Salmonella* cultures, they were streaked onto tryptic soy agar (TSA; Sigma-Aldrich) and then incubated at 37°C for 18–20 h. Subsequently, single bacterial colonies were streaked onto TSA, and isolated colonies were then cultured in TSB overnight for 18 h.

2.2 Quantification of minimum inhibitory concentration (MIC) of acetic acid

The MIC of acetic acid against the evolutionary lineages of *S.* Enteritidis was determined using the broth dilution method (Wiegand et al., 2008) with minor modifications. Briefly, in 50 mL tubes, bacterial inoculum taken from the evolutionary lineages (EL) of 10^7-10^8 CFU/mL was added in 20 mL TSB containing acetic acid concentrations from 20 to 40 mM. These tubes were incubated under agitation at 37°C for ∼18 h. As control measures, bacterial cultures were also introduced into TSB containing 200 mM acetic acid (negative control) and TSB without acetic acid (positive control). The lowest concentration of acetic acid that completely inhibited visible bacterial growth and exhibited $OD_{600} \leq 0.1$ using the spectrophotometer was defined as MIC. This procedure was replicated three times for each lineage. The MIC of acetic acid for the ELs was assessed every 5 days during the ALE study.

2.3 Adaptive laboratory evolution of *S.* Enteritidis in acetic acid

Adaptive laboratory evolution of *S.* Enteritidis in acid stress was performed by continuing the method described in our previous study with some additions (Ghoshal et al., 2023). Briefly, at the beginning of the ALE study, MIC of WT (wild type) *S.* Enteritidis was quantified, and an inoculum of 10^7-10^8 CFU/mL was added to three 50 mL conical tubes containing 20 mL of TSB to generate the three replicates of EL1. Inoculum from the WT was also added to three tubes containing 26 mM acetic acid (sub-MIC acetic acid; MIC acetic acid for WT was 27 mM) in TSB, which served as the three replicates of EL2. The six tubes were incubated at 37°C for 18–20 h under shaking. After 20 h, the OD_{600} of the six tubes

was measured and recorded, and an inoculum of 10^7-10^8 CFU/mL from each tube was serially transferred into six new tubes for day 2. This process was repeated till day 70 of the ALE study, and a similar inoculum was transferred to fresh tubes for each of the ELs daily. Samples from the previous day were stored at −80°C over the course of the evolutionary process. The number of bacterial generations "n" was calculated using the following equation (Lee et al., 2011):

$$n = log(N/N_0)/log(2)$$

where N is the final number of cells in the conical tube after 20 h at the time of passage to the next day's tubes. N_0 is the initial number of cells that are transferred to each conical tube at the beginning of ALE for that day. The initial and final numbers of cells were estimated daily by measuring the OD_{600} with a spectrophotometer and using plate counts. The generation number calculation assumes that each cell is viable, the death rate is negligible, the cells are growing exponentially throughout the ALE experiment, and the cells are dividing by binary fission. The MIC of acetic acid and several antibiotics was quantified against all three replicates of EL1-EL4 every 5 days.

After ALE day 70, the continuous adaptation of evolutionary lineages EL1-EL4 was halted, and the ELs were frozen at −80°C. EL4 was recovered from freezing and adapted in TSB without acid stress for 48 h with a serial transfer at 20 h. This culture of EL4 was denoted as EL4-FR (EL4 frozen and recovered without stress). Subsequently, EL4-FR was exposed to 30 mM acetic acid for 20 h, and this culture was termed EL4-A2 (EL4 that had undergone a second exposure to acid stress). EL4-FR (three replicates) was also reintroduced into 30 mM acetic acid and adapted through the ALE process for 20 more days, and this culture was denoted as EL5. The MIC of acetic acid and several antibiotics was quantified against all three replicates of EL5 every 5 days until ALE day 90.

2.4 Quantification of MIC of antibiotics against *S.* Enteritidis evolutionary lineages

The MIC of the antibiotics meropenem, ciprofloxacin, gentamycin, and streptomycin were determined using MTS strips (MIC test strips, Liofilchem) (Matuschek et al., 2018; van den Bijllaardt et al., 2018). The test strips for each antibiotic had a concentration range of 0.016–256 μg/mL, and MIC was determined using the method previously described (Ghoshal et al., 2023). Briefly, overnight cultures of the various evolutionary lineages were cultivated using their respective acetic acid concentrations. The following day, OD_{600} was adjusted to ∼10^7CFU/mL in TSB. Bacterial cultures were applied onto cotton swab applicators and streaked onto TSA plates to establish uniform bacterial growth. The MTS test strips were then meticulously positioned at the center of the TSA plates using sterile forceps. Subsequently, the plates were incubated at 37°C for 18 h−20 h, and the zones of inhibition encircling the test strips were documented. The point where the zone of inhibition intersected the MIC test strip was designated as the MIC. This procedure was replicated three times for each replicate in all evolutionary lineages.

2.5 Bacterial whole genome sequencing

Genomic DNA extraction and sequencing were performed at Seqcenter (Pittsburgh, PA, USA). Illumina sequencing of the WT was conducted on a NextSeq 2000 sequencer, generating 151-bp paired-end reads. Afterward, low-quality reads were trimmed, and adapter sequences were eliminated from Illumina sequences using bcl-convert version 3.9.3. In addition, long sequence reads were generated through Oxford Nanopore (ONT) PCR-free ligation library preparation. These ONT reads were subject to trimming of adapters and quality assessment using porechop33 version 0.2.3_seqan2.1.1 (RRID:SCR_016967). A hybrid assembly, incorporating both Illumina and ONT reads, was generated using Unicycler version 0.4.8. The quality of the assembly was evaluated using QUAST version 5.0.2 (Gurevich et al., 2013). Subsequently, the complete genome assembly was characterized, and plasmid sequences were identified through the NCBI Nucleotide BLAST database. Gene models were predicted, and functional annotations were added using Prokka version 1.14.5 with the default parameters along with "—rfam" (Tatusova et al., 2016; Haft et al., 2018). For EL4 and EL5, the same culturing and DNA extraction procedure as that of the WT strain was employed. However, for the evolved lineages, only Illumina sequencing was used, generating 151-bp paired-end libraries following the aforementioned steps.

2.6 Genome assembly and identification of polymorphism

Genomic analysis of EL5 from ALE day 90 was performed using the methods from our previous study (Ghoshal et al., 2023). The genomes of EL5 on ALE day 90 and EL4 on ALE day 70 were mapped to that of the WT genome. All software mentioned below was used with default parameters unless specified otherwise. BWA version 0.7.15 (RRID:SCR_010910) was used to map EL4 and EL5 to the ancestral genome (Li, 2013). Samtools (v 1.14) (RRID:SCR_002105) was used to index the sorted BAM files, and bamaddrg was used to add read groups to the indexed, sorted BAM files (Danecek et al., 2021). Joint genotyping of the evolved strains was then performed using Freebayes (v 1.3.1) (RRID:SCR_010761) (Garrison and Marth, 2012). Low-quality site filtering was performed using VCFtools (v 0.1.14) (RRID:SCR_001235) with the following parameters: –remove-filtered-all –minQ 20 –min-meanDP 50. GATK (v. 4.0.6) (RRID:SCR_001876) was used to convert the resulting VCF files into table format (McKenna et al., 2010). A SnpEff database was built for the *S.* Enteritidis BAA-1045 WT reference genome, and SNP annotation prediction was performed in the evolved strains using SnpEff (v. 4.1) (Cingolani et al., 2012). A second variant calling software, Breseq (v. 0.35.4) (RRID:SCR_010810), was used by SeqCenter to align and compare evolved lineage sequence reads to the ancestor (Deatherage and Barrick, 2014). Assemblies were generated from the Illumina reads for each evolved lineage using SPAdes (v. 3.13.1) (RRID:SCR_000131) with the parameters "-k 21,33,55 –careful" (Bankevich et al., 2012).

2.7 Bacterial RNA sequencing

Samples were DNAse treated with Invitrogen DNAse (RNAse free). Library preparation was performed by SeqCenter (Pittsburgh, PA, USA) using Illumina's Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit and 10bp IDT for Illumina indices. Sequencing was done on a NovaSeq 6000 with 2 × 51bp reads. Demultiplexing, quality control, and adapter trimming were performed with bcl-convert (v4.0.3) (Illumina, 2021). EL5 was grown in 30 mM acetic acid until ALE day 90, while WT *S.* Enteritidis was exposed to 26 mM acetic acid (sub-MIC of acetic acid for WT) for 18 h for RNA sequencing. Further, EL4-FR was also exposed to 30 mM acetic acid for 18 h before RNA sequencing. This sample has been described from here on as simply EL4-A2 to denote that EL4 was exposed to acid for the second time. EL5, EL4-A2, and WT were also grown for 18 h in TSB without acid stress and served as controls. Three biological replicates for each of the samples were sequenced.

2.8 Bacterial RNA sequencing analysis

Read mapping was performed with HISAT2 (Kim et al., 2019), and read quantification was performed using Subread's feature Counts (Liao et al., 2014) functionality. Read counts were loaded into R (R Core Team, 2020) and were normalized using edgeR's (Robinson et al., 2010) Trimmed Mean of M values (TMM) algorithm. Subsequent values were then converted to counts per million (cpm). Differential expression analysis was performed using edgeR's exact test for differences between two groups of negative-binomial counts with an estimated dispersion value of 0.1. The results of the qlfTest for all genes, in addition to the normalized cpm, were generated. The differentially expressed gene's normalized cpm were then used to create a heatmap. Genome indexing and mapping were performed using bwa (v0.7.17) with default parameters. Samtools (v1.14) was used to sort and index BAM files. Bedtools2 (v2.30.0) was used to extract read counts per transcript from gene coordinates in the sorted BAM files. The DESeq2 (v.1.38.3) package was used in R (version 4.2.3 and RStudio 2023.03.1446) to normalize read counts and identify differentially expressed genes. Differential expression thresholds were defined using log-fold change [log2(FC)] values >2 (upregulated) and <-2 (downregulated) with a P_{Adj} value ≤0.01. Principal component analysis (PCA) was performed on DESeq2 normalized read counts using JMP Pro (version 17) to examine the relationship between samples and replicates. For comparing the expression of the different classes of genes, annotations were generated from EggNOG and Blast2GO within the OmicsBox software (Götz et al., 2008; Cantalapiedra et al., 2021).

2.9 Statistical analyses

All bacterial growth measurements were biologically triplicated, and their differences were examined by a two-sided *t*-test assuming unequal variance (Welch's *t*-test). GraphPad Prism was used to generate most of the graphs in this project. Statistical significance was calculated with GraphPad Prism using two-way ANOVA. A $p \leq 0.05$ was considered statistically significant. Fisher's exact test was used to calculate the statistical significance for the percentage of genes upregulated and downregulated as revealed in the RNA seq data.

3 Results

3.1 Adaptive evolutionary process of *S.* Enteritidis to acetic acid

Salmonella Enteritidis was subjected to different concentrations of acetic acid using adaptive laboratory evolution (ALE). The evolutionary lineages (EL) EL1-EL4 were adapted with daily transfers for 70 ALE days, and the ALE process till day 70 has been described in detail in our previous study (Ghoshal et al., 2023). Briefly, the minimum inhibitory concentration (MIC) of acetic acid for the wild type (WT) *S.* Enteritidis was measured to be 27 mM. Evolutionary lineages EL1 and EL2 were initiated from WT *S.* Enteritidis. EL1 was grown without exposure to acid stress, while EL2 was cultivated in 26 mM acetic acid (sub-MIC of acetic acid). Over time, the MIC of EL2 increased to 29 mM, prompting the creation of EL3, which was subsequently grown in 28 mM acetic acid (new sub-MIC of acetic acid). After 30 days, EL3's MIC further rose to 31 mM, leading to the initiation of EL4, which was cultivated in 30 mM acetic acid and subjected to daily transfers in fresh media. The ELs were grown in triplicates, and transfers were carried out to create new evolutionary lines from corresponding replicates.

The current study describes the ALE process after 70 days. The adaptive evolutionary process was halted after 70 days, and all the ELs were frozen at−80°C. Subsequently, EL4 was recovered from freezing in TSB without acid stress (EL4-FR) and then readapted in 30 mM acetic acid for 20 more days. This readapted evolutionary lineage was denoted as EL5. In the previous study, we analyzed several phenotypic and genomic changes that occurred in EL2, EL3, and EL4 after exposure to acetic acid and compared it to EL1, which was not exposed to any stress and served as our control (Ghoshal et al., 2023). In this study, we focused on the phenotypic, genomic, and transcriptomic changes in EL5 after exposure to acid stress until ALE day 90. The entire evolutionary process is shown in Figure 1. ALE days refers to the total number of days since the beginning of our ALE study. Experiments were conducted with EL4 from ALE day 70 (EL4-D70) and EL5 from ALE day 90 (EL5-D90). Additionally, EL4 after ALE day 70 was grown in TSB without stress for 2 days and denoted as EL4-R (EL4 recovered in TSB without acid). EL4 that was frozen at −80°C after ALE day 70 and recovered in TSB for 2 days without acid was denoted as EL4-FR (EL4-frozen and recovered without acid). The growth rate of EL1-EL4 in the presence and absence of acetic acid has been described in the previous study (Ghoshal et al., 2023). On Day 90, the bacterial growth rate of EL5 in the absence and presence of acetic acid was determined to be 0.49 and 0.36 respectively.

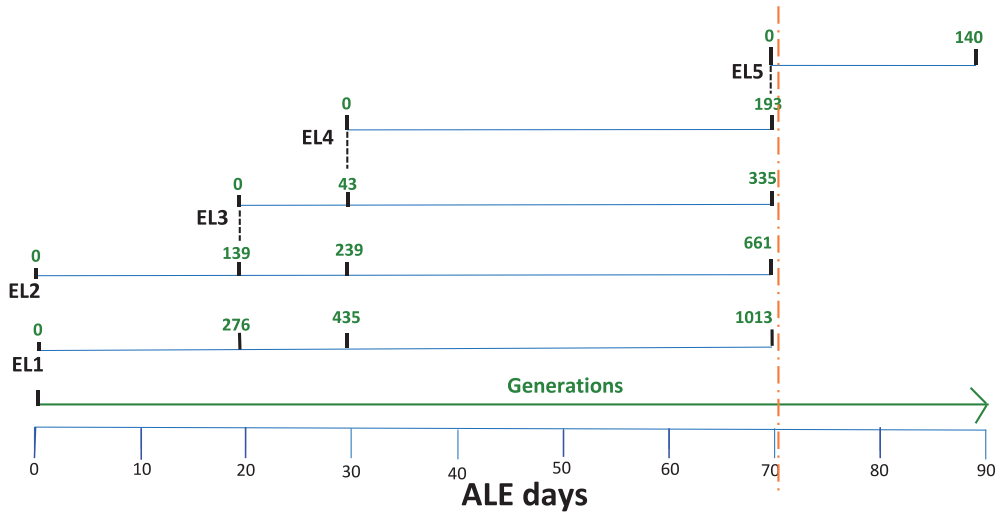

FIGURE 1
Adaptive evolution of *S.* Enteritidis evolutionary lineages (EL) in acetic acid. EL1-EL4 were adapted with daily transfers for 70 days. EL3 and EL4 were initiated from EL2 and EL3, respectively (indicated by black dotted lines). After 70 days, the evolutionary process was halted, and the ELs were frozen at −80°C (orange dotted line). Subsequently, EL4 was recovered in TSB without stress for 48 h and readapted to acetic acid until ALE day 90. This readapted culture was labeled EL5. The number of generations for each EL is shown in green.

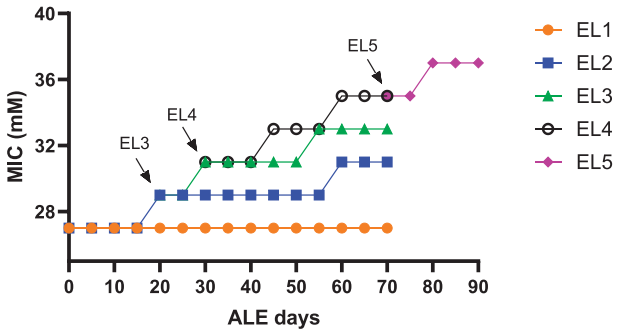

FIGURE 2
The MIC of acetic acid of the evolutionary lineages EL1-EL5. The acetic acid MICs of EL1-EL4 were measured every 5 days till day 70 of the ALE study. EL5 was initiated from EL4, and its acetic acid MIC was also measured every 5 days until ALE day 90. The arrows indicate the initiation of EL3, EL4, and EL5 from EL2, EL3, and EL4, respectively.

3.2 Change in MIC of acetic acid in the adapted evolutionary lineages

The acetic acid MIC of EL1-EL5 quantified during the study is shown in Figure 2. The MIC of acetic acid for EL1-EL4 was measured every 5 days as described in the previous study (Ghoshal et al., 2023). After 70 days, the MIC of EL5 was also similarly quantified every 5 days till 90 ALE days. The arrows in Figure 2 indicate the number of ALE days at which each EL was initiated from the previous one. EL5 was adapted in 30 mM acetic acid, and an increase in its MIC against acetic acid was observed on ALE day 80, which remained constant until ALE day 90.

3.3 Change in susceptibility to acetic acid and human antibiotics in the evolutionary lineages

We studied if freezing caused a change in the MIC against acetic acid in EL4. Acetic acid MIC of EL4 from ALE day 70 (EL4-D70), EL4-FR, and EL5 on ALE day 74 and ALE day 90 were compared. No significant differences were observed between the MIC of acetic acid in samples compared ($p > 0.05$), as shown in Table 1. This indicates that this phenotypic response to acid stress adaptation remained in the ELs even when the stress was removed, making it a stable phenotype. The same experiment was repeated with EL4-R (data not shown). Even in this case, no significant changes were observed in the acetic acid MIC of EL4-R as compared to EL4-D70 and EL4-FR. The only significant difference that was observed was between EL1-D70 (adapted in no stress for ALE day 70) and EL4-D70 ($p \leq 0.01$).

A significant increase in the MIC of various antibiotics against EL4-D70 as compared to EL1-D70 was observed for all the antibiotics tested, as seen in Table 1. We also analyzed how the MIC values against the same antibiotics changed for EL4-FR and EL5 as compared to EL4-D70. The MIC of antibiotics meropenem, ciprofloxacin, gentamycin, and streptomycin (Table 1) were studied. After recovery from freezing in the absence of stress, EL4-FR demonstrated reduced susceptibility to the human antibiotics tested. There was a significant reduction in the MIC values of all the antibiotics in EL4-FR as compared to EL4-D70 ($p < 0.01$). The MIC values of EL4-R were also similar to those of EL4-FR ($p > 0.05$, data not shown). However, after reintroduction in acetic acid, EL5 showed a recovery in the MIC values on ALE day 74 against the antibiotics, and its values were comparable to those of EL4-D70 ($p > 0.05$). Additionally, EL5 on ALE day 90 demonstrated

TABLE 1 Change in susceptibility to acetic acid and antibiotics in the evolutionary lineages.

	EL1-D70	EL4-D70	EL4-FR	EL5-D74	EL5-D90
Acetic acid (mM)	26	35	35	35	37
Meropenem (mg/L)	0.047	0.084	0.037	0.084	0.125
Ciprofloxacin (mg/L)	0.016	0.047	0.032	0.047	0.094
Gentamycin (mg/L)	0.38	0.83	0.46	0.83	1.5
Streptomycin (mg/L)	2	6	3	6	8

Mean MIC of acetic acid, meropenem, ciprofloxacin, gentamycin, and streptomycin against EL1 and EL4 on ALE day 70 and EL5 on ALE days 74 and 90 is shown. EL1 was initiated from WT S. Enteritidis and adapted in TSB without acetic acid for 70 ALE days. EL4 was adapted in 30 mM acetic acid from ALE day 30 to ALE day 70. EL4 on day 70 was frozen at −80°C and recovered in TSB for 2 days without acid stress and is referred to as EL4-FR (EL4 from day 70 frozen and recovered without stress). Finally, EL4-FR was readapted to 30 mM acetic acid for 20 days and named EL5.

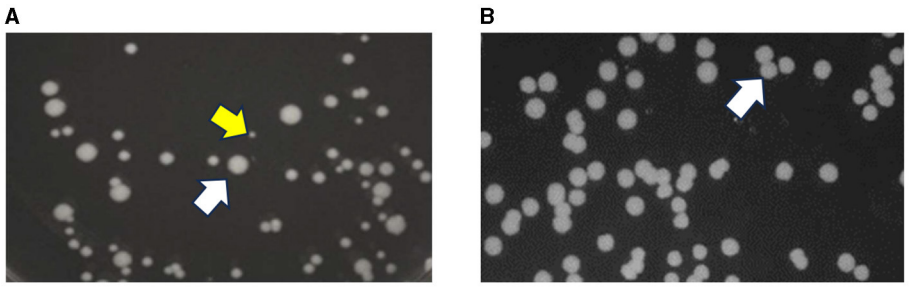

FIGURE 3
Stability of the phenotypic characteristics of EL4 before and after freezing. *Salmonella* small colony variant (SSCV) are pinpoint colonies observed in EL4 on ALE day 70 and EL5 on ALE day 90 after adaptation in acetic acid stress (yellow arrow) along with regular-sized colonies [normal colony phenotype, white arrow, **(A)**]. Homogeneous distribution of normal colony phenotype (white arrow) was observed in EL4-FR (EL4 from day 70 frozen and recovered without stress) and EL4-R (EL4 from day 70 recovered without stress) without the presence of SSCV **(B)**.

a significant increase in the MIC values of all the human antibiotics tested as compared to EL5-D74, EL4-D70 and EL4-FR ($p \leq 0.01$).

An inoculum from EL4-D70 plated on TSA showed the presence of two types of colonies: normal phenotype colonies (NPC) as well as pinpoint colonies known as *Salmonella* small colony variants (SSCV) (Figure 3A). The presence of SSCV along with NPC was also observed in EL5 on ALE days 74 and 90. However, the SSCV phenotype was not observed when the inoculum from EL4-R and EL4-FR were plated on TSA, which showed a homogeneous distribution of NPC of *Salmonella* (Figure 3B). The SSCV isolated from EL4-D70, EL5-D74, and EL5-D90 obtained after 24 h on TSA reverted back to NPC after subsequent subcultures.

3.4 Comparative genomics of the acid-adapted evolutionary lineages EL4 and EL5

The genomic analysis of the evolutionary lineages EL1-EL4 on ALE day 70 was performed and the results have been described in the previous study (Ghoshal et al., 2023). In the present study, we compared the genomic profile of EL5-D90 to EL4-D70, and the results are shown in Table 2. Only replicate 2 of EL5 maintained the *phoQ* mutation p.Ile421Leu from EL4. The other two replicates of EL5 lost that mutation but yielded a new *phoQ* mutation of p.Leu250Phe. Again, only replicate 2 of EL5 maintained the *thiB* mutation that occurred in all three replicates of EL4. These results suggest that replicate 2 of EL5 may have used a different pathway of adaptation than replicate 1 and 3. Additionally, the replicates of EL5 had novel mutations that were absent in EL4. Replicates 1 and 3 of EL5 showed mutations in the *spoT* gene that encodes for guanosine 3′,5′-bis(diphosphate) 3′-pyrophosphohydrolase (ppGppase), which is an enzyme that catalyzes the hydrolysis of guanosine tetraphosphate (ppGpp) to guanosine triphosphate (gtp) (Magnusson et al., 2005). Additionally, frameshift mutations were observed in the *wzc* gene in replicates 1 and 3 of EL5-D70, which is involved in capsule formation in *E. coli* (Yang et al., 2021).

3.5 Determining the transcriptomic profile of EL5 in the presence of acetic acid stress

RNA-sequencing and differential gene expression analyses were performed on EL4 and EL5 to investigate how ALE affected their transcriptomic profile in the presence of acetic acid over time. The transcriptomic profile of EL5 (grown in 30 mM acetic acid until ALE day 90) and EL4-A2 (described in Section 2.2) (grown in 30 mM acetic acid for 18 h) was compared to that of WT *S.* Enteritidis, which was exposed to 26 mM acetic acid (sub-MIC of acetic acid for WT) for 18 h before RNA sequencing. EL5, EL4-A2, and WT were also grown for 18 h in TSB without acid stress

TABLE 2 Mutations in EL5 after adaptation in acetic acid for 90 ALE days were identified using genomic analysis and compared to EL4 after adaptation in acetic acid for 70 ALE days.

Position	Gene	Mutation	Ancestor allele	Evolved allele	Protein Product	EL4	EL5
1,745,485	*rfbE*	Missense	A	T	CDP-paratose 2-epimerase	R1: p.Asn220Tyr R2: p.Asn220Tyr R3: p.Asn220Tyr	
1,972,868	*phoQ*	Missense	T	G	Virulence sensor histidine kinase PhoQ	R1: p.Ile421Leu R2: p.Ile421Leu R3: p.Ile421Leu	R2: p.Ile421Leu
1,973,381	*phoQ*	Missense	G	A	Virulence sensor histidine kinase PhoQ		R1: p.Leu250Phe R3: p.Leu250Phe
3,776,974	*thiB*	Missense	AACGGTGACGGTGA	AACGGTGACGGTGACGGTGA	Thiamine-binding periplasmic protein	R1: p.Val193_Thr194dup R2: p.Val193_Thr194dup R3: p.Val193_Thr194dup	R2: p.Val193_Thr194dup
439,516	*tuf1*	Synonymous	C	T	Elongation factor Tu 1	R3: p. His320His	
518,130	*oadB*	Synonymous	T	C	Oxaloacetate decarboxylase beta chain	R3: p. Leu85Leu	
1,477,186	*[ackA]–[hxpA]*	Deletion	Δ2,207 bp	x	Acetate kinase- Hexitol phosphatase	R1: Δ2,207 bp R2: Δ2,207 bp R3: Δ2,207 bp	R1: Δ2,207 bp R2: Δ2,207 bp R3: Δ2,207 bp
103,404	*spoT*	Missense	C	A	ppGpp (guanosine 3′-diphosphate 5-′ diphosphate)		R1: p.Ser368Ile R3: p.Ser368Ile
1,715,212	*wzc*	Frameshift	GCCCGCA	GCCGCA	putative tyrosine kinase		R1: p.Pro455fs R3: p.Pro455fs
2,024,345	*nimT*	Synonymous	C	T	CynX/NimT family MFS transporter		R1: p.Leu151Leu R3: p.Leu151Leu
4,453,078	*yiiM*	Synonymous	G	A	3-alpha domain		R1: p.Asp131Asp R3: p.Asp131Asp

All the evolutionary lineages were mapped to the parent genome of *Salmonella enterica* serovar Enteritidis BAA 1045. R1, R2, and R3 refer to the three replicates in each evolutionary lineage.

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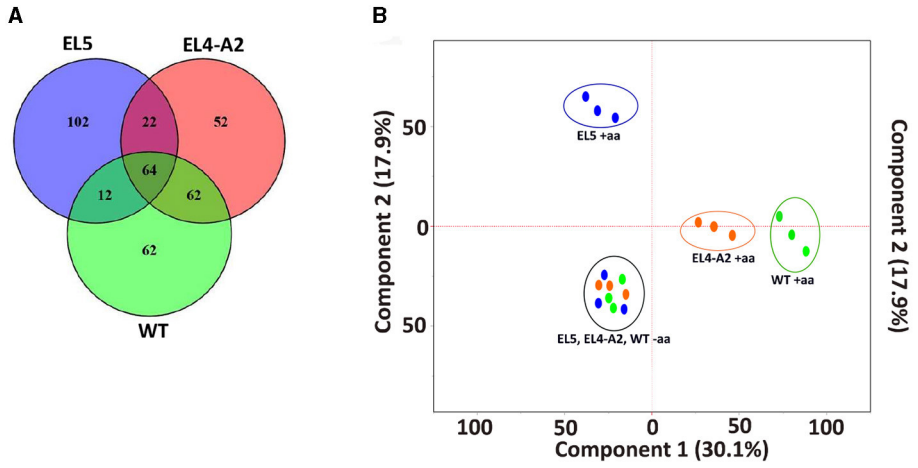

FIGURE 4
Similarities between the transcriptomic profiles of EL5, EL4-A2, and WT *S.* Enteritidis after growth in acetic acid. A Venn diagram of the top 200 upregulated genes in acetic acid stress is shown in **(A)**. PCA plot of the gene expression profiles of EL4, EL5, and WT *S.* Enteritidis with and without acetic acid stress **(B)**. The blue dots inside the blue circle are the three replicates of EL5 after treatment with 30 mM acetic acid until ALE day 90, the orange dots inside the orange circle are EL4-A2 adapted to 30 mM acetic acid for 20 h, and the green dots inside the green circle are WT *S.* Enteritidis grown in 26 mM acetic acid for 20 h. EL4-A2 refers to the EL4 that was exposed to acid stress the second time for 20 h. The blue, orange, and green dots inside the black circle are the three replicates of EL5, EL4-A2, and WT, respectively, grown in TSB for 20 h without acid stress. Here, "+aa" and "−aa" refer to the presence and absence of acetic acid, respectively.

and served as controls. Three biological replicates for each sample were sequenced.

The Venn diagram in Figure 4A shows 200 of the highest upregulated genes in EL4-A2, EL5, and WT with $\log_2$foldchange ≥ 2 and $p_{adj} \leq 0.01$. About 64 of the 200 genes were upregulated in all three lineages, most of which were related to cellular physiological functions such as elongation factor Tu (EF-Tu) and glutathione synthase (*gshB*). However, some of the genes were related to stress response, such as the small toxic protein IdrD and alternative sigma factors RpoS and RpoH. While 22 of the same genes were upregulated in EL5 and EL4-A2, 62 of the same genes were upregulated in EL4-A2 and WT, and only 12 of the same genes were upregulated between EL5 and WT. EL5 had the highest percentage of genes that were exclusively upregulated, without being upregulated in EL4-A2 or WT. The transcription profiles of the specific genes that were found mutated in EL1-EL4 in our previous study were analyzed in EL5 and EL4-A2 in acetic acid and compared to that of WT in acetic acid (Table 3).

The principal component analysis (PCA) of genome-wide DeSeq2 normalized gene counts for WT, EL4-A2, and EL5 is shown in Figure 4B. The PCA results reveal independent clustering of WT (green circle), EL4-A2 (orange circle), and EL5 (blue circle) replicates in acetic acid, which suggests distinct expression profiles between these three groups when grown under acid stress. All three replicates of EL4-A2, EL5, and WT grown in TSB were found to cluster together (black circle). Figures 4A, B demonstrate that when compared to WT in acetic acid, EL5 exhibits greater transcriptomic variation than EL4-A2. Information about the RNA sequencing alignment and RNA sequencing statistics have been provided in Supplementary Tables S1, S2, respectively.

Volcano plots showing the global changes in the expression of genes between WT, EL4-A2, and EL5 are shown in Figure 5. Genes having a minimum of two-fold upregulation or downregulation in their expression levels (p_{adj} value ≤ 0.01) have been characterized as differentially expressed. At a global level, many genes appear to be significantly upregulated and downregulated in acetic acid in EL5 as compared to the WT (Figure 5A) and EL4-A2 (Figure 5B). A total of 591 genes were upregulated, and 964 genes were downregulated in EL5 vs. WT in acetic acid stress. Further, 391 genes were upregulated, and 704 genes were downregulated in EL5 vs. EL4-A2. The differential expression of genes in EL4-A2 compared to WT was also statistically significant (p_{adj} value ≤ 0.01) (Figure 5C), with 208 genes being upregulated and 279 genes downregulated in EL4-A2 vs. WT.

Heat maps demonstrating the relative expression profiles of various genes involved in pathogenesis, stress response, and drug resistance after exposure to acetic acid stress are depicted in Figure 6A. A large fraction of these genes was found to be upregulated in EL4-A2 and EL5 with respect to WT. A few notable genes that were significantly upregulated in EL5 and EL4-A2 vs. the WT were acid shock protein and Na^+/H^+ antiporter NhaB, which is known to help regulate intracellular pH levels. Some of the genes that were upregulated in EL5 vs. both EL4-A2 and WT were virulence protein SpvA, nitrate reductase subunit alpha, addiction module toxin RelE, and radical SAM protein. Virulence protein SpvA and toxin RelE were found to be upregulated more than 8-fold and 6-fold, respectively, in EL5 with respect to the WT ($p_{adj} \leq 0.01$). Three genes that were significantly downregulated in EL5 and EL4-A2 vs. WT were the virulence membrane protein PagC, phage virulence protein, and virulence protein PagD. Further, virulence protein

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TABLE 3 Change in expression of specific genes in EL5 and EL4-A2 as compared to WT in acetic acid after ALE day 90, which were found to be mutated in EL1-EL4 after ALE day 70.

Gene	Mutation	EL with the mutation	Protein Product	EL5 vs. WT	EL5 vs. EL4-A2	EL4-A2 vs. WT
ptsP	Missense	EL1, EL2	Phosphoenolpyruvate-dependent phosphotransferase system	+ 3-fold	+ 2-fold	No change
nimT	Synonymous	EL1, EL2	2-nitroimidazole transporter	No change	No change	No change
barA	Missense	EL2	Signal transduction histidine-protein kinase BarA	+ 3-fold	+ 3-fold	No change
alaS	Missense	EL2	Alanine–tRNA ligase	+ 2-fold	+ 2-fold	No change
phoQ	Missense	EL3, EL4, EL5	Virulence sensor histidine kinase PhoQ	+ 2.3-fold	+ 2-fold	No change
phoP	Missense	EL2	Virulence transcriptional regulatory protein PhoP	No change	No change	No change
zinT	Frameshift	EL2	Metal-binding protein ZinT	- 2-fold	- 2.5-fold	No change
ybaY	Frameshift	EL2	putative lipoprotein YbaY	N/A	N/A	N/A
fhuA	Missense	EL2	Ferrichrome outer membrane transporter/phage receptor	No change	No change	No change
rpoB	Missense	EL2	DNA-directed RNA polymerase subunit beta	+ 2.5-fold	+ 2.3-fold	No change
rfbE	Missense	EL4	CDP-paratose 2-epimerase	No change	No change	No change
thiB	Missense	El4, EL5	Thiamine-binding periplasmic protein	N/A	N/A	N/A
tuf1	Synonymous	EL4	Elongation factor Tu 1	+ 3-fold	+ 3.6-fold	No change
oadB	Synonymous	EL4	Oxaloacetate decarboxylase beta chain	N/A	N/A	N/A
wzc	Frameshift	EL5	Putative tyrosine kinase	No change	No change	No change
spoT	Missense	EL5	ppGpp (guanosine 3′-diphosphate 5-′ diphosphate)	N/A	N/A	N/A
nimT	Synonymous	EL5	CynX/NimT family MFS transporter	N/A	N/A	N/A
yiiM	Synonymous	EL5	3-alpha domain	N/A	N/A	N/A

EL5 was exposed to acetic acid for 90 ALE days; EL4-A2 and WT were exposed to acetic acid for 18 h before RNA sequencing; '+' and '-' refer to up and down-regulation of the genes. At least a 2-fold change is considered to be change. N/A indicates that the specific genes in question did not show up in our RNA sequencing profile.

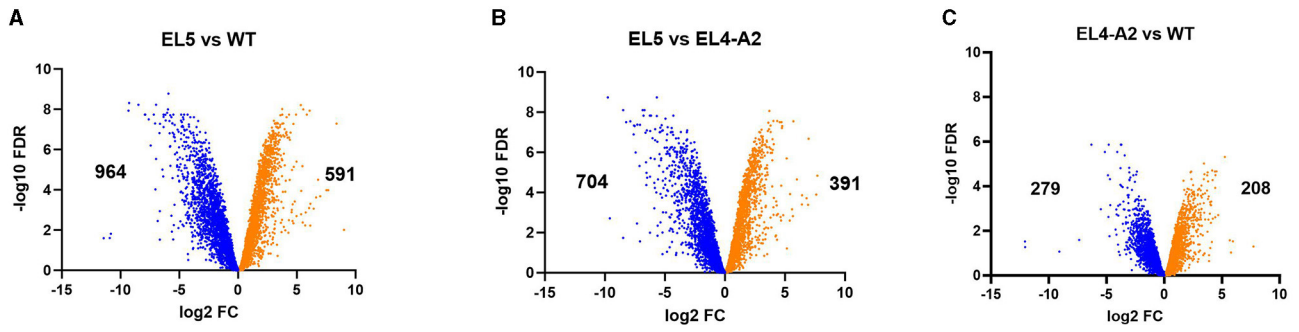

FIGURE 5
Volcano plots showing the overall changes in gene expression profiles between EL5 and WT **(A)**, EL5 and EL4-A2 **(B)**, and EL4-A2 and WT **(C)** *S.* Enteritidis. EL5 was grown in 30 mM acetic acid for 90 ALE days, EL4-A2 was exposed to 30 mM acetic acid for the second time for 20 h, and WT was grown in 26 mM acetic acid for 20 h. For each comparison, the downregulated and upregulated genes are shown in blue and orange, respectively.

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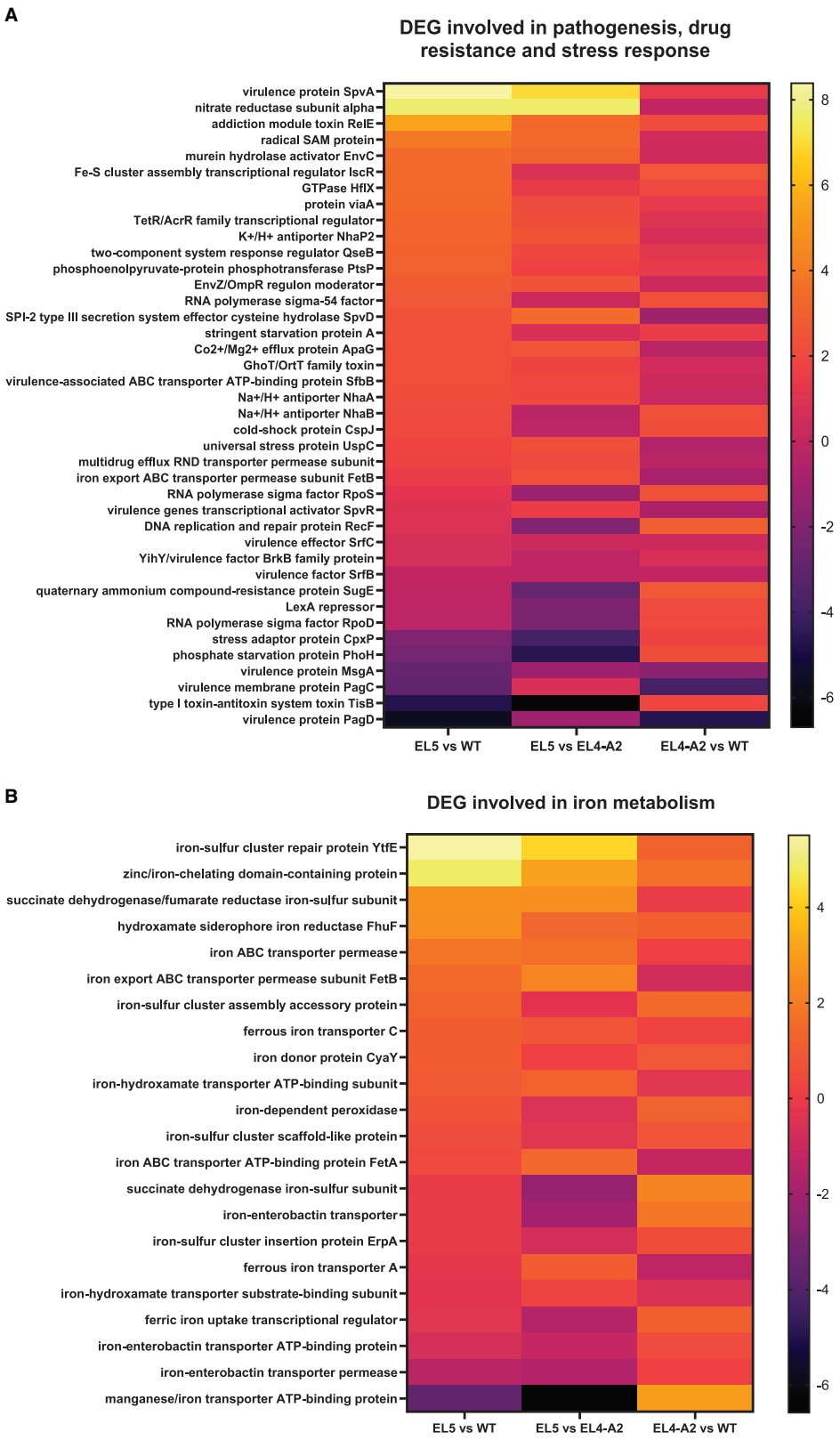

FIGURE 6
Heat maps demonstrating the differential expression of several classes of genes in EL5, EL4-A2, and WT *S.* Enteritidis after treatment with different concentrations of acetic acid. Differentially expressed genes (DEG) involved in pathogenesis, drug resistance, and stress response are shown in **(A).** The differential expression of genes involved in iron metabolism is shown in **(B)**. EL5 was grown in 30 mM acetic acid for 90 ALE days, EL4-A2 was exposed to 30 mM acetic acid for the second time for 20 h, and WT was grown in 26 mM acetic acid for 20 h.

MsgA was significantly downregulated in EL5 vs. both EL4-A2 and WT.

Several genes involved in iron metabolism were also differentially expressed in EL4-A2, EL5, and WT during acetic acid stress (Figure 6B). Most of the iron metabolism genes were upregulated in EL5 and EL4-A2 vs. the WT. A large number of these genes encode for iron transporters, especially ATP Binding Cassette (ABC) transporters. A high expression of iron transporters has been linked to the increased virulence of *S.* Enteritidis (Domínguez-Acuña and García-Del Portillo, 2022), indicating that EL4-A2 and EL5 might have higher virulence capabilities as compared to WT in acetic acid. These results indicate that ALE in acetic acid stress has the potential to increase virulence in *S.* Enteritidis. One significant observation was that a gene coding for a manganese/iron transporter ATP-binding protein was significantly downregulated in EL5 vs. EL4-A2.

In the presence of acid stress, WT showed an upregulation of stress proteins as compared to WT without acid stress (Figure 7A). The expression of different classes of genes in EL5 and EL4-A2 in acetic acid were compared to WT in acetic acid in Figure 7B. EL5 in acetic acid demonstrated an increase in the expression of TA systems, pathogenesis, as well as biofilm-forming genes and downregulation in the expression of genes involved in biosynthetic processes as compared to WT in acetic acid. EL4-A2 in acid demonstrated an increase in the expression of genes involved in stress and pathogenesis while showing a downregulation in the expression of genes involved in biosynthetic processes and biofilm formation as compared to WT in acetic acid.

4 Discussion

This study explored the interplay between ALE of *S.* Enteritidis under acid stress and its subsequent impact on susceptibility to acetic acid and human antibiotics. We studied if the bacteria can quickly regain their previously acquired adaptations upon stress reintroduction, providing insights into the reversibility and persistence of stress-induced changes. Freezing and reviving EL4-D70 in a neutral growth medium (TSB without acetic acid) allowed us to attribute any observed responses in EL5 primarily to the reintroduction of the stressor (acetic acid) rather than the effects of residual acetic acid from previous exposure. Although EL4 and EL5 shared some common genetic adaptations due to acid stress, labeling the reintroduced population (EL5) as a new EL also added clarity to our ALE study, acknowledging the potential complexity of adaptive processes.

The increase in acetic acid MIC against EL5 over time under acid stress aligns with that of the other ELs (EL2-EL4), indicating a consistent trend from EL2 to EL5 (Figure 2). Several other studies have also demonstrated that bacterial populations can evolve and adapt in response to prolonged exposure to specific stressors (Li et al., 2019; Sulaiman and Lam, 2020). Although the antibiotic MICs increased in EL4-D70, EL5-D74, and EL5-D90, all except ciprofloxacin remained within susceptible ranges as defined by established critical breakpoints (FDA, 2023). Elevated MICs suggest a shift toward reduced susceptibility, which is a potential precursor to future resistance. EL5 exhibited intermediate resistance to ciprofloxacin (FDA, 2023) on ALE day 90, indicating ongoing adaptation to acid stress. This is indicative of a correlation between the mechanisms of acid stress adaptation and ciprofloxacin adaptation and underscores the importance of understanding *Salmonella* acid adaptation to prevent reduced antibiotic efficacy.

The increase in acetic acid MIC in the ELs suggests the acquisition of genetic changes that confer a selective advantage under acidic conditions. This has also been described in our previous study where, after exposure to acetic acid for 70 ALE days, the ELs had acquired several genetic mutations in the form of base substitutions and deletions (Ghoshal et al., 2023). Building on these findings, our study explored the effects of removing acid stress on antibiotic MIC. The reversible nature of the observed changes in MIC against the antibiotics (Table 1) is intriguing. EL4 was recovered in TSB without acid stress for 2 days (EL4-R), and EL4 frozen and recovered in TSB without stress for 2 days (EL4-FR) exhibited significantly reduced antibiotic MICs compared to EL4-D70 ($p \leq 0.01$). This suggests that adaptation without acetic acid may have resulted in a loss of the antibiotic tolerance acquired during the initial adaptation to acetic acid. While stress adaptation provides short-term benefits, maintaining them in the absence of stress might be energetically costly or less compatible with optimal growth and functioning (Windels et al., 2020). EL4-R and EL4-FR prioritized growth over maintaining high antibiotic MICs, reflected in increased daily OD_{600} values of EL4-R and EL4-FR compared to EL4-D70 ($p < 0.01$; data not shown). This aligns with findings in *E. coli* after the removal of initial stress (Gottesman et al., 2009; Dunai et al., 2019), emphasizing the dynamic nature of bacterial responses to environmental pressures and their effects on antibiotic susceptibility. The observed reduction of MICs against antibiotics in the absence of acid stress also suggests that stress adaptation might involve specific transcriptional changes contributing to increased antibiotic MICs in the presence of acid stress. The rapid restoration of MICs in EL5 upon acid stress reintroduction (Table 1) indicates high phenotypic plasticity in the adapted ELs. It also implies retained adaptive changes even in the absence of continuous stress, suggesting stable genetic alterations.

The genomic analysis of EL5, as compared to EL4, revealed the presence of several mutations, such as mutations in the *phoP/Q* system, which help in acid adaptation (Table 2) (Lang et al., 2021; Han et al., 2023). This possibly explains why the increased acetic acid MIC of EL4-D70 was not lost in EL4-R or EL4-FR upon stress removal, and no significant differences in their acetic acid MIC values were observed ($p > 0.05$; Table 1). EL5 acquired a *spoT* gene mutation, potentially altering (p)ppGpp response, resulting in increased stress tolerance. (p)ppGpp is a signaling molecule that helps in bacterial survival during stress (Pacios et al., 2020) and causes large-scale transcriptional changes by binding directly to the RNA polymerase in gram-negative bacteria (Irving et al., 2021). The level of stress tolerance in *E. coli* is dependent on the amount of (p)ppGpp available in the cells (Spira and Ospino, 2020). We hypothesize that mutations in the *spoT* gene indirectly contributed to higher antibiotic resistance, as the stringent response by (p)ppGpp might affect various cellular processes, including those relevant to antibiotic susceptibility. EL5 also showed a mutation in the *wzc* gene, which is involved in capsule formation (Yang et al., 2021). Mutations in the *wzc* gene

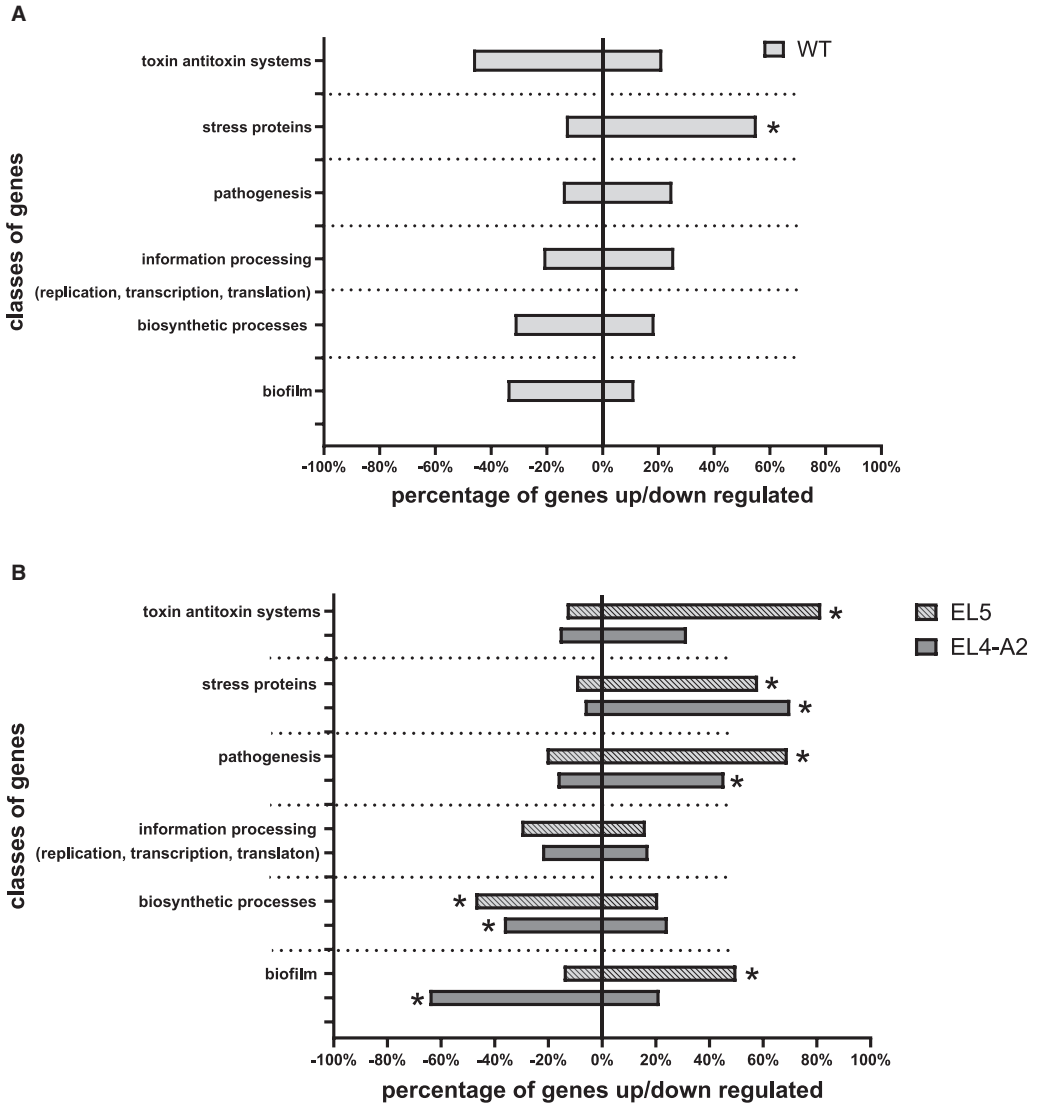

FIGURE 7
Overview of differentially expressed genes in EL5 vs. WT and EL4-A2 vs. WT according to functional categories after exposure to acetic acid. Changes in the expression of genes of WT grown in 26 mM acetic acid with respect to WT grown in TSB without acetic acid are shown in **(A)**. Changes in the expression of genes in EL4-A2 and EL5 grown in 30 mM acetic acid as compared to WT grown in 26 mM acetic acid are shown in **(B)**. The '*' in **(A)** refers to a statistically significant ($p < 0.01$) change in the expression of genes of a specific functional category in WT grown in 26 mM acetic acid with respect to WT grown in TSB without acetic acid. The '*' in **(B)** refers to a statistically significant ($p < 0.01$) change in the expression of genes of a specific functional category in EL4-A2 and EL5 grown in 30 mM acetic acid as compared to WT grown in 26 mM acetic acid.

have been reported to provide resistance against antibiotics in *E. coli* (Jazdarehee et al., 2017). These changes in the capsule structure might limit the penetration of antibiotics, thereby conferring resistance to antibiotics. The mutations in *spoT* and *wzc* genes in EL5 could synergistically enable EL5 to resist human antibiotics more effectively compared to EL4-D70. This could possibly explain why we observed an increase in the antibiotic MIC values in EL5-D90 as compared to EL4-D70, indicating further adaptation in acid stress (Table 1). Some of the mutations observed in EL4-D70 were not present in EL5-D90, as shown in Table 2. This could possibly be because these mutations were not homogenous throughout the EL4-D70 cultures and were subsequently lost during the propagation of EL5.

RNA-sequencing was performed on EL4 and EL5 to study the transcriptional changes caused during adaptation to acetic acid stress over varying durations. EL4-A2, representing the combined effects of prior long-term acid adaptation and short-term acid adaptation, was used, allowing us to investigate the interplay between pre-adaptation and short-term stress response. The PCA plot (Figure 4B) demonstrates that the expression profile of EL5 in acetic acid was more distantly related to those of EL4-A2 and WT in acetic acid. This indicates that EL5 possibly has more unique mechanisms for coping with acetic acid stress than EL4-A2 and WT. This could be attributed to the longer continuous ALE of EL5 in acid as opposed to EL4-A2 and WT. The volcano plots in Figure 5 indicate that EL5 exhibits significant downregulation

of numerous genes ($p \leq 0.01$), potentially due to a high number of persistor cells. We observed unstable *Salmonella* small colony variants (SSCV) in EL4 and EL5 (Figures 3A, B), consistent with findings in other studies highlighting their instability (Kahl et al., 2016; Li et al., 2016). SSCV are known to be persister cells that are less susceptible to antibiotics and environmental stresses than their wild-type counterparts (Proctor et al., 2006). For instance, acetic acid stress induces persistence in *E. coli* (Kawai et al., 2018). Persistence can be triggered during stress, and toxin-antitoxin systems are known to be the effectors of bacterial persistence under environmental stresses (Bakkeren et al., 2020; Jurenas et al., 2022). Evidence of toxin-encoding genes being upregulated in acetic acid stress was observed in EL5 as compared to WT (Figure 7). The Gho/Ort family toxin, toxin RelE, was upregulated in EL5 vs. WT, and the type 1 TA system toxin TisB was upregulated in EL4-A2 vs. WT (Figure 6). The Gho/Ort family toxins—RelE toxin and the TisB toxin —are known to promote persistence and tolerance to antibiotics in bacteria (Singh et al., 2010; Wang and Wood, 2011; Wang et al., 2012; Edelmann and Berghoff, 2022). The outer membrane virulence proteins SpvA and SpvD (a cysteine hydrolase) are a part of the *spvABCD* operon that is involved in bacterial pathogenesis (El-Gedaily et al., 1997; Grabe et al., 2016) and were significantly upregulated in EL5 vs. WT.

Proteins conferring antibiotic resistance, such as quaternary ammonium compound resistance protein SugE, were upregulated in EL4-A2 vs. WT, and the role of these proteins in antibiotic resistance has been reported in earlier studies (Jiang et al., 2020). EL4-A2 demonstrated a higher upregulation of drug resistance genes than the WT, indicating that short-term acid exposure, especially in lineages with prior acid exposure, also promotes drug resistance in *S.* Enteritidis. Heat maps (Figures 6A, B) reveal the two-component system EnvZ/OmpR was found to be upregulated in EL5 vs. WT in acetic acid stress. It is known to play an important role in *E. coli* and *S.* Enteritidis survival under acid stress and cellular acidification during osmotic stress (Kenney, 2019). Stress response kinase A and universal stress protein UspC were highly upregulated in EL5 vs. EL4-A2 and WT in acetic acid. The Usp family of proteins is known to be upregulated in the presence of acids and antibiotics and promote virulence in *S.* Enteritidis and *E. coli* (Liu et al., 2007; Luo et al., 2023). The two-component response regulator QseB, which promotes the expression of various virulence genes in *S.* Enteritidis (Weigel and Demuth, 2016), was upregulated in EL5 vs. WT and EL4-A2. The increase in expression of OmpR/EnvZ, UspC, and QseB, which are involved in stress adaptation and antibiotic resistance (Su et al., 2007; Viveiros et al., 2007; Deng et al., 2013; Zhu et al., 2023), possibly indirectly contributed to reduced antibiotic susceptibility in the ELs.

The upregulation of Na^+/H^+ antiporters, which regulate intracellular pH levels in EL5 and EL4-A2 vs. WT, indicates its possible involvement in bacterial survival under acidic conditions (Xu et al., 2014). The expression levels of the Na^+/H^+ transporter and K^+/H^+ antiporter in EL5 were higher than those of EL4-A2 and WT. Efflux pumps that transport various compounds, including different classes of antibiotics outside cells, contribute to bacterial multidrug resistance (El Meouche and Dunlop, 2018; Pandey et al., 2020). Upregulation of efflux pumps, which leads to the expulsion of antibiotics outside cells, contributed directly to an increase in antibiotic MIC values in the ELs. A multidrug RND efflux transporter and TetR/Acr transcriptional regulator were upregulated in EL5 as compared to EL4-A2. Resistance nodulation cell division (RND)-type efflux transporters provide resistance against various antimicrobial compounds in gram-negative bacteria (Matsuo et al., 2013). The TetR-family transcriptional regulators (TFTRs), such as AcrR, regulate multidrug efflux systems and stress response in *S.* Enteritidis (Colclough et al., 2019). This indicates that long-term adaptation to acid stress, as in the case of EL5, results in increased antibiotic resistance compared to shorter-term adaptation, even if previously exposed to acetic acid for a long time (as in EL4-A2).

Some genes were significantly downregulated in EL5 and EL4-A2 compared to the WT, including virulence membrane protein PagC and PagD, which are controlled by the PhoP/Q operon and MsgA. These proteins play a crucial role in macrophage survival and protecting *S.* Enteritidis from host complement-mediated killing (Nishio et al., 2005; Zhao et al., 2008; Bahramianfard et al., 2021). It is possible that in the absence of a host, the expression of several genes required for host immune evasion was downregulated, and instead, the bacteria prioritized the upregulation of genes required for survival in the current stress conditions. The RNA seq results provide insights into the transcriptomic responses during the adaptation of *S.* Enteritidis to different durations of acid stress.

The adaptive laboratory evolution of *S.* Enteritidis in acetic acid has significant implications for bacterial environmental responses. The increased acetic acid MIC reflects enhanced acid tolerance, which is critical for bacterial survival in acidic food industry settings. Changes in antibiotic MIC suggest cross-resistance, potentially impacting treatment efficacy. Genomic and transcriptomic changes in stress adaptation, virulence, and drug resistance pathways underscore bacterial complexity, potentially enhancing persistence and affecting food safety. The study highlights the bacteria's evolutionary potential under stress and emphasizes the importance of examining microbial responses to various environmental stresses. Together with our previous study, it offers a detailed view of the adaptations of *S.* Enteritidis during varied acetic acid exposures.

5 Conclusion

In conclusion, this study provides important insights into the process of adaptive evolution in bacterial populations and underscores the intricate relationship between stress adaptation, antibiotic resistance, and bacterial fitness. The reversible nature of antibiotic resistance highlights the adaptability of bacterial populations and the potential consequences of stress-induced adaptations. Transcriptomic analysis demonstrated the upregulation of drug resistance, virulence, iron metabolism, and stress adaptation genes during continuous acid stress. The results from the genomic analysis in our current and previous study and transcriptomic analysis in this current study highlight the importance of a comprehensive understanding of the adaptation of bacteria to stressful environments for the development of effective treatment strategies. The implications of these findings extend

beyond fundamental microbiology, emphasizing the need for a holistic approach to understanding bacterial responses to stress and their impact on public health and food safety.

Data availability statement

The RNA data generated in this project is deposited in the NCBI repository under BioSample accession numbers SAMN37104682 – WT replicates (aa and TSB); SAMN37188996 – EL4-A2 replicates (aa and TSB); and SAMN37188997 – EL5 replicates (aa and TSB). Here 'aa' refers to the presence of acetic acid and 'TSB' refers to the absence of acetic acid, the samples were grown in trypticase soy broth.

Author contributions

MG: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. TB: Data curation, Formal analysis, Methodology, Software, Writing – review & editing. JG: Methodology, Project administration, Software, Supervision, Writing – review & editing. LM: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1348063/full#supplementary-material

References

Aljumaah, M. R., Alkhulaifi, M. M., Abudabos, A. M., Alabdullatifb, A., El-Mubarak, A. H., Al Suliman, A. R., et al. (2020). Organic acid blend supplementation increases butyrate and acetate production in *Salmonella enterica* serovar Typhimurium challenged broilers. *PLoS ONE* 15, e0232831. doi: 10.1371/journal.pone.0232831

Bahramianfard, H., Derakhshandeh, A., Naziri, Z., and Khaltabadi Farahani, R. (2021). Prevalence, virulence factor and antimicrobial resistance analysis of *Salmonella* Enteritidis from poultry and egg samples in Iran. *BMC Vet. Res.* 17, 196. doi: 10.1186/s12917-021-02900-2

Bakkeren, E., Diard, M., and Hardt, W. D. (2020). Evolutionary causes and consequences of bacterial antibiotic persistence. *Nat. Rev. Microbiol.* 18, 479–490. doi: 10.1038/s41579-020-0378-z

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., and Kulikov, A. S. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comp. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Brown, E. W., Bell, R., Zhang, G., Timme, R., Zheng, J., Hammack, T. S., et al. (2021). *Salmonella* genomics in public health and food safety. *EcoSal Plus* 9, eESP-0008. doi: 10.1128/ecosalplus.ESP-0008-2020

Cantalapiedra, C. P., Hernández-Plaza, A., Letunic, I., Bork, P., and Huerta-Cepas, J. (2021). eggNOG-mapper v2: functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Mol. Biol. Evol.* 38, 5825–5829. doi: 10.1093/molbev/msab293

Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., et al. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff. *Fly* 6, 80–92. doi: 10.4161/fly.19695

Colclough, A. L., Scadden, J., and Blair, J. M. A. (2019). TetR-family transcription factors in Gram-negative bacteria: conservation, variation and implications for efflux-mediated antimicrobial resistance. *BMC Genomics* 20, 731. doi: 10.1186/s12864-019-6075-5

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., et al. (2021). Twelve years of SAMtools and BCFtools. *GigaScience* 10, giab008. doi: 10.1093/gigascience/giab008

Deatherage, D. E., and Barrick, J. E. (2014). Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. *Methods Mol. Biol.* 1151, 165–188. doi: 10.1007/978-1-4939-0554-6_12

Deng, W., Li, C., and Xie, J. (2013). The underling mechanism of bacterial TetR/AcrR family transcriptional repressors. *Cellular Sig.* 25, 1608–1613. doi: 10.1016/j.cellsig.2013.04.003

Domínguez-Acuña, L., and García-Del Portillo, F. (2022). Ferrous iron uptake is required for *Salmonella* to persist within vacuoles of host cells. *Infect Immun.* 90, e0014922. doi: 10.1128/iai.00149-22

Dunai, A., Spohn, R., Farkas, Z., Lázár, V., Györkei, Á., Apjok, G., et al. (2019). Rapid decline of bacterial drug-resistance in an antibiotic-free environment through phenotypic reversion. *eLife* 8, e47088. doi: 10.7554/eLife.47088.024

Edelmann, D., and Berghoff, B. A. (2022). A shift in perspective: a role for the type i toxin tisb as persistence-stabilizing factor. *Front. Microbiol.* 13, 871699. doi: 10.3389/fmicb.2022.871699

El Meouche, I., and Dunlop, M. J. (2018). Heterogeneity in efflux pump expression predisposes antibiotic-resistant cells to mutation. *Science* 362, 686–690. doi: 10.1126/science.aar7981

El-Gedaily, A., Paesold, G., and Krause, M. (1997). Expression profile and subcellular location of the plasmid-encoded virulence Spv proteins in wild-type *Salmonella dublin*. *Inf. Immun.* 65, 3406–3411. doi: 10.1128/iai.65.8.3406-3411.1997

Garrison, E., and Marth, G. (2012). Haplotype-based variant detection from short read sequencing. *bioRxiv*. doi: 10.48550/arXiv.1207.3907

FDA (2023). Available online at: https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/resources (accessed May 8, 2023).

Ghoshal, M., Bechtel, T. D., Gibbons, J. G., and McLandsborough, L. (2023). Adaptive laboratory evolution of *Salmonella enterica* in acid stress. *Front. Microbiol.* 14, 1285421. doi: 10.3389/fmicb.2023.1285421

Gottesman, B. S., Carmeli, Y., Shitrit, P., and Chowers, M. (2009). Impact of quinolone restriction on resistance patterns of *escherichia coli* isolated from urine by culture in a community setting. *Clin. Inf. Dis.* 49, 869–875. doi: 10.1086/605530

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., et al. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* 36, 3420–3435. doi: 10.1093/nar/gkn176

Grabe, G. J., Zhang, Y., Przydacz, M., Rolhion, N., Yang, Y., Pruneda, J. N., et al. (2016). The *Salmonella* effector SpvD is a cysteine hydrolase with a serovar-specific polymorphism influencing catalytic activity, suppression of immune responses, and bacterial virulence *J. Biol. Chem.* 291, 25853–25863. doi: 10.1074/jbc.M116.752782

Gurevich, A., Saveliev, V., Vyahhi, N., and Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 29, 1072–1075. doi: 10.1093/bioinformatics/btt086

Haft, D. H., DiCuccio, M., Badretdin, A., Brover, V., Chetvernin, V., O'Neill, K., et al. (2018). RefSeq: an update on prokaryotic genome annotation and curation. *Nucleic Acids Res.* 46, D851–d860. doi: 10.1093/nar/gkx1068

Han, J., Gao, X., Luo, X., Zhu, L., Zhang, Y., Dong, P., et al. (2023). The role of PhoP/PhoQ system in regulating stress adaptation response in *Escherichia coli* O157:H7. *Food Microbiol.* 112, 104244. doi: 10.1016/j.fm.2023.104244

He, S., Cui, Y., Qin, X., Zhang, F., Shi, C., Paoli, G. C., et al. (2018). Influence of ethanol adaptation on *Salmonella enterica* serovar Enteritidis survival in acidic environments and expression of acid tolerance-related genes. *Food Microbiol.* 72, 193–198. doi: 10.1016/j.fm.2017.12.005

Illumina (2021). *BCL Convert*. Available online at: https://support-docs.illumina.com/SW/BCL_Convert/Content/SW/FrontPages/BCL_Convert.htm (accessed August 20, 2023).

Irving, S. E., Choudhury, N. R., and Corrigan, R. M. (2021). The stringent response and physiological roles of (pp)pGpp in bacteria. *Nat. Rev. Microbiol.* 19, 256–271. doi: 10.1038/s41579-020-00470-y

Jazdarehee, A., Anderson, J., Morrison, D., and Pardoe, W. (2017). Deletion of *Escherichia coli* K30 type I capsule assembly gene WZC confers resistance to the antibiotic erythromycin in solid media. *Researchgate* 21, 108–112.

Jiang, X., Ren, S., Geng, Y., Yu, T., Li, Y., Liu, L., et al. (2020). The sug operon involves in resistance to quaternary ammonium compounds in *Listeria monocytogenes* EGD-e. *Appl Microbiol Biotechnol* 104, 7093–7104. doi: 10.1007/s00253-020-10741-6

Jurenas, D., Fraikin, N., Goormaghtigh, F., and Van Melderen, L. (2022). Biology and evolution of bacterial toxin–antitoxin systems. *Nat. Rev. Microbiol.* 20, 335–350. doi: 10.1038/s41579-021-00661-1

Kahl, B. C., Becker, K., and Löffler, B. (2016). Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections. *Clin. Microbiol. Rev.* 29, 401–427. doi: 10.1128/CMR.00069-15

Kawai, Y., Matsumoto, S., Ling, Y., Okuda, S., and Tsuneda, S. (2018). AldB controls persister formation in *Escherichia coli* depending on environmental stress. *Microbiol. Immunol.* 62, 299–309. doi: 10.1111/1348-0421.12587

Kenney, L. J. (2019). The role of acid stress in *Salmonella* pathogenesis. *Curr. Opin. Microbiol.* 47, 45–51. doi: 10.1016/j.mib.2018.11.006

Kim, D., Paggi, J. M., Park, C., Bennett, C., and Salzberg, S. L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* 37, 907–915. doi: 10.1038/s41587-019-0201-4

Lang, C., Zhang, Y., Mao, Y., Yang, X., Wang, X., Luo, X., et al. (2021). Acid tolerance response of *Salmonella* during simulated chilled beef storage and its regulatory mechanism based on the PhoP/Q system. *Food Microbiol.* 95, 103716. doi: 10.1016/j.fm.2020.103716

Lee, D. H., Feist, A. M., Barrett, C. L., and Palsson, B. (2011). Cumulative number of cell divisions as a meaningful timescale for adaptive laboratory evolution of *Escherichia coli*. *PLoS ONE* 6, e26172. doi: 10.1371/journal.pone.0026172

Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *bioRxiv*. doi: 10.48550/arXiv.1303.3997

Li, M., He, Y., Sun, J., Li, J., Bai, J., Zhang, C., et al. (2019). chronic exposure to an environmentally relevant triclosan concentration induces persistent triclosan resistance but reversible antibiotic tolerance in *Escherichia coli*. *Environ. Sci. Technol.* 53, 3277–3286. doi: 10.1021/acs.est.8b06763

Li, W., Li, Y., Wu, Y., Cui, Y., Liu, Y., Shi, X., et al. (2016). Phenotypic and genetic changes in the life cycle of small colony variants of *Salmonella enterica* serotype Typhimurium induced by streptomycin. *Ann. Clin. Microbiol. Antimicrob.* 15, 37. doi: 10.1186/s12941-016-0151-3

Liao, Y., Smyth, G. K., and Shi, W. (2014). Feature counts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–930. doi: 10.1093/bioinformatics/btt656

Liu, W. T., Karavolos, M. H., Bulmer, D. M., Allaoui, A., Hormaeche, R. D. C. E., Lee, J. J., et al. (2007). Role of the universal stress protein UspA of *Salmonella* in growth arrest, stress and virulence. *Microbial. Pathogenesis* 42, 2–10. doi: 10.1016/j.micpath.2006.09.002

Luo, D., Wu, Z., Bai, Q., Zhang, Y., Huang, M., Huang, Y., et al. (2023). Universal stress proteins: from gene to function. *Int. J. Mol. Sci.* 24, 25. doi: 10.3390/ijms24054725

Magnusson, L. U., Farewell, A., and Nyström, T. (2005). ppGpp: a global regulator in *Escherichia coli*. *Trends Microbiol.* 13, 236–242. doi: 10.1016/j.tim.2005.03.008

Matsuo, T., Nakamura, K., Kodama, T., Mikami, T., Hiyoshi, H., Tsuchiya, T., et al. (2013). Characterization of all RND-type multidrug efflux transporters in *Vibrio parahaemolyticus*. *Microbiologyopen* 2, 725–742. doi: 10.1002/mbo3.100

Matuschek, E., Åhman, J., Webster, C., and Kahlmeter, G. (2018). Antimicrobial susceptibility testing of colistin – evaluation of seven commercial MIC products against standard broth microdilution for *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. *Clin. Microbiol. Inf.* 24, 865–870. doi: 10.1016/j.cmi.2017.11.020

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., et al. (2010). The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 20, 1297–1303. doi: 10.1101/gr.107524.110

Nishio, M., Okada, N., Miki, T., and Haneda, T. (2005). Identification of the outer-membrane protein PagC required for the serum resistance phenotype in *Salmonella enterica* serovar Choleraesuis. *Microbiology* 151, 863–873. doi: 10.1099/mic.0.27654-0

Pacios, O., Blasco, L., Bleriot, I., Fernandez-Garcia, L., Ambroa, A., López, M., et al. (2020). (p)ppGpp and its role in bacterial persistence: new challenges. *Antimicrobial. Agents Chemother.* 64, 1220. doi: 10.1128/AAC.01283-20

Pandey, D., Kumari, B., Singhal, N., and Kumar, M. (2020). BacEffluxPred: a two-tier system to predict and categorize bacterial efflux mediated antibiotic resistance proteins. *Sci. Rep.* 10, 9287. doi: 10.1038/s41598-020-65981-3

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M., et al. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* 4, 295–305. doi: 10.1038/nrmicro1384

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Core Team.

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140. doi: 10.1093/bioinformatics/btp616

Sandberg, T. E., Salazar, M. J., Weng, L. L., Palsson, B. O., and Feist, A. M. (2019). The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. *Metab. Eng.* 56, 1–16. doi: 10.1016/j.ymben.2019.08.004

Singh, R., Barry, C., and Boshoff, H. (2010). The Three RelE homologs of *Mycobacterium tuberculosis* have individual, drug-specific effects on bacterial antibiotic tolerance. *J. Bacteriol.* 192, 1279–1291. doi: 10.1128/JB.01285-09

Spira, B., and Ospino, K. (2020). Diversity in *E. coli* (p)ppGpp levels and its consequences. *Front. Microbiol.* 11, 1759. doi: 10.3389/fmicb.2020.01759

Su, C. C., Rutherford, D. J., and Yu, E. W. (2007). Characterization of the multidrug efflux regulator AcrR from *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 361, 85–90. doi: 10.1016/j.bbrc.2007.06.175

Sulaiman, J. E., and Lam, H. (2020). Proteomic study of the survival and resuscitation mechanisms of filamentous persisters in an evolved *Escherichia coli* population from cyclic ampicillin treatment. *mSystems* 5, 420. doi: 10.1128/mSystems.00462-20

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., et al. (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res.* 44, 6614–6624. doi: 10.1093/nar/gkw569

van den Bijllaardt, W., Schijffelen, M. J., Bosboom, R. W., Cohen Stuart, J., Diederen, B., Kampinga, G., et al. (2018). Susceptibility of ESBL *Escherichia coli* and *Klebsiella pneumoniae* to fosfomycin in the Netherlands and comparison of several testing methods including Etest, MIC test strip, Vitek2, Phoenix and disc diffusion. *J. Antimicrob. Chemother.* 73, 2380–2387. doi: 10.1093/jac/dky214

Viveiros, M., Dupont, M., Rodrigues, L., Couto, I., Davin-Regli, A., Martins, M., et al. (2007). Antibiotic stress, genetic response and altered permeability of *E. coli*. *PLoS ONE* 2, e365. doi: 10.1371/journal.pone.0000365

Wang, X., Lord, D. M., Cheng, H. Y., Osbourne, D. O., Hong, S. H., Sanchez-Torres, V., et al. (2012). A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. *Nat. Chem. Biol.* 8, 855–861. doi: 10.1038/nchembio.1062

Wang, X., and Wood, T. K. (2011). Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. *Appl. Environ. Microbiol.* 77, 5577–5583. doi: 10.1128/AEM.05068-11

Weigel, W. A., and Demuth, D. R. (2016). QseBC, a two-component bacterial adrenergic receptor and global regulator of virulence in Enterobacteriaceae and Pasteurellaceae. *Mol. Oral Microbiol.* 31, 379–397. doi: 10.1111/omi.12138

Wiegand, I., Hilpert, K., and Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protocols* 3, 163–175. doi: 10.1038/nprot.2007.521

Windels, E. M., Van den Bergh, B., and Michiels, J. (2020). Bacteria under antibiotic attack: Different strategies for evolutionary adaptation. *PLOS Pathogens* 16, e1008431. doi: 10.1371/journal.ppat.1008431

Xu, N., Wang, L., Cheng, H., Liu, Q., Liu, J., Ma, Y., et al. (2014). In vitro functional characterization of the Na+/H+ antiporters in *Corynebacterium glutamicum*. *FEMS Microbiol. Lett.* 363, 237. doi: 10.1093/femsle/fnv237

Yang, Y., Liu, J., Clarke, B. R., Seidel, L., Bolla, J. R., Ward, P. N., et al. (2021). The molecular basis of regulation of bacterial capsule assembly by Wzc. *Nat. Commun.* 12, 4349. doi: 10.1038/s41467-021-24652-1

Zhao, G., Weatherspoon, N., Kong, W., Curtiss, R., and Shi, Y. (2008). A dual-signal regulatory circuit activates transcription of a set of divergent operons in *Salmonella typhimurium*. *Proc. Nat. Acad. Sci.* 105, 20924–20929. doi: 10.1073/pnas.0807071106

Zhu, Y., Dou, Q., Du, L., and Wang, Y. (2023). QseB/QseC: a two-component system globally regulating bacterial behaviors. *Trends Microbiol.* 31, 749–762. doi: 10.1016/j.tim.2023.02.001

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Antimicrobial resistance and genomic investigation of *Salmonella* isolated from retail foods in Guizhou, China

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Introduction: *Salmonella* is a major foodborne pathogen worldwide that causes severe morbidity and mortality. It is mainly caused by consuming contaminated food, with retail food considered the primary source.

Methods: In Guizhou, China, 102 *Salmonella* strains isolated from 2016 to 2021 underwent phenotypic antimicrobial resistance testing and whole-genome sequencing (WGS) to understand Salmonella diversity, including serotypes, sequencing types (STs), antimicrobial genes, virulence genes, plasmid types, multi-locus sequence types (MLST), and core genome MLST (cgMLST).

Results and discussion: *S.Typhimurium* was the dominant serotype, and O:4(B) was the leading serogroup. The most prevalent genotype was ST40. Phenotypic antimicrobial resistance identified 66.7% of the sampled isolates as multi-drug resistant (MDR). *S.Enteritidis* ($n = 7$), *S.Typhimurium* ($n = 1$), *S.Indiana* ($n = 1$), *S.*Kentucky ($n = 1$), *S.*Uganda ($n = 1$), all of which were MDR, were resistant to Colistin. Resistance rates varied significantly across different strains and food types, particularly meat products exhibiting higher resistance. Notably, significant increases in resistance were observed from 2016 to 2021 for the following: ≥ 1 resistant ($P = 0.001$), MDR ($P = 0.001$), ampicillin ($P = 0.001$), tetracycline ($P < 0.001$), chloramphenicol ($P = 0.030$), and trimethoprim/sulfamethoxazole ($P = 0.003$). The marked escalation in drug resistance over the recent years, coupled with the varying resistance rates among food sources, underscores the growing public health concern. Our findings highlight the need for a coordinated approach to effectively monitor and respond to Salmonella infections in Guizhou, China.

KEYWORDS

***Salmonella*, retail foods, Guizhou province, multi-drug resistance, whole genome sequencing**

1 Introduction

Salmonella *spp.*, highly prevalent foodborne pathogens, are responsible for a range of diseases from gastroenteritis to typhoid fever. They are mainly isolated from foods of animal origin, particularly meat, eggs, and their products (Liu et al., 2021; Alzahrani et al., 2023). Globally, approximately 200 million to over 1 billion cases of Salmonella occur each year, of

which there are 93 million cases of gastroenteritis and 155,000 fatalities and 85% are linked to food consumption (Hung et al., 2017; Chlebicz and Slizewska, 2018; Castro-Vargas et al., 2020). In the United States, data on non-typhoidal Salmonella infections show an estimated annual occurrence of approximately 1.2 million cases, 23,000 hospitalizations, and 450 deaths (Scallan et al., 2011; Medalla et al., 2021). While in the European Union (EU), Salmonella caused the most outbreaks and outbreak-related illnesses. In 2021, 30 EU countries reported 60,494 laboratory-confirmed cases of salmonellosis, 73 of which were fatal. Compared with the number of cases in 2020, the number of cases in 2021 increased by 14% (European CDC, 2022). In China, an estimated 9.87 million cases of gastroenteritis are caused by Salmonella each year (Mao et al., 2011).

Guizhou Province is situated in the mountainous region of southwest China, spanning from 24°37′N to 29°130′N and 103°360′E to 109°350′E (Zuo et al., 2022; Xie and Zhang, 2023). It is known as a "natural encyclopedia" of the karst landform, has a population of approximately 38.56 million inhabitants, and covers an expansive area of around $1.76 \times 105\,km^2$. The unique geological features and landscapes of the region make it a popular tourist destination for exploring the beauty of karst formations. This province has recently experienced a boost in tourism owing to its abundant natural, cultural, and environmental resources. However, this increase in tourism may have also led to the spread of pathogens that cause diarrhea and antimicrobial resistance (Koch et al., 2011; Boolchandani et al., 2022). According to the "National Foodborne Disease Surveillance Work Manual" from China's National Center for Food Safety Risk Assessment (Lu et al., 2023), *Salmonella* is the first-listed pathogen, along with *Vibrio parahaemolyticus, Diarrheagenic Escherichia coli, Shigella, and Norovirus*, as mandatory monitoring items for all provinces. This indicates the high priority given to *Salmonella* in the context of food safety and antimicrobial resistance. Our previous surveillance results have shown that food-related disease cases in Guizhou Province are mostly caused by *Salmonella typhimurium* (Wei et al., 2019; Bai et al., 2022; Long et al., 2022; Wei et al., 2023). Concerningly, we also have recently reported a foodborne outbreak in Guizhou caused by *Salmonella* (Zhou et al., 2023). Wide-ranging drug resistance and a high proportion of multi-drug resistant (MDR) strains have been isolated in meat, eggs, and their products. However, data on antimicrobial resistance and molecular genotyping of *Salmonella* spp. in Guizhou are lacking.

Recent advancements in genome analysis have significantly enhanced our understanding of *Salmonella*'s pathogenic mechanisms and epidemiology (Djordjevic et al., 2023). Genome sequencing and analysis provide insights into its genetic diversity, virulence factors, and antibiotic resistance patterns. This genomic information is crucial for developing effective therapies. Furthermore, comparative genomics among different *Salmonella* strains helps in tracing the source and transmission of outbreaks, thus playing a vital role in public health surveillance and response strategies (Kong et al., 2022; Fatima et al., 2023).

To better understand the characteristics of *Salmonella*, we characterized antimicrobial resistance, presence of antimicrobial resistance and virulence genes, and genetic characterization of *Salmonella* isolates in Guizhou between 2016 and 2021. These findings serve as valuable reference points for implementing effective measures for the prevention and control of *Salmonella* infections. Moreover, they promote the rational use of antimicrobial agents. By leveraging this information, appropriate strategies can be developed to mitigate the transmission of *Salmonella* and address the issue of antimicrobial resistance in the Guizhou Province.

2 Materials and methods

2.1 Strain source and collection

Strains were collected based on the Guizhou Foodborne Disease Surveillance Program. The study's sampling period extended from 2016 to 2021. In this process, we methodically chose retail food items from all nine areas and prefectures of Guizhou Province (Figure 1A) to isolate *Salmonella*. The diverse assortment of food items sampled encompassed a range of categories, including meat and meat products (encompassing cooked meat products, raw poultry, raw livestock meat, and minced meat products), aquatic products, eggs and egg products, baked foods, soy products, rice and flour-based items (both in cooked and raw forms), various catering foods (like sushi, salads, and traditional Chinese cold dishes), as well as street vendors. In adherence to the project's confidentiality protocols, specific details regarding the sample size and sampling plan are restricted internal information. Consequently, this study does not present the detection rate or other related findings.

2.2 *Salmonella* isolation and serotyping

Salmonella was isolated as previously described (GB4789.4-2016, 2016; Li et al., 2023). The suspected strains were subjected to CHROMagar *Salmonella* agar plates and a VITEK 2 compact automatic microbial identification system (BioMérieux, France). Positive colonies were subsequently confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Bruker Bremen, Germany). Single colonies obtained from the Swarm agar after overnight incubation at 37°C were selected for testing the phase 1 and phase 2 H antigens using slide agglutination. *Salmonella* antiserum Kits (60 V) were purchased from Statens Serum Institute (Copenhagen, Denmark) and Beijing Land Bridge Technology Ltd. (Beijing, China).

Based on the Guizhou Foodborne Disease Surveillance Program, from all retail food samples tested between 2016 and 2021, a total of 135 *Salmonella* strains were isolated. Due to limitations in detection capabilities and budget, a subset of 102 strains was subsequently selected for Antimicrobial Resistance (AMR) and Whole Genome Sequencing (WGS) analysis. Originally, the plan was to successfully sequence 100 of the 102 strains, acknowledging the possibility that 2 strains might fail in sequencing process. However, all 102 strains were successfully sequenced. Consequently, all 102 strains were utilized for the further antimicrobial susceptibility testing and whole genomic sequencing (Zhao et al., 2022; Petrin et al., 2023). Among these, 50 strains were from the northern region, and 52 were from the southern region. These strains were chosen for the following reasons: (1) complete coverage of all sampling geographic regions, (2) representation of positive isolates recovered from different retail foods, and (3) representation of positive isolates recovered from different years. Redundant strains with the same characteristics, such as the same serotype isolated from the same location and in the same

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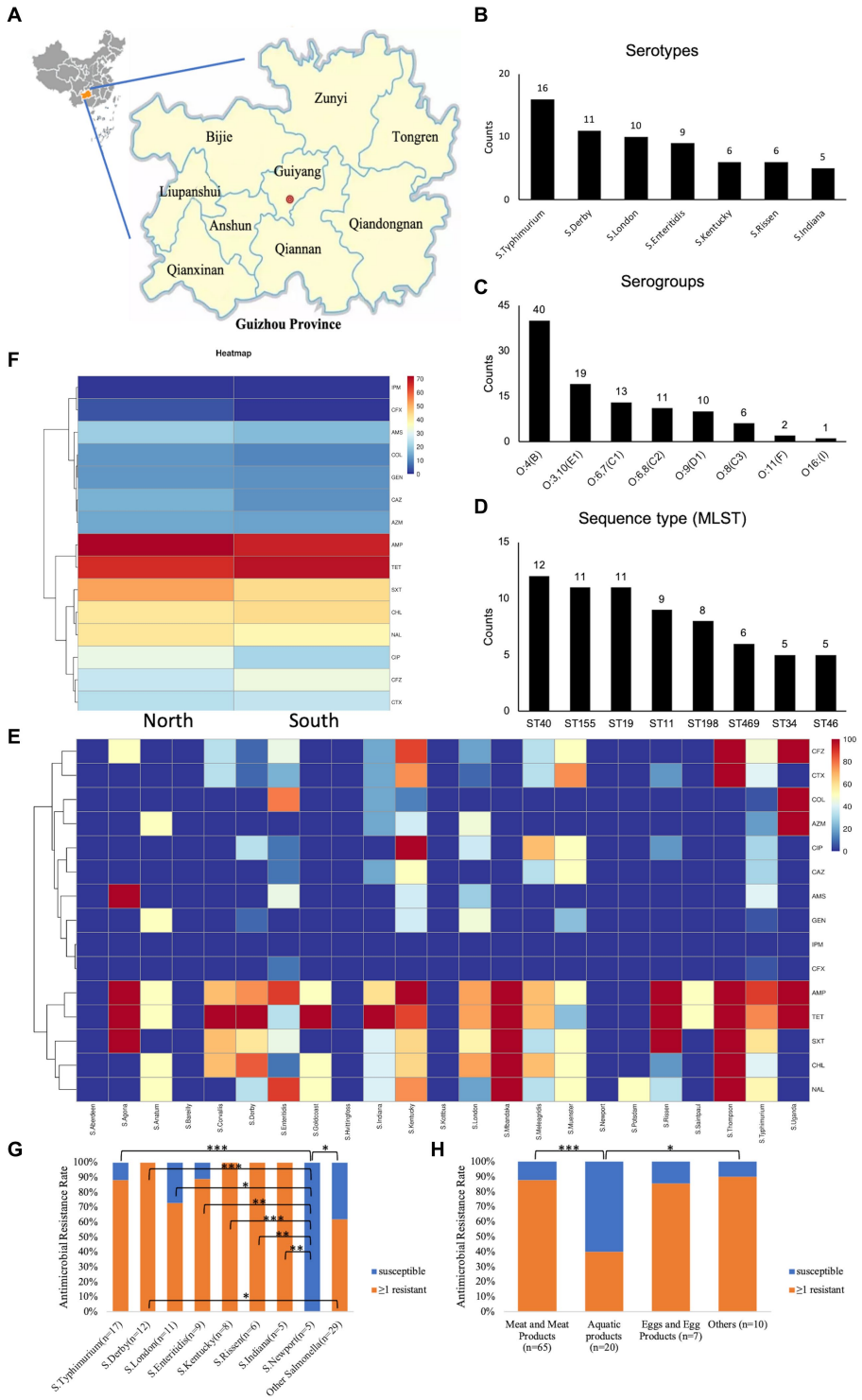

FIGURE 1
An overview of the isolated *Salmonella* strains' classification and antimicrobial resistance patterns. **(A)** Source; **(B)** main serotypes; **(C)** serogroup; **(D)** primary sequence type via MLST; **(E)** antimicrobial resistance heatmap for diverse strains; **(F)** antimicrobial resistance heatmap by isolate region; **(G)** frequency of antibiotic resistance in 102 *Salmonella* isolates, Scale 0–100, representing 0–100% proportions; **(H)** *Salmonella* resistance distribution by food sources; ≥1 resistant, with at least one resistant strain; $*p < 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

year, were excluded. Given the selection criteria and our selection encompassed more than 75% (102/135) of the total samples, we believe that the chosen samples for this study are likely to be representative.

2.3 Antimicrobial susceptibility testing

The antimicrobial resistance of all *Salmonella* isolates was examined using a previously reported micro broth dilution method

(Xingbai) (Kong et al., 2022). *Salmonella* isolates was tested against 15 antibiotics across 11 categories: ampicillin (AMP), ampicillin/sulbactam (AMS), tetracycline (TET), cefazolin (CFZ), cefotaxime (CTX), cephalosporin cefoxitin (CFX), ceftazidime (CAZ), imipenem (IMI), Gentamicin (GEN), Polymyxin E (Colistin, CT), Azithromycin (AZM), nalidixic acid (NAL), ciprofloxacin (CIP), chloramphenicol (CHL), and trimethoprim/sulfamethoxazole (SXT). MDR strains were defined as those resistance to three or more antimicrobial classes (Zhou et al., 2018, 2022).

Briefly, the operation was described as follows: A fresh, pure culture was taken and emulsified into a bacterial suspension with a turbidity equivalent to 0.5 McFarland standard in sterile water. Subsequently, 10 μL of this bacterial suspension was added to the Cation-Adjusted Mueller–Hinton Broth (CAMHB), which corresponded with the Thermo Sensititre™ Gram-negative drug susceptibility identification plate. In this broth, 50 μL of the bacterial broth solution was added to each microwell on the drug susceptibility plate. The solution was then incubated at 36°C for a duration of 18–24 h. Following incubation, the results of the drug susceptibility test were read using the Thermo Vizion automatic microbial susceptibility analysis system, in accordance with the guidelines provided by the Sensititre software. Blank and negative controls were also used. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains to validate the results of the antimicrobial susceptibility testing (Kong et al., 2022). Antimicrobial breakpoints were determined in line with the interpretive standards provided by the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2016).

2.4 Whole genome sequencing and bioinformatic analysis

The *Salmonella* strain was streaked onto Luria-Bertani (LB) plates. The plates were incubated overnight (approximately 18 h) at 37°C. Next, the single colony on the LB plate was inoculated into LB broth at 37°C under 150 rpm shaking conditions. Thereafter, *Salmonella* was harvested after 10 min centrifugation at 12,000 × g. Genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisc, United States) according to the manufacturer's protocol. Purified genomic DNA was quantified using the TBS-380 fluorometer (Turner BioSystems Inc., Sunnyvale, CA, United States). High-quality DNA (OD260/280 ≥ 1.5; ≥150 ng) was used for further analysis.

DNA samples were sheared into 400–500 bp fragments using a Covaris M220 Focused Acoustic Shearer following the manufacturer's protocol. Illumina sequencing libraries were prepared from the sheared fragments using a Rapid DNA-Seq Kit (NEXTflex, San Jose, CA, United States). After end-repair, A-tailing, adapter ligation, and PCR enrichment, the libraries underwent paired-end sequencing (2 × 150 bp). Draft genome sequence analysis of the *Salmonella* strain was performed using an Illumina NovaSeq6000 sequencing platform.

Bioinformatic analysis of the Illumina data involved filtering raw reads with Fastp (version 0.19.6) (Chen et al., 2018), assembling with SOPA *de novo* version 2.04 (Luo et al., 2012), and predicting CDS, tRNA, and rRNA with Glimmer, tRNA-scan-SE, and Barrnap, respectively (Xu et al., 2023). Annotations were derived from NR, Swiss-Prot, Pfam, GO, COG, and KEGG databases using tools like BLASTP, Diamond, and HMMER. Briefly, each set of query proteins was aligned with the databases, and annotations of the best-matched subjects (*e*-value < 10^{-5}) were obtained for gene annotation. Based on the Comprehensive Antibiotic Resistance Database (CARD) and the Virulence Factor Database (VFDB), the antibiotic resistance and virulence genes for each *Salmonella* isolate were evaluated (Akter et al., 2023). The Plasmid replicons for the genome sequences were studied using PlasmidFinder (Akter et al., 2023). The sequencing results for *Salmonella* strains were uploaded to the National Foodborne Disease Molecular Tracing Network (TraNet). Multi-locus sequence typing (MLST) and core genome MLST (cgMLST) analysis were performed using BioNumerics version 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium) (Kong et al., 2022). Complete linkage was used to construct a cluster analysis dendrogram. For these analyses, default software parameters were consistently utilized.

2.5 Statistical analysis

All reported data were audited, checked, exported, and managed using Microsoft Excel 2016. Variable values are reported either as counts or percentages (%) for categorical data, or as mean ± standard deviation for continuous data. Statistical analyses of resistance rates and MDR profiles were conducted using R version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) or SPSS version 25 (IBM Corp, Armonk, NY, United States). Besides, stratified analyses (Mukherjee et al., 2019; Marchello et al., 2022) by year were also conducted. Based on the characteristics of the data, appropriate statistical tests were selected for analysis, including the Chi-square test/Fisher's test for categorical data and the *t*-test/Mann–Whitney U test for continuous data. The Mantel–Haenszel χ^2 test was employed to assess trends. A p-value < 0.05 was deemed statistically significant.

3 Results

3.1 Distribution of *Salmonella* isolates

From 2016 to 2021, 102 strains of *Salmonella* were isolated from retail foods in nine distinct regions of Guizhou Province: Bijie, Zunyi, Tongren, Liupanshui, Anshun, Guiyang, Qianxinan (Miao and Dong autonomous prefectures in southeast Guizhou), Qiannan (Buyi and Miao autonomous prefectures in south Guizhou), and Qiandongnan (Buyi and Miao autonomous prefectures in southwest Guizhou, Figure 1A).

Table 1 shows the distribution of *Salmonella* isolates among food types and sampling sites. In total, 102 *Salmonella* isolates were obtained. No significant differences were observed between the sampling sites in northern and southern Guizhou. However, considering food types, *Salmonella* was more common in meat and meat Products (63.70%; 65/102) than in aquatic products 19.60% (20/102) and "other origin" samples 9.80% (10/102).

The slide agglutination test revealed the presence of 32 different serotypes and eight serogroups among the 102 studied *Salmonella* strains. The dominant serotype was *Typhimurium* (15.7%; 16/102, Figure 1B), the dominant serogroup was O:4 (B) (39.2%; 40/102, Figure 1C), and among the *Salmonella* strains under investigation,

sequence type ST40 was the most prevalent (Figure 1D). Moreover, approximately one-third of agglutination-based serotypes did not match the MSLT-based serotypes (Supplementary Data Sheet 1_"AST" sheet). To enhance accuracy and reduce potential human errors in serotype determination from hemagglutination tests, which could lead to misjudgment, our subsequent analysis focused on analyzing predicted serotypes derived from sequencing types.

According to Supplementary Figure S1, in meat, the main *Salmonella* strains are *S.* London, *S.* Derby, *S.* Kentucky, *S. enteritidis*, *S.* Rissen, and *S. typhimurium*. In aquatic food, the main strains are *S.* Newport and *S. typhimurium*. In eggs and other food, the main strains are *S.* London and *S. typhimurium*, respectively.

3.2 Phenotypic antimicrobial resistance

Table 2 shows that the highest resistance was observed for ampicillin (69.6%; 71/102), followed by tetracycline (67.7%; 69/102) and trimethoprim/sulfamethoxazole (48.0%; 49/102), whereas no instance of resistance was observed for imipenem (0.0%; 0/102). Of the isolated strains, 78.4% (80/102) of the isolated strains were resistant to at least one antimicrobial class, 72.5% (74/102) were resistant to at least two antimicrobial classes, and 66.7% (68/102) were resistant to three or more than three antimicrobial classes, namely MDR strains. Moreover, our results showed that 16.7% (17/102) of isolates were resistant to eight antimicrobial classes, 10.8% (11/102) were resistant to nine antimicrobial classes, and no isolates (0/102) were resistant to all tested antimicrobial agents (Supplementary Data Sheet 1_"AST" sheet).

The distribution of antimicrobial resistance showed that some *S. Kentucky* (16.7%, 1/6) and *S. typhimurium* (11.8%, 2/17) isolates were the most resistant (resistant to ≥11 antibiotics), while *S.* Aberdeen ($n=1$), *S.* Bareilly ($n=1$), *S.* Hvittingfoss ($n=1$), *S.* Kottbus ($n=1$), and *S. Newport* ($n=5$) were sensitive to all tested antimicrobial agents (Figure 1E). *S. enteritidis* ($n=7$), *S. typhimurium* ($n=1$), *S. Indiana* ($n=1$), *S.* Kentucky ($n=1$), and *S.* Uganda ($n=1$), all of which were MDR, were resistant to colistin (Supplementary Data Sheet 1_"AST" sheet). Concerning the impact of the sampling location on the antimicrobial resistance of isolates, our findings demonstrated that the *Salmonella* strains obtained from North Guizhou displayed similar resistance levels to those from South Guizhou (Figure 1F, $p>0.05$). In the stratified analysis of the major *Salmonella* strains ($n\geq5$), it was observed that except for *S.* Newport, which had a 0% drug resistant rate, the resistance rates of the other strains were all ≥70% (Figure 1G). Specifically, the resistance rate in *S.* Newport was significantly lower compared to *S.* Kentucky, *S. typhimurium*, those with isolates ≥5, and the other *Salmonella* strains with fewer than 5 isolates ($p<0.05$, Figure 1G). In contrast, all 12 *S.* Derby strains exhibited 100% resistance, significantly higher than other *Salmonella* strains with fewer than 5 isolates ($p<0.05$, Figure 1G). In the stratified analysis by food sources, the resistance rate of *Salmonella* isolated from aquatic products (40.0%) was significantly lower than that from meat (87.7%) and other sources (90.0%, $p<0.05$, Figure 1H).

TABLE 1 Distribution of *Salmonella* isolates among food types and sampling sites.

Food types	North Guizhou	South Guizhou	Total
Meat and meat products	70.00% (35/50)	57.69% (30/52)	63.70% (65/102)
Aquatic products	14.00% (7/50)	25.00% (13/52)	19.60% (20/102)
Eggs and egg products	4.00% (2/50)	9.62% (5/52)	6.90% (7/102)
Others	12.00% (6/50)	7.69% (4/52)	9.80% (10/102)
Total	49.02% (50/102)	50.98% (52/102)	100% (102/102)

TABLE 2 Drug resistance patterns of *Salmonella* isolates.

Antimicrobial classes	Antimicrobial agents	Abbreviations	Resistant criteria (μg/mL)	Resistant rate (%)
Penicillin	Ampicillin	AMP	≥32	69.61% (71/102)
Tetracyclines	Tetracycline	TET	≥16	67.65% (69/102)
Folate pathway inhibitors	Trimethoprim/Sulfamethoxazole	SXT	≥4/76	48.04% (49/102)
Phenylpropanol	Chloramphenicol	CHL	≥32	43.14% (44/102)
Quinolones and fluoroquinolones	Nalidixicacid	NAL	≥32	40.20% (41/102)
	Ciprofloxacin	CIP	≥1	26.47% (27/102)
Cephalosporins	Cefazolin	CFZ	≥8	29.41% (30/102)
	Cefotaxime	CTX	≥4	24.51% (25/102)
	Ceftazidime	CAZ	≥16	13.73% (14/102)
	Cefoxitin	CFX	≥32	1.96% (2/102)
β-lactams combination	Ampicillin/Sulbactam	AMS	≥32/16	18.63% (19/102)
Macrolide	Azithromycin	AZM	≥32	13.73% (14/102)
Aminoglycosides	Gentamicin	GEN	≥16	11.76% (12/102)
Lipopeptide	Colistin	COL	≥4	10.78% (11/102)
Carbapenems	Imipenem	IPM	≥4	0.00% (0/102)

To address the trends in antimicrobial resistance in retail foods from 2016 to 2021, we evaluated the yearly changes in resistance. Significant increases in resistance were observed from 2016 to 2021 for the following: ≥1 resistant (Figure 2A, $p = 0.001$), MDR (Figure 2B, $p = 0.001$), AMP (Figure 2C, $p = 0.001$), TET (Figure 2C, $p < 0.001$), CHL (Figure 2C, $p = 0.030$), and SXT (Figure 2C, $p = 0.003$). However, no significant increase in resistance rates was detected for the other drugs examined. Due to the limited sample size in different retail food types, it was not feasible to stratify these trends further by each category.

3.3 Genotypic antimicrobial resistance

Figures 3, 4 present the findings of antimicrobial resistance gene prediction, revealing a total of 24 drug-resistant genes in all *Salmonella* strains studied: *acrB*, *acrD*, *bacA*, *baeR*, *cpxA*, *CRP*, *emrB*, *emrR*, *E. coli acrA*, *E. coli ampH beta-lactamase*, *golS*, *H-NS*, *kdpE*, *marA*, *mdsA*, *mdsB*, *mdsC*, *mdtB*, *mdtC*, *mdtG*, *mdtH*, *MdtK*, *sdiA*, and *YojI*. Furthermore, 60.8% (62/102) of isolates harbored the *AAC (6′)-Iy gene*, which was responsible for aminoglycoside resistance. Approximately 49.0% (50/102) of strains had the *tet (A)* gene for tetracycline resistance, whereas 39.2% (40/102) carried the *AAC (6′)-Iaa* gene. The *TEM-1* gene was found in 37.3% (38/102) of isolates, the *floR* gene in 30.4% (31/102), and *sul2* gene in 29.4% (30/102). These genes confer resistance to ampicillin/penicillin, florfenicol, and sulphonamides, respectively.

Concerning the distribution of antimicrobial resistance genes among the predicted serotypes based on sequence type, our data revealed that the presence of antimicrobial resistance genes was more prevalent in *S.* Agona, *S.* Derby, *S.* Kentucky, and *S.* Muenster than in the other serotypes, whereas *S.* Aberdeen, *S.* Bareilly, *S.* Hvittingfoss, *S.* Newport, and *S.* Thompson contained the least number of resistance genes (Figure 3, Supplementary Data Sheet 1_"CARD" sheet). Moreover, the distribution of antimicrobial resistance genes in *Salmonella* strains isolated from North Guizhou did not significantly differ from those isolated from South Guizhou (Supplementary Figure S2, $p > 0.05$).

3.4 Virulence gene prediction

Supplementary Data Sheet 1 ("VFDB" sheet) provides predictions for virulence genes using the Virulence Factors Database; 35 potential virulence genes were identified. Among the individual *Salmonella* strains, the highest and lowest counts of virulence genes were found in *S. typhimurium* (28 genes) and *S.* Derby (20 genes), respectively. Our data indicated that *cdtB*, which encodes typhoid toxins, was found in *S. Indiana* (5 strains), *S.* Muenster (4 strains), and *S.* Goldcoast (2 strains). In contrast, 10 *S. typhimurium* strains and 7 *S. enteritidis* strains carried the *plasmid-encoded fimbriae* (*Pef; VF0104*) and *Salmonella plasmid virulence* (*Spv; VF0107*) genes, which encode fimbriae and significantly contribute to the virulence of non-typhoidal *Salmonella* strains. Every strain examined possessed key virulence factors from *Salmonella* Pathogenicity Islands 1 and 2 (*SPI-1* and *SPI-2*) and peritrichous flagella (*AI145*), as well as *AcrAB* (*VF0568*), *Agf* (*VF0103*), *Bcf* (*AI058*), *OmpA* (*VF0236*), *Enterobactin* (*IA019*), *AGF* (*AI094*), *Capsule* (*VF0560*), *Enterobactin* (*VF0228*), *MgtBC* (*VF0106*), *RcsAB* (*VF0571*), *SinH* (*VF0400*), *Type 1 fimbriae* (*VF0102*), and *MisL* (*VF0397*).

3.5 Plasmid profiles

Figure 5 shows the predicted plasmid types of the studied *Salmonella* isolates. Forty-nine different plasmids were identified in the *Salmonella* isolates. Our results showed that *IncHI2_1* and *Col (pHAD28)_1* were the most prevalent plasmids (present in 32 isolates), followed by *IncFII (pKP91)_1*, *IncFII (S)_1*, and *IncFII (Yp)_1* ($n = 30$ isolates). Additionally, *S.* Agona possessed an average of 10 plasmid types, followed by *S.* Derby (average plasmid types = 8), *S.* Kentucky, *S.* Rissen, and *S. typhimurium* (average plasmid types = 7). In contrast, *S.* Bareilly and *S.* Mbandaka did not harbor any plasmids.

3.6 Comparative and correlation analyses

Table 3 shows the Comparative analysis of drug susceptibility, drug resistance genes, virulence genes, and plasmids of 2 3-year periods. We found that the number of drug-resistant agents (3.13 ± 3.10 vs. 5.15 ± 3.10, $p = 0.002$, Table 3) and drug-resistant genes (29.19 ± 5.89 vs. 32.19 ± 6.61, $p = 0.006$, Table 3) of *Salmonella* in 2016–2018 was significantly lower than that in 2019–2021, suggesting a marked escalation in drug resistance over the recent 3 years. On the contrary, the virulence genes of *Salmonella* in 2016–2018 were significantly higher than those in 2019–2021 (23.96 ± 2.41 vs. 22.96 ± 2.07, $p = 0.035$, Table 3), indicating that the virulence of *Salmonella* has declined in recent years. In addition, compared with 2016–2018, the number of plasmids carried by *Salmonella* in 2019–2021 increased, but no statistical difference was found (7.06 ± 5.21 vs. 9.31 ± 7.31, $p > 0.05$, Table 3).

Supplementary Figure S3 demonstrates the correlation between phenotypic and genotypic antimicrobial resistance. Significant correlations were observed between *Colistin* and *aadA5* ($\rho = 0.581$, $p < 0.001$), *dfrA17* ($\rho = 0.581$, $p < 0.001$), *CMY-2* ($\rho = 0.286$, $p = 0.004$), *FosA3* ($\rho = 0.255$, $p = 0.010$), and *BalTEM-1* ($\rho = 0.255$, $p = 0.010$). Supplementary Figure S4 shows the correlation between phenotypic antimicrobial resistance and plasmid. Significant correlations were observed between *Colistin* and *IncFIB (S)_1* ($\rho = 0.523$, $p < 0.001$), *IncX3_1* ($\rho = 0.507$, $p < 0.001$), *IncX1_4* ($\rho = 0.407$, $p < 0.001$), *IncFIA_1* ($\rho = 0.286$, $p = 0.004$), *IncFIB (pB171)_1* ($\rho = 0.286$, $p = 0.004$), and *IncI2_1* ($\rho = 0.286$, $p = 0.004$). Significant correlations were also observed between phenotypic antimicrobial resistance and virulence genes (Supplementary Figure S5), between genotypic antimicrobial resistance and plasmids (Supplementary Figure S6), and between genotypic antimicrobial resistance and virulence genes (Supplementary Figure S7).

3.7 Phylogenetic analyses

We performed phylogenetic analyses using MLST and cgMLST.

Figure 6 showed that the 102 *Salmonella* isolates were divided into 28 STs using *in silico* MLST, which revealed that ST40 was the most common (*S.* Derby, $n = 12$), followed by ST19 (*S. typhimurium*, $n = 11$), ST155 (*S.* London, $n = 11$), ST11 (*S. enteritidis*, $n = 9$), ST198

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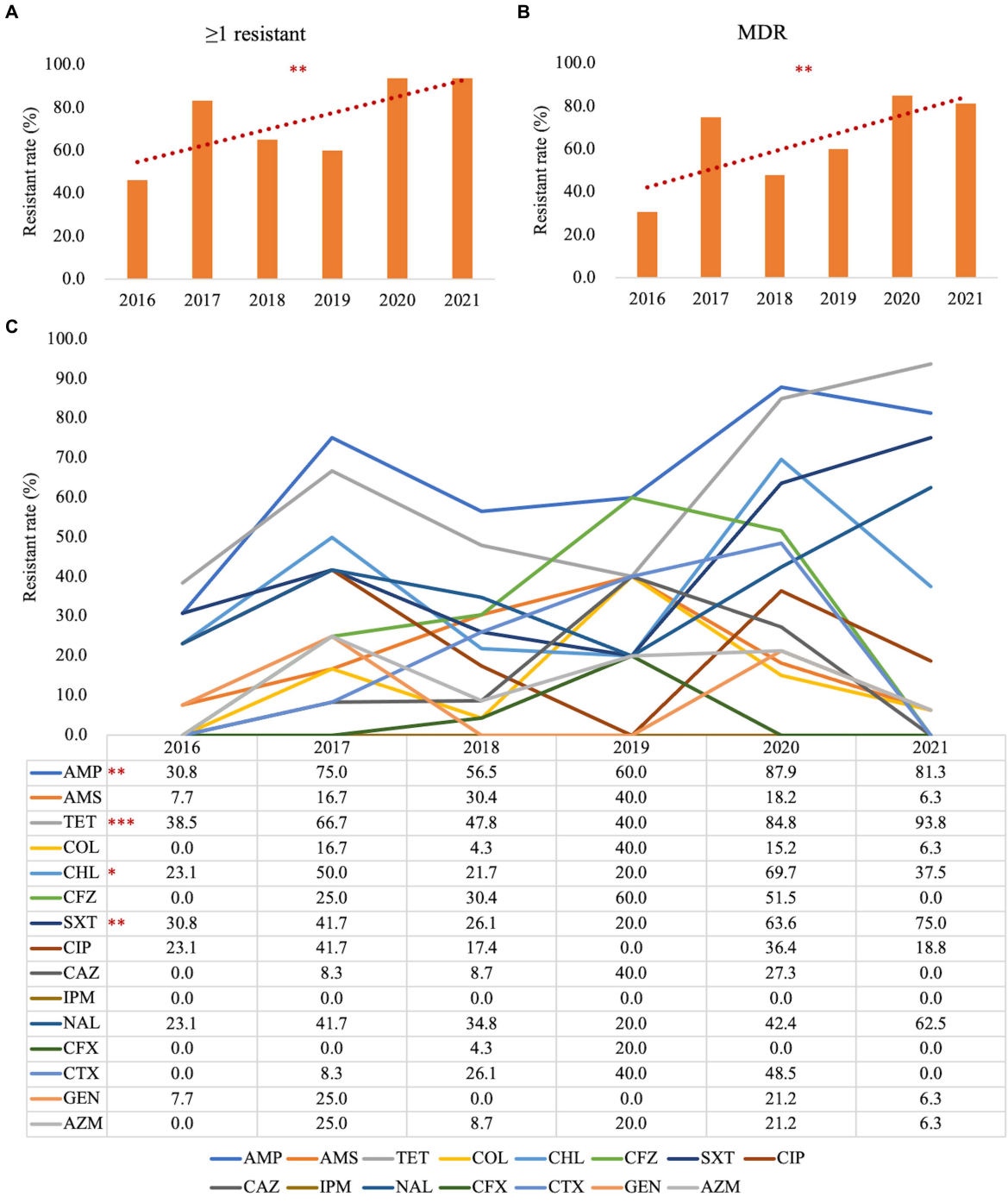

FIGURE 2
Stratified analysis by year for antimicrobial susceptibility testing in *Salmonella*. **(A)** Trends in the *Salmonella* antimicrobial resistance rate with ≥1 resistant from 2016 to 2021; **(B)** Trends in *Salmonella* antimicrobial resistance rate for MDR from 2016 to 2021; **(C)** Trends in *Salmonella* antimicrobial resistance rate for each antimicrobial reagent from 2016 to 2021; ≥1 resistant, with at least one resistant strain; MDR, multi-drug resistant; AMP, ampicillin; AMS, ampicillin/sulbactam; TET, tetracycline; CFZ, cefazolin; CTX, cefotaxime; CFX, cephalosporin cefoxitin; CAZ, ceftazidime; IMI, imipenem; GEN, gentamicin; CT, polymyxin E; AZM, azithromycin; NAL, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole. *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; $n = 102$.

(*S.* Kentucky, $n = 8$), ST469 (*S.* Rissen, $n = 6$), ST34 (*S. typhimurium*, $n = 5$), ST46 (*S.* Newport, $n = 5$), ST321 (*S.* Muenster, $n = 4$), and ST2040 (*S. Indiana*, $n = 4$). Among the 28 STs, 14 were observed in 2 or more regions. ST155, ST40, ST19, ST11, and ST34 appeared in more than five regions, indicating that these five STs were prevalent and had the potential for transregional infection.

Statistics of the isolation sources revealed that (1) the 65 isolates from "meat and meat products" comprised 19 different STs, among

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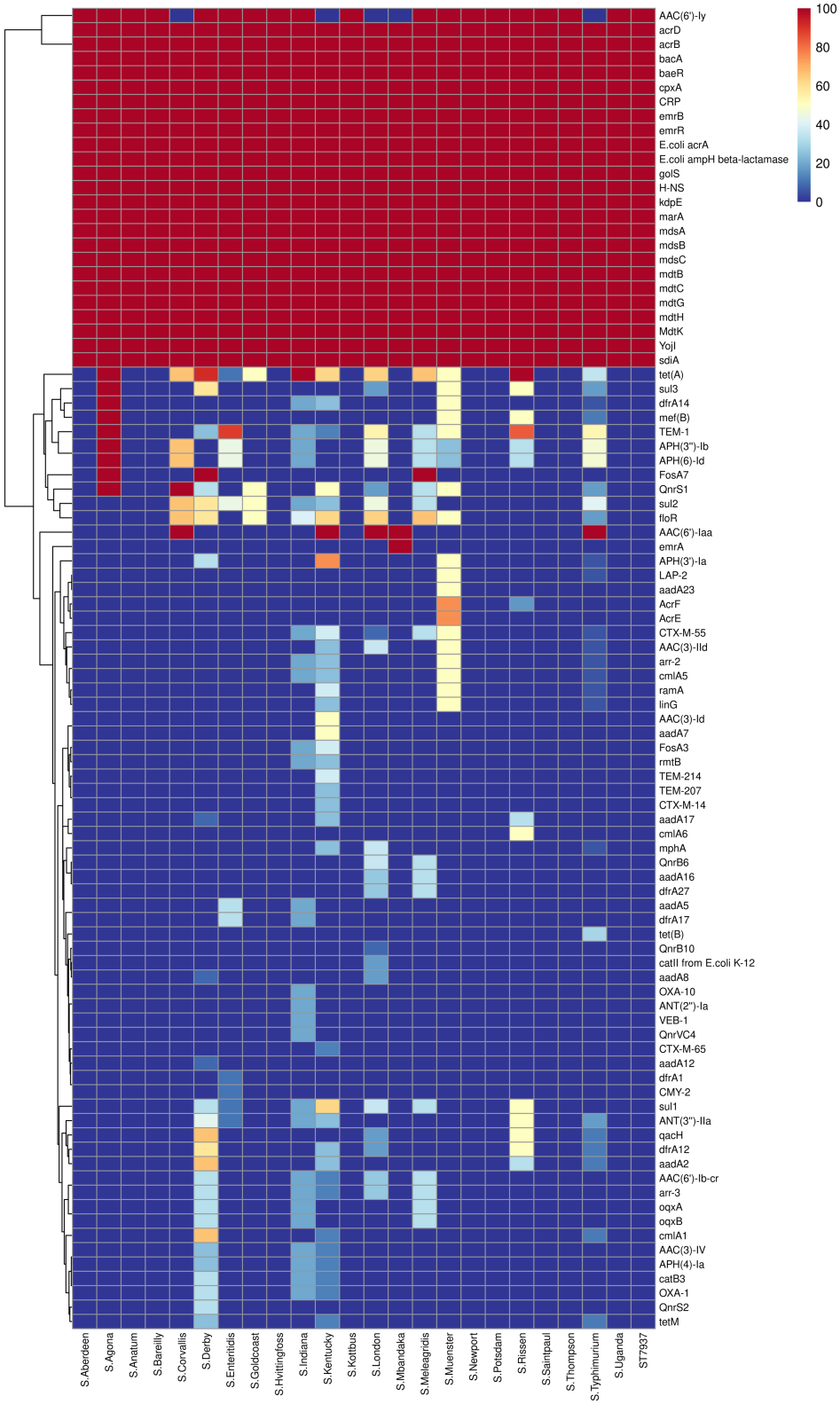

FIGURE 3
The heatmap of antimicrobial resistance genes in the studied *Salmonella* strains. Scale 0–100, representing 0–100% proportions.

which ST155 had the highest number of 10 isolates, followed by ST40 ($n = 9$) and ST198 ($n = 7$); (2) the 20 isolates from "aquatic products" belonged to 13 STs, among which ST46 had the highest number ($n = 5$), followed by ST11 ($n = 2$), ST19 ($n = 2$), and ST426 ($n = 2$); (3) the 7 isolates from "eggs and egg products" contained 6 STs, among which ST19 had the highest number ($n = 2$); and (4) the 10 isolates

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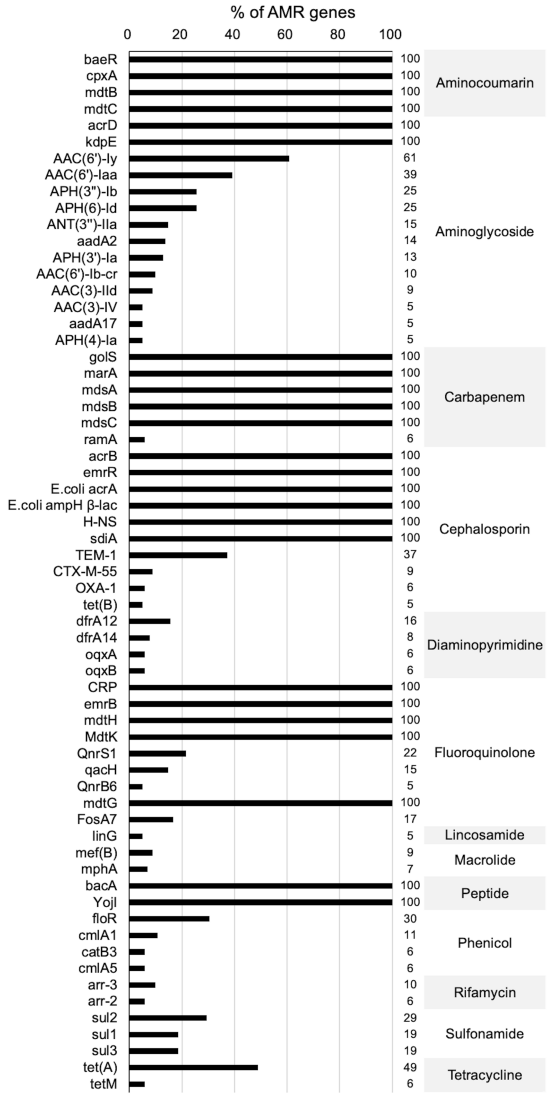

FIGURE 4
Main antibiotic resistance genes profiles. (The proportion of drug resistance genes ≥5%).

from "other" belonged to 7 different STs. Here, we defined "the STs variation index" as the number of STs/isolates. Thus, the ST variation indexes of "meat and meat products," "aquatic products," "eggs and egg products," and "other" were 29.2, 65.0, 85.7, and 70%, respectively.

The statistics of isolation years showed that the major ST differed every year between 2016 and 2021. ST19 ($n = 7$), ST40 ($n = 5$), and ST46 ($n = 5$) were the major STs for 2016, 2017, and 2018, respectively ($n = 48$), while ST155 ($n = 8$), ST40 ($n = 7$), and ST469 ($n = 6$) were the major ST for 2019, 2020, and 2021, respectively ($n = 54$).

Comparisons of MLST and serotyping results showed that, among the top 10 MLST types mentioned above, only ST469 and ST2040 comprised one serotype. Approximately one-third (32/102) of the serotypes did not match their STs. Results indicated that MLST exhibited higher accuracy compared with serotyping. Moreover, two serotypes included more than one ST: 17 isolates with serotype Typhimurium contained three STs (ST19, ST34, and ST1544), and five isolates of *S. Indiana* contained two STs (ST17 and ST2040).

We performed cgMLST analysis on the 102 isolates based on their genomic sequences. Allelic differences were calculated with assembly-free and assembly-based allele calling using default settings. Supplementary Figure S8 illustrates the relatedness between all sequenced strains via CL, revealing that the 102 *Salmonella* isolates were grouped into 96 clusters. Supplementary Data Sheet 2 shows that 3,002 target loci (Garcia-Soto et al., 2021) were identified in the *Salmonella* genome. We found an allelic difference of 99.2% (2,979/3002) between the 102 isolates.

4 Discussion

Salmonella infection is a major public health concern in Guizhou Province. In this study, we first described the antimicrobial and phylogenetic characteristics of foodborne Salmonella in Guizhou Province between 2016 and 2021.

4.1 Distribution

In our study, the proportion of *Salmonella* was higher in "meat and meat products" (63.70%; 65/102) than in "aquatic products" (19.60%; 20/102) and "other origin" samples (9.80%; 10/102). Meat and meat products have been identified as the primary carriers of *Salmonella*, which are pathogenic to humans (Liu et al., 2021). Generally, the prevalence of *Salmonella* in samples collected from meat and meat products is 12–19% in China (Li et al., 2013; Yang et al., 2019; Jiang et al., 2021; Liu et al., 2021). The contamination of meat products with *Salmonella* can occur at various stages within the retail food chain—from breeding on farms to slaughter, transportation, and eventually sale in different locations and areas. It is crucial to emphasize the control and epidemiological monitoring of *Salmonella* throughout the retail food system as a priority to ensure consumer safety.

Serotype prediction analysis revealed that *S. typhimurium* was the most prevalent serotype among *Salmonella* isolates. According to Supplementary Figure S1, *S. typhimurium* was detected in all four main sources: meat and meat products, eggs and egg products, aquatic products, and other-origin products. In a previous study conducted in China, *S. typhimurium* has also been identified as the predominant isolate in pork meat (Campos et al., 2019), egg samples (Hu et al., 2021), and aquatic food products (Yang et al., 2015). Additionally, in our study, no single source covered all predicted serotypes. Approximately 70% of predicted serotypes were found in "meat and meat products." *S.* Rissen ($n = 6$) and *S. Corvallis* ($n = 3$) were found only in meat and meat products. *S.* Newport ($n = 5$) and *S.* Aberdeen ($n = 2$) were only found in aquatic products. These observations suggest that cross-contamination of local retail foods may contribute to the presence of *S. typhimurium*. However, different *Salmonella* serotypes exhibit different source preferences.

Moreover, we found that approximately one-third of serotypes determined through agglutination did not correspond to the serotypes identified via MSLT. Generally, the discriminatory power of MLST is comparable to or better than traditional serotyping. If there is a discrepancy between the slide agglutination test and MLST outcomes, many studies would generally lean toward MLST results owing to their superior resolution and reproducibility (Achtman et al., 2012; Pearce

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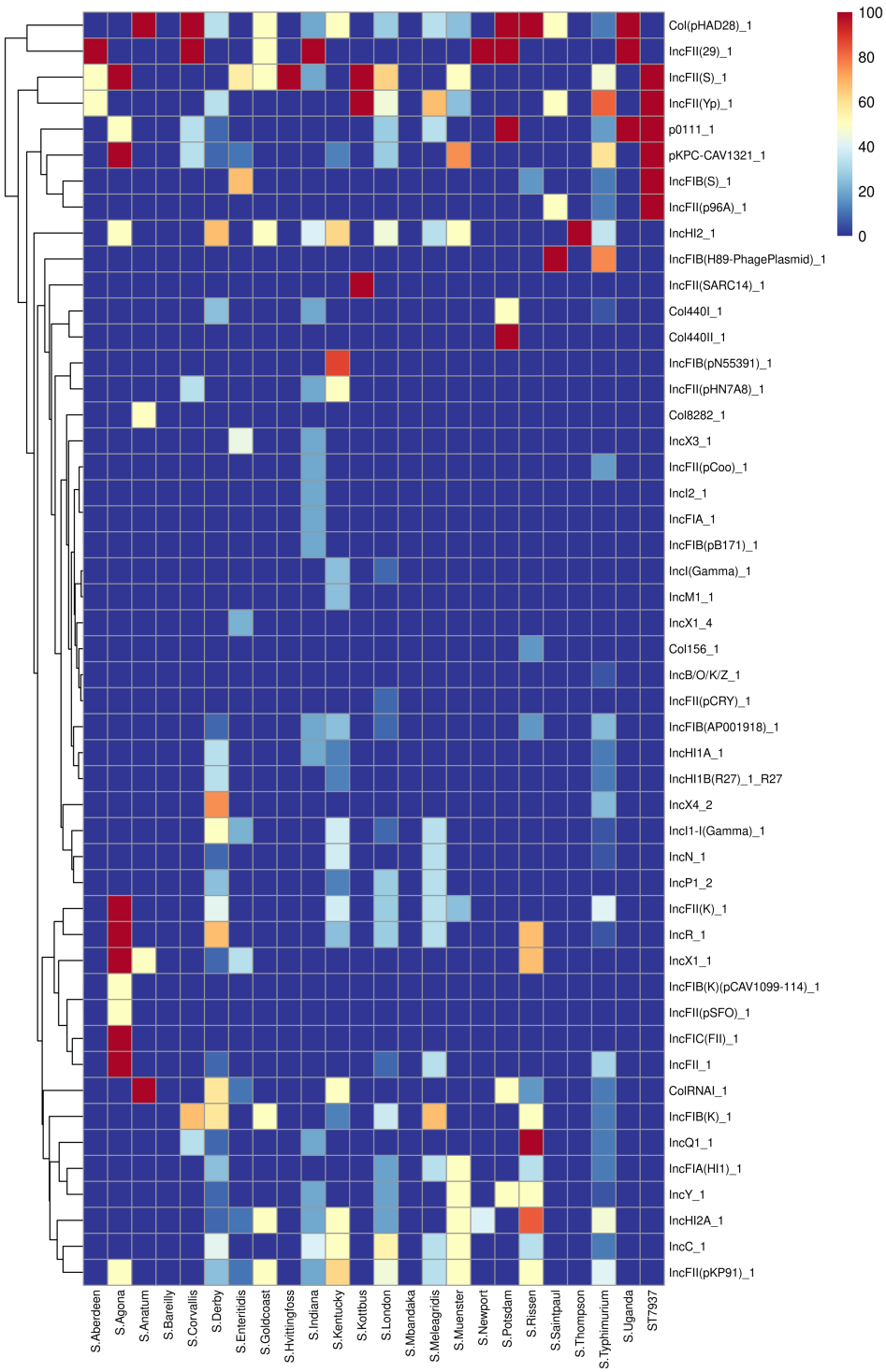

FIGURE 5
The heatmap of plasmids distribution in different *Salmonella* isolates. Scale 0–100, representing 0–100% proportions.

et al., 2018; Uelze et al., 2020; Yan et al., 2021). In our study, over 99% (101/102) had their predicted serotypes derived from sequencing types. Thus, in the same way, we chose to analyze the predicted serotypes. MLST is a more reliable and precise method for identifying bacterial strain relationships because it relies on the sequences of multiple housekeeping genes and can even distinguish closely related strains (Yan et al., 2021). However, MLST detects changes at the DNA level that cannot be inferred from the phenotype, such as serotyping or multi-locus enzyme electrophoresis (Uelze et al., 2020). The decision may also depend on the specific context, nature of the

TABLE 3 Comparative analysis of drug susceptibility, drug resistance genes, virulence genes, and plasmids between two groups (2016–2018 vs. 2019–2021).

Items	Groups	*N*	Mean ± std.	*p*-value
MIC(R)_SUM	2016–2018	48	3.13 ± 3.10	0.002
	2019–2021	54	5.15 ± 3.10	
CARD_SUM	2016–2018	48	29.19 ± 5.89	0.006
	2019–2021	54	32.19 ± 6.61	
VFDB_SUM	2016–2018	48	23.96 ± 2.41	0.035
	2019–2021	54	22.96 ± 2.07	
Plasmid_SUM	2016–2018	48	7.06 ± 5.21	0.199
	2019–2021	54	9.31 ± 7.31	

Std: standard deviation; MIC (R)_SUM: In the drug sensitivity test, the total count of antibiotic resistances exhibited by each isolated Salmonella bacterium; CARD_SUM: Utilizing the Comprehensive Antibiotic Resistance Database (CARD), the total counts of antibiotic resistance genes present in each isolated Salmonella bacterium were calculated; VFDB_SUM: Based on the Virulence Factor Database (VFDB), the total number of virulence genes for each Salmonella isolate were calculated; Plasmid_SUM: Employing the PlasmidFinder, the total count of plasmids for every individual Salmonella isolate were calculated.

discrepancy, and other available evidence. For example, the slide agglutination test results matched other phenotypic or biochemical characteristics of the isolate. If the MLST results do not, additional tests or analyses may be necessary. Here, we illustrated the advantages of using molecular typing methods and routine surveillance in China.

4.2 MDR

Our findings revealed that a significant proportion (66.7%) of the *Salmonella* isolates examined ($n = 102$) displayed resistance to at least three classes of antimicrobial agents, indicating MDR. This high percentage is alarming, as it indicates a continuous increase in the prevalence of MDR *Salmonella* strains in retail foods and their surrounding environments. The increase in the MDR rate could potentially be attributed to the inappropriate and excessive use of antibiotics on animal farms for therapeutic and prophylactic purposes (Liu et al., 2021), which contributes to the development and spread of antimicrobial resistance among *Salmonella* strains, ultimately leading to higher MDR rates. Furthermore, many *Salmonella* isolates demonstrated resistance to ampicillin and tetracycline, which are antimicrobial agents widely used in animal farms worldwide (Lekagul et al., 2019). Resistance to quinolones and beta-lactams, two important antimicrobial classes used to treat salmonellosis, has also been identified in numerous *Salmonella* isolates (Castanheira et al., 2020). This substantially threatens public health as these drugs are currently considered the preferred treatment options. Resistance to colistin, previously regarded as a last-resort therapeutic option for treating carbapenem-resistant Enterobacteriaceae, has been reported in several *Salmonella* isolates.

Moreover, a concerning trend emerged during 2019 and 2021, marked by a substantial increase in the prevalence of drug-resistant agents and genes in *Salmonella*. The rise in resistance not only underscores the evolving nature of *Salmonella* but also highlights the need for enhanced surveillance and innovative strategies in antibiotic management in Guizhou. This trend poses significant implications for both healthcare practices and food safety protocols in the province, calling for a coordinated response to address this emerging public health issue.

In addition, noteworthy differences exist between Guizhou's southern and northern regions regarding population demographics and economic development. The southern areas have a population of approximately 20.2 million, with a higher concentration of ethnic minorities, such as the Miao, Dong, and Buyi communities. These regions generally display lower levels of economic development. In contrast, the northern region has a population of about 18.4 million and is inhabited by more Han Chinese and fewer ethnic minorities. This area had an approximately 1.7 times higher GDP than the south (150 vs. 89 USD billion in 2020) (Guizhou Provincial Bureau of Statistics, 2021; Guizhou Provincial Bureau of Statistics NBS Survey Office in Guizhou, 2021). Although these disparities between the north and south could potentially influence local *Salmonella* characteristics (Bhattacharya et al., 2023; Wang et al., 2023), our study did not find any significant differences between the two regions.

4.3 Antimicrobial resistance genes

The prediction of antimicrobial resistance genes in the studied *Salmonella* isolates revealed the presence of several genes that could contribute to phenotypic resistance. The results of this study showed that all isolates harbored 24 drug-resistant genes: *acrB*, *acrD*, *bacA*, *baeR*, *cpxA*, *CRP*, *emrB*, *emrR*, *E. coli acrA*, *E. coli ampH beta-lactamase*, *golS*, *H-NS*, *kdpE*, *marA*, *mdsA*, *mdsB*, *mdsC*, *mdtB*, *mdtC*, *mdtG*, *mdtH*, *MdtK*, *sdiA*, and *YojI*. Few studies have reported that all *Salmonella* strains in a certain area in China are resistant to more than 20 drug-resistant genes (Liu et al., 2021; Chen et al., 2022; Sun et al., 2022). Additionally, other notable antimicrobial-resistant genes, such as *tet (A)* (49.0%, 50/102), which encodes resistance to tetracycline, and *AAC (6′)-Iaa* (39.2%, 40/102), which encodes resistance to aminoglycosides, were also identified. However, the presence of these genes in bacterial genomes does not guarantee phenotypic resistance or vice versa (Piddock, 2016). Antimicrobial resistance cannot be attributed solely to the presence or absence of resistance genes. Additional factors, including enzyme activation, target modification/protection, regulation of gene expression related to antimicrobial resistance, and even changes in the cell wall charge, significantly contribute to the development of antimicrobial resistance. Consequently, when comparing only the presence of antimicrobial-resistant genes, some degree of discordance is anticipated. Given the multitude of variables and intricate connections between genotypic and phenotypic data, a comprehensive evaluation of genotype–phenotype correlations provides a more accurate and comprehensive understanding of antimicrobial resistance (Liu et al., 2020). Therefore, phenotypic analysis was necessary to confirm the antimicrobial-resistant profiles of the studied isolates.

4.4 Virulence genes

In our study, several detected plasmids carried virulence genes associated with the virulence system of *Salmonella*. Notably, *cdtB*, which encodes typhoid toxins, was detected in 5 *S. Indiana*, 4 *S.* Muenster, and 2 *S.* Goldcoast strains. Moreover, the *spv* and *pef* genes were identified in 10 *S. typhimurium* and 7 *S. enteritidis* isolates.

FIGURE 6
MLST of 102 *Salmonella* isolates.

The detection of the *cdtB* gene in non-typhoidal *Salmonella* strains is notable because its presence has been linked to severe instances of bloodstream infections and invasive conditions in humans (Xu et al., 2020). Moreover, the spv locus has been closely linked to strains that cause non-typhoidal bacteremia (Fierer, 2022). *Pef* also plays an essential role in the virulence mechanisms of non-typhoidal *Salmonella* strains (Ben Hassena et al., 2021). The presence of *Salmonella* strains carrying these virulence factors in the retail food supply chain poses a considerable threat to public health, potentially leading to severe illness in consumers.

Meanwhile, an intriguing pattern emerged in the behavior of *Salmonella* strains over two distinct timeframes: 2016–2018 and 2019–2021. During the earlier period, the virulence genes of *Salmonella* were significantly higher, suggesting a greater potential for causing severe illness. However, this trend was reversed in the latter period, where there was a notable decrease in virulence genes. In contrast, this period saw a significant rise in the number of drug-resistant agents and drug-resistant genes, signaling an emerging challenge in combating these infections with standard antibiotics. This shift, with decreasing virulence but increasing drug resistance, highlights the complex and evolving nature of *Salmonella* in the region, necessitating a multifaceted and dynamic approach to public health interventions and antibiotic management strategies in Guizhou.

4.5 Plasmid

Analysis of plasmid distribution among *Salmonella* isolates revealed the presence of 49 different plasmids. The most prevalent plasmids detected were *IncHI2_1* and *Col (pHAD28)_1*, which were observed in 32 isolates, followed by *IncFII (pKP91)_1*, *IncFII (S)_1*, and *IncFII (Yp)_1*, which were present in 30 isolates. Notably, transferable plasmids, such as *IncQ1_1*, *IncR_1*, *IncHI2_1*, and *IncX1_1*, were detected in the MDR strains. These mobile plasmids confer resistance against various antimicrobial groups, encompassing β-lactams, aminoglycosides, sulfonamides, tetracycline, and others (Chen et al., 2016; McMillan et al., 2020). Moreover, we detected the *IncFIB (S)_1* and *IncFII (S)_1* plasmids, which encode virulence factors, in the four *S. enteritidis* samples. These plasmids have been documented in *S. enteritidis* isolates sourced from clinical and food samples (Mansour et al., 2020; Liu et al., 2021).

4.6 Correlation analyses

Our study observed that the colistin significantly correlated to *aadA5* and *drfA17* ($\rho > 0.5$, Supplementary Figure S3). Currently, the *aadA5* and *drfA17* genes are not recognized as causative agents for colistin resistance. These genes are predominantly linked to resistance against other antibiotic types: The *aadA5* gene is known for conferring resistance to aminoglycosides (Wang et al., 2017). Similarly, the *drfA17* gene is primarily associated with resistance to trimethoprim (Gu et al., 2020). Like *aadA5*, *drfA17* does not have a known role in colistin resistance. Colistin resistance, in contrast, is frequently connected to alterations in the bacterial cell membrane, especially changes in the lipid A structure of the lipopolysaccharide layer in Gram-negative bacteria. Genes commonly implicated in colistin resistance include *mcr-1* to *mcr-10* and those involved in two-component regulatory systems, such as PmrAB and PhoPQ (Li et al., 2022). The significant correlation between colistin resistance and the presence of *aadA5* and *drfA17* genes, evidenced by a correlation coefficient (ρ) exceeding 0.5, is noteworthy given their traditional lack of association with colistin resistance. Potential explanations for this correlation could include co-selection, cross-resistance, genetic linkage, and novel resistance mechanisms (Pal et al., 2017). Further investigation and experimental validation are needed to comprehend the underlying mechanisms of these correlations.

4.7 Phylogenetic analyses

In this study, we compared the evolutionary trees generated by MLST and cgMLST. Both methods yielded similar clustering results. However, cgMLST provides a higher resolution, allowing the detection of minor differences between isolates and generation of more detailed clustering patterns. MLST, which relies on the analysis of 7 housekeeping genes, is a precise and dependable typing method suitable for routine microbial surveillance (Yan et al., 2021). Since MLST is advantageous for establishing associations with specific serotypes, high-resolution molecular methods, including WGS-based cgMLST, cannot easily replace this method (Kimura, 2018). Different cgMLST classification schemes influence *Salmonella* strains (Li et al., 2008). Among the various cgMLST schemes available, the widely accepted 3,002 core loci cgMLST scheme for *Salmonella*, promoted in EnteroBase, is favored owing to its default settings that align with the preferences of many microbiologists, promoting consistency and accuracy between laboratories and jurisdictions. With over 270,000 *Salmonella* genomic sequences included, EnteroBase facilitates the cgMLST analysis of *Salmonella* and provides robust data for pathogen evolution analysis (Achtman et al., 2021). Based on thousands of genomic alleles, WGS-based cgMLST has shown significant potential for enhancing typing accuracy and facilitating convenient data sharing and comparison across international laboratories (Yan et al., 2021). The advancement of WGS-based cgMLST for traceability typing holds immense promise for improving our understanding and management of microorganisms with significant public health and ecological implications.

5 Conclusion

This study presents an in-depth analysis of *Salmonella* in retail food products from 2016 to 2021 in Guizhou province, China, uncovering a marked increasing trend in MDR *Salmonella* strains. Meanwhile, the resistance rates differed among strains and food sources, with strains from meat products showing significantly higher drug resistance than those from other sources. Our data also showed that *S. typhimurium* and *S. enteritidis* were the most prevalent strains, with a notable presence of MDR strains and key virulence genes such as *cdtB*, *Pef*, and *Spv*. This study underscores the alarming escalation in both the number of drug-resistant agents and resistance genes in *Salmonella*, particularly in meat-derived strains, highlighting the urgent need for enhanced surveillance measures and innovative antibiotic management strategies in Guizhou, China.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors. The data presented in the study are deposited in the NCBI's Sequence Read Archive repository, accession number PRJNA1063165 (SAMN39337012-SAMN39337113).

Ethics statement

This study only involved food samples. No ethical approval was deemed required for the experiments conducted in current study.

Author contributions

LZ: Funding acquisition, Resources, Writing – review & editing, Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation. QY: Data curation, Formal analysis, Writing – review & editing, Visualization. QZ: Writing – review & editing. JW: Writing – original draft. GL: Writing – original draft. JX: Writing – original draft. JH: Writing – original draft. YZ: Writing – original draft. TZ: Writing – original draft. HZ: Data curation, Formal analysis, Funding acquisition, Resources, Software, Writing – original draft, Writing – review & editing. SL: Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1345045/full#supplementary-material

References

Achtman, M., Van den Broeck, F., Cooper, K. K., Lemey, P., Parker, C. T., Zhou, Z., et al. (2021). Genomic population structure associated with repeated escape of *Salmonella enterica* ATCC14028s from the laboratory into nature. *PLoS Genet.* 17:e1009820. doi: 10.1371/journal.pgen.1009820

Achtman, M., Wain, J., Weill, F. X., Nair, S., Zhou, Z., Sangal, V., et al. (2012). Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. *PLoS Pathog.* 8:e1002776. doi: 10.1371/journal.ppat.1002776

Akter, T., Haque, M. N., Ehsan, R., Paul, S. I., Foysal, M. J., Tay, A. C. Y., et al. (2023). Virulence and antibiotic-resistance genes in *Enterococcus faecalis* associated with streptococcosis disease in fish. *Sci. Rep.* 13:1551. doi: 10.1038/s41598-022-25968-8

Alzahrani, K. O., Al-Reshoodi, F. M., Alshdokhi, E. A., Alhamed, A. S., Al Hadlaq, M. A., Mujallad, M. I., et al. (2023). Antimicrobial resistance and genomic characterization of *Salmonella enterica* isolates from chicken meat. *Front. Microbiol.* 14:1104164. doi: 10.3389/fmicb.2023.1104164

Bai, G., You, L., Long, L., Wang, D., Wang, M., Wang, J., et al. (2022). The CRISPR genotypes and genetic diversity of different serogroups of nontyphoidal *Salmonella* in Guizhou Province, 2013-2018. *PLoS One* 17:e0278321. doi: 10.1371/journal.pone.0278321

Ben Hassena, A., Haendiges, J., Zormati, S., Guermazi, S., Gdoura, R., Gonzalez-Escalona, N., et al. (2021). Virulence and resistance genes profiles and clonal relationships of non-typhoidal food-borne *Salmonella* strains isolated in Tunisia by whole genome sequencing. *Int. J. Food Microbiol.* 337:108941. doi: 10.1016/j.ijfoodmicro.2020.108941

Bhattacharya, R., Bose, D., Gulia, K., and Jaiswal, A. (2023). Impact of antimicrobial resistance on sustainable development goals and the integrated strategies for meeting environmental and socio-economic targets. *Environ. Prog. Sustain. Energy* 43:e14320. doi: 10.1002/ep.14320

Boolchandani, M., Blake, K. S., Tilley, D. H., Cabada, M. M., Schwartz, D. J., Patel, S., et al. (2022). Impact of international travel and diarrhea on gut microbiome and resistome dynamics. *Nat. Commun.* 13:7485. doi: 10.1038/s41467-022-34862-w

Campos, J., Mourao, J., Peixe, L., and Antunes, P. (2019). Non-typhoidal *Salmonella* in the pig production chain: a comprehensive analysis of its impact on human health. *Pathogens* 8:19. doi: 10.3390/pathogens8010019

Castanheira, S., Lopez-Escarpa, D., Pucciarelli, M. G., Cestero, J. J., Baquero, F., and Garcia-Del Portillo, F. (2020). An alternative penicillin-binding protein involved in *Salmonella* relapses following ceftriaxone therapy. *EBioMedicine* 55:102771. doi: 10.1016/j.ebiom.2020.102771

Castro-Vargas, R. E., Herrera-Sanchez, M. P., Rodriguez-Hernandez, R., and Rondon-Barragan, I. S. (2020). Antibiotic resistance in *Salmonella* spp. isolated from poultry: A global overview. *Vet. World* 13, 2070–2084. doi: 10.14202/vetworld.2020.2070-2084

Chen, J., Ed-Dra, A., Zhou, H., Wu, B., Zhang, Y., and Yue, M. (2022). Antimicrobial resistance and genomic investigation of non-typhoidal *Salmonella* isolated from outpatients in Shaoxing city, China. *Front. Public Health* 10:988317. doi: 10.3389/fpubh.2022.988317

Chen, W., Fang, T., Zhou, X., Zhang, D., Shi, X., and Shi, C. (2016). Inc HI2 plasmids are predominant in antibiotic-resistant *Salmonella* isolates. *Front. Microbiol.* 7:1566. doi: 10.3389/fmicb.2016.01566

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Chlebicz, A., and Slizewska, K. (2018). Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *Int. J. Environ. Res. Public Health* 15:863. doi: 10.3390/ijerph15050863

CLSI (2016). *Performance standards for antimicrobial susceptibility testing. 26* (Wayne, PA: Clinical and Laboratory Standards Institute).

Djordjevic, S. P., Jarocki, V. M., Seemann, T., Cummins, M. L., Watt, A. E., Drigo, B., et al. (2023). Genomic surveillance for antimicrobial resistance - a One Health perspective. *Nat. Rev. Genet.* 25, 142–157. doi: 10.1038/s41576-023-00649-y

European CDC (2022). "Salmonellosis" in *ECDC. Annual Epidemiological Report for 2021* (Stockholm: ECDC), 2022. Available at: https://www.ecdc.europa.eu/en/publications-data/salmonellosis-annual-epidemiological-report-2021

Fatima, S., Ishaq, Z., Irfan, M., AlAsmari, A. F., Achakzai, J. K., Zaheer, T., et al. (2023). Whole-genome sequencing of multidrug resistance *Salmonella Typhi* clinical strains isolated from Balochistan. *Front. Public Health* 11:1151805. doi: 10.3389/fpubh.2023.1151805

Fierer, J. (2022). Invasive non-typhoidal *Salmonella* (iNTS) infections. *Clin. Infect. Dis.* 75, 732–738. doi: 10.1093/cid/ciac035

Garcia-Soto, S., Tomaso, H., Linde, J., and Methner, U. (2021). Epidemiological analysis of *Salmonella enterica* subsp. enterica serovar dublin in German cattle herds using whole-genome sequencing. *Microbiol. Spectr.* 9:e0033221. doi: 10.1128/Spectrum.00332-21

GB4789.4-2016 (2016). National Food Safety Standard-Food Microbiological Examination: *Salmonella* test. Available at: http://tradechina. dairyaustralia.com.au/wp-content/uploads/2018/08/GB-4789.4-2016-Safety-Standard-Food-Microbiological-Examination-Salmonella.pdf.

Gu, D., Wang, Z., Tian, Y., Kang, X., Meng, C., Chen, X., et al. (2020). Prevalence of *Salmonella* Isolates and Their Distribution Based on Whole-Genome Sequence in a Chicken Slaughterhouse in Jiangsu, China. *Front. Vet. Sci.* 7:29. doi: 10.3389/fvets.2020.00029

Guizhou Provincial Bureau of Statistics (2021). Bulletin of the Seventh National Census of Guizhou Province (No. 2) [Online]. Available at: https://www.guizhou.gov.cn/home/tzgg/202109/t20210913_70093116.html.

Guizhou Provincial Bureau of Statistics NBS Survey Office in Guizhou. (2021). *Guizhou Statistical Yearbook*. Beijing, China: China Statistics Press. Available at: http://hgk.guizhou.gov.cn/publish/tj/2021/zk/indexeh.htm

Hu, Y., Fanning, S., Nguyen, S. V., Wang, W., Liu, C., Cui, X., et al. (2021). Emergence of a *Salmonella enterica* serovar Typhimurium ST34 isolate, CFSA629, carrying a novel mcr-1.19 variant cultured from egg in China. *J. Antimicrob. Chemother.* 76, 1776–1785. doi: 10.1093/jac/dkab090

Hung, Y. T., Lay, C. J., Wang, C. L., and Koo, M. (2017). Characteristics of nontyphoidal *Salmonella* gastroenteritis in Taiwanese children: A 9-year period retrospective medical record review. *J. Infect. Public Health* 10, 518–521. doi: 10.1016/j.jiph.2016.09.018

Jiang, Z., Anwar, T. M., Peng, X., Biswas, S., Elbediwi, M., Li, Y., et al. (2021). Prevalence and antimicrobial resistance of *Salmonella* recovered from pig-borne food products in Henan, China. *Food Control* 121:107535. doi: 10.1016/j.foodcont.2020.107535

Kimura, B. (2018). Will the emergence of core genome MLST end the role of *in silico* MLST? *Food Microbiol.* 75, 28–36. doi: 10.1016/j.fm.2017.09.003

Koch, K., Kristensen, B., Holt, H. M., Ethelberg, S., Molbak, K., and Schonheyder, H. C. (2011). International travel and the risk of hospitalization with non-typhoidal *Salmonella* bacteremia. A Danish population-based cohort study, 1999-2008. *BMC Infect. Dis.* 11:277. doi: 10.1186/1471-2334-11-277

Kong, X., Chen, J., Yang, Y., Li, M., Wang, J., Jia, Q., et al. (2022). Phenotypic and genotypic characterization of *salmonella Enteritidis* isolated from two consecutive Food-Poisoning outbreaks in Sichuan, China. *J. Food Safety* 43:e13015. doi: 10.1111/jfs.13015

Lekagul, A., Tangcharoensathien, V., and Yeung, S. (2019). Patterns of antibiotic use in global pig production: A systematic review. *Vet. Anim. Sci.* 7:100058. doi: 10.1016/j.vas.2019.100058

Li, F., Cheng, P., Li, X., Liu, R., Liu, H., and Zhang, X. (2022). Molecular epidemiology and colistin-resistant mechanism of MCR-positive and MCR-negative *Escherichia coli* isolated from animal in Sichuan Province, China. *Front. Microbiol.* 13:818548. doi: 10.3389/fmicb.2022.818548

Li, W., Han, H., Liu, J., Ke, B., Zhan, L., Yang, X., et al. (2023). Antimicrobial resistance profiles of *Salmonella* isolates from human diarrhea cases in China: an eight-year surveilance study. *One Health Adv.* 1, 1–8. doi: 10.1186/s44280-023-00001-3

Li, R., Lai, J., Wang, Y., Liu, S., Li, Y., Liu, K., et al. (2013). Prevalence and characterization of *Salmonella* species isolated from pigs, ducks and chickens in Sichuan Province, China. *Int. J. Food Microbiol.* 163, 14–18. doi: 10.1016/j.ijfoodmicro.2013.01.020

Li, R., Li, Y., Kristiansen, K., and Wang, J. (2008). SOAP: short oligonucleotide alignment program. *Bioinformatics* 24, 713–714. doi: 10.1093/bioinformatics/btn025

Liu, Q., Chen, W., Elbediwi, M., Pan, H., Wang, L., Zhou, C., et al. (2020). Characterization of *Salmonella* resistome and plasmidome in pork production system in Jiangsu, China. *Front. Vet. Sci.* 7:617. doi: 10.3389/fvets.2020.00617

Liu, Y., Jiang, J., Ed-Dra, A., Li, X., Peng, X., Xia, L., et al. (2021). Prevalence and genomic investigation of *Salmonella* isolates recovered from animal food-chain in Xinjiang, China. *Food Res. Int.* 142:110198. doi: 10.1016/j.foodres.2021.110198

Long, L., You, L., Wang, D., Wang, M., Wang, J., Bai, G., et al. (2022). Highly prevalent MDR, frequently carrying virulence genes and antimicrobial resistance genes in *Salmonella enterica* serovar 4,[5],12: i: - isolates from Guizhou Province, China. *PLoS ONE* 17:e0266443. doi: 10.1371/journal.pone.0266443

Lu, D., Liu, J., Liu, H., Guo, Y., Dai, Y., Liang, J., et al. (2023). Epidemiological features of foodborne disease outbreaks in catering service facilities-China, 2010-2020. *China CDC Wkly* 5, 479–484. doi: 10.46234/ccdcw2023.091

Luo, R., Liu, B., Xie, Y., Li, Z., Huang, W., Yuan, J., et al. (2012). SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. *Gigascience* 1:18. doi: 10.1186/2047-217X-1-18

Mansour, M. N., Yaghi, J., El Khoury, A., Felten, A., Mistou, M. Y., Atoui, A., et al. (2020). Prediction of *Salmonella* serovars isolated from clinical and food matrices in Lebanon and genomic-based investigation focusing on Enteritidis serovar. *Int. J. Food Microbiol.* 333:108831. doi: 10.1016/j.ijfoodmicro.2020.108831

Mao, X., Hu, J., and Liu, X. (2011). Estimation on disease burden of foodborne non-typhoid salmonellosis in China using literature review method. *Chin. J. Dis. Control Prev.* 15, 622–625.

Marchello, C. S., Birkhold, M., and Crump, J. A.Vacc-iNTS (2022). Complications and mortality of non-typhoidal *Salmonella* invasive disease: a global systematic review and meta-analysis. *Lancet Infect. Dis.* 22, 692–705. doi: 10.1016/S1473-3099(21)00615-0

McMillan, E. A., Jackson, C. R., and Frye, J. G. (2020). Transferable plasmids of *Salmonella enterica* associated with antibiotic resistance genes. *Front. Microbiol.* 11:562181. doi: 10.3389/fmicb.2020.562181

Medalla, F., Gu, W., Friedman, C. R., Judd, M., Folster, J., Griffin, P. M., et al. (2021). Increased incidence of antimicrobial-resistant nontyphoidal *Salmonella* infections, United States, 2004-2016. *Emerg. Infect. Dis.* 27, 1662–1672. doi: 10.3201/eid2706.204486

Mukherjee, S., Anderson, C. M., Mosci, R. E., Newton, D. W., Lephart, P., Salimnia, H., et al. (2019). Increasing frequencies of antibiotic resistant non-typhoidal *Salmonella* infections in Michigan and risk factors for disease. *Front. Med. (Lausanne)* 6:250. doi: 10.3389/fmed.2019.00250

Pal, C., Asiani, K., Arya, S., Rensing, C., Stekel, D. J., Larsson, D. G. J., et al. (2017). Metal resistance and its association with antibiotic resistance. *Adv. Microb. Physiol.* 70, 261–313. doi: 10.1016/bs.ampbs.2017.02.001

Pearce, M. E., Alikhan, N. F., Dallman, T. J., Zhou, Z., Grant, K., and Maiden, M. C. J. (2018). Comparative analysis of core genome MLST and SNP typing within a European *Salmonella* serovar Enteritidis outbreak. *Int. J. Food Microbiol.* 274, 1–11. doi: 10.1016/j.ijfoodmicro.2018.02.023

Petrin, S., Wijnands, L., Beninca, E., Mughini-Gras, L., Delfgou-van Asch, E. H. M., Villa, L., et al. (2023). Assessing phenotypic virulence of *Salmonella enterica* across serovars and sources. *Front. Microbiol.* 14:1184387. doi: 10.3389/fmicb.2023.1184387

Piddock, L. J. (2016). Assess drug-resistance phenotypes, not just genotypes. *Nat. Microbiol.* 1:16120. doi: 10.1038/nmicrobiol.2016.120

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States--major pathogens. *Emerg. Infect. Dis.* 17, 7–15. doi: 10.3201/eid1701.p11101

Sun, R. Y., Guo, W. Y., Zhang, J. X., Wang, M. G., Wang, L. L., Lian, X. L., et al. (2022). Phylogenomic analysis of *Salmonella* Indiana ST17, an emerging MDR clonal group in China. *J. Antimicrob. Chemother.* 77, 2937–2945. doi: 10.1093/jac/dkac243

Uelze, L., Grutzke, J., Borowiak, M., Hammerl, J. A., Juraschek, K., Deneke, C., et al. (2020). Typing methods based on whole genome sequencing data. *One Health Outlook* 2:3. doi: 10.1186/s42522-020-0010-1

Wang, Y., Liu, Y., Lyu, N., Li, Z., Ma, S., Cao, D., et al. (2023). The temporal dynamics of antimicrobial-resistant *Salmonella enterica* and predominant serovars in China. *Natl. Sci. Rev.* 10:nwac 269. doi: 10.1093/nsr/nwac269

Wang, W., Peng, Z., Baloch, Z., Hu, Y., Xu, J., Zhang, W., et al. (2017). Genomic characterization of an extensively-drug resistance *Salmonella enterica* serotype Indiana strain harboring bla (NDM-1) gene isolated from a chicken carcass in China. *Microbiol. Res.* 204, 48–54. doi: 10.1016/j.micres.2017.07.006

Wei, X., Long, L., You, L., Wang, M., Wang, D., Liu, C., et al. (2023). Serotype distribution, trend of multidrug resistance and prevalence of beta-lactamase resistance genes in human *Salmonella* isolates from clinical specimens in Guizhou, China. *PLoS One* 18:e0282254. doi: 10.1371/journal.pone.0282254

Wei, X., You, L., Wang, D., Huang, H., Li, S., and Wang, D. (2019). Antimicrobial resistance and molecular genotyping of *Salmonella enterica* serovar Enteritidis clinical isolates from Guizhou province of Southwestern China. *PLoS One* 14:e0221492. doi: 10.1371/journal.pone.0221492

Xie, B., and Zhang, M. (2023). Spatio-temporal evolution and driving forces of habitat quality in Guizhou Province. *Sci. Rep.* 13:6908. doi: 10.1038/s41598-023-33903-8

Xu, X., Chen, Y., Pan, H., Pang, Z., Li, F., Peng, X., et al. (2020). Genomic characterization of *Salmonella* Uzaramo for human invasive infection. *Microb. Genom.* 6:mgen000401. doi: 10.1099/mgen.0.000401

Xu, R., Zhang, S., Ma, Z., Rao, Q., and Ma, Y. (2023). Characterization and genome analysis of Neobacillus mesonae NS-6, a ureolysis-driven strain inducing calcium carbonate precipitation. *Front. Microbiol.* 14:1277709. doi: 10.3389/fmicb.2023.1277709

Yan, S., Zhang, W., Li, C., Liu, X., Zhu, L., Chen, L., et al. (2021). Serotyping, MLST, and core genome MLST analysis of *Salmonella enterica* from different sources in China during 2004-2019. *Front. Microbiol.* 12:688614. doi: 10.3389/fmicb.2021.688614

Yang, X., Wu, Q., Zhang, J., Huang, J., Chen, L., Liu, S., et al. (2015). Prevalence, enumeration, and characterization of *Salmonella* isolated from aquatic food products from retail markets in China. *Food Control* 57, 308–313. doi: 10.1016/j.foodcont.2015.03.046

Yang, X., Wu, Q., Zhang, J., Huang, J., Chen, L., Wu, S., et al. (2019). Prevalence, bacterial load, and antimicrobial resistance of *Salmonella* serovars isolated from retail meat and meat products in China. *Front. Microbiol.* 10:2121. doi: 10.3389/fmicb.2019.02121

Zhao, W., Li, X., Shi, X., Li, K., Shi, B., Sun, J., et al. (2022). Whole genome sequencing, antibiotic resistance, and epidemiology features of nontyphoidal *Salmonella* isolated from diarrheic children: evidence from North China. *Front. Microbiol.* 13:882647. doi: 10.3389/fmicb.2022.882647

Zhou, M., Liu, S., Li, Q., Wang, Q., Zhu, M., Cao, L., et al. (2018). Drug resistance characteristics of *Mycobacterium tuberculosis* isolates between 2014 and 2017 in Sichuan, China: A retrospective study. *PLoS One* 13:e0209902. doi: 10.1371/journal.pone.0209902

Zhou, R., Zheng, T., Luo, D., Zhu, M., Li, Q., Xu, Y., et al. (2022). Drug resistance characteristics of *Mycobacterium tuberculosis* isolates obtained between 2018 and 2020 in Sichuan, China. *Epidemiol. Infect.* 150:e27. doi: 10.1017/S0950268822000127

Zhou, Q., Zhong, Y., Shan, Z., Pan, X., Huang, J., Xiang, J., et al. (2023). Aetiological survey and traceability analysis of a foodborne disease outbreak of *Salmonella* senftenberg in Guizhou Province. *Foodborne Pathog. Dis.* 20, 351–357. doi: 10.1089/fpd.2023.0012

Zuo, H., Zheng, T., Wu, K., Yang, T., Wang, L., Nima, Q., et al. (2022). High-altitude exposure decreases bone mineral density and its relationship with gut microbiota: Results from the China multi-ethnic cohort (CMEC) study. *Environ. Res.* 215:114206. doi: 10.1016/j.envres.2022.114206

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Glossary

MDR	Multi-drug resistant
MALDI-TOF-MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
AMP	Ampicillin
AMS	Ampicillin/sulbactam
TET	Tetracycline
CFZ	Cefazolin
CTX	Cefotaxime
CFX	Cephalosporin cefoxitin
CAZ	Ceftazidime
IMI	Imipenem
GEN	Gentamicin
CT	Colistin (Polymyxin E)
AZM	Azithromycin
NAL	Nalidixic acid
CIP	Ciprofloxacin
CHL	Chloramphenicol
SXT	Trimethoprim/sulfamethoxazole
CLSI	Clinical Laboratory Standards Institute
CARD	Comprehensive Antibiotic Resistance Database
VFDB	Virulence Factor Database
TraNet	National Foodborne Disease Molecular Tracing Network
MLST	Multi-locus sequence typing
cgMLST	Core genome MLST

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Genome-based surveillance reveals cross-transmission of MRSA ST59 between humans and retail livestock products in Hanzhong, China

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized in hospitals, community and livestock animals and the epidemiology of MRSA is undergoing a major evolution among humans and animals in the last decade. This study investigated the prevalence of MRSA isolates from ground pork, retail whole chicken, and patient samples in Hanzhong, China. The further characterization was performed by antimicrobial susceptibility testing and in-depth genome-based analysis to identify the resistant determinants and their phylogenetic relationship. A total of 93 MRSA isolates were recovered from patients (n = 67) and retail livestock products (n = 26) in Hanzhong, China. 83.9% (78/93) MRSA isolates showed multiple drug resistant phenotype. Three dominant livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) sequence types were identified: ST59-t437 (n = 47), ST9-t899 (n = 10) and ST398 (n = 7). There was a wide variation among sequence types in the distribution of tetracycline-resistance, *scn*-negative livestock markers and virulence genes. A previous major human MRSA ST59 became the predominant interspecies MRSA sequence type among humans and retail livestock products. A few LA-MRSA isolates from patients and livestock products showed close genetic similarity. The spreading of MRSA ST59 among livestock products deserving special attention and active surveillance should be enacted for the further epidemic spread of MRSA ST59 in China. Data generated from this study will contribute to formulation of new strategies for combating spread of MRSA.

KEYWORDS

interspecies transmission, livestock-associated methicillin-resistant *Staphylococcus aureus*, ST59, ST9, ST398

1 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one bacteria type that may cause numerous clinical manifestations ranging from mild skin and soft tissue infections to life-threatening fulminant invasive diseases (Turner et al., 2019; Tuffs et al., 2022). MRSA has been recognized in hospitals, community and livestock animals and the epidemiology of

MRSA is undergoing a major evolution among humans and animals in the last decade (Chen et al., 2021; Yu et al., 2021). Since the livestock-associated MRSA (LA-MRSA) was first recognized in Europe in 2003, LA-MRSA has been identified in numerous countries around the world, including China (Cui et al., 2009; Silva et al., 2023). Multilocus sequence typing (MLST) analyzes seven constitutively expressed (housekeeping) genes that are essential to cellular functioning of organisms. LA-MRSA of sequence type (ST) 398 dominates in Europe, Australia and the United States, while LA-MRSA-ST9 is the main epidemic lineage in Asia (Yu et al., 2021; Silva et al., 2023). Several studies have identified specific LA-MRSA within the community acquired-MRSA category (Bisdorff et al., 2012; Mascaro et al., 2018; Sun et al., 2019). Recently, LA-MRSA-ST398 and other sequence types previously widely disseminated among human beings (such as ST59) were also identified in livestock animals and meat samples from China (Wang et al., 2014; Li et al., 2021; Zhang et al., 2021).

Because of the huge volume of livestock animals and the consumption of livestock products in the community, there is a concern that MRSA may be spreading and concentrating in livestock animals with subsequent dissemination into the community population through contact with livestock, farm environment or retail livestock products (Yu et al., 2021; Li et al., 2022). Especially retail livestock products, such as pork and poultry, are susceptible to MRSA contamination during slaughtering process and can become an ideal media of MRSA transmission to the kitchens (Hennekinne et al., 2012; Li et al., 2021).

In 2009, LA-MRSA-ST9 was firstly recognized from the swine and farm worker samples in Hanzhong city, Shaanxi province and all MRSA isolates in the previous study were grouped into ST9-spa899 (Cui et al., 2009). Hanzhong city lies in the center of the Hanzhong Basin. The south of the city is the Daba mountains and the north of the city is the Qinling mountains which limits the human migration and economic exchange with other areas. The location factor makes this city an ideal place for LA-MRSA transmission study. Since the pilot study in 2009 (Cui et al., 2009), no follow-up study was conducted on the transmission of LA-MRSA in this area.

The objective of this study was to determine the prevalence of MRSA isolates in ground pork, retail whole chicken and patient samples in Hanzhong city. The isolates were further characterized by in-depth genome-based analysis to identify the resistant determinants and their phylogenetic relationship.

2 Materials and methods

2.1 Sample collection and MRSA isolation

From July 2019 to May 2020, retail ground pork (n = 88) and retail whole chicken carcasses (n = 87) were collected from seven supermarkets in Hanzhong, China. Each supermarket was visited once a month. On each sampling day, no more than two whole chicken carcasses or two ground pork samples were randomly selected from each sampling site. All samples were transported to the laboratory and processed within 4 h. Each whole chicken carcass was immediately aseptically removed from the package and placed in a 3500 stomach bag (Seward, UK) followed by the addition of 500-mL buffered peptone water (BPW; Becton-Dickinson, Beijing, China). The bag was manually massaged for 3–5 min and the rinse were used for MRSA isolation. 25 ml of the rinse or 25 g of ground pork samples were added into 225 ml enrichment broth containing 1% tryptone, 7.5% sodium chloride, 1% mannitol and 0.25% yeast extract and incubated at 35 ± 1°C. After 22–24 h incubation, a loopful of the culture was inoculated onto selective MRSA agar plates (BBL CHROMagar MRSA) and incubated at 35 ± 1°C for 24–48 h. Purple colonies on the selective plates were screened for coagulase activity. All MRSA isolates were confirmed by the API Staph ID test (BioMe′rieux, Beijing, China) and PCR screening for the carriage of *nuc* and *mecA* (Merlino et al., 2002). One confirmed MRSA isolate from each sample was selected for further study. All MRSA isolates were kept in brain heart infusion broth (BD, China) with 50% glycerol at −80°C freezer for further analysis.

2.2 Collection of MRSA isolates from patients

MRSA isolates from patients were collected from the 3201 hospital which is the largest hospital in Hanzhong, Shaanxi, China. During the food sample collection, this hospital tested 7033 independent blood samples, 3517 cerebrospinal fluid samples, 128 wound secretion samples from inpatients for bacteria infection and 406 fecal samples from outpatients for *S. aureus*. The MRSA isolates from outpatient and inpatient samples were obtained and included in this study.

2.3 Antibiotic susceptibility testing

The antimicrobial susceptibility of all the MRSA isolates was determined by the micro-broth dilution method and interpreted according to the clinical and laboratory standards institute guidelines (Clinical and Laboratory Standards Institute [CLSI], 2022). The MICs of 13 antimicrobials were measured, including oxacillin (OXA), gentamicin (GEN), erythromycin (ERY), clindamycin (CLI), levofloxacin (LEV), vancomycin (VAN), teicoplanin (TEC), linezolid (LZD), trimethoprim-sulfamethoxazole (SXT), rifampin (RIF), nitrofurantoin (NIT), daptomycin (DAP) and tetracycline (TET). *S. aureus* ATCC 29213 was included as the quality control organism in antimicrobial susceptibility test to ensure that the concentration for each antimicrobial agent was properly controlled.

2.4 Genome sequencing and assembly

Bacterial genomic DNA of each isolate was extracted from 2 mL fresh culture using QIAamp DNA Mini Kit (Qiagen, Germany) following manufacturer's instructions. DNA was subjected to quality control by visualizing electrophoresis products on a 1% agarose gel and quantifying them using a Qubit fluorometer (Invitrogen, Shanghai, China). WGS was performed with massively parallel sequencing (MPS) Illumina technology at Beijing Novogene Bioinformatics Technology Co., Ltd. A paired-end

library with a 350 bp insert size was constructed and sequenced by Illumina NovaSeq using PE150 strategy. Illumina PCR adapter reads and low-quality reads were filtered by Readfq (version:10) and the filtered reads were assembled using SOAP denovo (version 2.04), SPAdes (version 3.10.0) and Abyss (version 1.3.7) to generate scaffolds (Li et al., 2008; Simpson et al., 2009; Bankevich et al., 2012) which were integrated by CISA software (Lin and Liao, 2013). The initial assembly results were optimized and matched using Gapclose (version 1.12) software to obtain the final assembly results (Luo et al., 2012). WGS data of 93 MRSA isolates were deposited into GenBank under BioProject accession number PRJNA966921 (**Supplementary Table 3**).

2.5 Bioinformatic analysis

The assembled contigs were subjected and analyzed on publicly available ResFinder 4.1, VirulenceFinder 2.0, MLST 2.0, spaTyper 1.0 and SCCmecFinder 1.2 server using default thresholds from the Center for Genomic Epidemiology (CGE) (Camacho et al., 2009; Larsen et al., 2012; Bartels et al., 2014; Bortolaia et al., 2020). The core genome alignment and SNPs calculation of genome in this research was performed using the snippy pipeline (version 4.4.5).[1] The maximum likelihood tree was generated using the iqtree pipeline (version 2.1.2) (Kalyaanamoorthy et al., 2017) by including online isolates (**Supplementary Table 4**) and visualized using iTOL (Letunic and Bork, 2016).

2.6 Statistical analysis

The chi-square test was used to determine differences in the resistance rate of *S. aureus*. All statistical analyses were performed using the SPSS 18.0 software package.

3 Results

3.1 MRSA isolates isolation and confirmation

26 MRSA isolates were recovered from retailed ground pork (13/88) and retail whole chicken carcasses (13/87). A total of 67 MRSA isolates from outpatient fecal swabs (n = 18) and inpatient blood (n = 28), cerebrospinal fluid (n = 1) and wound secretion (n = 20) samples were obtained. All 93 isolates harbored *nuc* and *mecA*.

3.2 Susceptibility of MRSA isolates

All 93 MRSA isolates were resistant to oxacillin and susceptible to daptomycin, linezolid, nitrofurantoin, teicoplanin and vancomycin. Most MRSA isolates were also resistant to erythromycin (86.0%, 80/93), clindamycin (75.3%, 70/93) and tetracycline (50.5%, 47/93). 76.9% (20/26) food isolates were resistant to tetracycline which was significantly higher than the human isolates (40.3%, 27/67) ($P < 0.01$) and similar trend was found for clindamycin (**Table 1**). 83.9% (78/93) MRSA isolates showed multiple drug resistant phenotype. CLI-ERY-OXA (n = 30) and CLI-ERY-OXA-TET (n = 22) were two predominant multidrug resistant phenotypes. Only two of the CLI-ERY-OXA multidrug resistant isolates were from retail livestock product samples, whereas ten of the CLI-ERY-OXA-TET multidrug resistant isolates were from retail livestock product samples and the difference of this distribution pattern was significant ($P < 0.05$) (**Supplementary Table 2**).

3.3 MLST analysis of MRSA isolates

93 MRSA isolates were grouped into 11 STs, including ST59 (n = 57), ST9 (n = 10), ST239 (n = 9), ST398 (n = 7), ST88 (n = 3), ST6576 (n = 2), ST338 (n = 1), ST45 (n = 1), ST5 (n = 1), ST5052 (n = 1) and ST509 (n = 1). ST59 was the dominant sequence type among ground pork (n = 7/13), whole chicken (n = 6/13), outpatient fecal swabs (n = 15/18), inpatient sterile sites (n = 15/29) and wound secretion (n = 14/20) samples. ST9 (n = 10), ST398 (n = 7) and ST88 (n = 3) isolates were also identified from both food and patient samples (**Supplementary Table 1**).

3.4 Characterization and phylogenetic analysis of ST59 isolates

Eight resistance phenotypes were identified among 57 ST59 isolates. Among isolates of CLI-ERY-OXA resistant phenotype, 92.8% (26/28) isolates were sourced from human samples. However, among isolates of CLI-ERY-OXA-TET resistant phenotype, 57.9% (11/19) isolates were sourced from human samples and the distribution difference was highly significant ($P < 0.01$). One isolate from patient secretion sample showed CLI-ERY-GEN-LEV-OXA-RIF-TET resistant phenotype. *Staphylococcus* chromosomal cassette mec (*SCCmec*) *_type_IVa(2B)* was identified among 54 ST59 isolates. *SCCmec_type_Vb(5C2&5)* was identified in two isolates from inpatient blood samples and *SCCmec_type_IVg(2B)* was identified in one isolate from chicken sample. The common resistant determinants identified among ST59 isolates including *aph(3′)-III* (42/57), *blaZ* (49/57), *ermB* (46/57), *ermC* (13/57) and *tet(K)* (28/57) (**Supplementary Table 1**).

Six Staphylococcal protein a (*spa*) typing were identified among 57 ST59 MRSA isolates, including t437 (n = 47), t441 (n = 6), t13774 (n = 1), t3515 (n = 1), t4193 (n = 1) and t8391 (n = 1). *Spa* t437 was identified among ground pork (n = 7/7), whole chicken (n = 2/6), outpatient fecal swabs (n = 13/15), inpatient sterile sites (n = 14/15) and wound secretion (n = 11/14) samples. *Spa* t441 was identified among whole chicken (n = 1), outpatient fecal swabs (n = 2), inpatient wound secretion (n = 2) and sterile site (n = 1) samples.

After SNP analysis, eight isolates harbored *lukF/S-PV* were grouped into one cluster, comprising isolates 5-23, 5-19, 7-20, Y3, 5-31, Y13, 7-4 and 7-32 (**Figure 1**). Human isolates from blood, fecal and wound secretion samples were crossly distributed.

1 https://github.com/tseemann/snippy

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TABLE 1 Resistance phenotypes of MRSA isolates (n = 93) from food and patient samples, Hanzhong, China.

Antimicrobial agents	MIC* mg/L	No. (%) of resistant isolates					
		Total (N = 93)	Patients (N = 67)	Food(N = 26)	ST59(N = 57)	ST9(N = 10)	ST398(N = 7)
Clindamycin	≥4	70 (75.3)	47 (70.1)	23 (88.5)	49 (86.0)	10 (100)	4 (57.1)
Erythromycin	≥8	80 (86.0)	56 (83.6)	24 (92.3)	54 (94.7)	10 (100)	4 (57.1)
Gentamicin	≥16	21 (22.6)	12 (18.0)	9 (34.6)	1 (1.8)	10 (100)	0 (0.0)
Levofloxacin	≥4	16 (17.2)	10 (15.0)	6 (23.1)	2 (3.5)	5 (50.0)	0 (0.0)
Tetracycline	≥16	47 (50.5)	27 (40.3)	20 (76.9)	24 (42.1)	10 (100)	3 (42.9)
Rifampin	≥4	10 (10.8)	10 (14.9)	0 (0.0)	1 (1.8)	0 (0.0)	0 (0.0)
Trimethoprim-sulfamethoxazole	≥4/76	10 (10.8)	1 (1.5)	9 (34.6)	0 (0.0)	9 (90.0)	0 (0.0)

*MIC, minimal inhibitory concentration.

A cluster of five ground pork isolates (isolates 2884, 3115, 3116, 3117 and 3118) of CLI-ERY-OXA-TET multidrug resistant phenotype was identified. Two ground pork isolates were scattered among patient isolates with isolate 1-22 from ground pork showing a close relationship with isolate 7-5 from patient blood sample, while another ground pork isolate 2885 showed a close relationship with pork isolate 3025 of online reference. Six isolates from whole chicken carcasses were scattered among human isolates. Genetic similarities were found among five pairs of chicken and human isolates (Figure 1).

3.5 Characterization and phylogenetic analysis of ST9 isolates

ST9 isolates (n = 10) were identified from whole chicken (n = 6), ground pork (n = 3) and inpatient secretion (n = 1) samples. All ST9 isolates were resistant to clindamycin, erythromycin, gentamicin, oxacillin, tetracycline. Additionally, the strains were also intermediate or resistant to levofloxacin (MIC was found to be 2 or 4 μg/ml). Nine food isolates were also found to be resistant to trimethoprim/sulfamethoxazole. All 10 ST9 MRSA isolates displayed the same *spa* type (allelic profile, 07-16-23-02-34, t899) and contained *SCCmec_type_XII(9C2)*.

All isolates harbored the following resistant determinants: *aac(6′)*, *aadD*, *aph(2″)*, *blaZ*, *dfrG*, *erm(C)*, *lsa(E)*, *lnu(B)*, *mecA* and *tet(L)*. Eight isolates also harbored florfenicol-chloramphenicol resistant determinant *fexA* (Supplementary Table 1). After SNP analysis, six chicken isolates were grouped together. Genetic similarities were found between one retail ground pork isolate 2887 and one wound secretion isolate S8 (Figure 2).

3.6 Characterization and phylogenetic analysis of ST398 isolates

ST398 isolates (n = 7) were identified from ground pork (n = 2), whole chicken (n = 1), inpatient secretion (n = 2) and blood (n = 2) samples. All ST398 isolates (n = 7) were susceptible to gentamycin, linezolid, nitrofurantoin, rifampin, teicoplanin, tigecycline, trimethoprim/sulfamethoxazole, vancomycin and resistant to oxacillin. Clindamycin and tetracycline resistance were observed in three food isolates and two isolates were also resistant to erythromycin. All four human isolates were susceptible to tetracycline and two isolates were resistant to erythromycin and one isolate was also resistant to clindamycin. Four human isolates were grouped into three *spa* types: t034 (n = 2), t571(n = 1) and t1928 (n = 1). t034 (n = 2) and t1928 isolates contained *SCCmec_type_V(5C2)*, t571(n = 1) isolate contained *SCCmec_type_III(3A)*. All three food isolates shared the same *spa* type (t011) and contained *SCCmec_type_Vc(5C2&5)*. All ST398 isolates harbored *blaZ* and *mecA*. All three food isolates also harbored *dfrG*, *lnu(B)*, *lsa(E)*, *tet(K)* and *tet(M)* (Supplementary Table 1). After SNP analysis, four human isolates showed genetic similarities and were grouped independently from three food isolates (Figure 3).

3.7 Virulence genes among ST59, ST9 and ST398 isolates

All ST59, ST9, and ST398 isolates were found to contain the following virulence factors, including the metalloprotease (*aur*) and the hemolysins *hlgA*, *hlgB*, and *hlgC*. PVL was present in eight ST59-*spa* t437 human isolates (8/44, 18.2%) and six isolates were staphylokinase (*sak*) negative, but all the PVL negative ST59 MRSA isolates were *sak* positive. The distribution of *scn*, *sak* and enterotoxin encoding genes showed different patterns across different STs. All ST59 (n = 57) and patient ST398 (n = 4) isolates were *scn* positive, all ST9 (n = 10) and the food ST398 (n = 3) isolates were *scn* and *sak* negative.

Among ST59 isolates, *sak* was identified among 51 isolates and enterotoxins *seb*, *sek* and *seq* were identified among 50 isolates. Both *sak and* enterotoxins *seb*, *sek* and *seq* were identified among 45 isolates from ground pork (n = 7/7), whole chicken (n = 5/6), fecal swabs (n = 13/15), sterile sites (n = 10/15) and wound secretion (n = 10/14) samples. All ST9 isolates harbored enterotoxin gene *seo,* and eight ST9 food isolates also harbored more enterotoxin gene (*seg*, *sei*, *sem*, *sen* and *seu*), but these genes were absent in ST398 isolates (Supplementary Table 1).

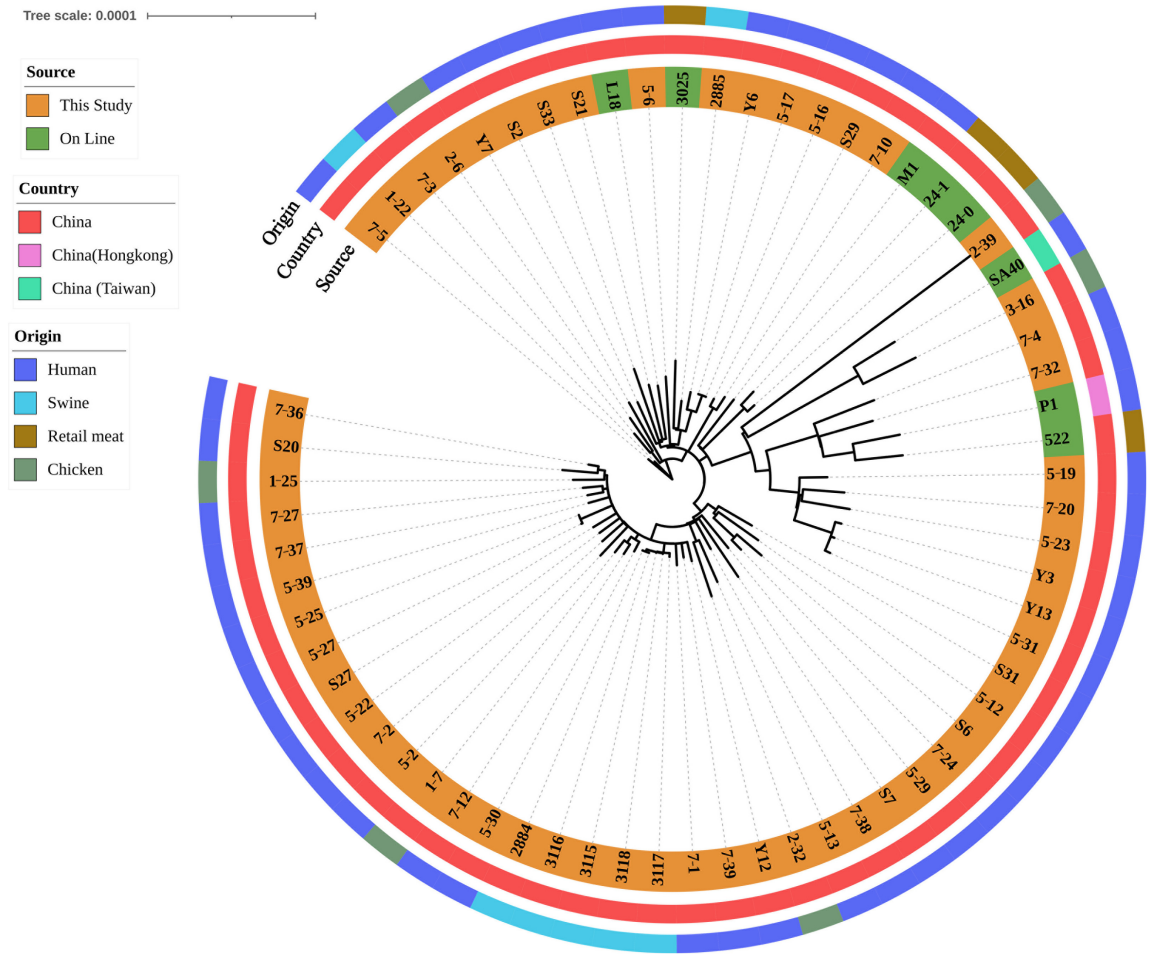

FIGURE 1
Phylogenetic relationship of ST59 isolates from retail livestock products and patients, Hanzhong, China. The genome sequences of 65 ST59 isolates were aligned through SNP analysis, including 8 online isolates. The information of online isolates was provided in **Supplementary Table 4**.

3.8 Characterization of the remaining MRSA isolates

Except one isolate recovered from the ground pork sample, the other leftover MRSA isolates (n = 18) were recovered from patient samples. These 19 isolates were grouped into eight STs. All ST239 (n = 9) isolates showed multiple resistant phenotypes and carried *SCCmec_type_III(3A)* and were grouped into *spa* t030 (n = 5), *spa* t459 (n = 4) types. *SCCmec_type_IV* (n = 8) and *SCCmec_type_V* (n = 3) were identified among the other isolates. Except one ST45 isolate from the fecal sample, the other MRSA isolates (n = 18) were all *scn* and *sak* positive. *lukE/D* was present in 15 isolates.

4 Discussion

From patients and retail livestock products in Hanzhong, China, three dominant LA-MRSA sequence types were identified: ST59-t437 (n = 47), ST9-t899 (n = 10), and ST398 (n = 7). There was a wide variation among sequence types in the distribution of tetracycline-resistance, *scn*-negative livestock markers and virulence genes, indicating different origins and evolutionary processes. We found that in Hanzhong, China, a major human MRSA ST59, not LA-MRSA ST9 or ST398, became the predominant interspecies MRSA sequence type among humans and retail livestock products. Some MRSA-ST59 isolates from patients and livestock products showed close genetic similarity. Our study further indicated that certain human MRSA may become LA-MRSA and transfer from humans to livestock animals or vice versa, which demonstrated the importance of continuous MRSA surveillance among humans and livestock animals.

Our data showed ST59 MRSA was dominant not only among patients in Hanzhong (44/67), but also among livestock product isolates (13/26). Initially being reported in North America in the early 2000s (Enright et al., 2002), ST59 MRSA has gradually been replacing ST239 and ST5 human clones and become the predominant sequence type in most hospitals of China since 2010 (Li et al., 2018; Jin et al., 2021; Wang et al., 2022). However, ST59 MRSA remains geographically confined and is low prevalence in Europe and North America (Pimentel de Araujo et al., 2021; Di Gregorio et al., 2023). Previous studies have shown ST9 is the dominant LA-MRSA in China and other Asian countries as opposed to ST398 in Europe and North America (Silva et al., 2023). Recent studies have also reported ST59 MRSA isolates

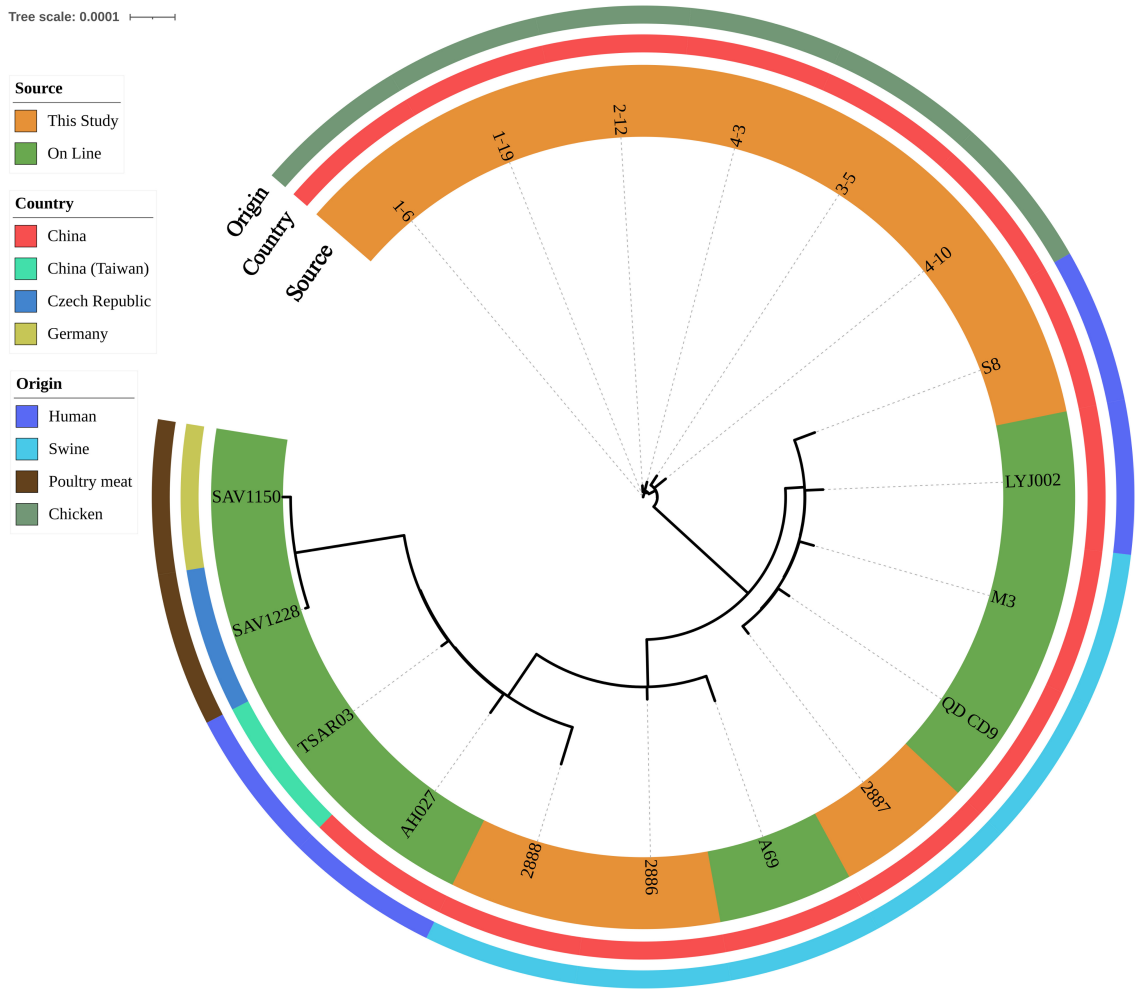

FIGURE 2
Phylogenetic relationship of ST9 isolates from retail livestock products and patients, Hanzhong, China. The genome sequences of 18 ST9 isolates were aligned through SNP analysis, including 8 online isolates. The information of online isolates was provided in **Supplementary Table 4**.

were scattered among livestock animals in China (Wang et al., 2021; Li et al., 2022), our data indicated that the detection rate of ST59 in livestock products in Hanzhong was higher than ST9 and 398 isolates. ST59 MRSA isolates displayed a greater overall phylogenetic diversity than LA-MRSA ST9 isolates did which indicated the multiple origins of ST59 isolates or higher adaptability within different hosts, suggesting that the ST59 isolates might undergo multiple and continuous evolutionary events. Usually, ST59 isolates carry *SCCmec_type_IV* (55/57) or *SCCmec_type_V*, both are smaller *SCCmec* cassettes that may reduce the host fitness burden. Coculture experiment in a previous study showed that ST59 isolates displayed higher growth rates and competitive capacity than MRSA ST239 *in vitro* which provided further evidence that ST59 clones may have a better capacity for surviving outside the host and promote ST59 transmission among livestock animals and human beings (Li et al., 2018). In this study, some ST59 isolates from livestock products were scattered among patient isolates in phylogenetic tree suggesting that frequent exchanges might occur between livestock animals and patients. Because of the higher adaptability and competitive power of ST59 isolates, a detailed study of the genetic basis for the successful dissemination of ST59 among both humans and livestock animals in China should be conducted.

Multiple livestock-association markers, such as *scn*-negative, tetracycline-resistance, CC9, CC398 have been reported in different studies (Verkaik et al., 2011; Cuny et al., 2015). There is growing evidence that accessory genes carried by prophages of *S. aureus* significantly modulate bacterial fitness as they carry multiple virulence factors (VFs). These VFs include human immune evasion cluster (IEC) comprising the genes sak, chp, scn and sea/sep (Nepal et al., 2021). As a marker of IEC, *scn* has been recognized as the indicator of human *S. aureus* isolates and might be useful for differentiating livestock isolates from human isolates (Rinsky et al., 2013). In this study, all human ST398 (n = 4) isolates were *scn* positive, but all ST9 (n = 10) and food ST398 (n = 3) isolates were *scn* negative. This was consistent with a recent study that the loss of IEC might happen after their shift from human to animals because antimicrobials in feed can induce *scn* prophage loss (Allen et al., 2011; Price et al., 2012; Yu et al., 2021). All food (n = 13) ST59 isolates in this study were *scn*-positive which indicated these isolates might jump from human into the livestock animals recently. A recent study also found *scn*-positive ST59 was

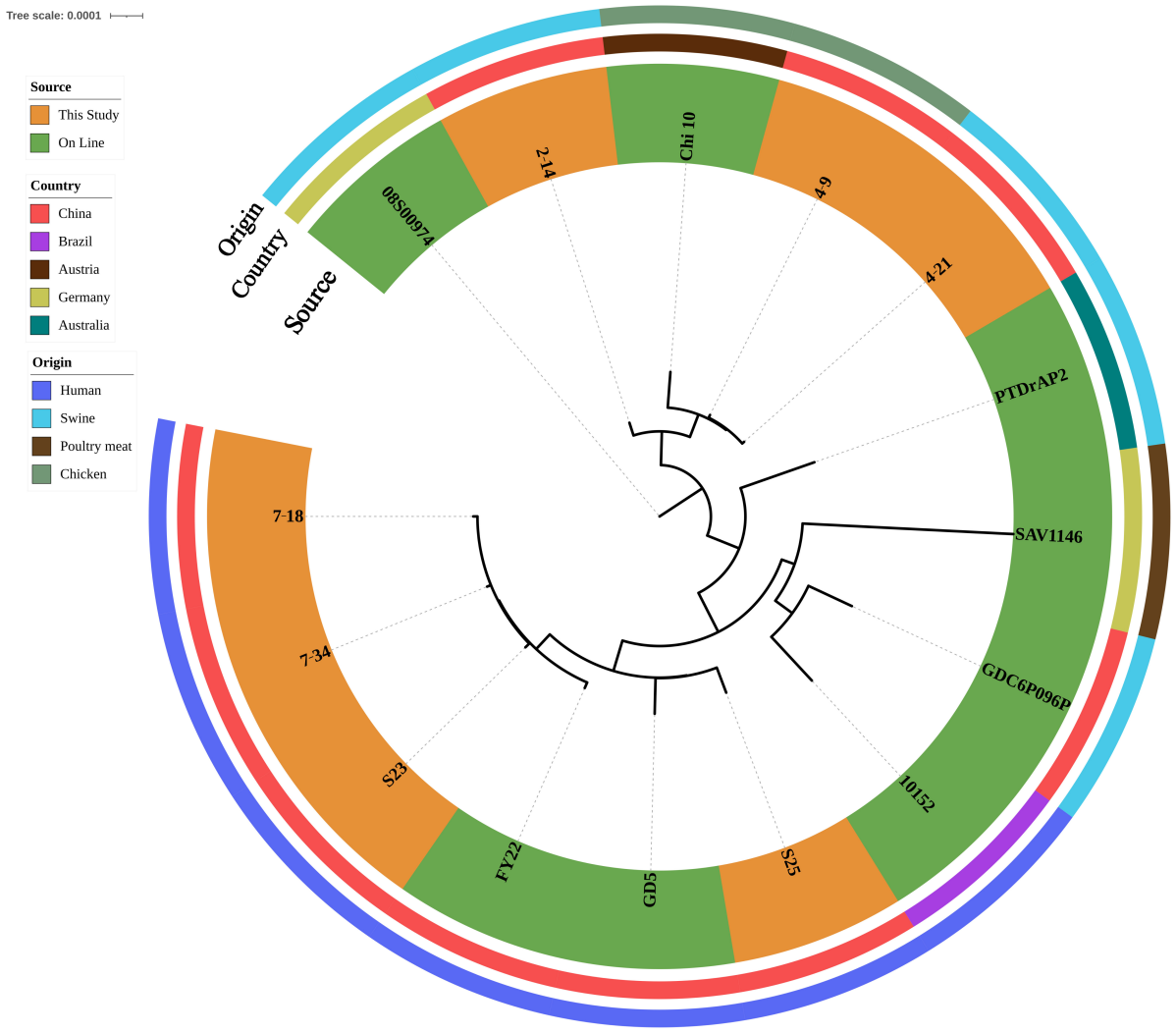

FIGURE 3
Phylogenetic relationship of ST398 isolates from retail livestock products and patients, Hanzhong, China. The genome sequences of 15 ST398 isolates were aligned through SNP analysis, including 8 online isolates. The information of online isolates was provided in **Supplementary Table 4**.

the major MRSA among Yak (Bos grunniens) herds in Tibetan, China (Zou et al., 2022). A continuous surveillance study should be carried out to find out how long ST59 isolates would keep *scn* after it was transmitted to livestock hosts and the factors that might influence the speed of *scn* loss.

Tetracycline-resistance is another livestock-association marker different from human isolates (Rinsky et al., 2013). Tetracyclines are a class of broad-spectrum antibiotics, including naturally occurring and semi-synthetic tetracyclines (Roberts, 2003). Due to their side-effects, the use of tetracyclines is limited in human clinics, but they are still been used globally as important infection treatment measures and growth promoters among livestock animals which promoted the tetracycline resistant determinants transmission in livestock products (Inglis et al., 2019; Chen et al., 2023). Since animals cannot fully absorb or metabolize tetracyclines, they excrete a significant fraction of such drugs or of their breakdown products into the environment via feces or urine which may also promote the transmission of resistant determinants (Mackie et al., 2006). More than 30 tetracycline specific resistant determinants have been recognized and some of them can be transferred horizontally among different bacteria through mobile genetic elements, such as plasmids, phages or integrons (Roberts, 2003). In this study, the food isolates (20/26) showed significantly higher frequency of tetracycline resistance than isolates from human beings (27/67) which indicated the food isolates circulating among livestock animals for considerable time. The genotypes of tetracycline resistant determinants were corresponding to the sequence types which further indicated different evolution processes of these isolates.

Genetic advantages might contribute to the replacement of LA-MRSA from ST9 to ST59 locally. Diversified genetic context was found among 57 ST59 MRSA isolates which were group into six *spa* types with t437 (n = 47) as the local dominant LA-MRSA type which was more diversified than ST9 (n = 10) isolates that were all belonged to *spa* t899 (Cui et al., 2009; Wang et al., 2017). Other studies also reported much more diverse genetic context of ST59 isolates that probably reflected active and extensive genetic recombination of this clone (Jin et al., 2021; Wang et al., 2021).

The diversified genetic context might have more exchange and adaption opportunities and speed up the transmission of this clone. More virulence factors might be another advantage that could contribute to ST59 higher transmission compacity than ST9 isolates (Li et al., 2016). ST9 was originated from human-adapted strains which had lost genes related to the evasion of the immune system (Yu et al., 2021). In this study, all ST9 (n = 10) isolates were *scn* and *sak* negative which further confirmed this theory.

There are several shortages of this study. All MRSA isolates were from patients and retail livestock product samples in Hanzhong city, China and no isolates recovered from livestock animals were included. Limited isolates of ST9 and ST398 were analyzed from human and retail livestock product samples in this study. More isolates from other areas should be analyzed to confirm the distribution patterns found in this study.

5 Conclusion

Because of the high adaption and transmission capacity, MRSA ST59 may be widespread and become the dominant clones among livestock animals in the future. Therefore, active surveillance should be enacted for the further epidemic spread of MRSA ST59 in China.

Data availability statement

The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA966921.

Ethics statement

The studies involving humans were approved by 3201 Hospital Ethics Committee, Affiliated with 3201 Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

WZ: Conceptualization, Methodology, Software, Validation, Visualization, Formal analysis, Investigation, Resources, Data curation, Funding acquisition, Writing – original draft, Writing – review and editing. XW: Formal analysis, Visualization, Writing – original draft. LZ: Formal analysis, Visualization, Writing – original draft. YG: Methodology, Resources, Writing – original draft. YiC: Formal analysis, Writing – original draft. NL: Formal analysis, Writing – original draft. LA: Formal analysis, Writing – original draft. LB: Conceptualization, Methodology, Writing – review and editing. YaC: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software, Writing – review and editing, Supervision, Validation. SC: Conceptualization, Methodology, Software, Writing – review and editing, Supervision, Validation, Funding acquisition, Project administration.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1392134/full#supplementary-material

References

Allen, H. K., Looft, T., Bayles, D. O., Humphrey, S., Levine, U. Y., Alt, D., et al. (2011). Antibiotics infeed induce prophages in swine fecal microbiomes. *mBio* 2:e00260-11. doi: 10.1128/mbio.00260-11.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Bartels, M. D., Petersen, A., Worning, P., Nielsen, J. B., Larner-Svensson, H., Johansen, H. K., et al. (2014). Comparing whole-genome sequencing with Sanger sequencing for spa typing of methicillin-resistant *Staphylococcus aureus*. *J. Clin. Microbiol.* 52, 4305–4308. doi: 10.1128/jcm.01979-14

Bisdorff, B., Scholholter, J. L., Claussen, K., Pulz, M., Nowak, D., and Radon, K. (2012). MRSA-ST398 in livestock farmers and neighbouring residents in a rural area in Germany. *Epidemiol. Infect.* 140, 1800–1808. doi: 10.1017/S0950268811002378

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., et al. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother.* 75, 3491–3500. doi: 10.1093/jac/dkaa345

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics* 10:421. doi: 10.1186/1471-2105-10-421

Chen, H., Yin, Y., van Dorp, L., Shaw, L. P., Gao, H., Acman, M., et al. (2021). Drivers of methicillin-resistant *Staphylococcus aureus* (MRSA) lineage replacement in China. *Genome Med.* 13:171. doi: 10.1186/s13073-021-00992-x

Chen, Y., Sun, L., Hong, Y., Chen, M., Zhang, H., Peng, Y., et al. (2023). Exploring the third-generation tetracycline resistance of multidrug-resistant livestock-associated methicillin-resistant *Staphylococcus aureus* ST9 across healthcare settings in China. *J. Antimicrob. Chemother.* 78, 1871–1881. doi: 10.1093/jac/dkad174

Clinical and Laboratory Standards Institute [CLSI] (2022). *Performance standards for antimicrobial susceptibility testing. CLSI approved standard M100-S32*. Wayne, IL: Clinical and Laboratory Standards Institute.

Cui, S., Li, J., Hu, C., Jin, S., Li, F., Guo, Y., et al. (2009). Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from swine and workers in China. *J. Antimicrob. Chemother.* 64, 680–683. doi: 10.1093/jac/dkp275

Cuny, C., Abdelbary, M., Layer, F., Werner, G., and Witte, W. (2015). Prevalence of the immune evasion gene cluster in *Staphylococcus aureus* CC398. *Vet. Microbiol.* 177, 219–223. doi: 10.1016/j.vetmic.2015.02.031

Di Gregorio, S., Vielma, J., Haim, M. S., Rago, L., Campos, J., Kekre, M., et al. (2023). Genomic epidemiology of *Staphylococcus aureus* isolated from bloodstream infections in South America during 2019 supports regional surveillance. *Microb. Genom.* 9:mgen001020. doi: 10.1099/mgen.0.001020

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., and Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci. U.S.A.* 99, 7687–7692. doi: 10.1073/pnas.122108599

Hennekinne, J. A., De Buyser, M. L., and Dragacci, S. (2012). *Staphylococcus aureus* and its food poisoning toxins: Characterization and outbreak investigation. *FEMS Microbiol. Rev.* 36, 815–836. doi: 10.1111/j.1574-6976.2011.00311.x

Inglis, G. D., Gusse, J. F., House, K. E., Shelton, T. G., and Taboada, E. N. (2019). Tetracycline resistant *Campylobacter jejuni* subtypes emanating from beef cattle administered non-therapeutic chlortetracycline are longitudinally transmitted within the production continuum but are not detected in ground beef. *Microorganisms* 8:23. doi: 10.3390/microorganisms8010023

Jin, Y., Zhou, W., Zhan, Q., Zheng, B., Chen, Y., Luo, Q., et al. (2021). Genomic epidemiology and characterization of methicillin-resistant *Staphylococcus aureus* from bloodstream infections in China. *mSystems* 6:e0083721. doi: 10.1128/mSystems.00837-21

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., and Jermiin, L. S. (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat. Methods* 14, 587–589. doi: 10.1038/nmeth.4285

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., et al. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* 50, 1355–1361. doi: 10.1128/jcm.06094-11

Letunic, I., and Bork, P. (2016). Interactive tree of life (iTOL) v3: An online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res.* 44, W242–W245. doi: 10.1093/nar/gkw290

Li, H., Tang, T., Stegger, M., Dalsgaard, A., Liu, T., and Leisner, J. J. (2021). Characterization of antimicrobial-resistant *Staphylococcus aureus* from retail foods in Beijing, China. *Food Microbiol.* 93:103603. doi: 10.1016/j.fm.2020.103603

Li, M., Dai, Y., Zhu, Y., Fu, C. L., Tan, V. Y., Wang, Y., et al. (2016). Virulence determinants associated with the Asian community-associated methicillin-resistant *Staphylococcus aureus* lineage ST59. *Sci. Rep.* 6:27899. doi: 10.1038/srep27899

Li, R., Li, Y., Kristiansen, K., and Wang, J. (2008). SOAP: Short oligonucleotide alignment program. *Bioinformatics* 24, 713–714. doi: 10.1093/bioinformatics/btn025

Li, S., Sun, S., Yang, C., Chen, H., Yin, Y., Li, H., et al. (2018). The changing pattern of population structure of *Staphylococcus aureus* from bacteremia in China from 2013 to 2016: ST239-030-MRSA replaced by ST59-t437. *Front. Microbiol.* 9:332. doi: 10.3389/fmicb.2018.00332

Li, Y., Li, W., Pan, Y., Liu, C., Liang, S., and Zeng, Z. (2022). The emergence and molecular study of methicillin-resistant *Staphylococcus aureus* ST239, ST59, ST9, and ST630 in food animals, Chongqing, China. *Vet. Microbiol.* 265:109329. doi: 10.1016/j.vetmic.2021.109329

Lin, S. H., and Liao, Y. C. (2013). CISA: Contig integrator for sequence assembly of bacterial genomes. *PLoS One* 8:e60843. doi: 10.1371/journal.pone.0060843

Luo, R., Liu, B., Xie, Y., Li, Z., Huang, W., Yuan, J., et al. (2012). SOAPdenovo2: An empirically improved memory-efficient short-read de novo assembler. *Gigascience* 1:18. doi: 10.1186/2047-217X-1-18

Mackie, R. I., Koike, S., Krapac, I., Chee-Sanford, J., Maxwell, S., and Aminov, R. I. (2006). Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. *Anim. Biotechnol.* 17, 157–176. doi: 10.1080/10495390600956953

Mascaro, V., Leonetti, M., Nobile, C. G. A., Barbadoro, P., Ponzio, E., Recanatini, C., et al. (2018). Prevalence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) among farm and slaughterhouse workers in Italy. *J. Occup. Environ. Med.* 60, e416–e425. doi: 10.1097/JOM.0000000000001385

Merlino, J., Watson, J., Rose, B., Beard-Pegler, M., Gottlieb, T., Bradbury, R., et al. (2002). Detection and expression of methicillin/oxacillin resistance in multidrug-resistant and non-multidrug-resistant *Staphylococcus aureus* in Central Sydney, Australia. *J. Antimicrob. Chemother.* 49, 793–801. doi: 10.1093/jac/dkf021

Nepal, R., Houtak, G., Shaghayegh, G., Bouras, G., Shearwin, K., Psaltis, A. J., et al. (2021). Prophages encoding human immune evasion cluster genes are enriched in *Staphylococcus aureus* isolated from chronic rhinosinusitis patients with nasal polyps. *Microb. Genom.* 7:000726. doi: 10.1099/mgen.0.000726

Pimentel de Araujo, F., Monaco, M., Del Grosso, M., Pirolo, M., Visca, P., and Pantosti, A. (2021). *Staphylococcus aureus* clones causing osteomyelitis: A literature review (2000-2020). *J. Glob. Antimicrob. Resist.* 26, 29–36. doi: 10.1016/j.jgar.2021.03.030

Price, L. B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P. S., et al. (2012). *Staphylococcus aureus* CC398: Host adaptation and emergence of methicillin resistance in livestock. *mBio* 3, e305–e311. doi: 10.1128/mbio.00305-11

Rinsky, J. L., Nadimpalli, M., Wing, S., Hall, D., Baron, D., Price, L. B., et al. (2013). Livestock-associated methicillin and multidrug resistant *Staphylococcus aureus* is present among industrial, not antibiotic-free livestock operation workers in North Carolina. *PLoS One* 8:e67641. doi: 10.1371/journal.pone.0067641

Roberts, M. C. (2003). Tetracycline therapy: Update. *Clin. Infect. Dis.* 36, 462–467. doi: 10.1086/367622

Silva, V., Araujo, S., Monteiro, A., Eira, J., Pereira, J. E., Maltez, L., et al. (2023). *Staphylococcus aureus* and MRSA in livestock: Antimicrobial resistance and genetic lineages. *Microorganisms* 11:124. doi: 10.3390/microorganisms11010124

Simpson, J. T., Wong, K., Jackman, S. D., Schein, J. E., Jones, S. J., and Birol, I. (2009). ABySS: A parallel assembler for short read sequence data. *Genome Res.* 19, 1117–1123. doi: 10.1101/gr.089532.108

Sun, C., Chen, B., Hulth, A., Schwarz, S., Ji, X., Nilsson, L. E., et al. (2019). Genomic analysis of *Staphylococcus aureus* along a pork production chain and in the community, Shandong Province, China. *Int. J. Antimicrob. Agents* 54, 8–15. doi: 10.1016/j.ijantimicag.2019.03.022

Tuffs, S. W., Goncheva, M. I., Xu, S. X., Craig, H. C., Kasper, K. J., Choi, J., et al. (2022). Superantigens promote *Staphylococcus aureus* bloodstream infection by eliciting pathogenic interferon-gamma production. *Proc. Natl. Acad. Sci. U.S.A.* 119:e2115987119. doi: 10.1073/pnas.2115987119

Turner, N. A., Sharma-Kuinkel, B. K., Maskarinec, S. A., Eichenberger, E. M., Shah, P. P., Carugati, M., et al. (2019). Methicillin-resistant *Staphylococcus aureus*: An overview of basic and clinical research. *Nat. Rev. Microbiol.* 17, 203–218. doi: 10.1038/s41579-018-0147-4

Verkaik, N. J., Benard, M., Boelens, H. A., de Vogel, C. P., Nouwen, J. L., Verbrugh, H. A., et al. (2011). Immune evasion cluster-positive bacteriophages are highly prevalent among human *Staphylococcus aureus* strains, but they are not essential in the first stages of nasal colonization. *Clin. Microbiol. Infect.* 17, 343–348. doi: 10.1111/j.1469-0691.2010.03227.x

Wang, B., Xu, Y., Zhao, H., Wang, X., Rao, L., Guo, Y., et al. (2022). Methicillin-resistant *Staphylococcus aureus* in China: A multicentre longitudinal study and whole-genome sequencing. *Emerg. Microbes Infect.* 11, 532–542. doi: 10.1080/22221751.2022.2032373

Wang, W., Baker, M., Hu, Y., Xu, J., Yang, D., Maciel-Guerra, A., et al. (2021). Whole-genome sequencing and machine learning analysis of *Staphylococcus aureus* from multiple heterogeneous sources in China reveals common genetic traits of antimicrobial resistance. *mSystems* 6:e0118520. doi: 10.1128/msystems.01185-20

Wang, W., Liu, F., Baloch, Z., Zhang, C. S., Ma, K., Peng, Z. X., et al. (2017). Genotypic characterization of methicillin-resistant *Staphylococcus aureus* isolated from pigs and retail foods in China. *Biomed. Environ. Sci.* 30, 570–580. doi: 10.3967/bes2017.076

Wang, X., Li, G., Xia, X., Yang, B., Xi, M., and Meng, J. (2014). Antimicrobial susceptibility and molecular typing of methicillin-resistant *Staphylococcus aureus* in retail foods in Shaanxi, China. *Foodborne Pathog. Dis.* 11, 281–286. doi: 10.1089/fpd.2013.1643

Yu, F., Cienfuegos-Gallet, A. V., Cunningham, M. H., Jin, Y., Wang, B., Kreiswirth, B. N., et al. (2021). Molecular evolution and adaptation of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) sequence type 9. *mSystems* 6:e0049221. doi: 10.1128/msystems.00492-21

Zhang, T., Jia, M., Cheng, Y., Zhang, W., Lu, Q., Guo, Y., et al. (2021). First report of ST9-MRSA-XII from a chicken farm in China. *J. Glob. Antimicrob. Resist.* 27, 292–293. doi: 10.1016/j.jgar.2021.10.018

Zou, G., Matuszewska, M., Bai, F., Wang, S., Wang, S., Li, H., et al. (2022). Genomic analyses of *Staphylococcus aureus* isolated from yaks in Ganzi Tibetan autonomous prefecture, China. *J. Antimicrob. Chemother.* 77, 910–920. doi: 10.1093/jac/dkac011

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