frontiers | Research Topics

Reviews in antibiotic resistance and new antimicrobial drugs

Edited by
Sandra Aguiar, Falguni Debnath and Sanket Kaushik

Published in
Frontiers in Cellular and Infection Microbiology

ISSN 1664-8714
ISBN 978-2-8325-5025-0
DOI 10.3389/978-2-8325-5025-0

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Reviews in antibiotic resistance and new antimicrobial drugs

Topic editors

Sandra Aguiar — University of Lisbon, Portugal

Falguni Debnath — National Institute of Cholera and Enteric Diseases (ICMR), India

Sanket Kaushik — Amity University Jaipur, India

Citation

Aguiar, S., Debnath, F., Kaushik, S., eds. (2024). *Reviews in antibiotic resistance and new antimicrobial drugs*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-5025-0

Table of contents

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Editorial
PUBLISHED 04 June 2024
DOI 10.3389/fcimb.2024.1434140

OPEN ACCESS

EDITED AND REVIEWED BY
Costas C. Papagiannitsis,
University of Thessaly, Greece

*CORRESPONDENCE
Sanket Kaushik
skaushik@jpr.amity.edu;
sanketkaushik@gmail.com

RECEIVED 17 May 2024
ACCEPTED 28 May 2024
PUBLISHED 04 June 2024

CITATION
Kaushik S (2024) Editorial: Reviews in antibiotic resistance and new antimicrobial drugs.
Front. Cell. Infect. Microbiol. 14:1434140.
doi: 10.3389/fcimb.2024.1434140

Editorial: Reviews in antibiotic resistance and new antimicrobial drugs

Sanket Kaushik*

Amity Institute of Biotechnology, Amity University Rajasthan, Jaipur, Rajasthan, India

KEYWORDS

multi drug resistance (MDR), biofilm, antimicrobial resistance, ESKAPE, bacterial infections

Editorial on the Research Topic
Reviews in antibiotic resistance and new antimicrobial drugs

At the time of their discovery, antibiotics were considered a wonder drug for the treatment of bacterial infections, but the way microorganisms have involved several robust mechanisms to encounter the antibiotics has given rise to multi-drug-resistant (MDR) and extensively drug-resistant (XDR) strains (Eichenberger and Thaden, 2019; Terreni et al., 2021; Yadav et al., 2022). There are several ways by which the pathogen acquires resistance to antibiotics, including genetic variation in the genome of the pathogen, unselective usage of antibiotics, the development of biofilms, etc (Santos-Lopez et al., 2019; Singh et al., 2019). Since it has become difficult to treat such infections, it is essential to understand the mechanism of the development of anti-microbial resistance (AMR) so that strategies to prevent such infections can be developed (Lomazzi et al., 2019; Hu et al., 2020; Moo et al., 2020).

The goal of this Research Topic was to highlight recent advances in the field of antibiotic resistance while emphasising important directions and new possibilities for future inquiries. We anticipate the research being presented here will ignite conversations within the community about novel antimicrobial medications and antibiotic resistance, which will lead to the application of best practices in clinical, public health, and policy settings.

Overall, four research articles and six review articles were published in this Research Topic. A research article published by Wang et al. reported the epidemiological studies of nontuberculous mycobacteria (NTM) in tuberculosis suspects in the southwest of China from 2017 to 2022. In this study, the main NTM isolates, MAC and *M. chelonae/M. abscessus*, were identified, and it was observed that the isolation rate of NTM in southwest China has shown an increasing trend in the last few years. The infected cases were elderly patients, individuals with compromised immune systems who had an HIV infection. On evaluation, it was observed that antibiotics like amikacin, moxifloxacin, clarithromycin, and linezolid demonstrated effective antibacterial activity against slow-growing mycobacteria, whereas linezolid and amikacin exhibited relatively better antibacterial activity against rapid-growing mycobacteria. Another research article published by Shi et al. studied the prevalence and resistance characteristics of MDR *Streptococcus pneumoniae* isolated from the respiratory tracts of hospitalised children in Shenzhen, China. It was observed that the non-vaccine serotype strains accounted for 46.6% of all the pneumococcal isolated strains. The multidrug resistance rates (MDR) of vaccine serotypes were 19F (99.36%), 19A (100%), 23F (98.08%), 6B (100%), and 6C (100%), and the MDR of non-vaccine serotypes were 15B (100.00%), 6E

(100%), 15C (100%), 34 (95.24%), and 23A (98.31%), respectively. Data indicated that there has been a notable rise and spread of multidrug-resistant non-vaccine serotypes among children. Another research paper is entitled "*PqsA* mutation-mediated enhancement of phage-mediated combat against *Pseudomonas aeruginosa.*" In this study, the *PqsA* gene was highlighted as a potential drug target to enhance phage therapy, as the deletion of the *pqsA* gene could significantly promote the lysis of phages on *Pseudomonas aeruginosa. A research* article by Janc et al. highlighted the impact of *Klebsiella pneumoniae* NDM (New Delhi metallo-B-lactamase) infection and other bacterial infections on mortality in patients treated in ICUs due to COVID-19. It was reported that in patients treated for SARS-CoV-2 infection, acquiring a bacterial infection due to prolonged hospitalisation associated with the treatment of COVID-19 did not elevate mortality rates. The data also suggested that in severe COVID-19 patients who survived beyond the first week of hospitalisation, bacterial infections, particularly *Klebsiella pneumoniae* NDM, do not significantly impact mortality.

A review article published by Ari et al. highlighted the properties and potential of nitrofurantoin (NF) in the treatment of urinary tract infections (UTI). The author has studied the detailed pharmacokinetic and pharmacodynamic properties along with the antibacterial activity and mechanism of action of the drug and concluded that NF can be considered the most effective drug in the treatment of acute urinary infection, but due to the long-term side effects of this drug, especially in elderly patients, it is essential to introduce some criteria for prescribing NF in cases of chronic UTI. Another review article by Wang et al. highlighted antimicrobial resistance and its mechanism of epigenetic regulation. The author has extensively focused on the effects of DNA modification, histone modification, rRNA methylation, and the regulation of non-coding RNA expression on antimicrobial resistance. Bacterial epigenetics, modifications of DNA and rRNA, ncRNAs, as well as nucleoid-associated proteins, have been shown to regulate the development of AMR. Another review paper entitled "Application of the CRISPR-Cas system in the diagnosis and therapy of ESKAPE infections" elaborated on the applications of the CRISPR-Cas system for the study of ESKAPE pathogens. The author highlighted that although currently no direct CRISPR-based anti-infective treatment methods are available, the CRISPR-Cas method can be a promising alternative to treatment because of its specificity.

A review paper by Schami et al. focused on the cell envelope profiles of drug-resistant strains of *Mycobacterium tuberculosis* and their interactions with the host. The composition and complexity of the cell envelope were discussed, along with its importance as a drug target for the development of anti-bacterial drugs. The author has also described the current knowledge regarding the influence of drug resistance on infection outcomes. Another review paper by Pai et al. has highlighted the need to design novel compounds for the eradication of infections caused by MDR bacteria. Different strategies by which bacteria gain resistance to several antibiotics have been discussed, along with the pathways that can be targeted for development by antimicrobial drugs with better potency. One more review paper by Ramirez et al. emphasised the application of anti-microbial peptides in livestock farming and how they can mitigate the impact of this practice within the One Health framework. Despite several challenges, the pace at which bacteria adapt to these peptides is very slow as compared to other methods. Therefore, AMPs offer a potential solution to the scarcity of effective antibiotics against MDR bacteria.

Author contributions

SK: Writing – original draft, Writing – review & editing.

Conflict of interest

The author declares 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

Eichenberger, E. M., and Thaden, J. T. (2019). Epidemiology and mechanisms of resistance of extensively drug resistant Gram-negative bacteria. *Antibiotics* 8, 37. doi: 10.3390/antibiotics8020037

Hu, X. Y., Logue, M., and Robinson, N. (2020). Antimicrobial resistance is a global problem–a UK perspective. *Eur. J. Integr. Med.* 36, 101136. doi: 10.1016/j.eujim.2020.101136

Lomazzi, M., Moore, M., Johnson, A., Balasegaram, M., and Borisch, B. (2019). Antimicrobial resistance–moving forward? *BMC Public Health* 19, 1–6. doi: 10.1186/s12889-019-7173-7

Moo, C. L., Yang, S. K., Yusoff, K., Ajat, M., Thomas, W., Abushelaibi, A., et al. (2020). Mechanisms of antimicrobial resistance (AMR) and alternative approaches to overcome AMR. *Curr. Drug Discovery Technol.* 17, 430–447. doi: 10.2174/1570163816666190304122219

Santos-Lopez, A., Marshall, C. W., Scribner, M. R., Snyder, D. J., and Cooper, V. S. (2019). Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. *Elife* 8, e47612. doi: 10.7554/eLife.47612.024

Singh, H., Das, S., Yadav, J., Srivastava, V. K., Jyoti, A., and Kaushik, S. (2019). In search of novel protein drug targets for treatment of Enterococcus faecalis infections. *Chem. Biol. Drug Design* 94, 1721–1739. doi: 10.1111/cbdd.13582

Terreni, M., Taccani, M., and Pregnolato, M. (2021). New antibiotics for multidrug-resistant bacterial strains: latest research developments and future perspectives. *Molecules* 26, 2671. doi: 10.3390/molecules26092671

Yadav, J., Das, S., Karthikeyan, D., Chug, R., Jyoti, A., Srivastava, V. K., et al. (2022). Identification of protein drug targets of biofilm formation and quorum sensing in multidrug resistant enterococcus faecalis. *Curr. Protein Pept. Sci.* 23, 248–263. doi: 10.2174/1389203723666220526155644

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 14 June 2023
DOI 10.3389/fcimb.2023.1199646

OPEN ACCESS

EDITED BY
Falguni Debnath,
National Institute of Cholera and Enteric Diseases (ICMR), India

REVIEWED BY
Sushmita Bhattacharya,
National Institute of Cholera and Enteric Diseases (ICMR), India
Sandra C. Viegas,
Universidade Nova de Lisboa, Portugal

*CORRESPONDENCE
Lu Chen
cl_chen0909@163.com

RECEIVED 03 April 2023
ACCEPTED 26 May 2023
PUBLISHED 14 June 2023

CITATION
Wang X, Yu D and Chen L (2023) Antimicrobial resistance and mechanisms of epigenetic regulation.
Front. Cell. Infect. Microbiol. 13:1199646.
doi: 10.3389/fcimb.2023.1199646

Antimicrobial resistance and mechanisms of epigenetic regulation

Xinrui Wang[1,2], Donghong Yu[1,2] and Lu Chen[1,2]*

[1]Medical Research Center, Fujian Maternity and Child Health Hospital, College of Clinical Medicine for Obstetrics and Gynecology and Pediatrics, Fujian Medical University, Fuzhou, Fujian, China, [2]National Health Commission Key Laboratory of Technical Evaluation of Fertility Regulation for Non-Human Primate, Fujian Maternity and Child Health Hospital, Fuzhou, Fujian, China

The rampant use of antibiotics in animal husbandry, farming and clinical disease treatment has led to a significant issue with pathogen resistance worldwide over the past decades. The classical mechanisms of resistance typically investigate antimicrobial resistance resulting from natural resistance, mutation, gene transfer and other processes. However, the emergence and development of bacterial resistance cannot be fully explained from a genetic and biochemical standpoint. Evolution necessitates phenotypic variation, selection, and inheritance. There are indications that epigenetic modifications also play a role in antimicrobial resistance. This review will specifically focus on the effects of DNA modification, histone modification, rRNA methylation and the regulation of non-coding RNAs expression on antimicrobial resistance. In particular, we highlight critical work that how DNA methyltransferases and non-coding RNAs act as transcriptional regulators that allow bacteria to rapidly adapt to environmental changes and control their gene expressions to resist antibiotic stress. Additionally, it will delve into how Nucleolar-associated proteins in bacteria perform histone functions akin to eukaryotes. Epigenetics, a non-classical regulatory mechanism of bacterial resistance, may offer new avenues for antibiotic target selection and the development of novel antibiotics.

KEYWORDS

antimicrobial resistance, epigenetics, DNA modification, rRNA methylation, non-coding RNAs, epigenetic drugs

1 Introduction

The discovery and widespread use of antibiotics have greatly advanced modern medicine, significantly improving the treatment of bacterial infections. However, long-term exposure to antibiotics can pose a serious risk of antimicrobial resistance (AMR), where pathogenic microorganisms become resistant to the drugs. The emergence of AMR is a growing concern, particularly with the increasing detection of clinical resistant bacteria. According to the 2019 U.S. Antibiotic Resistance Threat Report, antibiotic-resistant bacteria and fungi are responsible for over 2.8 million infections and 35,000 deaths annually in the USA alone

(Centers for Disease Control and Prevention, 2019). Furthermore, predictive statistical models from the Institute for Health Metrics and Evaluation at the University of Washington, estimate that there may have been 4.95 million deathes worldwide in 2019 due to AMR (Antimicrobial Resistance Collaborators, 2022). Clearly, AMR has become a critical threat to global public health security, compounded by the onset of the post-antibiotic age and the inappropriate use of antibiotics.

Despite more than 80 years of antibiotics use, bacteria have evolved AMR mechanisms over billions of years that allow them to escape the impact of antibiotics (Hall and Barlow, 2004). The classical AMR mechanisms include chromosomal resistance, changes in cell membrane permeability, enzyme production, target modification or mutation, active efflux pump system changes, and horizontal or vertical transfer of AMR genes (Figure 1) (Cox and Wright, 2013; Yelin and Kishony, 2018). These mechanisms primarily involve well-documented biochemical mechanisms and gene alterations, which are diverse, specific and heritable. However, in addition to genome changes, environmental factors and genetic context also impact the development of AMR. Antibiotics can have multiple activities, including as a resistant inducer, an inducer of resistance determinant dissemination, and an antibacterial agent (Depardieu et al., 2007). Studies demonstrate that antibiotics can induce epigenetic changes in bacterial resistance, indicating the role of epigenetics (Motta et al., 2015). While much research has focused on classical AMR mechanisms, these mechanisms fall short in explaining the emergence and spread of drug resistance due to factors such as bacterial adaptive evolution, heterogeneity, and late retention (Depardieu et al., 2007; Depardieu et al., 2007; Zhang, 2014; Becker et al., 2018; Nolivos et al., 2019; Lv et al., 2021; Yuan et al., 2022). Therefore, epigenetics may provide useful answers to these questions.

There has been growing interest in non-classical models of epigenetic-mediated bacterial AMR in recent years. In this review, we will explore the latest research on AMR in the field of epigenetics, with a focus on how epigenetic regulation influences the emergence of AMR, as well as how epigenetic regulators can reverse epigenetic phenomena and eliminate AMR. This is critical for understanding the mechanisms of AMR and for developing the potential of epigenetic regulators as direct or indirect targets for new drug therapies.

2 What is epigenetics?

Epigenetics refers to the study of the heritable phenotypic changes in an organism that are caused by environmental factors and genetic context, without any alterations to the DNA sequence. Epigenetic research is broadly divided into two categories (Willbanks et al., 2016): (1) Regulation of selective gene transcription, which includes DNA methylation, histone modification, chromatin

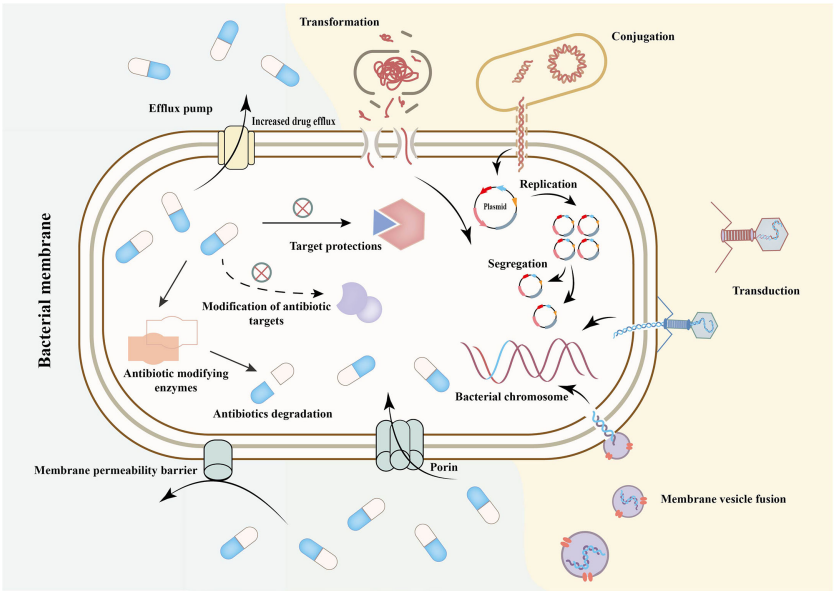


FIGURE 1
Mechanism of antimicrobial resistance and its transmission. Transformation is the intra- and inter- species exchange of naked DNA released by cell lysis or gene sequences actively effluxed by some bacteria. Conjuction is the direct transfer of DNA molecules (such as plasmids) from donor bacteria to recipient bacteria through the pipeline formed by sex pilus. Transduction is the transfer of DNA from donor bacteria to recipient bacteria by bacteriophages (Depardieu et al., 2007; Zhang, 2014; Yuan et al., 2022). Membrane vesicle fusion means that vesicles secreted which includes nucleic acids, enzymes and drug resistance genes and other substances, can enter another bacteria or host cells through direct fusion with host cell membrane or endocytosis (Depardieu et al., 2007; Zhang, 2014; Yuan et al., 2022). The outer membrane porin mediates the entry and exit of antibiotics into and out of bacteria as a permeability barrier. When the porin is missing or reduced, some antibiotics reduce influx and the host bacteria become resistant. The production of antibiotic hydrolases, inactivating enzymes and modifying enzymes can lead to the inactivation of antibiotics. The mutation or modification of related targets makes it impossible for antibiotics to bind to the corresponding sites to play a bactericidal or bacteriostatic role (Depardieu et al., 2007; Zhang, 2014; Yuan et al., 2022).

remodeling and DNA phosphorothioation; (2) Post-transcriptional gene regulation, which includes regulation by non-coding RNAs (ncRNAs), RNA modification, and nucleosome positioning.

Prokaryotes have a circular, double-stranded DNA chromosome without histones, which distinguishes them from eukaryotes and ancient karyotes. This lack of key elements, such as histones and nucleosomes, that can modify DNA structure makes the epigenetic regulation mode of prokaryotes relatively simple.

2.1 DNA modification

2.1.1 DNA methylation

In contrast to eukaryotes, bacteria lack a complete nucleus, which initially led to the theory that DNA methylation was the only type of bacterial epigenetic mechanism (Ghosh et al., 2020). Bacterial DNA methylation has been extensively studied over the past half century, revealing its involvement in chromosome replication, DNA degradation, mismatch repair, gene expression regulation, and other important physiological activities (Table 1) (Heusipp et al., 2007; Muhammad et al., 2022). Bacteria have three major forms of DNA methylation: 5-methylcytosine (m^5C), N6-methyladenosine (m^6A), and N4-methylcytosine (m^4C). DNA methyltransferase (MTase) add methyl groups to specific DNA locations, such as the C5 or N4 position of cytosine and the N6 position of adenine (Figure 2) (Dunn and Smith, 1955; Holliday and Pugh, 1975). The most commonly known DNA MTases are associated with the restriction-modification (R-M) system, which is a widely known defense mechanism in bacteria. While m^5C and m^6A are found in most bacteria, m^4C is specific to bacteria and archaea (Sánchez and Casadesús, 2020).

2.1.2 DNA phosphorothioation

In addition to DNA methylation, DNA modifications also include DNA phosphorothioation (PT) modification, which is a lesser known defence system that works in a way similar to that of the R-M system (Table 1) (Wang et al., 2019; Muhammad et al., 2022). PT modification, in which the nonbridging oxygen in the phosphate moiety of the DNA sugar-phosphate backbone is replaced by sulfur, was originally developed *via* chemically synthesized for decades (Tong et al., 2018). However, some research have discovered that PT modification can occur naturally in bacteria (Zou et al., 2018). Previously, it has been reported that DNA PT system consists of two parts: a five-gene *dndABCDE* cluster function as the M component to control DNA modification in a stereo- and sequence-selective manner, whereas products of the *dndFGH* cluster function as the R component to distinguish and restrict non-PT-protected foreign DNA (Tong et al., 2018). Among them, *dndA* possesses cysteine desulfurase activity and assembles DndC in bacteria (An et al., 2012). The IscS (a DndA homolog) can perform the same function as DndA to collaborate with DndBCDE in generating DNA PT modification (An et al., 2012). DndB can bind to the promoter region of the *dnd* operon to regulate the transcription of *dnd* genes. *dndCDE* function as modification genes: DndC is an iron-sulfur cluster protein that has ATP pyrophosphatase activity; DndD has ATPase activity and possibly provide energy for PT modification, and DndE is involved in binding nicked dsDNA (Hu et al., 2012; Wang et al., 2019). According to some research, the defence mechanism of PT modification has been revealed roughly (Figure 3). At first, the DndB function as a regulator to make response of environmental or cellular cues, and binds to the promoter region of the *dnd* operon. The DndA/IscS, DndC, DndD and DndE form a protein complex. Under the action of DndA/IscS, L-cysteine is used as a substrate to generate a persulphide group. Then, the sulphur is transferred to the DndACDE complex to complete the DNA PT modification (Wang et al., 2019). DNA PT modification has been reported in many bacteria. Except for function the similar way as the R-M system, DNA PT modification also plays important roles in antioxidant defenses, cellular redox homeostasis maintenance, environmental stress resistance, antibiotic resistance and cross talk with DNA methylation modification (Xie et al., 2012; Gan et al., 2014; Wu et al., 2017; Wu et al., 2020; Xu et al., 2023).

TABLE 1 Summary of bacterial epigenetics through DNA and RNA modifications.

Modifications	Type	Enzymatic Systems	Functions	Examples
DNA	Methylation	R-M system	Defense mechanism, regulate gene expression, virulence, biofilm formation	M.EcoGII, ModS, ModM, ModA, M.HpyIII, M2.HpyAII
		Orphan methyltransferases	Maintain *Eco*RII plasmid stability, DNA repair, chromosome replication, Adenine and Cytosine methyltransferases cause regulation of cell cycle	Dam, CcrM, Dcm, VchM, YhdJ,
	Phosphorothioation	DNA degradation	Defense mechanism, oxidative stress, balance intracellular redox homeostasis, influence the transcriptional efficiency	*dndABCDEFGH*
RNA	Methylation	N^6-methyladenosine modification, N^1-methyladenosine modification, 2-methylthiocytidine modification, 5-methylcytosine modification	Regulate RNA stability, localization, transport, splicing, antibiotic resistance and translation	RlmF, RlmJ, RlmCD
	Non-coding RNAs	Suppress or activate translation	Prevent RNA degradation	Fino/ProQ family, CsrA/RsmA family, OmpACF, MicACF

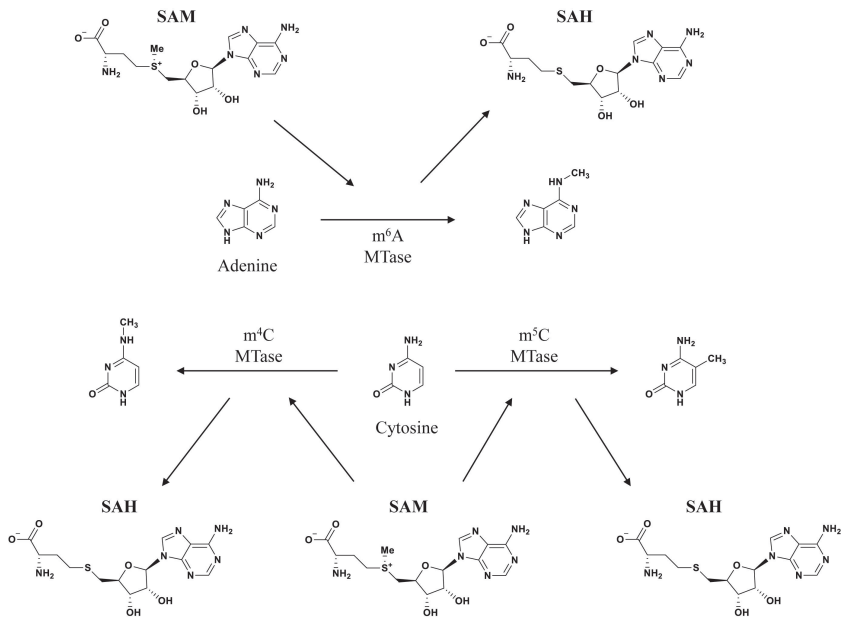


FIGURE 2
Position of DNA methylation. Adenine can add methyl at N6. Cytosine can add methyl at either endocyclic (C5) or exocyclic (N4) (Kumar et al., 2018).

2.2 Histone modification

Histone modification is a significant epigenetic modus that plays an important role in regulating gene expression. German scientist Kossel discovered histones in the nucleus in 1884, but it wasn't until the 1960s that their biological significance began to be investigated in depth (Doenecke and Karlson, 1984; Verdin and Ott, 2015). Histones are structural proteins that make up eukaryotic nucleosomes, which are essential for maintaining chromosomal structure and negative regulation of gene expression (Muhammad et al., 2020). Histone modification can involve methylation, acetylation, phosphorylation, and ubiquitination, each of which performs different functions (Zhang et al., 2020). Notably, bacterial genomes are packed into nucleoids through nucleoid-associated

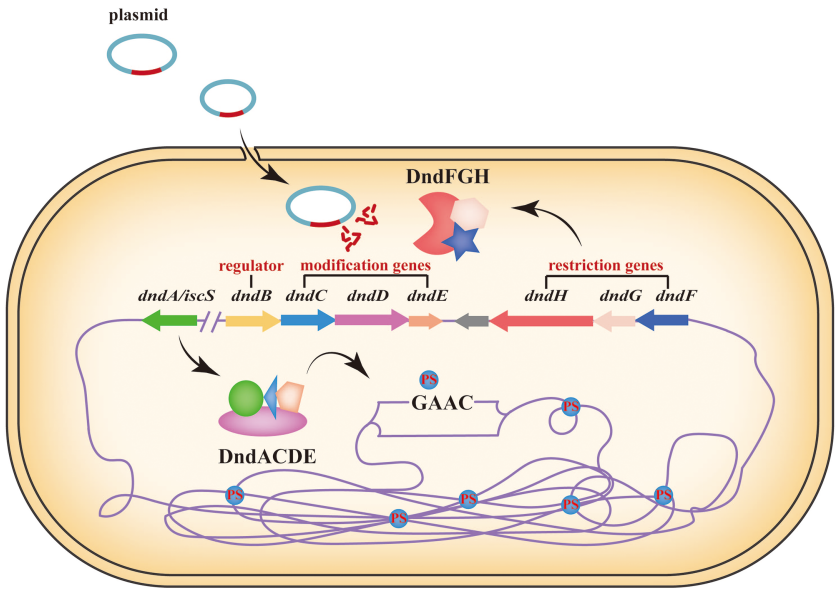


FIGURE 3
DNA phosphorothioation modification simple diagram. Based on R-M system, DNA PT modification recognize and restrict non-PT-protected foreign DNA, such as plasmids. The sulfur is transferred from L-cycsteine to DndA, and then to the cysteine residues in DndC and through DndDE complex protein to insert into the DNA backbone (Tang et al., 2022). DndB function as a negative regulator controlling the expression of *dndCDE*. DndFGH function as a restriction module to affect the acquisition of exogenous DNA (Wang et al., 2019).

proteins (NAPs) in distinct cytoplasmic regions, rather than having a membrane-bound nucleus like eukaryotic cells (Muhammad et al., 2022).

Mounting evidence supports the idea that NAPs play crucial roles in DNA structuring and can perform functions similar to eukaryotic histones (Swinger and Rice, 2007; Stojkova et al., 2019; Amemiya et al., 2021). These structural proteins have important regulatory functions, including in bacterial virulence and pathogenesis (Table 2). NAPs form numerous aggregated structures with bacterial genomic DNA and participate in processes such as replication, separation, translation, and repair of prokaryotic genomic DNA. Among the primary NAPs studied are histone-like protein (HU), leucine-responsive regulatory protein (Lrp), virulence factor transcriptional regulator (Mga*Spn*) and Histone-like nucleoid-structuring (H-NS) (Casadesús and Low, 2006; Xiao et al., 2021; Ziegler and Freddolino, 2021; Ramamurthy et al., 2022; Stojkova and Spidlova, 2022).

2.3 RNA modification

RNA modification is an emerging area of research that has gained significant attention in recent years, which is conceptually analogous to the modifications of DNA and protein. Along with DNA methylation, RNA modification is widely found in both bacteria and eukaryotes, and over 100 types of RNA modifications have been identified, including m^6A, N1-methyladenosine (m^1A), m^5C, and 2-methylthiocytidine (ms^2C) (Lopez et al., 2020). These modifications have been shown to play a critical role in regulating RNA stability, localization, transport, splicing, and translation, ultimately affecting gene regulation and biological function (Shi et al., 2019). RNA modifications are distributed on various RNA molecules, including transfer RNA (tRNA), messenger RNA (mRNA), ribosomal RNA (rRNA) and other small RNA species such as ncRNAs. RNA modification is almost found in tRNA (Jackman and Alfonzo, 2013). Though, not as common as in tRNA, rRNA contain numerous distinct types of post-transcriptional modifications, especially rRNA methylation. Research has shown that rRNA methylation can impact antibiotic resistance development, as many antibiotic targets are located on the ribosome and ncRNAs frequently adopt central roles in regulatory networks (Laughlin et al., 2022; Wang et al., 2022; Papenfort and Melamed, 2023). Of those, RNA methylation and ncRNAs modification have been reported as the most frequent type of modification in a wide range of bacteria (Table 1). In this section, we will discuss the research of rRNA methylation and ncRNAs in bacterial resistance.

2.3.1 Ribosomal RNA methylation

rRNA, a conserved macromolecule, is a structural component of the most abundant cellular molecule, the ribosome. In bacteria, ribosomes are composed of 16S, 23S, 5S rRNA and proteins. In eukaryotic cells, ribosomes are composed of 28S, 5S, 5.8S, 18S rRNA and proteins. In ribosomes, the rRNA is the main structural component and the core of structure and function, including (1) Synthesizing amino acids into peptide chains under the guidance of mRNA; (2) Providing binding sites for a variety of protein factors; (3) Having the activity of peptidyl transferase; (4) Providing binding sites for tRNA; (5) Targets of some antibiotics (Korobeinikova et al., 2012; Tafforeau, 2015; Srinivas et al., 2023). These functions are under tight transcriptional control to serve to meet cellular needs. Therefore, rRNA from all organisms undergoes post-transcriptional modifications that increase the diversity of its composition and activity.

Methylation of rRNA is a ubiquitous feature, and takes place during ribosomal biogenesis either by enzymes guided by an antisense small nucleolar RNA (snoRNA) or conventional protein enzymes (Lopez et al., 2020). Generally, rRNA methylation may promote the conformational rearrangement of rRNA, and regulate ribosome biogenesis and post-transcriptional modification (Wang et al., 2020). There are 25 rRNA modifications have been found in the 23S rRNA, including 13 methylations in *Escherichia coli* (*E. coli*) (Sergeeva et al., 2015). Wang et al. found that the absence of a single methylation in 23S rRNA affected 50S assembly and impaired translation initiation and elongation (Wang et al., 2020). In addition, rRNA methylation has emerged as a significant mechanism of AMR in pathogenic bacterial infections, such as aminoglycoside and macrolide resistance (Bhujbalrao et al., 2022; Srinivas et al., 2023).

TABLE 2 Bacteria exert epigenetic regulation through nucleoid-associated proteins.

Bacterial Species	NAPs	Functions	References
Francisella tularensis	HU	Regulates the adaptive growth of bacteria and resistance to oxidative stress	(Stojkova et al., 2018; Pavlik and Spidlova, 2022)
Streptococcus pneumoniae	HU, Mga*Spn*	Maintains DNA supercoil, regulates bacterial viability and virulence	(Solano et al., 2016; Ferrándiz et al., 2018)
Escherichia coli	HU, Lrp, H-NS	Promotes bacterial invasiveness and replication in host cells, accelerates phagosome escape; regulates metabolism, virulence, exercise, nutrient transport, stress tolerance and antibiotic resistance.	(Koli et al., 2011; Ziegler and Freddolino, 2021; Norris et al., 2022)
Porphyromonas gingivalis	HU, IHF	Regulates biofilm formation	(Rocco et al., 2017)
Salmonella	Fis	Regulates the supercoiling response to bacterial growing in macrophages and virulence	(C et al., 2006)

2.3.2 Non-coding RNAs

Post-transcriptional gene regulation, which includes ncRNAs, is another important epigenetic modification. There are various types of ncRNAs: including housekeeping ncRNAs such as tRNA, rRNA, and regulatory ncRNAs such as micro RNA (miRNA) and long non-coding RNA (lncRNA) (Gusic and Prokisch, 2020). These RNAs play significant roles in transcription and translation, and in eukaryotes, they are involved in regulatory processes such as development, cell death, and chromosomal silencing. Although three regulatory RNAs contained *E. coli* 6S RNA, Spot 42 and the eukaryotic 7SK RNA were first discovered by sequencing in the 1970s, but were uncharacterized until decades later (Griffin, 1971; Delihas, 2015). Until the 1980s, the *E. coli micF* RNA gene was the first regulatory RNA discovered and characterized. Recent research has shown that ncRNAs regulate various cellular processes in bacteria, including multidrug resistance, glucose metabolism, and biofilm formation (Hirakawa et al., 2003; Vanderpool and Gottesman, 2004; Zhao et al., 2022). As a result, the regulatory mode of ncRNAs has become a major focus in the bacterial regulatory network.

3 Bacterial epigenetics mediating antibiotic resistance

Bacteria have evolved to adapt to the environment over time, leading to increased antimicrobial resistance (AMR) or tolerance upon long-term exposure to antibiotics. Interestingly, bacteria can quickly restore susceptibility after returning to a normal antibiotic exposure (Figure 4). It is evident that gene mutations alone can not adequately explain this phenomenon.

Recent research has shown that bacteria can change the phenotypes of AMR through epigenetic intrinsic heterogeneity and transiently without the need for gene mutations (Foster, 2007; Adam et al., 2008). In order to adapt the environmental stress and ensure survival, bacteria has envolved molecular mechanisms for generating variation, such as *Helicobacter pylori* (*H. pylori*), *Haemophilus influenzae* (*H. influenzae*) and *Neisseria gonorrhoeae* (*N. gonorrhoeae*) (De et al., 2002; Srikhanta et al., 2009; Srikhanta et al., 2011). One mechanism is phase-variation, which is to randomly switch the expression of individual genes to generate a phenotypically diverse population to adapt to challenges (Seib et al., 2020). Genes can phase-vary by various of genetic mechanisms. Some studies consider that phase-variation is the high frequency reversible on/off switching of gene expression to evade antibiotic effects (Srikhanta et al., 2011). It has been reported that one way by which bacteria modulate the genes related to phase variation is *via* DNA hypermethylation or hypomethylation. However, variation in the length of hypermutable simple sequence repeats (SSRs) are a important source of phase variation, which facilitates adaptation to changing environments, immune and antibiotic escape of pathogens (Zhou et al., 2014; Pernitzsch et al., 2021). Recent studies have found that RepG (regulator of SSRs) ncRNA mediates the G-repeat length (rather than ON/OFF) and gradual control of lipopolysaccharide biosynthesis to affect AMR in *H. pylori* (Pernitzsch et al., 2021). Therefore, phenotypic variation, selection, and inheritance are necessary for evolution of bacteria. In this chapter, we summarize studies discussing the role of epigenetics in regulating AMR.

3.1 DNA modification

3.1.1 DNA methylation

Bacterial DNA methylation plays a vital role in epigenetic regulation by controlling gene expression, genome modification, virulence, mismatch repair, transcriptional regulation, cell cycle control, and AMR (Marinus and Casadesus, 2009). The most well-known DNA MTases are associated with the defense mechanisms in bacteria known as restriction-modification systems (R-M systems).

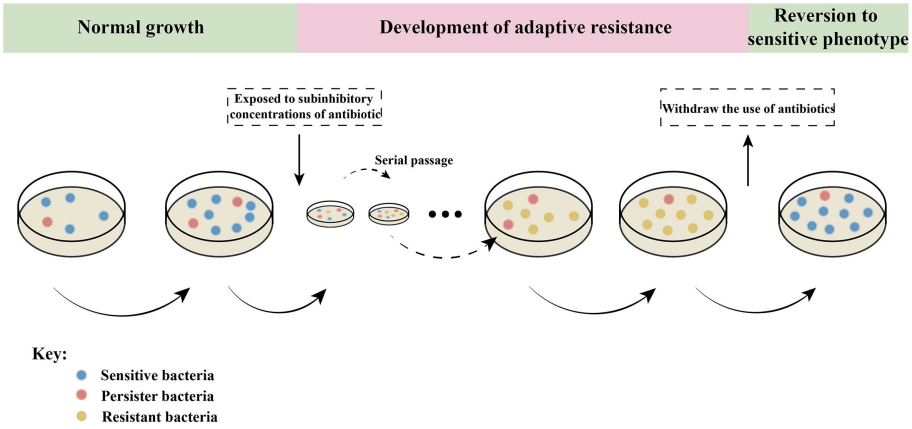


FIGURE 4
Epigenetic effects on adaptive resistance. When bacteria are continuously exposed to sub-inhibitory concentrations of antibiotics, they undergo adaptive evolution and gradually acquire resistance, which can be inherited. When antibiotics are withdrawn, the bacteria with adaptive resistance phenotype will immediately return to sensitivity (Marinus and Casadesus, 2009; Ghosh et al., 2020). Persistent bacteria are only a small part of the bacterial community that is stunted or slow to grow. Persistent bacteria can survive without mutation when exposed to antibiotic pressure (Marinus and Casadesus, 2009). These indicate that bacterial adaptive resistance is epigenetically regulated.

R-M systems prevent lethal cleavage of intracellular DNA by identifying their own DNA and methylating the same sequence as the restriction endonuclease cleavage site (Ghosh et al., 2020). However, foreign DNA such as plasmids carrying AMR genes, transposons, and insertable sequences cannot be methylated and will be recognized and degraded by endonucleases of the R-M systems. This defense mechanism can be circumvented if the foreign DNA carries a homolog methylase with the same specificity, and the sequence will be inserted into the genomic locus rather than degraded (Casadesús and Low, 2006; Ishikawa et al., 2010). This mechanism could explain why plasmids, phages, transposons, integrons, and gene islands can insert into bacterial genomes and contribute to the widespread dissemination of AMR genes.

The R-M systems are classified into four types (I, II, III and IV) based on their functional localization of restriction endonuclease (Rease), activity of MTases, and requirement for specific subunits or cofactors (Roberts et al., 2003). The R-M systems have reported to function as a barrier to horizontal gene transfer in many bacteria (Figure 5) (Vasu and Nagaraja, 2013; Kumar et al., 2018). Li et al. found a carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (*K. pneumoniae*) strain with a bla_{kpc} harboured conjugative plasmid and a pLVPK-like plasmid from the patient, and the type I R-M system on plasmids protected the plasmids from cleavage (Li et al., 2020). Bubendorfer et al. concluded that R-M systems inhibited genomic integration of exogenous sequencs, while they pose no effects to homeologous recombination in *H. pylori* (Bubendorfer et al., 2016).

The type I and III includes genes encoding the DNA MTase *mod*. Many studies have described that *mod* gene-mediated DNA methylation can regulate phase-variable expression associated with various resistant clinical strains (Table 3) (Phillips et al., 2019). For instance, the ability of *N. gonorrhoeae* to form biofilms is affected by allele *modA13* ON/OFF switching (Srikhanta et al., 2009); *Neisseria meningitidis* susceptibility to ceftazidime and ciprofloxacin result from ON/OFF of *modA11* and *modA12* OFF switching (Jen et al., 2014). A typical *H. influenzae* expressing *modA2* MTase produces more biofilms in an alkaline environment than *modA2*-deficient populations, and these biofilms have a larger biomass and less apparent structure (Brockman et al., 2018). Bacterial biofilms and AMR are closely connected. Biofilms are organized multicellular communities surrounded by an extracellular polymeric substances and can decrease bacterial metabolism, growth rate, and resistance to antibiotic penetration, all of which contribute to biofilm resistance (davies, 2003). Even in *Streptococcus suis*, Tram et al. found biaphasic switching of phase-variable DNA MTase ModS2 results in the expression of distinct phase varions. Proteins involved in general metabolism increased expression in ModS2 ON. Adversely, a glyoxalase/bleomycin resistance/extradiol dioxygenase family protein which has been described as involved

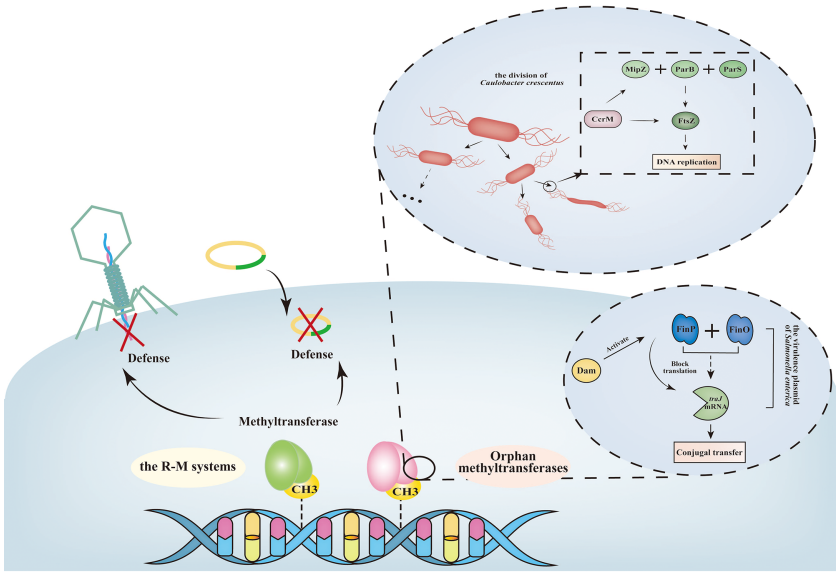


FIGURE 5
Overview of the function of bacterial DNA methylation. The R-M systems function as a barrier to recognize host genome and defenses foreign DNA, such as phage, plasmid (Phillips et al., 2019). Unlike R-M systems, orphan methyltransferases exist with no association with any restriction enzymes, and always function as regulators of DNA replication, gene transfer. Particularly, some orphan methyltransferases are not essential for most bacteria (Srikhanta et al., 2009). FinOP system regulates the conjugal transfer operon (*tra*) of plasmids. Specifically, *traJ* activates the transcription of *tra* operon (encodes the elements of pilus and products required for mating and DNA transfer). Synthesis of TraJ is controlled by FinP, a regulator that blocks *traJ* mRNA translation, and by FinO, a regulator that maintains the stability of FinP RNA-*traJ* mRNA complex (Jen et al., 2014). Dam methylation function as a conjugation repressor by activating FinP RNA synthesis. During the cell division process in bacteria, the essential FtsZ protein polymerizes into a Z-ring like structure at the future division site (Brockman et al., 2018). MipZ protein, which co-ordinates the initiation of chromosome replication with cell division, is important for the assembly of the Z-ring. MipZ interacts with the partitioning protein ParB, which then binds to the ParS locus near the chromosomal origin (davies, 2003). CcrM methylation activates the transcriptions of *ftsZ* and *mipZ*. When lacking the CcrM enzyme, the syntheses of FtsZ protein and MipZ protein are strongly downregulated, leading to a severe defect in cell division. In *Caulobacter crescentus* Δ*ccrM* strain, most Δ*ccrM* cells are filamentous with high cell length variability and frequent membrane defects (davies, 2003).

TABLE 3 Phase-variation of gene expression through DNA methyltransferases.

Bacterial Species	Type	Name	Number of alleles	Functions	References
Haemophilus influenzae	Type III R-M systems	*modA*	21	Antibiotic resistance, biofilm formation, immunoevasion and virulence	(Atack et al., 2015)
Neisseria species	Type III R-M systems	*modA*, *modB*, *modD*	8, 19, 10	Resistance to oxidative stress, biofilm formation, antibiotic resistance and survival	(Tan et al., 2016)
Helicobacter pylori	Type III R-M systems	*modH*	21	Colonization, persistent infection, motility	(Srikhanta et al., 2011)
	Type HpyAII R-M system	*M2.hpyAII*	–	DNA uptake, lipopolysaccharide profile, membrane Components, virulence, evolutionary fitness and adhesion	(Kumar et al., 2018)
Moraxella catarrhalis	Type III R-M systems	*modM*	6	biofilm formation, fitness cost of resistance, survival, colonization, infection, and protection against host defenses	(Blakeway et al., 2019)
Streptococcus suis	Type III R-M systems	*modS*	3	ABC transporters, alkylphosphonate utilization, transcriptional repressor, resistance to antimicrobials	(Tram et al., 2021)
	Type I R-M systems	*hsdS*,	4	Adhesion, virulence	(Atack et al., 2018; Roodsant et al., 2023)
Streptococcus pneumoniae	Type I R-M systems	*hsdS*	3	Adhesion, invasive infection, plasmid transformation rates and colony morphology	(Debroy et al., 2021)
Escherichia coli	Orphan methyltransferases	*dam*	–	Alter the *pap* promoter to influence the affinity of the *lrp* regulatory protein for DNA,	(Hernday et al., 2002; Zamora et al., 2020)

in resistance to beta-lactam and glycopeptide antibiotics was upregulated in strains that did not express ModS2 OFF (Tram et al., 2021).

In addition to the well-known R-M systems, there exists a group of bacterial DNA MTases called orphan MTases, which function independently without association with any R-M system (Ishikawa et al., 2010). Orphan MTases are unique, as they do not have functional counterparts in the restriction enzyme (Reases) family. The common categories of orphan MTases include DNA adenine methyltransferase (Dam), cell cycle regulated methyltransferase (CcrM) and DNA cytosine methyltransferase (Dcm). Bacteria exhibit complex stress responses when exposed to antibiotics, leading to the phenomenon of adaptive resistance. Recent research has revealed that these three orphans MTases play a crucial role in regulating adaptive resistance and the genetic pathways involved in drug sensitivity.

3.1.1.1 DNA adenine methyltransferase

Dam was the first orphan MTase identified in *E. coli*, where it modifies 5′-GATC-3′ sites (Marinus and Morris, 1973). Studies have shown that Dam-mediated DNA methylation is crucial for bacterial survival under antibiotic stress, and *E. coli* K12 Δ*dam* strains exhibit increased sensitivity to beta-lactams and quinolones (Cohen et al., 2016). Epigenetic factors, such as Dam methylation or the regulation of efflux pump expression, have been suggested to contribute to adaptive AMR (Mazzariol et al., 2000; Casadesús and Low, 2006; Adam et al., 2008). Adam et al. treated *E. coli* XL1-Blue strains with nalidixic acid and found that the expression of *dam* increased bacterial survival by approximately five-fold. This increased resistance was consistent with a two-fold rise in the expression of efflux pumps (Adam et al., 2008). Recent research has confirmed that the non-essential dam gene can be a potential target for enhancing antibiotic resistance. Chen et al. demonstrated that the *dam* deletion strain of *E.coli* MG1655 exhibited lower effective concentrations (EC50) than the wild-type strain when exposed to 20 antibiotics in five categories (Chen and Wang, 2021). This confirms that Dam plays a vital role in regulating drug sensitivity and can be utilized as a target for enhancing AMR. Dam in *Salmonella enteritidis* (*S. enteritidis*) has been found to repress the transcription of *traJ*, which encodes a transcriptional activator of the transfer (*tra*) operon of the pLST (Camacho and Casadesús, 2002). In addition, Dam activates the transcription of *finP*, which encodes a ncRNA that contributes to repression of *traJ* expression (Gorrell and Kwok, 2017). Evidence exists to suggest that in a strain with chromosomal mechanisms of quinolone resistance, a synergistic sensitization effect can be observed when the Dam methylation system and the *recA* gene were suppressed (Diaz et al., 2023).

3.1.1.2 Cell cycle regulated methyltransferase

CcrM is a significant orphan MTase that modifies 5′-GANTC-3′ sites, first discovered in *Caulobacter crescentus* (*C. crescentus*). Unlike the ubiquitous Dam enzyme, CcrM expression is limited to the last stage of chromosome replication (Albu et al., 2012). In *C. crescentus*, at least four genes are directly affected by the methylation status of GANTC, including *ftsZ*, which is necessary for cell division, *ctrA* and *dnaA*, the primary regulators of the cell cycle (Reisenauer and Shapiro, 2002; Collier et al., 2007). FtsZ is an essential regulatory protein for cell division and proliferation, forming a z-ring structure at the division site. In *C. crescentus* Δ*ccrM* strain, *ftsZ* expression is significantly downregulated, leading to a severe defect in cell division (Gonzalez and Collier, 2013). The

vertical transmission of heritable transfer elements carrying AMR genes is dependent on cell division and proliferation. When CcrM regulates the expression of the cytoskeleton *ftsZ* gene, it can affect bacterial division and proliferation and impact the vertical transfer of AMR genes.

3.1.1.3 DNA cytosine methyltransferase

Dcm is a typical DNA MTase in *E. coli* and has two targets: 5′-CCAGG-3′ and 5′-CCTGG-3′ sites. As a result, Dcm can protect the DNA sequences from restriction enzyme ECORII activity even if the R-M system is disturbed (Gómez and Ramírez, 1993). In bacteria, Dcm is typically associated with the transcription of active genes. However, the methylation of promoter DNA is frequently associated with gene silencing in higher eukaryotes (Zemach et al., 2010). The role of Dcm in prokaryotes remains unclear, but Kahramanoglou et al. suggested that Dcm controls gene expression in the stationary phase in *E. coli (*Kahramanoglou et al., 2012). Militello et al. demonstrated that the AMR transporter SugE was overexpressed in an *E. coli* Δ*dcm* strain, indicating that Dcm may affect the drug tolerance of SugE-mediated medicines by altering the level of *sugE* gene expression (Militello et al., 2014). Furthermore, Dcm promotes plasmid loss and protects against post-segregational killing by EcoRII (which cleaves DNA at the same site as Dcm methylates) (Takahashi et al., 2002; Ohno et al., 2008).

3.1.2 DNA phosphorothioation

The DNA PT modification, a novel R-M system, has been discovered widely in bacteria and archaea. As a defense barriers, DNA PT modification plays a vital part in bacterial AMR. Nonetheless, the potential role of the DNA PT modification in AMR is still unclear. By analyzing the functions of DNA PT modification in AMR with a serious of clinical pathogenic bacteria, Xu et al. demonstrated DNA PT modification reduced the distribution of horizontal gene transfer (HGT)-derived AMR genes in the genome, meanwhile the modification could suppress HGT frequence (Xu et al., 2023). To understand the mechanism of antibiotic resistance genes (ARGs) in drinking water supply systems, Khan et al. found the relative abundance of *dndB* and ARGs increased in the effluent, as well as, considered that DNA PT modification protected *mcr-1* and $bla_{\text{NDM-1}}$ carrying bacteria from chloramine disinfection during the water treatment process (Khan et al., 2021). DNA PT modification can recognize and cleave unmodified exogenous DNA, such as HGT, ARGs and phage. Therefore, the modification is significant for bacteria to resist foreign invasion and maintain own genetic stability. Up to now, there is few systematic studies on AMR base on DNA PT modification, while we need to study the impact on AMR further.

3.2 Nucleoid-associated protein modifications

NAPs can perform histone-like functions in bacteria and affect DNA structure and transcription, unlike histones in eukaryotes. Gram-negative and Gram-positive bacteria have different NAPs, but most research focuses on Gram-negative bacteria. NAPs are essential global regulators that play a significant role in AMR (Table 4), as demonstrated in *Salmonella*. Yan's research suggests that the Fis protein, known as a global regulator in *S. Typhi*, can mediated persistence by controlling glutamate metabolism (Yan et al., 2021). Additionally, the H-NS DNA binding protein can act as a transcriptional inhibitor to silence genes expression, control plasmid conjugative transfer, silence foreign genes, and inhibit conjugative transfer to reduce fitness costs (Dorman, 2007; Dorman, 2014). Cai et al. found that the IncX1 plasmid, which carries the tigecycline resistance gene *tet* (X4) and encodes the H-NS protein, results in little to no fitness cost in *E. coli* and *K. pneumoniae*. It's also noteworthy that some plasmids can help host bacteria form biofilms and enhance virulence (Cai et al., 2021).

Compared to DNA methylation, histone modification has greater plasticity. The H-NS protein can regulate the expression of genes encoding efflux pumps in multidrug-resistant *Acinetobacter baumannii* (*A. baumannii*) and down-regulate the expression of AMR genes for beta-lactams, aminoglycosides, quinolones, chloramphenicol, trimethoprim, and sulfonamides (Rodgers et al., 2021). Similarly, deleting *hns* lowers the expression of biofilm-related genes in *A. baumannii* (Rodgers et al., 2021). A recent study found that H-NS affects the stability of $bla_{\text{NDM-1}}$-bearing IncX3 plasmid and inhibits its plasmid conjugative transfer in *E. coli (*Liu et al., 2020). These indicate the complexity and breadth of the regulatory network controled by H-NS for genes involved in AMR and persistence.

In view of the biofilms play a major role in some chronic and recurrent infections and are associated with the failure of antibiotic therapy, antibiotic therapy is the first -line treatment of bacterial infections (Devaraj et al., 2018). The DNA-binding (DNABII) protein family includes two well-known NAPs, integration host factor (IHF) and HU. These proteins bind to DNA with high affinity and bend it, thereby playing essential roles in the structure and function of the bacterial nucleoid (Browning et al., 2010). While IHF binds to specific DNA sequences, HU does not. In addition to their structural functions, IHF and HU are also crucial for biofilm formation and the integrity of community structure (Devaraj et al., 2015). In uropathogenic *E. coli*, both subunits of IHF aid in biofilm formation, while HupB (HUβ), one of the subunits of HU, is required for biofilm formation (Devaraj et al., 2015). IHF and HU could be potential therapeutic targets for biofilm therapy, as antimicrobial agents and the host immune system have difficulty attacking biofilms. A research has found that the HU protein subunit HupB, post-translationally modified by lysine acetylation and methylation, is a breakthrough in treating multidrug-resistant *Mycobacterium tuberculosis* (*M. tuberculosis*) (Ghosh et al., 2016). Mutating a single post-translational modification site eliminates a drug-resistant cell subset of isoniazid-resistant *M. tuberculosis* (Sakatos et al., 2018). Additionally, it has been reported that using anti-*Porphyromonas gingivalis* (*P. gingivalis*) HUβ antibodies to specifically target the oral *Streptococcus* biofilm for preventing *P. gingivalis* organisms from entering into preexisting biofilms formed by oral *Streptococcal* species (Rocco et al., 2018). Therefore, HU, for instance HupB, could be a promising therapeutic target for bacterial therapy. Recent research has reported that targeting HU, Zhang

TABLE 4 Summary of representative Nucleoid-associated proteins in AMR.

Species	Nucleoid-associated proteins	Genes been regulated	Functions	References
Salmonella typhi	Fis	*gltK*, *gltJ*, *gltL*, *gltS*, *gltH* and *gltP*	Regulate glutamate metabolism to reduce persister formation	(Yan et al., 2021)
Salmonella typhi	H-NS, Hha, StpA	pathogenicity islands (SPIs), *pef*	Inhibite the expression of SPI2 to improve the fitness,	(Hurtado et al., 2019)
Escherichia coli	Fis	*fimS*, *fimA*, *fimB*, *acs*, *acnB*, *fum*	Function as a negative regulator in the *fimS* phase variation, enhanced growth ftness under acetate metabolism, regulate biofilm formation	(Jindal et al., 2022; Saldaña et al., 2022)
Escherichia coli	H-NS	*pilx1-11*, *taxB*, *taxC*, *actX*, *parB*	Facilitate horizontal plasmid transfer, affect the stability of plasmid	(Liu et al., 2020)
Escherichia coli	HU, IHF	*fim*, *pap*	Promote biofilm formation, Gp46 function as HU inhibitor	(Justice et al., 2012; Devaraj et al., 2015; Zhang et al., 2022)
Shigella	H-NS	*virB*	Silence the *virB* promoter and influence virulence plasmid trasnsfer	(Colonna et al., 1995)
Acinetobacter baumannii	H-NS	*aidA*, *abaI*, *kar*, *fadD*, bla_{OXA-23}, $bla_{OXA-51-like}$, bla_{ADC}, bla_{GES-14}, *carO*, *pbp1*, and *advA*	Regulate the expression of genes encoding efflux pumps and the formation of biofilm; modulate the expression of resistance-related genes	(Rodgers et al., 2021)
Klebsiella pneumoniae	H-NS	*tet* (X4),	Modulate the fitness cost of plasmids, promote the virulence and biofilm formation,	(Cai et al., 2021)
Mycobacterium tuberculosis	HU, HupB	*eis*, *arsR*, *marR*, *tetR*	Regulate the sensitivities of aminoglycosides, alter gene expression and phenotypic state in a subpopulation	(Zaunbrecher et al., 2009; Ghosh et al., 2016; Sakatos et al., 2018; Rodgers et al., 2021)
Porphyromonas gingivalis	HU	*ssP*, *fimA*	Disperse oral streptococcus biofilm and prevent *P. gingivalis* entry into oral *Streptococcus* biofilm	(Rocco et al., 2018)

et al. used Gp46 (an HU protein inhibitor from phages) to inhibit HU of many resistant pathogens by occupying DNA binding site, and preventing chromosome segregation during cell division (Zhang et al., 2022).

3.3 RNA modification

3.3.1 Ribosomal RNA methylation

RNA modifications, such as rRNA methylation, have emerged as important mechanisms associated with AMR. Ribosomes are a common target for antibiotics. Methylation of specific sites in rRNA can prevent antibiotics from binding to their target sites, thereby leading to antibiotic resistance. Thus AMR *via* rRNA methylation is one of the most common strategies adopted by multidrug resistant pathogens. One such example is 16S rRNA methylation, which is a major mechanism of aminoglycoside resistance in clinical pathogens (Tada et al., 2013; Liu et al., 2015). Two different methylation sites in 16S rRNA lead to different aminoglycoside-resistant phenotypes. Methylation of residue A1408 confers resistance to kanamycin and apramycin in *E. coli*, but sensitivity to gentamicin, while methylation of residue G1405 confers resistance to kanamycin and gentamicin, but sensitivity to apramycin (Liu et al., 2015). The multidrug resistance gene *cfr*, found in *Staphylococcus*, encodes an MTase that modifies the A2503 site in 23S rRNA, leading to resistance to antibiotics such as amide alcohols, lincomycins, oxazolidinones, pleuromutilin, and streptogramin A (Long et al., 2006). In *S. pneumoniae*, U747 methylation mediated by RlmCD promotes efficient G748 methylation by the MTase $RlmA^{II}$ in 23S rRNA, affecting the susceptibility to telithromycin (Shoji et al., 2015). Another research indicated the erythromycin-resistance MTase methylates rRNA at the conserved A2058 position, and imparts resistance to macrolides, such as erythromycin (Bhujbalrao et al., 2022). Up to now, the number of rRNA MTases related to AMR mechanisms have increased, but the source of MTases and the exact mechanisms of AMR are still unclear.

3.3.2 Non-coding RNAs

Advancements in high-throughput sequencing technology and bioinformatics have facilitated the discovery of various ncRNAs and their functions in bacteria. Recent studies have found that exposure to environmental stress, especially antibiotics, bacteria produce specific ncRNAs profiles, which may regulate the expression of downstream genes. When bacteria sense antibacterial stress, a large number of ncRNA regulators are upregulated, and one of their roles is to improve bacterial adaptation in a dynamic environment (Morita and Aiba, 2007). Thus, ncRNAs play an essential role in the bacterial regulatory network that controls the expression of bacterial genes through regulating proteins and target mRNAs. In comparison to regulatory proteins, ncRNAs are considered a better class of regulatory molecules for controlling gene expression (Toledo et al., 2007).

ncRNAs play an essential role in the regulation of bacterial gene expression and can affect AMR mechanisms. Although ncRNAs are

a major form of post-transcriptional gene control in bacteria, some research indicate ncRNAs also influence transcription (Rodgers et al., 2023). For instance, Majdalani et al. found that RprA ncRNA reduced type IV secretion-mediated transfer of pSLT (*Salmonella* virulence plasmid) (Papenfort and Melamed, 2023). In particular, RrpA controls the transcription and translation of *ricI*, which encodes a membrane protein that interacts with and suppresses the anchor protein Trav of the type IV secretion apparatus (Majdalani et al., 2001). It is reported that antisense *vicR* (a kind of ncRNAs) is transcribed from the opposite strand of *vicR* mRNA and regulates the biofilm formation of *Streptococcus mutans via* affecting the production and function of VicR protein (Lei et al., 2018).

The incomplete complementary pairing of most ncRNAs with the target mRNA sequence can lead to two results: (1) Blocking the ribosome binding sites and suppressing translation; (2) Secondary structure melting, exposing the nucleose binding site and translation start site, leading to translation activation (Vogel and Sharma, 2005; Fröhlich and Vogel, 2009). Moreover, since the instabilized base pairing between the ncRNAs and their target mRNAs, the RNA chaperone protein Hfq, binding protein Fino/ProQ family, CsrA/RsmA family and other regulators usually facilitate imperfect base pairing between ncRNAs and mRNAs, leading to regulate the translation initiation frequency or the stability of target mRNAs (Liao and Smirnov, 2023; Wang et al., 2023; Yu and Zhao, 2023). In this chapter, we will explore some research on ncRNAs that regulate the mechanisms of AMR from two perspectives.

3.3.2.1 Translation suppression

ncRNAs regulate bacterial cell wall or membrane to alter the sensitivity of antibiotics. Bacteria can control membrane permeability by regulating the expression of outer membrane proteins OmpF, OmpA, and OmpC. Studies have shown that ncRNAs such as MicF, MicA, and MicC inhibit the expression of these mRNAs by partial complementary pairing, interfering with antibiotic exposure (Chen et al., 2004; Udekwu et al., 2005). Therefore, ncRNAs represent a promising target for the development of new strategies to combat AMR in bacteria.

ncRNAs have been shown to affect AMR by targeting the efflux pumps. For instance, overexpression of SdsR has been found to decrease the mRNA and protein levels of the TolC,which encodes the outer membrane protein of many multidrug resistance efflux pumps, resulting in increased sensitivity to fluoroquinolones in *E. coli* (Kim et al., 2015; Parker and Gottesman, 2016). However, in *Shigella sonnei*, overexpression of SdsR leads to lower mRNA levels of *tolC* and increased survival rates at sub-MIC norfloxacin (Gan and Tan, 2019). *Pseudomonas aeruginosa* (*P. aeruginosa*) is a common source of hospital infections and has important adaption abilities to various environmental exposures (Jurado et al., 2021). A recent study found that overexpressing of AS1974 ncRNA restores the sensitivity of MDR clinical strains by down-regulating the expression of MexC-MexD-OprJ, a component of the multidrug efflux system (Law et al., 2019). On the other hand, overexpression of PA08051 and PA2952.1 ncRNAs leads to up-regulation of the drug efflux system mexGHI-opmD, resulting in increased resistance of aminoglycoside (Coleman et al., 2020; Coleman et al., 2021).

Bacterial biofilms, which are microcolonies formed by adhesion on solid surfaces or between bacteria, can secrete extracellular matrix to create a natural barrier. This multicellular-like lifestyle allows resistance to environmental and cell-intrinsic stresses, such as antibiotics exposure. For example, Falcone et al. found that based on RNA-seq analysis, the ErSA ncRNA of *P. aeruginosa* complementary pairs with *amrZ* mRNA to influence the expression of AmrZ, promoting biofilm development (Falcone et al., 2018). The RNA-binding protein ProQ has been shown to regulate mRNA-expression levels by interactions with 5′ and 3′ UTRs (Holmqvist et al., 2018). In an early study found that ProQ was necessary for robust biofilm formation, and this phenotype was independent of ProP (Sheidy and Zielke, 2013). Infections caused by *Staphylococcus aureus* (*S. aureus*) are often associated with adverse therapeutic outcomes due to various reasons, such as an antibiotic penetration barrier by bacterial biofilms (Singh et al., 2016). By sensing and responding to multifarious environmental exposure, bacteria carry out corresponding adaptive regulation. For instance, the *teg58* ncRNA have specific interaction with *argGH* mRNA (arginine biosynthesis genes) to repress arginine synthesis and biofilm formation in *S. aureus (*Manna et al., 2022). Raad et al. found that during stationary phase of *E. coli*, the 3' UTR-derived FimR2 ncRNA interacted with CsrA, antagonizing its post-transcriptional functions of flagellar and fimbrial biosynthesis, and firmly strengthening the control of bacterial motility and biofilm formation (Raad et al., 2022).

ncRNAs affect AMR by regulating the functions of plasmids carrying resistance genes, including fitness and conjugation. HGT refers to the transfer of genes between unrelated species, which increases genetic diversity and accelerates bacterial evolution (Gogarten and Townsend, 2005). Conjugative plasmids are typical representatives of HGT and promote the spread of AMR among pathogens. Due to plasmid reception, intergration, replication and the expression of genes, the antibiotic-resistant plasmids produce fitness costs in host bacteria (San and Maclean, 2017). Therefore, it seems that plasmids gradually lost over time during bacterial evolution without corresponding antibiotic exposure. In contrast to this conjecture, antibiotic-resistant plasmids can stably persist in host bacteria for long periods without any antibiotics (Zhang et al., 2022). There may be some mechanisms that regulate the bacteria fitness cost. Some research have found that ProQ/FinO family proteins encoded by the IncI2 plasmid carrying *mcr-1*, balanced *mcr-1* expression and bacteria fitness by inhibiting plasmid copy number (Yang et al., 2021). As well as, the RNA-binding protein ProQ has identified three distinct domains, one is a large conserved N-terminal Fino-like domain (Gulliver et al., 2022). The FinO-like domain facilitates binding to the RNA, shares similar structural and functional characteristics with the FinO RNA chaperone in IncF plasmid (Pandey et al., 2020). FinO was named so to reflect its fertility inhibition function observed in IncF plasmid conjugation (Finnegan and Willetts, 1972). These plasmids regulate conjugation through RNA antisense mechanisms, whereby the *cis*-encoded ncRNA FinP inhibits protein synthesis of conjugative transfer regulator TraJ (Timmis et al., 1978; Van Biesen and Frost, 1994;

El et al., 2021). The synthesis of TraJ is inhibited, and leads to higher conjugation of plasmids without FinO (El et al., 2021). El Mouali et al. found that the binding protein FinO encoded in virulence plasmid of *Salmonella* also regulated the replication of a cohabitating plasmid carrying antibiotic gene, which may suggest cross-regulation of plasmids in RNA level (El et al., 2021).

3.3.2.2 Translation activation

ncRNAs affect AMR by activating translation. ncRNAs commonly down-regulate gene expression, however, also have the ability to activate genes by multifarious mechanisms in bacteria. Several ncRNAs act as direct translational activators by preventing the formation of translation-inhibited stem-loop structures through antisense pairing translation in the 5′mRNA region (Fröhlich and Vogel, 2009). After being activated by the main regulators LuxO/HapR of the quorum sensing system, the Qrr ncRNA (quorum regulatory RNAs) of *Vibrio* species binds to the chaperone Hfq and regulates downstream gene expressions (Hammer and Bassler, 2007). One of the pathways is the HapR-independent pathway: the Qrr ncRNA interaction with *vca0939* mRNA prevents formation of inhibitory stem-loop structures, allows access to ribosomes and promote translation (Hammer and Bassler, 2007). Moreover, after the translational activation, *vca0939* encodes GGDEF proteins and induces virulence factors and biofilm formation (Camilli and Bassler, 2006).

4 Epigenetic drugs as treatment of antimicrobial resistance

Epigenetic drugs are small molecules that have been designed or studied based on epigenetic mechanisms, such as selective transcription or post-transcriptional regulation of genes. Some epigenetic drugs have been found to alter gene expression by inhibiting specific enzymes. Given the current situation of AMR, epigenetic drugs have important implications for the treatment of infectious diseases caused by multidrug-resistant bacteria. For instance, low concentrations of SAM analogues, such as SGC0946, JNJ-64619178, and SGC8158 were found to inhibit the activity of *C. difficile*-specific DNA adenine MTase, selectively affecting biofilm and spore production and quickly eradicating *C. difficile* infection (Zhou et al., 2022). Moreover, UVI5008, a derivative of the natural substance psammaplin A, was found to reduce the DNA gyrase activity of methicillin-resistant *S. aureus*, and reverse AMR by damaging the bacterial cell wall (Franci et al., 2018). Similarly, epigallocatechin-3-gallate (EGCG) can damage the integrity of the cell wall and reverse the resistance of imipenem, tetracycline, and amoxicillin in *S. aureus* (Sudano et al., 2004; Zeferino et al., 2022). With the deepening of research, Serra et al. thought that EGCG directly interfered with the assembly of curli fimbriae into amyloid fibrils and reduced the synthesis of CsgD (activator of curli fimbriae and cellulose biosynthesis) by promoting the expression of RybB ncRNA, ultimately inhibited the formation of cell membranes and affected biofilm-mediated antibiotic resistance and host defense (Serra et al., 2016) As well as, EGCG was found to be a suitable natural drug targeting LuxS/AI-2 system of *H. pylori* by high-throughput screening and molecular dynamics simulation (Ashok et al., 2023). Zhang et al. found that EGCG prevented *Shigella flexneri* biofilm extracellular polysaccharide from forming through reducing the expression of *mdoH* gene (Zhang et al., 2023). These findings suggest that epigenetic drugs have the potential to be used as a treatment for patients with multidrug-resistant bacterial infections.

5 Conclusions

AMR is an ancient and natural phenomenon, that has evolved in bacteria over millions of years. While biochemical and genetic alterations are known to contribute to AMR, non-classical mechanisms such as epigenetics have recently gained attention. Bacterial epigenetics, which involves modifications to DNA and rRNA, ncRNAs, as well as nucleoid-associated proteins, has been shown to regulate the formation and enrichment of AMR. This regulatory mechanism controls gene expression switching, phase variation, bacterial tolerance, and persistent bacteria. The epigenetic regulatory mechanisms of bacteria are complex which may have long term implications. Although our current understanding of bacterial epigenetics is still limited, recent advances in sequencing technologies are enabling high-resolution mapping of epigenetic landscapes in prokaryotes, which is expected to shed light on the complex regulatory mechanisms of AMR. With the advent of the post-antibiotic era, the discovery of epigenetic mechanisms in multidrug-resistant pathogens also helps to search for antibiotic potentiators or provide new targets for the development of newer drugs.

Author contributions

XW and DY researched data for the manuscript. LC provided conceptualization and was responsible for the first draft of the manuscript. XW provided conceptualization, review, comment and editing. All authors discussed the results and reviewed and commented on the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by: Key Project on the Integration of Industry, Education and Research Collaborative Innovation of Fujian Province (No. 2021YZ034011); the Key Project on Science and Technology Program of Fujian Health Commission (No. 2021ZD01002); Joint Funds for the innovation of science and Technology, Fujian province (Grant number: 2021Y9184).

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

Adam, M., Murali, B., Glenn, N. O., and Potter, S. S. (2008). Epigenetic inheritance based evolution of antibiotic resistance in bacteria. *BMC Evol. Biol.* 8, 52. doi: 10.1186/1471-2148-8-52

Albu, R., Jurkowski, T., and Jeltsch, A. (2012). The caulobacter crescentus DNA-(adenine-N6)-methyltransferase CcrM methylates DNA in a distributive manner. *Nucleic Acids Res.* 40 (4), 1708–1716. doi: 10.1093/nar/gkr768

Amemiya, H., Schroeder, J., and Freddolino, P. (2021). Nucleoid-associated proteins shape chromatin structure and transcriptional regulation across the bacterial kingdom. *Transcription* 12 (4), 182–218. doi: 10.1080/21541264.2021.1973865

An, X., Xiong, W., Yang, Y., Li, F., Zhou, X., Wang, Z., et al. (2012). A novel target of IscS in *Escherichia coli*: participating in DNA phosphorothioation. *PLoS One* 7 (12), e51265. doi: 10.1371/journal.pone.0051265

Ashok, A. K., Gnanasekaran, T. S., Santosh, K. H. S., Srikanth, K., Prakash, N., and Gollapalli, P. (2023). High-throughput screening and molecular dynamics simulations of natural products targeting LuxS/AI-2 system as a novel antibacterial strategy for antibiotic resistance in helicobacter pylori. *J. biomolecular structure dynamics*, 1–16. doi: 10.1080/07391102.2023.2210674

Atack, J. M., Srikhanta, Y. N., Fox, K. L., Jurcisek, J. A., Brockman, K. L., Clark, T. A., et al. (2015). A biphasic epigenetic switch controls immunoevasion, virulence and niche adaptation in non-typeable *Haemophilus influenzae*. *Nat. Commun.* 6 (1), 7828. doi: 10.1038/ncomms8828

Atack, J. M., Weinert, L. A., Tucker, A. W., Husna, A., Wileman, T. M., Hadjirin, N. F., et al. (2018). *Streptococcus suis* contains multiple phase-variable methyltransferases that show a discrete lineage distribution. *Nucleic Acids Res.* 46 (21), 11466–11476. doi: 10.1093/nar/gky913

Antimicrobial Resistance Collaborators. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet (London England)* 399 (10325), 629–655. doi: 10.1016/S0140-6736(21)02724-0

Becker, K., Van, A. S., Idelevich, E., Schleimer, N., Seggewiß, J., Mellmann, A., et al. (2018). Plasmid-encoded transferable *mecB*-mediated methicillin resistance in *Staphylococcus aureus*. *Emerging Infect. Dis.* 24 (2), 242–248. doi: 10.3201/eid2402.171074

Bhujbalrao, R., Gavvala, K., Singh, R. K., Singh, J., Boudier, C., Chakrabarti, S., et al. (2022). Identification of allosteric hotspots regulating the ribosomal RNA binding by antibiotic resistance-conferring erm methyltransferases. *J. Biol. Chem.* 298 (8), 102208. doi: 10.1016/j.jbc.2022.102208

Blakeway, L. V., Tan, A., Jurcisek, J. A., Bakaletz, L. O., Atack, J. M., Peak, I. R., et al. (2019). The moraxella catarrhalis phase-variable DNA methyltransferase ModM3 is an epigenetic regulator that affects bacterial survival in an *in vivo* model of otitis media. *BMC Microbiol.* 19 (1), 276. doi: 10.1186/s12866-019-1660-y

Brockman, K., Azzari, P., Branstool, M., Atack, J. M., Schulz, B. L., Jen, F. E-C., et al. (2018). Epigenetic regulation alters biofilm architecture and composition in multiple clinical isolates of nontypeable *Haemophilus influenzae*. *mBio* 9 (5), e01682–e01618. doi: 10.1128/mBio.01682-18

Browning, D., Grainger, D., and Busby, S. (2010). Effects of nucleoid-associated proteins on bacterial chromosome structure and gene expression. *Curr. Opin. Microbiol.* 13 (6), 773–780. doi: 10.1016/j.mib.2010.09.013

Bubendorfer, S., Krebes, J., Yang, I., Hage, E., Schulz, T. F., Bahlawane, C., et al. (2016). Genome-wide analysis of chromosomal import patterns after natural transformation of *Helicobacter pylori*. *Nat. Commun.* 7, 11995. doi: 10.1038/ncomms11995

C, T. O., Carroll, R. K., Kelly, A., Dorman, C. J., et al. (2006). Roles for DNA supercoiling and the Fis protein in modulating expression of virulence genes during intracellular growth of *Salmonella enterica* serovar typhimurium. *Mol. Microbiol.* 62 (3), 869–882. doi: 10.1111/j.1365-2958.2006.05416.x

Cai, W., Tang, F., Jiang, L., Li, , Wang, Z., Liu, Y., et al. (2021). tetHistone-like nucleoid structuring protein modulates the fitness of (X4)-bearing IncX1 plasmids in gram-negative bacteria. *Front. Microbiol.* 12, 763288. doi: 10.3389/fmicb.2021.763288

Camacho, E. M., and Casadesús, J. (2002). Conjugal transfer of the virulence plasmid of *Salmonella enterica* is regulated by the leucine-responsive regulatory protein and DNA adenine methylation. *Mol. Microbiol.* 44 (6), 1589–1598. doi: 10.1046/j.1365-2958.2002.02981.x

Camilli, A., and Bassler, B. L. (2006). Bacterial small-molecule signaling pathways [J]. *Science* 311 (5764), 1113–1116. doi: 10.1126/science.1121357

Casadesús, J., and Low, D. (2006). Epigenetic gene regulation in the bacterial world. *Microbiol. Mol. Biol. Rev.* 70 (3), 830–856. doi: 10.1128/MMBR.00016-06

Centers for Disease Control and Prevention (2019). *Antibiotic resistance threats in the united states, 2019* (Atlanta, Georgia: US Department of Health and Human Services, Centres for Disease Control and Prevention).

Chen, Z., and Wang, H. (2021). *Escherichia coli* antibiotic toxicity profiles of strains lacking DNA methyltransferases. *ACS omega* 6 (11), 7834–7840. doi: 10.1021/acsomega.1c00378

Chen, S., Zhang, A., Blyn, L., and Storz, G. (2004). MicC, a second small-RNA regulator of omp protein expression in escherichia coli. *J. bacteriol* 186 (20), 6689–6697. doi: 10.1128/JB.186.20.6689-6697.2004

Cohen, N., Ross, C., Jain, S., Shapiro, R. S., Gutierrez, A., Belenky, P., et al. (2016). A role for the bacterial GATC methylome in antibiotic stress survival. *Nat. Genet.* 48 (5), 581–586. doi: 10.1038/ng.3530

Coleman, S., Bains, M., Smith, M., Spicer, V., Lao, Y., Taylor, P., et al. (2021). The small RNAs PA2952.1 and PrrH as regulators of virulence, motility, and iron metabolism in *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 87 (3), e02182–e02120. doi: 10.1128/AEM.02182-20

Coleman, S., Smith, M., Spicer, V., Lao, Y., Mookherjee, N., Hancock, R., et al. (2020). Overexpression of the small RNA PA0805.1 in *Pseudomonas aeruginosa* modulates the expression of a Large set of genes and proteins, resulting in altered motility, cytotoxicity, and tobramycin resistance. *mSystems* 5 (3), e00204–e00220. doi: 10.1128/mSystems.00204-20

Collier, J., Mcadams, H., and Shapiro, L. (2007). A DNA methylation ratchet governs progression through a bacterial cell cycle. *Proc. Natl. Acad. Sci. U.S.A* 104 (43), 17111–17116. doi: 10.1073/pnas.0708112104

Colonna, B., Casalino, M., Fradiani, P. A., Zagaglia, C., Naitza, S., Leoni, L., et al. (1995). H-NS regulation of virulence gene expression in enteroinvasive *Escherichia coli* harboring the virulence plasmid integrated into the host chromosome. *J. Bacteriol* 177 (16), 4703–4712. doi: 10.1128/jb.177.16.4703-4712.1995

Cox, G., and Wright, G. (2013). Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. *Int. J. Med. Microbiol.* 303, 287–292. doi: 10.1016/j.ijmm.2013.02.009

Davies, D. (2003). Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discovery* 2 (2), 114–122. doi: 10.1038/nrd1008

De, V. N., Duinsbergen, D., Kuipers, E. J., Pot, R. G. J., Wiesenekker, P., Penn, C. W., et al. (2002). Transcriptional phase variation of a type III restriction-modification system in helicobacter pylori. *J. Bacteriol* 184 (23), 6615–6623. doi: 10.1128/JB.184.23.6615-6624.2002

Debroy, S., Shropshire, W. C., Tran, C. N., Hao, H., Gohel, M., Galloway-Peña, J., et al. (2021). Characterization of the type I restriction modification system broadly conserved among group a *Streptococci*. *mSphere* 6 (6), e0079921. doi: 10.1128/mSphere.00799-21

Delihas, N. (2015). Discovery and characterization of the first non-coding RNA that regulates gene expression, *micF* RNA: a historical perspective. *World J. Biol. Chem.* 6 (4), 272–280. doi: 10.4331/wjbc.v6.i4.272

Depardieu, F., Podglajen, I., Leclercq, R., Collatz, E., and Courvalin, P. (2007). Modes and modulations of antibiotic resistance gene expression. *Clin. Microbiol. Rev.* 20 (1), 79–114. doi: 10.1128/CMR.00015-06

Devaraj, A., Buzzo, J., Rocco, C. J., Bakaletz, L. O., and Goodman, S. D. (2018). The DNABII family of proteins is comprised of the only nucleoid associated proteins required for nontypeable *Haemophilus influenzae* biofilm structure. *MicrobiologyOpen* 7 (3), e00563. doi: 10.1002/mbo3.563

Devaraj, A., Justice, S., Bakaletz, L., and Goodman, S. D. (2015). DNABII proteins play a central role in UPEC biofilm structure. *Mol. Microbiol.* 96 (6), 1119–1135. doi: 10.1111/mmi.12994

Diaz, D. S., Recacha, E., Pulido, M. R., Romero-Muñoz, M., Gregorio-Iaria, B., Docobo-Pérez, F., et al. (2023). Synergistic effect of SOS response and GATC methylome suppression on antibiotic stress survival in *Escherichia coli*. *Antimicrobial Agents Chemother* 67 (3), e01392–e01322. doi: 10.1128/aac.01392-22

Doenecke, D., and Karlson, P. (1984). Albrecht Kossel and the discovery of histones. *Trends Biochem. Sci.* 9 (9), 404–405. doi: 10.1016/0968-0004(84)90226-3

Dorman, C. H-NS (2007). The genome sentinel. *Nat. Rev. Microbiol.* 5 (2), 157–161. doi: 10.1038/nrmicro1598

Dorman, C. (2014). H-NS-like nucleoid-associated proteins, mobile genetic elements and horizontal gene transfer in bacteria. *Plasmid* 75, 1–11. doi: 10.1016/j.plasmid.2014.06.004

Dunn, D., and Smith, J. (1955). Occurrence of a new base in the deoxyribonucleic acid of a strain of *Bacterium coli*. *Nature* 175 (4451), 336–337. doi: 10.1038/175336a0

El, M. Y., Gerovac, M., Mineikaitė, R., and Vogel, J. (2021). *In vivo* targets of *Salmonella* FinO include a FinP-like small RNA controlling copy number of a cohabitating plasmid. *Nucleic Acids Res.* 49 (9), 5319–5335. doi: 10.1093/nar/gkab281

Falcone, M., Ferrara, S., Rossi, E., Johansen, H. K., Molin, S., Bertoni, G., et al. (2018). The small RNA ErsA of *Pseudomonas aeruginosa* contributes to biofilm development and motility through post-transcriptional modulation of AmrZ. *Front. Microbiol.* 9, 238. doi: 10.3389/fmicb.2018.00238

Ferrándiz, M. J., Carreño, D., Ayora, S., et al. (2018). HU of *Streptococcus pneumoniae* is essential for the preservation of DNA supercoiling. *Front. Microbiol.* 9, 493. doi: 10.3389/fmicb.2018.00493

Finnegan, D., and Willetts, N. (1972). The nature of the transfer inhibitor of several f-like plasmids. *Mol. Gen. Genet. MGG* 119 (1), 57–66. doi: 10.1007/BF00270444

Foster, P. L. (2007). Stress-induced mutagenesis in bacteria. *Crit. Rev. Biochem. Mol. Biol.* 42 (5), 373–397. doi: 10.1080/10409230701648494

Franci, G., Folliero, V., Cammarota, M., Zannella, C, Zannella, C, Schiraldi, C, et al. (2018). Epigenetic modulator UVI5008 inhibits MRSA by interfering with bacterial gyrase. *Sci. Rep.* 8 (1), 13117. doi: 10.1038/s41598-018-31135-9

Fröhlich, K. S., and Vogel, J. (2009). Activation of gene expression by small RNA. *Curr. Opin. Microbiol.* 12 (6), 674–682. doi: 10.1016/j.mib.2009.09.009

Gan, I., and Tan, H. (2019). A small RNA decreases the sensitivity of *Shigella sonnei* to norfloxacin. *BMC Res. Notes* 12 (1), 97. doi: 10.1186/s13104-019-4124-4

Gan, R., Wu, X., He, W., Liu, Z., Wu, S., Chen, C., et al. (2014). DNA Phosphorothioate modifications influence the global transcriptional response and protect DNA from double-stranded breaks. *Sci. Rep.* 4, 6642. doi: 10.1038/srep06642

Ghosh, S., Padmanabhan, B., Anand, C., and Nagaraja, V. (2016). Lysine acetylation of the *Mycobacterium tuberculosis* HU protein modulates its DNA binding and genome organization. *Mol. Microbiol.* 100 (4), 577–588. doi: 10.1111/mmi.13339

Ghosh, D., Veeraraghavan, B., Elangovan, R., and Vivekanandan, P. (2020). Antibiotic resistance and epigenetics: more to it than meets the eye. *Antimicrob. Agents Chemother.* 64 (2), e02225–e02219. doi: 10.1128/AAC.02225-19

Gogarten, J. P., and Townsend, J. P. (2005). Horizontal gene transfer, genome innovation and evolution. *Nat. Rev. Microbiol.* 3 (9), 679–687. doi: 10.1038/nrmicro1204

Gómez, E. M., and Ramírez, S. J. (1993). Methylated cytosine at dcm (CCATGG) sites in *Escherichia coli*: possible function and evolutionary implications. *J. Mol. Evol.* 37 (1), 11–24. doi: 10.1007/BF00170457

Gonzalez, D., and Collier, J. (2013). DNA Methylation by CcrM activates the transcription of two genes required for the division of caulobacter crescentus. *Mol. Microbiol.* 88 (1), 203–218. doi: 10.1111/mmi.12180

Gorrell, R., and Kwok, T. (2017). The *Helicobacter pylori* methylome: roles in gene regulation and virulence. *Curr. topics Microbiol. Immunol.* 400, 105–127. doi: 10.1007/978-3-319-50520-6_5

Griffin, B. (1971). Separation of ^{32}P-labelled ribonucleic acid components. the use of polyethylenimine-cellulose (TLC) as a second dimension in separating oligoribonucleotides of '4.5 s' and 5 s from *E. coli*. *FEBS Lett.* 15 (3), 165–168. doi: 10.1016/0014-5793(71)80304-6

Gulliver, E. L., Sy, B. M., Wong, J. L., Lucas, D. S. D., Powell, D. R., Harper, M., et al. (2022). The role and targets of the RNA-binding protein ProQ in the gram-negative bacterial pathogen pasteurella multocida. *J. Bacteriol* 204 (4), e0059221. doi: 10.1128/jb.00592-21

Gusic, M., and Prokisch, H. (2020). ncRNAs: new players in mitochondrial health and disease? *Front. Genet.* 11, 95. doi: 10.3389/fgene.2020.00095

Hall, B., and Barlow, M. (2004). Evolution of the serine beta-lactamases: past, present and future [J]. *Drug resistance updates* 7 (2), 111–123. doi: 10.1016/j.drup.2004.02.003

Hammer, B. K., and Bassler, B. L. (2007). Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic vibrio cholerae. *Proc. Natl. Acad. Sci. U.S.A* 104 (27), 11145–11149. doi: 10.1073/pnas.0703860104

Hernday, A., Krabbe, M., Braaten, B., and Low, D. (2002). Self-perpetuating epigenetic pili switches in bacteria. *Proc. Natl. Acad. Sci.* 99 (suppl_4), 16470–16476. doi: 10.1073/pnas.182427199

Heusipp, G., Fälker, S., and Schmidt, M. A. (2007). DNA Adenine methylation and bacterial pathogenesis. *Int. J. Med. Microbiol.* 297 (1), 1–7. doi: 10.1016/j.ijmm.2006.10.002

Hirakawa, H., Nishino, K., Hirata, T., and Yamaguchi, A. (2003). Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. *J. bacteriol* 185 (6), 1851–1856. doi: 10.1128/JB.185.6.1851-1856.2003

Holliday, R., and Pugh, J. (1975). DNA Modification mechanisms and gene activity during development. *Sci. (New York NY)* 187 (4173), 226–232.

Holmqvist, E., Li, L., Bischler, T., Barquist, L., and Vogel, J. (2018). Global maps of ProQ binding *In vivo* reveal target recognition *via* RNA structure and stability control at mRNA 3' ends. *Mol. Cell* 70 (5), 971–82.e6. doi: 10.1016/j.molcel.2018.04.017

Hu, W., Wang, C., Liang, J., Zhang, T., Hu, Z., Wang, Z., et al. (2012). Structural insights into DndE from *Escherichia coli* B7A involved in DNA phosphorothioation modification. *Cell Res.* 22 (7), 1203–1206. doi: 10.1038/cr.2012.66

Hurtado, E. G. A., Grépinet, O., Raymond, P., Abed, N., Velge, P., Virlogeux, P. I., et al. (2019). H-NS is the major repressor of *Salmonella* typhimurium pef fimbriae expression. *Virulence* 10 (1), 849–867. doi: 10.1080/21505594.2019.1682752

Ishikawa, K., Fukuda, E., and Kobayashi, I. (2010). Conflicts targeting epigenetic systems and their resolution by cell death: novel concepts for methyl-specific and other restriction systems. *DNA Res.* 17 (6), 325–342. doi: 10.1093/dnares/dsq027

Jackman, J. E., and Alfonzo, J. D. (2013). Transfer RNA modifications: nature's combinatorial chemistry playground. *Wiley Interdiscip. Rev. RNA*, 4(1), 35–48. doi: 10.1002/wrna.1144

Jen, F., Seib, K., and Jennings, M. (2014). Phasevarions mediate epigenetic regulation of antimicrobial susceptibility in *Neisseria meningitidis*. *Antimicrobial Agents chemother* 58 (7), 4219–4221. doi: 10.1128/AAC.00004-14

Jindal, S., Iyer, M. S., Jyoti, P., Masakapalli, S. K., and Venkatesh, K. V. (2022). Mutants lacking global regulators, *fis* and *arcA*, in *Escherichia coli* enhanced growth fitness under acetate metabolism by pathway reprogramming. *Appl. Microbiol. Biotechnol.* 106 (8), 3231–3243. doi: 10.1007/s00253-022-11890-6

Jurado, M. I., Sainz, M. M., and Mcclean, S. (2021). *Pseudomonas aeruginosa*: an audacious pathogen with an adaptable arsenal of virulence factors. *Int. J. Mol. Sci.* 22 (6), 3128. doi: 10.3390/ijms22063128

Justice, S. S., Li, B., Downey, J. S., Dabdoub, S. M., Brockson, M. E., Probst, G. D., et al. (2012). Aberrant community architecture and attenuated persistence of uropathogenic *Escherichia coli* in the absence of individual IHF subunits. *PLoS One* 7 (10), e48349. doi: 10.1371/journal.pone.0048349

Kahramanoglou, C., Prieto, A., Khedkar, S., Haase, B., Gupta, A., Benes, A., et al. (2012). Genomics of DNA cytosine methylation in *Escherichia coli* reveals its role in stationary phase transcription. *Nat. Commun.* 3, 886. doi: 10.1038/ncomms1878

Khan, H., Liu, M., Kayani, M. U. R., Ahmad, S., Liang, J., Bai, X., et al. (2021). DNA Phosphorothioate modification facilitates the dissemination of *mcr-1* and bla_{NDM-1} in drinking water supply systems. *Environ. pollut.* 268, 115799. doi: 10.1016/j.envpol.2020.115799

Kim, T., Bak, G., Lee, J., and Kim, K. (2015). Systematic analysis of the role of bacterial hfq-interacting sRNAs in the response to antibiotics. *J. antimicrobial chemother* 70 (6), 1659–1668. doi: 10.1093/jac/dkv042

Koli, P., Sudan, S., Fitzgerald, D., Adhya, S., and Kar, S. (2011). Conversion of commensal *Escherichia coli* K-12 to an invasive form *via* expression of a mutant histone-like protein. *mBio* 2 (5), e00182–e00111. doi: 10.1128/mBio.00182-11

Korobeinikova, A. V., Garber, M. B., and Gongadze, G. M. (2012). Ribosomal proteins: structure, function, and evolution. *Biochem. Biokhimiia* 77 (6), 562–574. doi: 10.1134/S0006297912060028

Kumar, S., Karmakar, B. C., Nagarajan, D., et al. (2018). N4-cytosine DNA methylation regulates transcription and pathogenesis in *Helicobacter pylori*. *Nucleic Acids Res.* 46 (7), 3429–3445. doi: 10.1093/nar/gky126

Laughlin, Z. T., Nandi, S., Dey, D., Zelinskaya, N., Witek, M. A., Srinivas, P., et al. (2022). 50S subunit recognition and modification by the *Mycobacterium tuberculosis* ribosomal RNA methyltransferase TlyA. *Proc. Natl. Acad. Sci. U.S.A* 119 (14), e2120352119. doi: 10.1073/pnas.2120352119

Law, C., Huang, C., Pan, Q., Lee, J., Hao, Q., Chan, T., et al. (2019). A small RNA transforms the multidrug resistance of *Pseudomonas aeruginosa* to drug susceptibility. *Mol. Ther. Nucleic Acids* 16, 218–228. doi: 10.1016/j.omtn.2019.02.011

Lei, L., Stipp, R. N., Chen, T., Wu, S. Z., Hu, T., Duncan, M. J., et al. (2018). Activity of streptococcus mutans VicR is modulated by antisense RNA. *J. Dental Res.* 97 (13), 1477–1484. doi: 10.1177/0022034518781765

Li, C., Ma, G., Yang, T., Wen, X., Qin, C., Yue, L., et al. (2020). A rare carbapenem-resistant hypervirulent K1/ST1265 *Klebsiella pneumoniae* with an untypeable bla_{KPC}-harboured conjugative plasmid. *J. Global Antimicrobial Resistance* 22, 426–433. doi: 10.1016/j.jgar.2020.04.009

Liao, Z., and Smirnov, A. (2023). FinO/ProQ-family proteins: an evolutionary perspective. *Bioscience Rep.* 43 (3):BSR20220313. Online . doi: 10.1042/BSR20220313

Liu, Z., Ling, B., and Zhou, L. (2015). Prevalence of 16S rRNA methylase, modifying enzyme, and extended-spectrum beta-lactamase genes among *Acinetobacter baumannii isolates*. *J. chemother* 27 (4), 207–212. doi: 10.1179/1973947814Y.0000000190

Liu, B., Shui, L., Zhou, K., Jiang, Y., Li, X., Guan, J., et al. (2020). Impact of plasmid-encoded h-NS-like protein on bla_{NDM-1}-Bearing IncX3 plasmid in *Escherichia coli*. *J. Infect. Dis.* 221, S229–SS36. doi: 10.1093/infdis/jiz567

Long, K. S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S., and Vester, B. (2006). The cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin a antibiotics. *Antimicrob. Agents Chemother.* 50 (7), 2500–2505. doi: 10.1128/AAC.00131-06

Lopez, S. M. I. G., Cipullo, M., Gopalakrishna, S., Khawaja, A., and Rorbach, J. (2020). Methylation of ribosomal RNA: a mitochondrial perspective. *Front. Genet.* 11, 761. doi: 10.3389/fgene.2020.00761

Lv, F., Cai, J., He, Q., Wang, W., Luo, Y., Wang, X., et al. (2021). *Klebsiella pneumoniae* overexpression of efflux pumps mediate pan resistance of sequence type 11. *Microbial Drug resistance (Larchmont NY)* 27 (10), 1405–1411. doi: 10.1089/mdr.2020.0395

Majdalani, N., Chen, S., Murrow, J., John, K. S., and Gottesman, S. (2001). Regulation of RpoS by a novel small RNA: the characterization of RprA. *Mol. Microbiol.* 39 (5), 1382–1394. doi: 10.1111/j.1365-2958.2001.02329.x

Manna, A. C., Leo, S., Girel, S., González, R. V., Rudaz, S., Francois, P., et al. (2022). Teg58, a small regulatory RNA, is involved in regulating arginine biosynthesis and biofilm formation in *Staphylococcus aureus*. *Sci. Rep.* 12 (1), 14963. doi: 10.1038/s41598-022-18815-3

Marinus, M., and Casadesus, J. (2009). Roles of DNA adenine methylation in host-pathogen interactions: mismatch repair, transcriptional regulation, and more. *FEMS Microbiol. Rev.* 33 (3), 488–503. doi: 10.1111/j.1574-6976.2008.00159.x

Marinus, M., and Morris, N. (1973). Isolation of deoxyribonucleic acid methylase mutants of *Escherichia coli* K-12. *J. bacteriol* 114 (3), 1143–1150. doi: 10.1128/jb.114.3.1143-1150.1973

Mazzariol, A., Tokue, Y., Kanegawa, T. M., Cornaglia, G., and Nikaido, H. (2000). High-level fluoroquinolone-resistant clinical isolates of escherichia coli overproduce multidrug efflux protein AcrA. *Antimicrobial Agents Chemother* 44 (12), 3441–3443. doi: 10.1128/AAC.44.12.3441-3443.2000

Militello, K., Mandarano, A., Varechtchouk, O., and Simon, R. D. (2014). Cytosine DNA methylation influences drug resistance in *Escherichia coli* through increased *sugE* expression. *FEMS Microbiol. Lett.* 350 (1), 100–106. doi: 10.1111/1574-6968.12299

Morita, T., and Aiba, H. (2007). Small RNAs making a small protein. *Proc. Natl. Acad. Sci. U.S.A* 104 (51), 20149–20150. doi: 10.1073/pnas.0710634105

Motta, S. S., Cluzel, P., and Aldana, M. (2015). Adaptive resistance in bacteria requires epigenetic inheritance, genetic noise, and cost of efflux pumps. *PloS One* 10 (3), e0118464. doi: 10.1371/journal.pone.0118464

Muhammad, J., Bajbouj, K., Shafarin, J., and Hamad, M. (2020). Estrogen-induced epigenetic silencing of FTH1 and TFRC genes reduces liver cancer cell growth and survival. *Epigenetics* 15 (12), 1302–1318. doi: 10.1080/15592294.2020.1770917

Muhammad, J. S., Khan, N. A., Maciver, S. K., Alharbi, A. M., Alfahemi, H., Siddiqui, R., et al. (2022). Epigenetic-mediated antimicrobial resistance: host versus pathogen epigenetic alterations. *Antibiotics (Basel)* 11 (6), 809. doi: 10.3390/antibiotics11060809

Nolivos, S., Cayron, J., Dedieu, A., Page, A., Delolme, F., Lesterlin, C. , et al. (2019). Role of AcrAB-TolC multidrug efflux pump in drug-resistance acquisition by plasmid transfer. *Sci. (New York NY)* 364 (6442), 778–782. doi: 10.1126/science.aav6390

Norris, V., Kayser, C., Muskhelishvili, G., and Konto, G. Y. (2022). The roles of nucleoid-associated proteins and topoisomerases in chromosome structure, strand segregation and the generation of phenotypic heterogeneity in bacteria. *FEMS Microbiol. Rev* fauc049. Online ahead of print. doi: 10.1093/femsre/fuac049

Ohno, S., Handa, N., Watanabe, M. M., Takahashi, N., and Kobayashi, I. (2008). Maintenance forced by a restriction-modification system can be modulated by a region in its modification enzyme not essential for methyltransferase activity. *J. Bacteriol* 190 (6), 2039–2049. doi: 10.1128/JB.01319-07

Pandey, S., Gravel, C. M., Stockert, O. M., Wang, C. D., Hegner, C. L., LeBlanc, H., et al. (2020). Genetic identification of the functional surface for RNA binding by *Escherichia coli* ProQ. *Nucleic Acids Res.* 48 (8), 4507–4520. doi: 10.1093/nar/gkaa144

Papenfort, K., and Melamed, S. (2023). Small RNAs, Large networks: post-transcriptional regulons in gram-negative bacteria. *Annu. Rev. Microbiol.* 77. Online. doi: 10.1146/annurev-micro-041320-025836

Parker, A., and Gottesman, S. (2016). Small RNA regulation of TolC, the outer membrane component of bacterial multidrug transporters. *J. bacteriol* 198 (7), 1101–1113. doi: 10.1128/JB.00971-15

Pavlik, P., and Spidlova, P. (2022). Arginine 58 is indispensable for proper function of the *Francisella tularensis* subsp. *holarctica* FSC200 HU protein, and its substitution alters virulence and mediates immunity against wild-type strain. *Virulence* 13 (1), 1790–1809. doi: 10.1080/21505594.2022.2132729

Pernitzsch, S. R., Alzheimer, M., Bremer, B. U., Marie Robbe, S. M., Reuse, H. D., Sharma, C. M., et al. (2021). Small RNA mediated gradual control of lipopolysaccharide biosynthesis affects antibiotic resistance in helicobacter pylori. *Nat. Commun.* 12 (1), 4433. doi: 10.1038/s41467-021-24689-2

Phillips, Z. N., Tram, G., Seib, K. L., and Atack, J. M. (2019). Phase-variable bacterial loci: how bacteria gamble to maximise fitness in changing environments. *Biochem. Soc. Trans.* 47 (4), 1131–1141. doi: 10.1042/BST20180633

Raad, N., Tandon, D., Hapfelmeier, S., and Polacek, N. (2022). The stationary phase-specific sRNA FimR2 is a multifunctional regulator of bacterial motility, biofilm formation and virulence. *Nucleic Acids Res.* 50 (20), 11858–11875. doi: 10.1093/nar/gkac1025

Ramamurthy, T., Ghosh, A., Chowdhury, G., Mukhopadhyay, A. K., Dutta, S., Miyoshi, S. I., et al. (2022). Deciphering the genetic network and programmed regulation of antimicrobial resistance in bacterial pathogens. *Front. Cell Infect. Microbiol.* 12, 952491. doi: 10.3389/fcimb.2022.952491

Reisenauer, A., and Shapiro, L. (2002). DNA Methylation affects the cell cycle transcription of the CtrA global regulator in *Caulobacter*. *EMBO J.* 21 (18), 4969–4977. doi: 10.1093/emboj/cdf490

Roberts, R., Belfort, M., Bestor, T., Bhagwat, A. S., Bickle, T. A., Bitinaite, J., et al. (2003). A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res.* 31 (7), 1805–1812. doi: 10.1093/nar/gkg274

Rocco, C. J., Bakaletz, L. O., and Goodman, S. D. (2018). Targeting the HUβ protein prevents porphyromonas gingivalis from entering into preexisting biofilms. *J. Bacteriol* 200 (11), e00790–e00717. doi: 10.1128/JB.00790-17

Rocco, C. J., Davey, M. E., Bakaletz, L. O., and Goodman, S. D. (2017). Natural antigenic differences in the functionally equivalent extracellular DNABII proteins of bacterial biofilms provide a means for targeted biofilm therapeutics. *Mol. Oral. Microbiol.* 32 (2), 118–130. doi: 10.1111/omi.12157

Rodgers, D., Le, C., Pimentel, C., Tuttobene, M. R., Subils, T., Escalante, J., et al. (2021). Histone-like nucleoid-structuring protein (H-NS) regulatory role in antibiotic resistance in *Acinetobacter baumannii*. *Sci. Rep.* 11 (1), 18414. doi: 10.1038/s41598-021-98101-w

Rodgers, M. L., O'brien, B., and Woodson, S. A. (2023). Small RNAs and hfq capture unfolded RNA target sites during transcription. *Mol. Cell* 83 (9), 1489–501.e5. doi: 10.1016/j.molcel.2023.04.003

Roodsant, T. J., Putten, B. V. D., Brizuela, J., Coolen, J. P., Baltussen, T. J., Schipper, K., et al. (2023). The streptococcal phase-variable type I restriction-modification system SsuCC20p dictates the methylome of *Streptococcus suis* and impacts virulence. *bioRxiv*. Preprint. doi: 10.1101/2023.03.17.533248

Sakatos, A., Babunovic, G., Chase, M., Dills, A., Leszyk, J., Rosebrock, T., et al. (2018). Post-translational modification of a histone-like protein regulates phenotypic resistance to isoniazid in mycobacteria. *Sci. Adv.* 4 (5), eaao1478. doi: 10.1126/sciadv.aao1478

Saldaña, A. Z., Soria, B. J., Martínez, S. V. I., Yañez, J. A. S., Martínez, Y. L., Cedillo, M. L. R., et al. (2022). The Fis nucleoid protein negatively regulates the phase variation *fimS* switch of the type 1 pilus operon in enteropathogenic *Escherichia coli*. *Front. Microbiol.* 13, 882563. doi: 10.3389/fmicb.2022.882563

San, M. A., and Maclean, R. C. (2017). Fitness costs of plasmids: a limit to plasmid transmission. *Microbiol. Spectr.* 5 (5):MTBP-0016-2017. doi: 10.1128/microbiolspec

Sánchez, R. M. A., and Casadesús, J. (2020). The bacterial epigenome. *Nat. Rev. Microbiol.* 18 (1), 7–20. doi: 10.1038/s41579-019-0286-2

Seib, K. L., Srikhanta, Y. N., Atack, J. M., and Jennings, M. P. (2020). Epigenetic regulation of virulence and immunoevasion by phase-variable restriction-modification systems in bacterial pathogens. *Annu. Rev. Microbiol.* 74, 655–671. doi: 10.1146/annurev-micro-090817-062346

Sergeeva, O. V., Bogdanov, A. A., and Sergiev, P. V. (2015). What do we know about ribosomal RNA methylation in *Escherichia coli*? *Biochimie* 117, 110–118. doi: 10.1016/j.biochi.2014.11.019

Serra, D. O., Mika, F., Richter, A. M., and Hengge, R. (2016). The green tea polyphenol EGCG inhibits e. coli biofilm formation by impairing amyloid curli fibre assembly and downregulating the biofilm regulator CsgD *via* the σ(E) -dependent sRNA RybB. *Mol. Microbiol.* 101 (1), 136–151. doi: 10.1111/mmi.13379

Sheidy, D. T., and Zielke, R. A. (2013). Analysis and expansion of the role of the *Escherichia coli* protein ProQ. *PLoS One* 8 (10), e79656. doi: 10.1371/journal.pone.0079656

Shi, H., Wei, J., and He, C. (2019). Where, when, and how: context-dependent functions of RNA methylation writers, readers, and erasers. *Mol. Cell* 74 (4), 640–650. doi: 10.1016/j.molcel.2019.04.025

Shoji, T., Takaya, A., Sato, Y., Kimura, A., Suzuki, T., Yamamoto, T., et al. (2015). RlmCD-mediated U747 methylation promotes efficient G748 methylation by methyltransferase RlmAII in 23S rRNA in *Streptococcus pneumoniae*; interplay between two rRNA methylations responsible for telithromycin susceptibility. *Nucleic Acids Res.* 43 (18), 8964–8972. doi: 10.1093/nar/gkv609

Singh, R., Sahore, S., Kaur, P., Rani, A., and Ray, P. (2016). Penetration barrier contributes to bacterial biofilm-associated resistance against only select antibiotics, and exhibits genus-, strain- and antibiotic-specific differences. *Pathog. Dis.* 74 (6), ftw056. doi: 10.1093/femspd/ftw056

Solano, C. V., Hüttener, M., Espinosa, M., Juárez, A., and Bravo, A. (2016). MgaSpn and h-NS: two unrelated global regulators with similar DNA-binding properties. *Front. Mol. Biosci.* 3, 60. doi: 10.3389/fmolb.2016.00060

Srikhanta, Y. N., Dowideit, S. J., Edwards, J. L., Falsetta, M. L., Wu, H.J., Harrison, O. B., et al. (2009). Phasevarions mediate random switching of gene expression in pathogenic *Neisseria*. *PloS Pathog.* 5 (4), e1000400. doi: 10.1371/journal.ppat.1000400

Srikhanta, Y. N., Gorrell, R. J., Steen, J. A., Gawthorne, J. A., Kwok, T., Grimmond, S. M., et al. (2011). Phasevarion mediated epigenetic gene regulation in *Helicobacter pylori*. *PloS One* 6 (12), e27569. doi: 10.1371/journal.pone.0027569

Srinivas, P., Nosrati, M., Zelinskaya, N., Dey, D., Comstock, L. R., Dunham, C. M., et al. (2023). 30S subunit recognition and G1405 modification by the aminoglycoside-resistance 16S ribosomal RNA methyltransferase RmtC. *bioRxiv* 532395. doi: 10.1101/2023.03.13.532395

Stojkova, P., and Spidlova, P. (2022). Bacterial nucleoid-associated protein HU as an extracellular player in host-pathogen interaction. *Front. Cell Infect. Microbiol.* 12, 999737. doi: 10.3389/fcimb.2022.999737

Stojkova, P., Spidlova, P., Lenco, J., Rehulkova, H., Kratka, L., Stulik, J., et al. (2018). HU protein is involved in intracellular growth and full virulence of *Francisella tularensis*. *Virulence* 9 (1), 754–770. doi: 10.1080/21505594.2018.1441588

Stojkova, P., Spidlova, P., and Stulik, J. (2019). Nucleoid-associated protein HU: a Lilliputian in gene regulation of bacterial virulence. *Front. Cell. infection Microbiol.* 9, 159. doi: 10.3389/fcimb.2019.00159

Sudano, R. A., Blanco, A. R., GIuliano, F., Rusciano, D., and Enea, V. (2004). Epigallocatechin-gallate enhances the activity of tetracycline in staphylococci by

inhibiting its efflux from bacterial cells. *Antimicrob. Agents Chemother.* 48 (6), 1968–1973. doi: 10.1128/AAC.48.6.1968-1973.2004

Swinger, K., and Rice, P. (2007). Structure-based analysis of HU-DNA binding [J]. *J. Mol. Biol.* 365 (4), 1005–1016. doi: 10.1016/j.jmb.2006.10.024

Tada, T., Miyoshi, A. T., KATO, Y., Ohmagari, N., Takeshita, N., Hung, N. V., et al. (2013). Emergence of 16S rRNA methylase-producing *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates in hospitals in Vietnam. *BMC Infect. Dis.* 13, 251. doi: 10.1186/1471-2334-13-251

Tafforeau, L. (2015). About the ribosomal biogenesis in human. *Medecine Sci. M/S* 31 (6-7), 622–628. doi: 10.1051/medsci/20153106015

Takahashi, N., Naito, Y., Handa, N., and Kobayashi, I. (2002). A DNA methyltransferase can protect the genome from postdisturbance attack by a restriction-modification gene complex. *J. Bacteriol* 184 (22), 6100–6108. doi: 10.1128/JB.184.22.6100-6108.2002

Tan, A., Hill, D. M. C., Harrison, O. B., Srikhanta, Y. N., Jennings, M. P., Maiden, M. C. J., et al. (2016). Distribution of the type III DNA methyltransferases *modA*, *modB* and *modD* among *Neisseria meningitidis* genotypes: implications for gene regulation and virulence. *Sci. Rep.* 6 (1), 21015. doi: 10.1038/srep21015

Tang, Y., Hong, Y., Liu, L., Du, X., Ren, Y., Jiang, S., et al. (2022). Involvement of the DNA phosphorothioation system in TorR binding and anaerobic TMAO respiration in *Salmonella enterica*. *mBio* 13 (3), e0069922. doi: 10.1128/mbio.00699-22

Timmis, K. N., Andrés, I., and Achtman, M. (1978). Fertility repression of f-like conjugative plasmids: physical mapping of the R6-5 *finO* and *finP* cistrons and identification of the *finO* protein. *Proc. Natl. Acad. Sci. U.S.A* 75 (12), 5836–5840. doi: 10.1073/pnas.75.12.5836

Toledo, A. A., Repoila, F., and Cossart, P. (2007). Small noncoding RNAs controlling pathogenesis. *Curr. Opin. Microbiol.* 10 (2), 182–188. doi: 10.1016/j.mib.2007.03.004

Tong, T., Chen, S., Wang, L., Tang, Y., Ryu, J. Y., Jiang, S., et al. (2018). Occurrence, evolution, and functions of DNA phosphorothioate epigenetics in bacteria. *Proc. Natl. Acad. Sci. U.S.A* 115 (13), E2988–E2e96. doi: 10.1073/pnas.1721916115

Tram, G., Jen, F. E., Phillips, Z. N., Timms, J., Husna, A. U., Jennings, M. P., et al. (2021). *Streptococcus suis* encodes multiple allelic variants of a phase-variable type III DNA methyltransferase, ModS, that control distinct phasevarions. *mSphere* 6 (3), e00069–e00021. doi: 10.1128/mSphere.00069-21

Udekwu, K., Darfeuille, F., Vogel, J., Reimegård, J., Holmqvist, E., Wagner, E. G. H., et al. (2005). Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev.* 19 (19), 2355–2366. doi: 10.1101/gad.354405

Van Biesen, T., and Frost, L. S. (1994). The FinO protein of IncF plasmids binds FinP antisense RNA and its target, traJ mRNA, and promotes duplex formation. *Mol. Microbiol.* 14 (3), 427–436. doi: 10.1111/j.1365-2958.1994.tb02177.x

Vanderpool, C., and Gottesman, S. (2004). Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. *Mol. Microbiol.* 54 (4), 1076–1089. doi: 10.1111/j.1365-2958.2004.04348.x

Vasu, K., and Nagaraja, V. (2013). Diverse functions of restriction-modification systems in addition to cellular defense. *Microbiol. Mol. Biol. Rev.* 77 (1), 53–72. doi: 10.1128/MMBR.00044-12

Verdin, E., and Ott, M. (2015). 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nat. Rev. Mol. Cell Biol.* 16 (4), 258–264. doi: 10.1038/nrm3931

Vogel, J., and Sharma, C. M. (2005). How to find small non-coding RNAs in bacteria. *Biol. Chem.* 386 (12), 1219–1238. doi: 10.1515/BC.2005.140

Wang, N., Chen, X., Luo, J., Deng, F., Shi, F., Wu, Q., et al. (2022). Artemisinin derivative DHA27 enhances the antibacterial effect of aminoglycosides against *Pseudomonas aeruginosa* by inhibiting mRNA expression of aminoglycoside-modifying enzymes. *Front. Pharmacol.* 13, 970400. doi: 10.3389/fphar.2022.970400

Wang, L., Jiang, S., Deng, Z., Dedon, P. C., and Chen, S. (2019). DNA Phosphorothioate modification-a new multi-functional epigenetic system in bacteria. *FEMS Microbiol. Rev.* 43 (2), 109–122. doi: 10.1093/femsre/fuy036

Wang, W., Li, W., Ge, X., Yan, K., Mandava, C. S., Sanyal, S., et al. (2020). Loss of a single methylation in 23S rRNA delays 50S assembly at multiple late stages and impairs translation initiation and elongation. *Proc. Natl. Acad. Sci. U.S.A* 117 (27), 15609–15619. doi: 10.1073/pnas.1914323117

Wang, Y., Teng, Y., Geng, J., Long, J., Yang, H., Duan, G., et al. (2023). Involvement of RNA chaperone hfq in the regulation of antibiotic resistance and virulence in *Shigella sonnei*. *Res. Microbiol.* 104047. doi: 10.1016/j.resmic.2023.104047

Willbanks, A., Leary, M., Greenshields, M., Tyminski, C., Heerboth, S., Lapinska, K., et al. (2016). The evolution of epigenetics: from prokaryotes to humans and its biological consequences. *Genet. Epigenet.* 8, 25–36. doi: 10.4137/GEG.S31863

Wu, X., Cao, B., Aquino, P., Chiu, T. P., Chen, C., Jiang, S., et al. (2020). Epigenetic competition reveals density-dependent regulation and target site plasticity of phosphorothioate epigenetics in bacteria. *Proc. Natl. Acad. Sci. U.S.A* 117 (25), 14322–14330. doi: 10.1073/pnas.2002933117

Wu, T., Huang, Q., Wang, X. L., Shi, T., Bai, L., Liang, J., et al. (2017). Mechanistic investigation on ROS resistance of phosphorothioated DNA. *Sci. Rep.* 7, 42823. doi: 10.1038/srep42823

Xiao, S., Suo, W., Zhang, J., Zhang, X., Yin, Y., Guo, X., et al. (2021). MgaSpn is a negative regulator of capsule and phosphorylcholine biosynthesis and influences the virulence of *Streptococcus pneumoniae* D39. *Virulence* 12 (1), 2366–2381. doi: 10.1080/21505594.2021.1972539

Xie, X., Liang, J., Pu, T., Xu, F., Yao, F., Yang, Y., et al. (2012). Phosphorothioate DNA as an antioxidant in bacteria. *Nucleic Acids Res.* 40 (18), 9115–9124. doi: 10.1093/nar/gks650

Xu, C., Rao, J., Xie, Y., Lu, J., Li, Z., Dong, C., et al. (2023). The DNA phosphorothioation restriction-modification system influences the antimicrobial resistance of pathogenic bacteria. *Microbiol. Spectr.* 11 (1), e0350922. doi: 10.1128/spectrum.03509-22

Yan, D., Zhang, Q., Fu, Q., Sun, M., and Huang, X. (2021). Disruption of Fis reduces bacterial persister formation by regulating glutamate metabolism in salmonella. *Microbial pathogenesis* 152, 104651. doi: 10.1016/j.micpath.2020.104651

Yang, J., Wang, H. H., Lu, Y., Yi, L. X., Deng, Y., Lv, L., et al. (2021). A ProQ/FinO family protein involved in plasmid copy number control favours fitness of bacteria carrying *mcr-1*-bearing IncI2 plasmids. *Nucleic Acids Res.* 49 (7), 3981–3996. doi: 10.1093/nar/gkab149

Yelin, I., and Kishony, R. (2018). Antibiotic resistance. *Cell* 172 (5), 1136–1136.e1. doi: 10.1016/j.cell.2018.02.018

Yu, M., and Zhao, Y. (2023). The posttranscriptional regulator CsrA affects multidrug resistance and biocontrol activity in lysobacter enzymogenes. *J. Appl. Microbiol.* 134 (3), lxad045. doi: 10.1093/jambio/lxad045

Yuan, P., Ling, J., Zhu, J., Peng, C., Chen, E. Z., Zhong, Y. X., et al. (2022). Proteomics profiling of ertapenem challenged major porin deficient carbapenem-resistant *Klebsiella pneumoniae*. *J. Proteomics* 268, 104715. doi: 10.1016/j.jprot.2022.104715

Zamora, M., Ziegler, C. A., Freddolino, P. L., and Wolfe, A. J. (2020). A thermosensitive, phase-variable epigenetic switch: pap revisited. *Microbiol. Mol. Biol. Rev.* 84 (3), e00030–e00017. doi: 10.1128/MMBR.00030-17

Zaunbrecher, M. A., Sikes, R D, JR., Metchock, B., Shinnick, T. M., and Posey, J. E. (2009). Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A* 106 (47), 20004–20009. doi: 10.1073/pnas.0907925106

Zeferino, A., Mira, A., Delgadinho, M., Brito, M., Ponte, T., Ribeiro, E., et al. (2022). Drug resistance and epigenetic modulatory potential of epigallocatechin-3-Gallate against *Staphylococcus aureus*. *Curr. Microbiol.* 79 (5), 149. doi: 10.1007/s00284-022-02841-5

Zemach, A., Mcdaniel, I., Silva, P., and Zilberman, D. (2010). Genome-wide evolutionary analysis of eukaryotic DNA methylation. *Sci. (New York NY)* 328 (5980), 916–919.

Zhang, Y. (2014). Persisters, persistent infections and the yin-yang model. *Emerging Microbes infections* 3 (1), e3. doi: 10.1038/emi.2014.3

Zhang, J. F., Fang, L. X., Chang, M. X., Cheng, M., Zhang, H., Long, T. F., et al. (2022). A trade-off for maintenance of multidrug-resistant IncHI2 plasmids in *Salmonella enterica* serovar typhimurium through adaptive evolution. *mSystems* 7 (5), e0024822. doi: 10.1128/msystems.00248-22

Zhang, L., Lu, Q., and Chang, C. (2020). Epigenetics in health and disease. *Adv. Exp. Med. Biol.* 1253, 3–55. doi: 10.1007/978-981-15-3449-2_1

Zhang, , Zhang , Y., Ma, R., Sun, W., and Ji, Z. (2023). Antibacterial activity of epigallocatechin gallate (EGCG) against *Shigella flexneri*. *Int. J. Environ. Res. Public Health* 20 (6), 4676. doi: 10.3390/ijerph20064676

Zhang, P., Zhao, X., Wang, Y., Du, K., Wang, Z., Yu, J., et al. (2022). Bacteriophage protein Gp46 is a cross-species inhibitor of nucleoid-associated HU proteins. *Proc. Natl. Acad. Sci. U. S. A* 119 (9), e2116278119. doi: 10.1073/pnas.2116278119

Zhao, Z., Xu, Y., Jiang, B., et al. (2022). Systematic identification of CpxRA-regulated genes and their roles in escherichia coli stress response. *mSystems* 7 (5), e00419-22. doi: 10.1128/msystems.00419-22

Zhou, K., Aertsen, A., and Michiels, C. W. (2014). The role of variable DNA tandem repeats in bacterial adaptation. *FEMS Microbiol. Rev.* 38 (1), 119–141. doi: 10.1111/1574-6976.12036

Zhou, J., Horton, J. R., Yu, D., Ren, R., Blumenthal, R. M., Zhang, X., et al. (2022). Repurposing epigenetic inhibitors to target the clostridioides difficile-specific DNA adenine methyltransferase and sporulation regulator CamA. *Epigenetics* 17 (9), 970–981. doi: 10.1080/15592294.2021.1976910

Ziegler, C. A., and Freddolino, P. L. (2021). The leucine-responsive regulatory proteins/feast-famine regulatory proteins: an ancient and complex class of transcriptional regulators in bacteria and archaea. *Crit. Rev. Biochem. Mol. Biol.* 56 (4), 373–400. doi: 10.1080/10409238.2021.1925215

Zou, X., Wang, L., Li, Z., Luo, J., Wang, Y., Deng, Z., et al. (2018). Genome engineering and modification toward synthetic biology for the production of antibiotics. *Medicinal Res. Rev.* 38 (1), 229–260. doi: 10.1002/med.21439

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 27 July 2023
DOI 10.3389/fcimb.2023.1148603

OPEN ACCESS

EDITED BY
Dongsheng Zhou,
Beijing Institute of Microbiology and Epidemiology, China

REVIEWED BY
Jiun-Ling Wang,
National Cheng Kung University, Taiwan
Majid Taati Moghadam,
Gilan University of Medical Sciences, Iran

*CORRESPONDENCE
Roya Ghanavati
R.Ghanavati@behums.ac.ir;
Qanavati.r@gmail.com
Atieh Darbandi
Atiehdarbandi86@gmail.com

†These authors have contributed equally to this work

RECEIVED 23 January 2023
ACCEPTED 23 June 2023
PUBLISHED 27 July 2023

CITATION
Mahdizade Ari M, Dashtbin S, Ghasemi F, Shahroodian S, kiani P, Bafandeh E, Darbandi T, Ghanavati R and Darbandi A (2023) Nitrofurantoin: properties and potential in treatment of urinary tract infection: a narrative review. *Front. Cell. Infect. Microbiol.* 13:1148603. doi: 10.3389/fcimb.2023.1148603

Nitrofurantoin: properties and potential in treatment of urinary tract infection: a narrative review

Marzie Mahdizade Ari[1], Shirin Dashtbin[1], Fatemeh Ghasemi[2], Soheila Shahroodian[1], Parisa kiani[3], Elnaz Bafandeh[4], Talieh Darbandi[5], Roya Ghanavati[6*†] and Atieh Darbandi[1,7*†]

[1]Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran, [2]Department of Pathobiology, Division of Microbiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, [3]Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, [4]Department of Medical Biotechnology, Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran, [5]Department of Pharmacy, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran, [6]School of Medicine, Behbahan Faculty of Medical Sciences, Behbahan, Iran, [7]Molecular Microbiology Research Center, Shahed University, Tehran, Iran

Nitrofurantoin (NF), a wide-spectrum antibiotic accessible since 1953, is utilized widely to treat urinary tract infections as it usually stays active against drug-resistant uropathogen. The use of Nitrofurantoin has increased exponentially since new guidelines have repositioned it as first-line therapy for uncomplicated lower urinary tract infection (UTI). To, although fluoroquinolones are usually used to re-evaluate the first- and second-line therapies for treating uncomplicated UTI, their level of utilization is thought to be inappropriately excessive and will eventually have a detrimental impact; thus, we hypothesize that NF might be the best choice for this condition, because of its low frequency of utilization and its high susceptibility in common UTI pathogens. It can be concluded from this review that NF can be considered as the most effective drug in the treatment of acute urinary infection, but due to the long-term side effects of this drug, especially in elderly patients, it is essential to introduce some criteria for prescribing NF in cases of chronic UTI.

KEYWORDS

Nitrofurantoin, urinary tract infection, drug-resistant uropathogen, fluoroquinolones, side effects

1 Introduction

Nitrofurantoin (NF) was identified in 1953 and was first recommended for the treatment of cystitis in 2010 according to the Infectious Diseases Society of America (IDSA) guideline (Sanchez et al., 2016).

Currently, 150 million Urinary Tract Infections (UTIs) are reported annually worldwide, and drug-resistant infections usually require more complex treatment regimens and are more likely to occur if treatment fails (Khoshbayan et al., 2022). NF is

outstanding because NF is most extensively utilized in humans, and its effectiveness in the treatment of lower UTIs and prophylaxis is well established (Conklin, 1978). It is presently prescribed as first-line UTI medical care due to the emergence of resistance to different antibiotics such as carbapenem resistance (Conklin, 1978; Garau, 2008; Gupta et al., 2011; Matthews et al., 2016; Mohebi et al., 2016; Kazemian et al., 2019). It has bacteriostatic and bactericidal effects and is instantly excreted in high concentrations by the kidneys (Komp Lindgren et al., 2015; Fransen et al., 2016). NF is bacteriostatic in low concentrations (5-10 pg/mL) and bactericidal in higher concentrations (Andriole, 1985). Other studies refer to the therapeutic or prophylactic use of this antibiotic. In therapeutic application, 50–100 mg q6h (regular-release formulation) or 100 mg q12h or q8h (slow-release formulation) and in prophylaxis, 50–100 mg q24h is recommended (McOsker and Fitzpatrick, 1994; Cunha et al., 2017; Fransen et al., 2017). With this background, we summarized the NF data available as a valuable choice in the treatment of acute urinary infection, but due to the long-term side effects of this drug, especially in elderly patients, it is essential to introduce some criteria for prescribing NF in cases of chronic UTI.

2 Pharmacology (Pharmacokinetic and Pharmacodynamics) and Biochemistry of NF

NF is a redox-active antibacterial agent with the molecular formula of C8H6N4O5 and the molecular weight of 238.16, and is an oral antibiotic based on nitrofurans (Dos Santos et al., 2021). NF, which is a member of the nitrofuran family composed of a furan ring [five-membered aromatic ring with four carbon (C) atoms and one oxygen (O)] is directly linked to a nitro group (-NO2) (FDA. Macrodantin® (NF Macrocrystals) Capsules Product Information. Cincinnati, OH, USA: Procter & Gamble Pharmaceuticals, 2009; 1–12.) (Figure 1). In the market, NF is available in oral forms of capsules, tablets, and suspension (oral suspension also known as furadantin). NF is often prescribed in a dose of 50-100 mg 4 times a day for 5 days (Gardiner et al., 2019; Dos Santos et al., 2021). Table 1 summarizes these characteristics of NF. In terms of PK, NF quickly reaches its therapeutic concentration level, so 90% of NF is quickly exerted through the urine and that is why all its therapeutic effects are restricted to the treatment of UTI. NF is 80-90% orally bioavailable and its bioavailability is about 38.8-44.3%. It has a short half-life (20 minutes), is active only in the urinary tract, and has no other systemic activity. Also, it is rapidly absorbed and eliminated, with low plasma protein binding to plasma proteins or tissues. It is well absorbed from the gastrointestinal tract and excreted unchanged in urine (25–40%) and bile. It has been reported that NF can accumulate in urine, with its effect enhanced by the acidic pH of urine. The anti-bacterial activity of NF and its metabolites is improved under acid conditions. Metabolites are formed by bacterial enzyme reduction, but the precise structure and antibacterial activity of each metabolite remains uncertain (Beckett and Robinson, 1958).

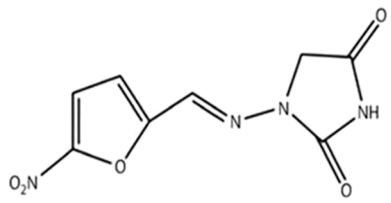


FIGURE 1
Chemical structure of NF (Wijma et al., 2018).

3 Effect of food on PK

Most absorption of NF is done in the duodenum, so the presence of food in GI tract leads to an increase in the time of gastric emptying. Therefore, more NF dissolves in gastric juice before it reaches the duodenum (Jaffe and JM, 1975).

The dissolution time hypothesis is supported by the results of Naggar and Khalil, who showed that absorption increased when the solubility of NF was increased by the addition of Mg2O8Si3 (Naggar and Khalil, 1979).

4 Impact of NF on UTI

UTI is one of the most common bacterial infections and has two complicated and uncomplicated forms which are differentiated by symptoms and causative agents (Figure 2).

The uncomplicated form of UTI is often caused by uropathogenic Escherichia coli (UPEC) strains in 80% of cases (Klein and Hultgren, 2020), followed by Klebsiella pneumoniae, Staphylococcus saprophyticus, Enterococcus spp., and group B Streptococcus (GBS). Dysuria, frequency and urgency, suprapubic pain and hematuria are the most common symptoms of UTI (Chew et al., 2019; Ghanavati et al., 2018a). This infection occurs mostly in all aged women (50-60), boys and the elderly. Moreover, the predisposing factors include age, level of sexual activity of people and pre-existing underlying disease (Klein and Hultgren, 2020). Untreated UTI cases can cause sepsis with or without pyelonephritis (Flores-Mireles et al., 2015), leading to death in 10-30%; therefore, UTI patients may sometimes need to be admitted to the hospital (Komagamine et al., 2022). Values less than or equal to 15 μg/mL are suitable for eliminating E. coli (common cause of UTI) and more than 100 μg/mL for eliminating Enterobacter spp. and Klebsiella spp (Cunha, 1988).

Conventional antibiotic therapy for acute uncomplicated UTI includes trimethoprim-sulfamethoxazole, Cefpodoxime, Cephalexin and Cefuroxime, Ciprofloxacin, Cefepime, Ampicillin, Imipenem/Cilastatin and Trimethoprim-Sulfamethoxazole are suitable choices for acute complicated form (Long and Koyfman, 2018). The emergence of antibiotic-resistant strains and elimination of the microbial flora of the gastrointestinal tract and vagina may occur following long-term use of these conventional antibiotics in patients suffering from UTI (Kostakioti et al., 2012; Flores-Mireles

TABLE 1 Characteristics of NF.

Antibiotic	NF
Formula	C8H6N4O5
Molecular weight	238.16
Bioavailability	38.8-44.3%.
PK	90% of NF quickly exerted through the urine,
Pharmacodynamics	Disruption of Krebs cycle and interference in metabolism of carbohydrates, biosynthesis of proteins, cell wall and DNA
Dosage	50-100 mg 4 times a day for 5 days
Clinical use	Urinary tract infection
Adverse effects	Renal, pulmonary, hepatic and nerve failure/Drug induced fever

et al., 2015). Fosfomycin and NF are two alternative antibiotics to prescribe in cases of resistant strains, but NF is more effective than fosfomycin and shows a greater effect on pregnant women (Gardiner et al., 2019; Ghanavati et al., 2018b). Oral prescription of NF in both liquid (25 mg/5 ml) and solid (100 mg) forms shows an optimum effect on the treatment of UTI. Studies have shown that oral NF is the best choice for prophylaxis before surgery and the treatment of patients over age 12. Therefore, NF is currently used

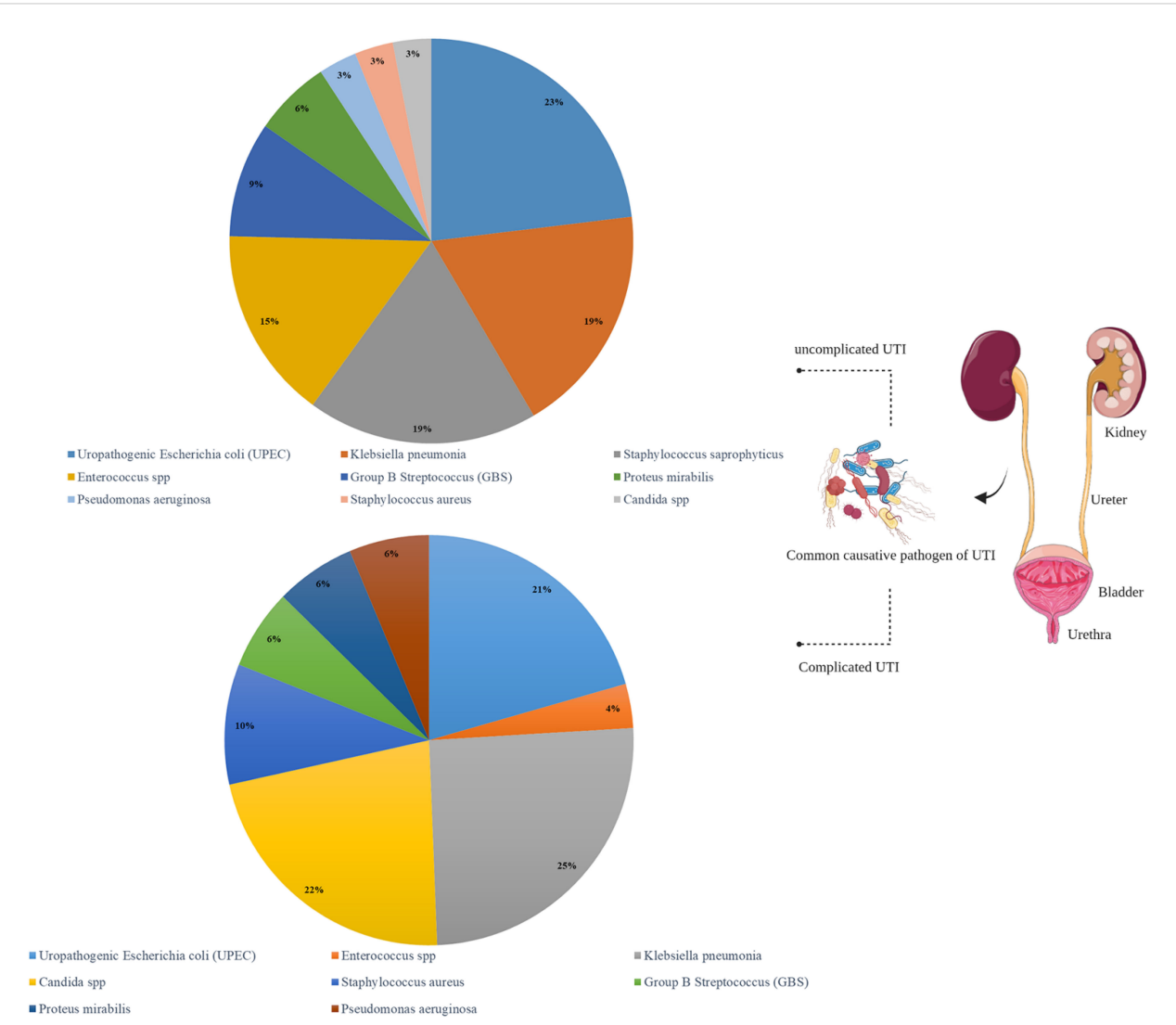


FIGURE 2
Complicated and uncomplicated UTI prevalence as well as causative pathogens.

prophylactically in UTI cases specially against vancomycin-sensitive and resistant strains (VRE and VRS) associated with catheters as well as fluoroquinolones and aminopenicillins resistant strains (Cunha, 2006). This can be the only antibiotic that is effective in treating enterococcal strains instead of ampicillin.

Twenty-seven trials consisting of 4807 patients have been conducted to analyze NF as a remedy for UTIs. NF was determined to be clinically and microbiologically effective, with clinical cure rates between 79% to 92% and microbiological eradication rates of 80%–92%.

5 Antimicrobial effect of NF

In addition to the greatest effect on uropathogens, NF has an inhibitory effect on a wide range of Gram-positive bacteria such as Staphylococcus and Enterococcus and Gram-negative bacteria such as Klebsiella and Citrobacter (Munoz-Davila, 2014). It seems that E. coli as the most bacteria isolated from the uncomplicated UTI cases is more inactivated by NF among other Gram-negatives, while Enterobacter, Klebsiella, Citrobacter and Providencia are less effective, and Pseudomonas, Proteus, Acinetobacter, Morganella and Serratia are completely ineffective and show resistance to NF (Naber et al., 2008; Gardiner et al., 2019). Mouse urinary tract infection models have shown that the MIC required for treatment with NF in an animal model is much lower than antibiotics such as Sulfamethoxazole/Trimethoprim, Fosfomycin, Mecillinam, Ciprofloxacin, and Cefdinir, and will eliminate more live bacteria (Nakagawa et al., 2021).

6 Mechanism of action and resistance rates

Following the activation of the nitro group in its molecular structure by the cytochrome P450 reductase (Figure 3), NF affects the protein synthesis machinery and ribosome in susceptible bacteria (Wang et al., 2008) and disrupts Krebs cycle (citric acid cycle) by inhibiting a series of enzymes involved in the metabolism of carbohydrates (Cunha, 1989; Munoz-Davila, 2014), as well as cell wall and DNA. This interference with vital processes leads to bacterial death (Munoz-Davila, 2014).

For the first time, the resistance to nitrofurantoin has been reported in *E. coli* which attributed to the presence of a mutation in the gene coding for nitrofurantoin (*nfsA*), an oxygen-insensitive enzyme. This mutation prevents the reduction of NF and the subsequent production of toxic compounds (Race et al., 2005). In addition to chromosomal gene, plasmid-mediated NF-resistant strains also shows higher MIC and target *nfsA* and *nfsB* genes (Sastry and Jayaraman, 1984; Ho et al., 2016). While the NF breakpoint is defining as 32 mg/L, some resistant strains in anaerobic conditions, shows decrease in the MIC value. This is explained by the activation of the oxygen system and the presence of oxygen-sensitive nitroreductase (type II), which are activated in the absence of oxygen-insensitive type I reductases. Gautam et al. in study which conducted in 2021, showed the increase in multi drug resistant strains while there is no effective drug, has led to an increase in the prescription of NF and its increasing resistance rate (Gautam et al., 2021). In response to whether NF-resistant isolates will emerge or not, it should be mentioned that although the frequency of mutations in resistance to NF is high, treatment failure seems to be rare, and considering that most urinary tract infections are treated empirically, the desired antibiotic first must be determined based on the sensitivity pattern therefore the importance is to investigate NF-resistant strains to see whether they are still treatable or not (Sandegren et al., 2008).

Bacterial flavoproteins can reduce the drug, leading to reactive electrophilic intermediates that change or inactivate bacterial molecules (Gleckman et al., 1979; McOsker and Fitzpatrick, 1994). As a prodrug, NF is activated by two kinds of oxygen-insensitive nitroreductases, nfsA and nfsB (Le and Rakonjac, 2021). High levels of resistance to NF (median MIC of 96 μg/ml) are principally mediated by mutations in nfsA and/or nfsB (encoding oxygen-insensitive nitroreductases) (Shakti and Veeraraghavan, 2015). Deletions in ribE also result in resistance due to inhibition synthesis of Riboflavin/Flavin (vital cofactor of NfsA and NfsB((Vervoort et al., 2014; Sekyere and Asante, 2018). Efflux pumps are the other factors that play a role in resistance to this antibiotic (Ho et al., 2016). A study reported resistance rates of nitrofurantoin from 2011 to 2019. In E. coli, Klebsiella spp, Proteus spp. and Enterococcus spp. resistance rates were 4.8%, 46.0%, 100.0% and 4.8%, respectively (Hrbacek et al., 2020). The low resistance rate in

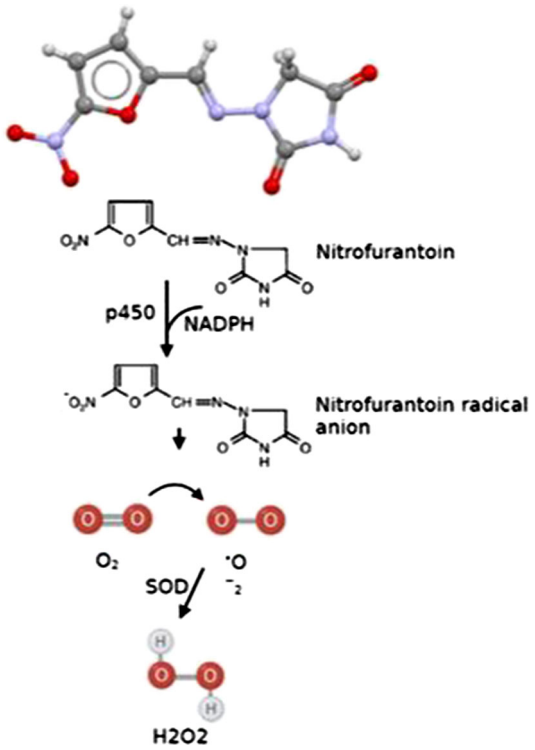


FIGURE 3
Scheme of NF mode of action and ROS generation. p450, cytochrome P450 reductase; NADPH, Nicotinamide Adenine Dinucleotide Phosphate Hydrogen; SOD, superoxide dismutase; H2O2, hydrogen peroxide.

E. coli and Extended Spectrum Beta-Lactamase (ESBL) producing Enterobacteriaceae may be due to different mechanisms of action (Huttner et al., 2015).

A study conducted by Ahmed et al. showed that the pattern of antibiotic resistance in E. coli as the most common pathogen causing UTI, was as follows: Ampicillin (86%), Amoxicillin (76%), Tetracycline (71%), Trimethoprim-Sulfamethoxazole (64%), Cephalexin (61%) and Cephalothin (60%), respectively. Also, this strain has the highest antibiotic sensitivity to Imipenem (86%), NF (82%), Amikacin (79%) and Ciprofloxacin (72%) (Ahmed et al., 2019). According to a systematic review by Bryce et al., the prevalence of antibiotic resistance to such common antibiotics in UTIs caused by E. coli such as Ampicillin, Trimethoprim, Co-amoxiclav, Ciprofloxacin and NF largely differ in different countries. In this way, the OECD (Organization for Economic Co-operation and Development) countries have much less antibiotic resistance, which is attributed to the availability of common antibiotics. In other words, NF in some countries have much lower antibiotic resistance than in non-OECD countries. When common antibiotics are routinely used in the treatment of UTIs, they have contributed more to antibiotic resistance (Bryce et al., 2016). Regarding the effect of NF on resistant pathogens, Tulara et al. evaluated the effect of Fosfomycin and NF on extended-spectrum-beta-lactamase-producing E. coli (ESBL-EC), and the results indicated the effectiveness of NF in the ESBL-EC (Tulara, 2018). Moreover, FQ-resistant E. coli, are not only affected by NF, but this antibiotic has also provided a cost-effective feature (McKinnell et al., 2011).

7 Adverse effects of NF

Antibiotic resistance is considered as one of the possible side effects of any antimicrobial agents. This issue has been reported in long-term prophylaxis cases for UTI and elderly patients with renal failure. Generally, NF is considered as a safe antimicrobial drug, but, in 1 per 100,000 patients (Vickery et al., 2022) in long-term use, there may be some risks. The non-drug resistance side effects of NF like hepatotoxicity, neuropathy and pulmonary damages are directly related to the long-term use of this drug (Wang et al., 2008). Three complications, Gastrointestinal (GI) and skin manifestations and peripheral neuropathy (Tan et al., 2012), are the most important and serious adverse effects of NF consumption, respectively. The first warning about the risks of NF in the elderly was given in 2003 (Fick et al., 2003), and then in 2012 NF was listed among the potentially dangerous drugs causing renal failure in elderly patients (Fick et al., 2012). It is important to mention that despite the serious reactions (e.g. renal, pulmonary, hepatic failure and nerve adverse effects) in elderly patients, the occurrence of these AEs is very rare (Rego et al., 2016). Pulmonary syndrome can also appear in the first hours to the first week of drug prescription which is accompanied by fever, chills, and cough (Munoz-Davila, 2014). This serious and lethal reaction to nitrofurantoin was diagnosed on the first day of admission by Kanji et al. which was eventually treated by intubation and corticosteroids (Kanji et al., 2011). In this case report, the patient had received Trimethoprim 100 mg for cystitis which was replaced by NF 100 mg prescription due to lack of any progress. Following another course of NF, an 82-year-old man (with no history of underlying disease or smoking) presented to the hospital with symptoms of dyspnea, fever, and cough (Kanji et al., 2011). The incidence rate of such complications is less than 1% following frequent and long-term use of NF (for 6 months to years), and mostly in women younger than 60 years old (Guidance et al., 2002; Vahid and Wildemore, 2006; Fenton et al., 2008). According to the American Geriatrics Society Beers Criteria Update of 2012, using NF for a long time in elderly patients with renal failure must be banned. Moreover, some researchers prefer nitrofurantoin to be considered as a second treatment choice not a primary agent (Fick et al., 2012).

According to *in vitro* studies, long-term consumption of NF damages DNA by inhibiting DNA synthetase and chromosomal mutations. Moreover, Tumorigenicity of NF has not yet been precisely proven, but there is no doubt that NF has the ability to produce toxic metabolites (Lawson et al., 2016). Further studies are needed in this field.

7.1 Liver injury due to NF

Drug-induced liver injury (DILI) can result in an acute or chronic hepatitis-like syndrome. The acute form is usually associated with 1 to 2 weeks of NF treatment and is rare (approximately 0.3 per 100,000 prescriptions). Acute liver injury usually occurs within weeks of starting treatment with NF and may occur within weeks of completing a defined course of treatment. The more common form of hepatotoxicity is due to chronic prophylactic use, occurring in 1 in 1,500 people (National Institute of Diabetes and Digestive and Kidney Diseases (US), 2012). A wide range of hepatotoxicity has been reported in association with NF use, including acute hepatitis, granulomatous reaction, cholestasis, or autoimmune hepatitis to chronic active hepatitis that can lead to cirrhosis or death. The mechanism is not fully understood, but is thought to be due to an immunological reaction or a direct cytotoxic response. It has been hypothesized that prolonged therapy to NF, female gender, older age, and impaired renal function increase the risk of hepatotoxicity. Corticosteroids have been used in conjunction with stopping NF to treat severe cases (Sakaan et al., 2014). Genetic predisposition including HLA-B8 appears to increase the risk of NF-induced liver injury (Burgert et al., 1995; Stine and Northup, 2016). The correlation between the dose and NF-induced liver injury is contradictory. Lower and higher doses of NF are effective in long-term prophylaxis (Muller et al., 2017). Prophylactic choice in recurrent cystitis is controversial, but must be based on evaluating the patient, risks and benefits as well as hepatotoxicity (Byron, 2019). Although there are no guidelines,

10.3389/fcimb.2023.1148603

TABLE 2 Patients with liver disorders due to the use of this antibiotic.

Author	Age/G	Medication	Dosage	Pattern	Latency	Recovery	Other Drug histories	Ref
Luk T et al.	53y, F	Nitrofurantoin	100 mg twice daily	Portal-hepatic vasculature, hepatic nodularity, prominent parenchymal necrosis and collapse and accompanying cholestatic hepatic encephalopathy and ascites	12 M after restarting	Death	LOR	(Luk et al., 2021)
Wonnacott S et al.	24y, F	Nitrofurantoin	50 mg every six hours	Epigastric pain	Past 3 D	After stopping	Not Reported	(Wonnacott et al., 2022)
Appleyard S et al.	65y, F	Nitrofurantoin	50 mg daily	Chronic inflammatory cell infiltrate with interface hepatitis and piecemeal necrosis in portal areas	6 Y	After only one M of prednisolone	Mb, dosulepin, LAN, PCT, inhaled SALB, and intermittent Fcz	(Appleyard et al., 2010)
	42y, F	Nitrofurantoin	50 mg daily	Marked chronic inflammation within the portal tracts and extensive fibrosis, and some features of cirrhosis	2 Y	After a few weeks of corticosteroid medication	FolA	
	74y, F	Nitrofurantoin	100 mg daily	Striking lobular inflammation, confluent and bridging necrosis, syncytial giant cells, minimal portal inflammation and minimal plasma cells	2 Y	Seven months after initial presentation	Not Reported	
Khan F et al.	56y, F	Nitrofurantoin	Not reported	Abdominal pain	14 D	Over the course of 3 D	Not Reported	(Khan et al., 2019)
Koulaouzidis et al.	57y, F	Nitrofurantoin	100 mg at night	Hepatitis	16 M	At 4 M follow up	SALB inhaler, beclathasone inhaler, AM, and LAN	(Koulaouzidis et al., 2007)
Hydes T et al.	50y, F	Nitrofurantoin	50 mg once daily	Biliary obstruction autoimmune chronic active hepatitis with mild fibrosis, in keeping with immune-mediated drug-induced liver injury	12 M prior to admission	Two months later	PIO, Met, AM, PAX, LAN, CPM, BUP, MSO4, temazepam and LOS	(Hydes et al., 2014)
	75y, F	Nitrofurantoin	50 mg once daily	Chronic active hepatitis with a florid inflammatory cell infiltrates consistent with primary or drug-induced AIH on a background of cirrhosis	6 M later	One month postadmission, the patient developed a pulmonary embolus	PRO, ramipril, BFTZ and AT	
Carvalho de Matos A et al.	68y, F	Nitrofurantoin	100 mg once daily	Portal tract moderate mononuclear cell inflammatory infiltrate, with mild plasmacytes and some eosinophils, severe interface hepatitis with focal emperipolesis, periportal hepatocellular rosetting and ballooning, and ductular reaction. Severe panlobular bilirubinostasis, focal lobular necroinflammatory activity, and mild to moderate portal fibrosis (Masson trichrome) were also observed as well as focal periportal copper deposit (rhodamine).	2 Y	Not Reported	AX, clavulanic acid, and Cla	(Carvalho de Matos et al., 2022)

D, day; M, month; Y, year; NR, not reported; LOR, lorazepam; Mb, Mebeverine; LAN, Lansoprazole; PCT, Paracetamol; SALB, salbutamol; Fcz, Fluconazole; FolA, folic acid; PIO, pioglitazone; Met, Metformin; AM, amitriptyline; PAX, paroxetine; CPM, Chlorphenamine; BUP, Buprenorphine; MSO4, morphine sulfate; LOS, lactulose; PRO, Propranolol; BFTZ, Bendroflumethiazide; AT, atorvastatin; AX, amoxicillin; Cla, clarithromycin; AM, amitriptyline.

monitoring liver enzymes at 1 to 3 months interval during therapy is recommended (Sherigar et al., 2012). Table 2 mentions the cases suffering from liver disorders due to the use of this antibiotic.

7.2 Fever following the consumption of nitrofurantoin

Drug-induced fevers are independent-infection conditions which are classified as the miscellaneous group and may induce fever which is known as fever of unknown origin (FUO). FUO is characterized as temperatures higher than 38.3°C which will elapse more than two weeks after they appear (Haidar and Singh, 2022). According to estimations, 4-7% of empirical antibiotic therapy performed in hospitalized patients induce FUO (Patel and Gallagher, 2010; Vickery et al., 2022). NF in oral non-suspension form may be associated with FUO (Roth and Basello, 2003). However, the occurrence of these fevers is directly related to patients with an impaired immune system, especially neutropenic patients (Patel and Gallagher, 2010), so it is important to discuss it.

TABLE 3 Clinical efficacy of Nitrofurantoin on UTI by investigation randomized clinical trials (RCTs).

Author	Year	Sex	Age (years)	Clinical use (prophylaxis or treatment)	Route of nitrofurantoin administration	Dosage/ Duration	Outcomes (recovery/bacterial colony count) If prophylaxis, do experience UTI or not?	Adverse effects (AEs)	Cure rate/ Incidence rate (IR) of UTI	Ref
Bastawros et al.	2021	F	61.6 ± 11.7	PEP	Capsule	5 D/100 mg twice-OD	Did not reduce the risk of UTI	NS/GI manif (nausea)	IR= 18%	(Bastawros et al., 2021)
Akinci et al.	2021	46/59 (F: M)	4.8 ± 3.9	PEP	Capsule	Single dose of 1 mg/kg	Significant reduction in risk of UTI	NG	3.8% reduction	(Akinci et al., 2021)
Lavelle et al.	2020	F	61.7 ± 61.9	PEP	Capsule	100 mg OD	Did not reduce the risk of UTI	AEs were common/RES to NF were found	IR= 17.3%	(Lavelle et al., 2019)
Fisher et al.	2018	115/88 (F: M)	59.1	PEP	Capsule	50 mg once OD	Significant reduction in risk of UTI	NS/ GI, skin and fungal manif. RES to NF were found commonly	IR=1·3 cases per person; 0·52	(Fisher et al., 2018)
Pickard et al.	2018	115/88 (F: M)	X≥18	PEP	Capsule	50 mg once OD	Significant reduction in risk of UTI	RES to NF were found commonly	IR=0·52	(Pickard et al., 2018)
Huttner et al.	2018	F	X≥18	Treatment	Tablet	5 D/100 mg 3 times OD	Improvement in symptoms/less than 10^3 CFU/ml	NS/ GI manif	72%	(Huttner et al., 2018)
Gupta et al.	2007	F	18-45	Treatment	Tablet	5 D/100 mg twice OD	Improvement in symptoms/less than 10^5 CFU/ml	NS/ GI, UG and NEUR manif	88%	(Gupta et al., 2007)
Christiaens et al.	2002	F	15-55	Treatment	Capsule	3 D/100 mg four times OD	Improvement in symptoms/less than 10^5 CFU/ml	NS/ GI, skin, UG and NEUR manif	81%	(Christiaens et al., 2002)
Stein	1999	F	X≥12	Treatment	Capsule	7 D/100 mg	Improvement in symptoms/less than 10^5 CFU/ml	NS/ GI and UG manif	69.5%	(Stein, 1999)
Iravani et al.	1999	F	X≥18	Treatment	Capsule	7 D/100 mg	Improvement in symptoms/less than 10^3 CFU/ml	NS/ GI and UG manif	83%	(Iravani et al., 1999)
Hooton et al.	1995	F	X≥18	Treatment	Capsule	3 D/100 mg four times OD	less than 10^2 CFU/ml	NS/ GI, UG and NEUR manif	61%	(Hooton et al., 1995)

PEP, Antibiotic post-exposure prophylaxis before surgery; D, day; OD, Once a day; NS, Not serious; NG, Not given; UG, Urogenital; NEURm Neurological; manif, manifestations; RES, Resistance.

A case report published in 2022 observed clozapine-induced fever (CIF) in a 60-year-old woman who was receiving medication related to schizoaffective disorder (Vickery et al., 2022). Fever was reported in this patient following antibiotic therapy with three doses of NF. The fever did not follow a regular pattern. Also, laboratory analyses have shown an increase in eosinophil and lactate dehydrogenase. Apart from the 39.3 C fever, use of nitrofurantoin by the patient indicated the occurrence of bradycardia. Similar to these results, another cohort study indicated an increase in the incidence of fever and allergy in the group receiving nitrofurantoin compared to the sulfonamide group (Koch-Weser et al., 1971). Forster et al. attributed the occurrence of such an adverse reaction to the frequent and repeated use of NF in their case report (Forster et al., 2009).

8 The response spectrum of patients with UTI to the prescription of nitrofurantoin

As shown in Table 3, NF is well-tolerated by patients with UTI following both prophylaxis and treatment (Fisher et al., 2018). NF is often given as prophylaxis for 3 days. Cohort studies have indicated the beneficial effect of nitrofurantoin on patients, like a study by Huttner et al. who reported the cure rate of nitrofurantoin at 70% (Huttner et al., 2018). NF is safe and effective for short-term treatment at younger ages which is in contrast with the low effect of nitrofurantoin in patients who use nitrofurantoin for the treatment of UTI for a longer period of time (Gardiner et al., 2019). Antibiotic treatment in symptomatic patients (i.e., UTI caused by uropathogens) seems more successful than in asymptomatic patients, probably due to the effective targeting of pathogens by antibiotics. However, in asymptomatic patients only uropathogens are colonized (do not show any activity), and the treatment is less successful. Having a series of virulence factors in bacteria which make them a target, tolerance in bacteria in the presence of antibiotics, and inaccessible areas to antibiotics activity (e.g., presence of uropathogens in the bladder) are the most important reasons for the ineffectiveness of antibiotics in preventing the occurrence of UTI (Fisher et al., 2018). Today, possible adverse effects of prophylaxis of nitrofurantoin in the emergence of antibiotic-resistant strains have become a concern as we will discuss in the next section (Goff and Mendelson, 2017).

9 Nitrofurantoin-resistant bacteria in patients with UTI

As shown in Table 2, improvement of symptoms in symptomatic UTI patients, incidence rate of UTI in prophylaxis use of nitrofurantoin and occurrence of antibiotic resistance are important outcomes in assessing the effects of nitrofurantoin. Generally, resistance to nitrofurantoin is less common even in drug-resistant strains (Sanchez et al., 2016). However, a few strains of E. coli isolated from urine and K. pneumoniae producing ESBL enzyme (Procop et al., 2003) have been reported to show resistance to nitrofurantoin. The prevalence of NF resistant in E. coli strains isolated from UTI cases in the United States and France has been reported at 1.1% and 1.8%, respectively (Zhanel et al., 2005; Honderlick et al., 2006). However, 99% of E. coli, 69% of Klebsiella strains and 63% of Enterobacter strains are still sensitive to nitrofurantoin, while the resistance level of conventional drugs used in UTI against E.coli such as ciprofloxacin and trimethoprim/sulfamethoxazole is reported at 25-29% (Vs 2.3%) which is more than nitrofurantoin's resistance rate (Mazzulli et al., 2001; Kashanian et al., 2008). On the other hand, antibiotics such as Fluoroquinolones and Cotrimoxazole, which were conventionally prescribed for the treatment of uncomplicated UTI, today seems to have lost their effectiveness due to the emergence of antibiotic resistance, so nitrofurantoin and fosfomycin are suitable alternatives due to less drug-resistant cases (Munoz-Davila, 2014). In addition to the clinical use of nitrofurantoin, antibiotic prophylaxis before surgery outcomes for prevention of UTI is shown in Table 3. Clinical trials have shown that the use of nitrofurantoin in the group that used this antibiotic as prophylaxis, compared to the control group which used conventional drugs for UTI such as trimethoprim and co-trimoxazole, led to more antibiotic resistance. This was the most important result of the study by Fisher et al. in which patients had received nitrofurantoin for 9-12 months before surgery (Fisher et al., 2018). Moreover, these findings are consistent with a study by Pickard et al. who found that bacteria isolated from patients who had taken oral nitrofurantoin prophylactically showed more antibiotic resistance (Pickard et al., 2018). Researchers pointed to complications caused by the long-term use of nitrofurantoin; therefore, the use of this drug as a preventive tool was avoided and fluoroquinolones were prescribed instead which led to an increase in fluoroquinolone-resistant strains (Slekovec et al., 2014). Therefore, it is advisable the use of nitrofurantoin be limited to the treatment of UTI cases which show resistance to other antibiotics.

10 Combined effects of nitrofurantoin with different antimicrobial agents

Extensive experimental use of antibiotics to treat various infectious diseases has increased antibacterial resistance among many strains of pathogenic bacteria worldwide (Ayaz et al., 2019). Combining antibiotic treatments with other treatments is becoming an increasingly important strategy for treating many of these infections, especially those caused by pathogens with antibiotic resistance (Fatsis-Kavalopoulos et al., 2020). One of the advantageous features of combination therapies is the synergism effect. The therapeutic effect is greater when an antibiotic is combined *in vitro* than the sum of each drug (Coates et al., 2020). The synthetic antibiotic NF is used to treat lower urinary tract infections orally (Dos Santos et al., 2021). By reviewing the studies conducted so far, which are summarized in Table 4, we found that

TABLE 4 Results of the combinations of nitrofurantoin with other Antibiotics.

Authors/References	Year	Study	Country	Pathogen	Source of pathogen	Combination antibiotics	Effects (synergistic, antagonistic, additive or no effect)
R Daza et al. (Daza et al., 1977)	1997	Mic St	Spain	Gram-Negative bacilli	Pathological products of hospital	NF +FOS	No effect
JL Descourouez et al. (Descourouez et al., 2013)	2013	Mic St	USA	VRE	USI	NF +FOS	No effect
Nikos Fatsis Kavalopouos et al. (Fatsis-Kavalopoulos et al., 2020)	2020	*CombiANT methodology	Sweden	E. coli	UTI	NF +CIP	Additive
						NF +TMP	Synergistic
						NF +MEC	Antagonistic
Alice Zhou et al. (Zhou et al., 2015)	2015	Mic St	USA	Escherichia coli mutants**	–	NF +VAN	Synergistic
Peng Cui et al. (Cui et al., 2016)	2016	Mic St and Ani St	China	E. coli Persisters	UTI	NF +COL	No effect *in vitro*, Additive *in vivo*
Abdulkareem H.ABD et al. (ABD et al., 2014)	2014	Mic St	Iraq	E. coli	UTI	NF +CN	Synergistic
						NF +CIP	No effect
Zi-Xing Zhong et al. (Zhong et al., 2020)	2020	Mic St and Ani St	China	MDR UPEC	UTI	NF +AK	Synergistic

Nitrofurantoin (NF), Fosfomycin (FOS), Ciprofloxacin (CIP), Gentamycin (CN), Vancomycin (VAN), amikacin (AK), colistin (COL), mecillinam (MEC), ciprofloxacin (CIP), trimethoprim (TMP), Microbiological Study (Mic St), animal study (Ani St), Vancomycin-Resistant Enterococcus faecium (VRE), Urinary Tract Infection (UTI), Urinary Stent Infections (USI), Uropathogenic E. coli (UPEC).
*CombiANT methodology: a 3D-printed agar plate insert that produces defined diffusion landscapes of 3 antibiotics, permitting synergy quantification between all 3 antibiotic pairs with a single test.
** mutant E. coli strains (dcd and surA mutants) that have increased sensitivity to VAN.

the combination of NF and other antibiotics has not been extensively studied. Moreover, most of the studies conducted in this field have been done in laboratory conditions. The results of these studies show that in most cases, the effect of NF increases in combination with other antibiotics (except in combination with mecillinam) (Fatsis-Kavalopoulos et al., 2020). It seems that NF antimicrobial combination therapy is superior to monotherapy, but using drug combinations has many challenges, including

TABLE 5 The effect of nitrofurantoin combination with some plant extracts and nanoparticles.

Authors/References	Year	Country	Pathogen	Combined treatment		Effects (synergistic, antagonistic, additive or no effect)
				Plant extract	Nanoparticles	
Ngalah Bidii Stephen et al. (Frank et al., 2022)	2022	Germany	Serratia marcescens	NF + Iso[1]		Synergistic
Shatha Mousa mlaghee Al-safi et al. (Al-safi et al., 2020)	2022	Iraq	salmonella	NF + Phoenix dactylifera[2]		Synergistic
Ali M Khlaifat et al. (Khlaifat et al., 2019)	2022	Jordan	Pseudomonas aeruginosa		NF + AgNPs	Synergistic
			Pseudomonas aeruginosa	NF + C. Sempervirens and A.graveolens[3]		Synergistic
			E. aerogenes	NF + C. Sempervirens and A.graveolens[3]		Synergistic
			S. aureus	NF + A.graveolens[3]	NF + AgNPs	Additive
Rajendran Mala et al. (Mala et al., 2017)	2017	India	Escherichia coli		NF + SNPs	Synergistic

[1] Isothiocyanates: Natural plant products generated by enzymatic hydrolysis of glycosylates; [2] Oily extraction of leaves; [3] Essential oils; Silver nanoparticles (AgNPs), Silver nanoparticles (SNPs).

simultaneous assessment of distribution and tissue penetration, among others (Zhong et al., 2020).

Another noteworthy point is that in recent years, researchers have done considerable research on the effect of various bioactive compounds in combination with antibiotics. The scientific and medical community has been exploring the possibility of creating synergistic therapeutic regimens by combining plant extracts and nanoparticles [especially silver nanoparticles (AgNPs)]. There is growing evidence that the use of these substances enhances the antibacterial properties of conventional antibiotics, repurposing them instead of replacing them (Cheesman et al., 2017; Vazquez-Muñoz et al., 2019). Combinations of natural compounds may make it possible for antimicrobial agents to interact better with their targets within pathogens and prevent resistance. Such a strategy can reduce toxicity, because lower concentrations of both agents can be used in this method (Betoni et al., 2006; Sanhueza et al., 2017). Furthermore, because nanoparticles are so small, they stick to the cell wall in addition to damaging it. For this reason, NPs are less resistant to antibiotics than antibiotics (Betoni et al., 2006). The antimicrobial action of NPs is also influenced by metal ions and reactive oxygen species (Khleifat et al., 2022). So far, many studies have been conducted on the combined effect of NF with these substances, and we mentioned a few of them (Table 5). The results of these studies showed that the combination of NF with nanomaterials or plant extracts has increased the effectiveness of this antibiotic. But since in other studies (Moussaoui and Alaoui, 2016; Paralikar et al., 2019), some materials in combination with antibiotics had an antagonistic effect, it is necessary to conduct more studies on the combination of substances with this antibiotic. Also, more studies on the mechanism of the antagonistic effect of these substances are necessary.

Generally, understanding the mechanism of action of antibiotics, AgNPs, plants and combined treatments allows predicting more feasible treatments or designing new ones more efficiently. Even if some aspects of the mechanism of action remain unknown, these results provide a more effective way to fight infectious diseases (Vazquez-Muñoz et al., 2019).

11 Conclusion

In this review, our goal was to obtain a comprehensive picture by considering the clinical use of nitrofurantoin and its adverse effects to inform physicians to manage UTI patients under long-term nitrofurantoin therapy. Nitrofurantoin should not be recommended for long-term prophylaxis in patients with UTI, especially elderly patients. The intention is not to discard nitrofurantoin prescription, but urologists must use nitrofurantoin as the most effective drug on acute UTI. Therefore, a series of supervisions and criteria regarding the prescription of nitrofurantoin in cases of chronic UTI are needed.

Author contributions

AD and RG conceived, designed and supervised the study. AD and MM contributed to data collection, interpretation and final approval of data for the work. SD and FG developed the first and final draft of the manuscript. SS and PK developed the second draft of the manuscript. All figures and tables were designed and checked by MM, EB and TD. All authors reviewed and contributed to the revisions and finalized the drafts.

Funding

This study was supported by a grant from Behbahan Faculty of Medical Sciences [grant number 401082].

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

Abd, A. H., Abd, M. H., and Obed, F. A. (2014). Efficacy of combination of nitrofurantoin with gentamicin, and ciprofloxacin against resistant *E. Coli* isolated from patients with urinary tract infections: *in vitro* study. *Mintage J. Pharm. Med. Sci.* 3 (2), 5–9.

Ahmed, S. S., Shariq, A., Alsalloom, AA., Babikir, IH., and Alhomoud, B. N. (2019). Uropathogens and their antimicrobial resistance patterns: relationship with urinary tract infections. *Int. J. Health Sci. (Qassim)* 13 (2), 48–55.

Akinci, A., Kubilay, E., Solak, V. T., Karaburun, M. C., Baklaci, C. U., Aydoğ, E., et al. (2021). Effect of continuous antibiotic prophylaxis in children with postoperative JJ stents: a prospective randomized study. *J. Pediatr. Urol.* 17 (1), 89–94. doi: 10.1016/j.jpurol.2020.10.004

Al-safi, S. M., fadhil hasson Al-Shabaa, K., AL-Zeiny, S. S., and Al-Dhalimy, A. M. (2020). Synergistic and antimicrobial effects of leaves oil (phoenix dactylifera) agonist gram negative bacteria. *System. Rev. Pharm.* 11 (11), 177–181.

Andriole, V. T. (1985). "Urinary tract agents: quinolones, nitrofurantoin, and methenamine," in *Principles and practice of infectious diseases* (New York: John Wiley and Sons), 244–253.

Appleyard, S., Saraswati, R., and Gorard, D. A. (2010). Autoimmune hepatitis triggered by nitrofurantoin: a case series. *J. Med. Case Rep.* 4 (1), 1–5. doi: 10.1186/1752-1947-4-311

Ayaz, M., Ullah, F., Sadiq, A., Ullah, F., Ovais, M., Ahmed, J., et al. (2019). Synergistic interactions of phytochemicals with antimicrobial agents: potential strategy to counteract drug resistance. *Chemico Biol. Interact.* 308, 294–303. doi: 10.1016/j.cbi.2019.05.050

Bastawros, D., Kaczmarski, K., Zhao, J., Bender, R., Myers, E., and Tarr, M. E. (2021). Twice-daily nitrofurantoin administration following short-term transurethral

catheterization after pelvic reconstructive surgery: a randomized clinical trial. *Female Pelvic Med. Reconstr. Surg.* 27 (3), 202–207. doi: 10.1097/SPV.0000000000000977

Beckett, A., and Robinson, A. E. (1958). The reaction of nitrofurans with bacteria–III. reduction of a series of antibacterial nitrofurans (Type b compounds) by aerobacter aerogenes. *J. Medicinal Chem.* 1 (2), 155–164.

Betoni, J. E. C., Mantovani, R. P., Barbosa, L. N., Di Stasi, L. C., and Fernandes Junior, A. (2006). Synergism between plant extract and antimicrobial drugs used on staphylococcus aureus diseases. *Memórias do Instituto Oswaldo Cruz* 101, 387–390. doi: 10.1590/S0074-02762006000400007

Bryce, A., Hay, A. D., Lane, I. F., Thornton, H. V., Wootton, M., and Costelloe, C. (2016). Global prevalence of antibiotic resistance in paediatric urinary tract infections caused by Escherichia coli and association with routine use of antibiotics in primary care: systematic review and meta-analysis. *Bmj* 352, i939. doi: 10.1136/bmj.i939

Burgert, S. J., Burke, J. P., and Box, T. D. (1995). Reversible nitrofurantoin-induced chronic active hepatitis and hepatic cirrhosis in a patient awaiting liver transplantation. *Transplantation* 59 (3), 448–449. doi: 10.1097/00007890-199502000-00029

Byron, J. K. (2019). Urinary tract infection. *Vet. Clin. North Am. Small Anim. Pract.* 49 (2), 211–221. doi: 10.1016/j.cvsm.2018.11.005

Carvalho de Matos, A., Pais Macedo, C., Afonso Mendes, P., Cipriano, MA., Paiva, A., and Simão, A. (2022). Concomitant nitrofurantoin-induced autoimmune hepatitis and interstitial lung disease. *GE Port J. Gastroenterol.* 29 (4), 273–279.

Cheesman, M. J., Ilanko, A., Blonk, B., and Cock, IE. (2017). Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution? *Pharmacogn. Rev.* 11 (22), 57.

Chew, A. B., Suda, KJ., Patel, U. C., Fitzpatrick, M. A., Ramanathan, S., Burns, S. P., et al. (2019). Long-term prescribing of nitrofurantoin for urinary tract infections (UTI) in veterans with spinal cord injury (SCI). *J. Spinal Cord Med.* 42 (4), 485–493.

Christiaens, T. C., De Meyere, M., Verschraegen, G., Peersman, W., Heytens, S., and De Maeseneer, J. M. (2002). Randomised controlled trial of nitrofurantoin versus placebo in the treatment of uncomplicated urinary tract infection in adult women. *Br. J. Gen. Pract.* 52 (482), 729–734.

Coates, A. R., Hu, Y., Holt, J., and Yeh, P. (2020). Antibiotic combination therapy against resistant bacterial infections: synergy, rejuvenation and resistance reduction. *Expert Rev. Anti Infect. Ther.* 18 (1), 5–15.

Conklin, J. D. (1978). The pharmacokinetics of nitrofurantoin and its related bioavailability. *Antibiot. Chemother. (1971)* 25, 233–252.

Cui, P., Niu, H., Shi, W., Zhang, S., Zhang, H., Margolick, J., et al. (2016). Disruption of membrane by colistin kills uropathogenic Escherichia coli persisters and enhances killing of other antibiotics. *Antimicrob. Agents Chemother.* 60 (11), 6867–6871.

Cunha, B. A. (1988). Nitrofurantoin–current concepts. *Urology* 32 (1), 67–71. doi: 10.1016/0090-4295(88)90460-8

Cunha, B. A. (1989). Nitrofurantoin: an update. *Obstet. Gynecol. Surv.* 44 (5), 399–406. doi: 10.1097/00006254-198905000-00034

Cunha, B. (2006). New uses for older antibiotics: nitrofurantoin, amikacin, colistin, polymyxin b, doxycycline, and minocycline revisited. *Med. Clinics North America* 90, 1089–1107. doi: 10.1016/j.mcna.2006.07.006

Cunha, B. A., Cunha, C. B., Lam, B., Giuga, J., Chin, J., Zafonte, V. F., et al. (2017). Nitrofurantoin safety and effectiveness in treating acute uncomplicated cystitis (AUC) in hospitalized adults with renal insufficiency: antibiotic stewardship implications. *Eur. J. Clin. Microbiol. Infect. Dis.* 36 (7), 1213–1216. doi: 10.1007/s10096-017-2911-1

Daza, R., Moreno-Lopez, M., and Damaso, D. (1977). Interactions of fosfomycin with other antibiotics. *Chemotherapy* 23 (Suppl. 1), 86–92. doi: 10.1159/000222031

Descourouez, J. L., Jorgenson, M. R., Wergin, J. E., and Rose, W. E. (2013). Fosfomycin synergy in vitro with amoxicillin, daptomycin, and linezolid against vancomycin-resistant enterococcus faecium from renal transplant patients with infected urinary stents. *Antimicrob. Agents Chemother.* 57 (3), 1518–1520.

Dos Santos, C., Dos Santos, L. S., and Franco, O. L. (2021). Fosfomycin and nitrofurantoin: classic antibiotics and perspectives. *J. Antibiot. (Tokyo)* 74 (9), 547–558.

Fatsis-Kavalopoulos, N., Roemhild, R., Tang, P.-C., Kreuger, J., and Andersson, D. I. (2020). CombiANT: antibiotic interaction testing made easy. *PloS Biol.* 18 (9), e3000856.

Fenton, M. E., Kanthan, R., and Cockcroft, D. W. (2008). *Nitrofurantoin-associated bronchiolitis obliterans organizing pneumonia: report of a case*. *Can. Respir. J.* 15(6), 311–312.

Fick, D. M., Cooper, J. W., Wade, W. E., Waller, J. L., Maclean, J. R., and Beers, MH. (2003). Updating the beers criteria for potentially inappropriate medication use in older adults: results of a US consensus panel of experts. *Arch. Intern. Med.* 163 (22), 2716–2724.

Fick, D., Semla, T., Beizer, J., Brandt, N., Dombrowski, R., DuBeau, C. E., et al. (2012). American geriatrics society updated beers criteria for potentially inappropriate medication use in older adults. *J. Am. Geriatr. Soc.* 60 (4), 616–631. doi: 10.1111/j.1532-5415.2012.03923.x

Fisher, H., Oluboyede, Y., Chadwick, T., Abdel-Fattah, M., Brennand, C., Fader, M., et al. (2018). Continuous low-dose antibiotic prophylaxis for adults with repeated urinary tract infections (AnTIC): a randomised, open-label trial. *Lancet Infect. Dis.* 18 (9), 957–968. doi: 10.1016/S1473-3099(18)30279-2

Flores-Mireles, A. L., Walker, J. N., Caparon, M., and Hultgren, S. J. (2015). Urinary tract infections: epidemiology, mechanisms of infection and treatment options. *Nat. Rev. Microbiol.* 13 (5), 269–284. doi: 10.1038/nrmicro3432

Forster, C. J., Cohee, B. M., Wood-Morris, R. N., and Hartzell, J. D. (2009). Nitrofurantoin-induced systemic inflammatory response syndrome. *Am. J. Med. Sci.* 338 (4), 338–340. doi: 10.1097/MAJ.0b013e3181abd9f6

Frank, U., Ngalah, B. S., Börner, K., Finkbeiner, J., Harter, A., and Biehler, K. (2022). Combination of isothiocyanates and antibiotics increases susceptibility against Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis and Serratia marcescens. *Escherichia Coli Klebsiella Pneumoniae Proteus Mirabilis Serratia Marcescens* p, 19. doi: 10.2139/ssrn.4200934

Fransen, F., Melchers, M. J., Meletiadis, J., and Mouton, J. W. (2016). Pharmacodynamics and differential activity of nitrofurantoin against ESBL-positive pathogens involved in urinary tract infections. *J. Antimicrob. Chemother.* 71 (10), 2883–2889. doi: 10.1093/jac/dkw212

Fransen, F., Melchers, M. J. B., Lagarde, C. M. C., Meletiadis, J., and Mouton, J. W. (2017). Pharmacodynamics of nitrofurantoin at different pH levels against pathogens involved in urinary tract infections. *J. Antimicrob. Chemother.* 72 (12), 3366–3373. doi: 10.1093/jac/dkx313

Garau, J. (2008). Other antimicrobials of interest in the era of extended-spectrum β-lactamases: fosfomycin, nitrofurantoin and tigecycline. *Clin. Microbiol. Infect.* 14, 198–202. doi: 10.1111/j.1469-0691.2007.01852.x

Gardiner, B. J., Stewardson, A., Abbott, I., and Peleg, A. (2019). Nitrofurantoin and fosfomycin for resistant urinary tract infections: old drugs for emerging problems. *Aust. Prescr.* 42 (1), 14–19. doi: 10.18773/austprescr.2019.002

Gautam, G., Gogoi, S., Saxena, S., Kaur, R., and Dhakad, M. S. (2021). Nitrofurantoin susceptibility pattern in gram-negative urinary isolates: in need of increased vigilance. *J. Lab. Physicians* 13 (3), 252–256. doi: 10.1055/s-0041-1731106

Ghanavati, R., Emaneini, M., Kalantar-Neyestanaki, D., Maraji , A. S., Dalvand, M., Beigverdi, R., et al. (2018a). Clonal relation and antimicrobial resistance pattern of extended-spectrum β-lactamase- and AmpC β-lactamase-producing Enterobacter spp. isolated from different clinical samples in Tehran, Iran. *Revista da Sociedade Brasileira de Medicina Tropical* 51, 88–93. doi: 10.1590/0037-8682-0227-2017

Ghanavati, R., Ohadi, E., Kazemian, H., Yazdani, F., Torki, A., Kalani, B. S., et al. (2018b). Evaluation of fosfomycin activity against extended spectrum beta lactamase (ESBL) producing Enterobacteriaceae isolated from three centers of Tehran, Iran. Recent patents on anti-infective. *Drug Discov.* 13(2), 180–186. doi: 10.2174/1574891X13666180517075803

Gleckman, R., Alvarez, S., and Joubert, D. W. (1979). Drug therapy reviews: nitrofurantoin. *Am. J. Hosp. Pharm.* 36 (3), 342–351. doi: 10.1093/ajhp/36.3.342

Goff, D., and Mendelson, M. (2017). Antibiotic stewardship hits a home run for patients. *Lancet Infect. Dis.* 17 (9), 892–893. doi: 10.1016/S1473-3099(17)30344-4

Guidance, R., Recalls, P., Reporting, S., Update, P., Advisory, M. A., Monitoring, S., et al. (2002). Pulmonary reactions with nitrofurantoin. *Prescriber Update* 23 (2), 24–25.

Gupta, K., Hooton, T. M., Roberts, P. L., and Stamm, W. E. (2007). Short-course nitrofurantoin for the treatment of acute uncomplicated cystitis in women. *Arch. Intern. Med.* 167 (20), 2207–2212. doi: 10.1001/archinte.167.20.2207

Gupta, K., Hooton, T. M., Naber, K. G., Wullt, B., Colgan, R., Miller, L. G., et al. (2011). International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: a 2010 update by the infectious diseases society of America and the European society for microbiology and infectious diseases. *Clin. Infect. Dis.* 52 (5), e103–e120.

Haidar, G., and Singh, N. (2022). Fever of unknown origin. *New Engl. J. Med.* 386 (5), 463–477. doi: 10.1056/NEJMra2111003

Ho, P. L., Ng, K. Y., Lo, W. U., Law, P. Y., Lai, E. L., Wang, Y., et al. (2016). Plasmid-mediated OqxAB is an important mechanism for nitrofurantoin resistance in Escherichia coli. *Antimicrob. Agents Chemother.* 60 (1), 537–543. doi: 10.1128/AAC.02156-15

Honderlick, P., Cahen, P., Gravisse, J., and Vignon, D. (2006). [Uncomplicated urinary tract infections, what about fosfomycin and nitrofurantoin in 2006?]. *Pathol. Biol. (Paris)* 54 (8-9), 462–466. doi: 10.1016/j.patbio.2006.07.016

Hooton, T. M., Winter, C., Tiu, F., and Stamm, W. E. (1995). Randomized comparative trial and cost analysis of 3-day antimicrobial regimens for treatment of acute cystitis in women. *Jama* 273 (1), 41–45. doi: 10.1001/jama.1995.03520250057034

Hrbacek, J., Cermak, P., and Zachoval, R. (2020). Current antibiotic resistance trends of uropathogens in central Europe: survey from a tertiary hospital urology department 2011-2019. *Antibiotics (Basel)* 9 (9), 630. doi: 10.3390/antibiotics9090630

Huttner, A., Verhaegh, E. M., Harbarth, S., Muller, A. E., Theuretzbacher, U., and Mouton, J. W. (2015). Nitrofurantoin revisited: a systematic review and meta-analysis of controlled trials. *J. Antimicrob. Chemother.* 70 (9), 2456–2464. doi: 10.1093/jac/dkv147

Huttner, A., Kowalczyk, A., Turjeman, A., Babich, T., Brossier, C., Eliakim-Raz, N., et al. (2018). Effect of 5-day nitrofurantoin vs single-dose fosfomycin on clinical resolution of uncomplicated lower urinary tract infection in women: a randomized clinical trial. *Jama* 319 (17), 1781–1789. doi: 10.1001/jama.2018.3627

Hydes, T., Wright, M., Jaynes, E., and Nash, K. (2014). Nitrofurantoin immune-mediated drug-induced liver injury: a serious complication of a commonly prescribed medication. *Case Rep.* 2014, bcr2013203136. doi: 10.1136/bcr-2013-203136

Iravani, A., Klimberg, I., Briefer, C., Munera, C., Kowalsky, S. F., and Echols, R. M. (1999). A trial comparing low-dose, short-course ciprofloxacin and standard 7 day

therapy with co-trimoxazole or nitrofurantoin in the treatment of uncomplicated urinary tract infection. *J. Antimicrob. Chemother.* 43 Suppl A, 67–75.

Jaffe, J. M., and JM, J. (1975). Effect of propantheline on nitrofurantoin absorption. *J. Pharm. Sci.* 64 (10), 1729-1730.

Kanji, Z., Su, V. C., and Mainra, R. (2011). Nitrofurantoin-induced pulmonary reaction involving respiratory symptoms: case report. *Can. J. Hosp. Pharm.* 64 (5), 362–365. doi: 10.4212/cjhp.v64i5.1072

Kashanian, J., Hakimian, P., Blute, M. Jr., Wong, J., Khanna, H., Wise, G., et al. (2008). Nitrofurantoin: the return of an old friend in the wake of growing resistance. *BJU Int.* 102 (11), 1634–1637. doi: 10.1111/j.1464-410X.2008.07809.x

Kazemian, H., Heidari, H., Ghanavati, R., Ghafourian, S., Yazdani, F., Sadeghifard, N., et al. (2019). Phenotypic and genotypic characterization of ESBL-, AmpC-, and carbapenemase-producing Klebsiella pneumoniae and Escherichia coli isolates. *Med. Princ. Pract.* 28 (6), 547–551. doi: 10.1159/000500311

Khan, F., Davis, M., and Khan, N. (2019). 2467 recognizing macrobid as a potential cause for major liver injury. *Off. J. Am. Coll. Gastroenterol. ACG* 114, S1360–S1361. doi: 10.14309/01.ajg.0000599400.11947.cc

Khlaifat, A. M., Al-limoun, M. O., Khleifat, K. M., Al Tarawneh, A. A., Qaralleh, H., Rayyan, E. A., et al (2019). Antibacterial synergy of tritirachium oryzae-produced silver nanoparticles with different antibiotics and essential oils derived from cupressus sempervirens and asteriscus graveolens (Forssk). *Trop. J. Pharm. Res.* 18 (12), 2605–2616.

Khleifat, K., Qaralleh, H., and Al-Limoun, M. (2022). Antibacterial activity of silver nanoparticles synthesized by aspergillus flavusand its synergistic effect with antibiotics. *J. Pure Appl. Microbiol* 16 (3), 1722-1735. doi: 10.22207/JPAM.16.3.13

Khoshbayan, A., Golmoradi Zadeh, R., Taati Moghadam, M., Mirkalantari, S., and Darbandi, A. (2022). Molecular determination of O25b/ST131 clone type among extended spectrum β-lactamases production Escherichia coli recovering from urinary tract infection isolates. *Ann. Clin. Microbiol. Antimicrob.* 21 (1), 35. doi: 10.1186/s12941-022-00526-2

Klein, R. D., and Hultgren, S. J. (2020). Urinary tract infections: microbial pathogenesis, host-pathogen interactions and new treatment strategies. *Nat. Rev. Microbiol.* 18 (4), 211–226. doi: 10.1038/s41579-020-0324-0

Koch-Weser, J., Sidel, VW., Dexter, M., Parish, C., Finer, D. C., and Kanarek, P. (1971). Adverse reactions to sulfisoxazole, sulfamethoxazole, and nitrofurantoin. manifestations and specific reaction rates during 2,118 courses of therapy. *Arch. Intern. Med.* 128 (3), 399–404. doi: 10.1001/archinte.1971.00310210075006

Komagamine, J., Yabuki, T., Noritomi, D., and Okabe, T. (2022). Prevalence of and factors associated with atypical presentation in bacteremic urinary tract infection. *Sci. Rep.* 12 (1), 5197.

Komp Lindgren, P., Klockars, O., Malmberg, C., and Cars, O. (2015). Pharmacodynamic studies of nitrofurantoin against common uropathogens. *J. Antimicrob. Chemother.* 70 (4), 1076–1082. doi: 10.1093/jac/dku494

Kostakioti, M., Hultgren, S. J., and Hadjifrangiskou, M. (2012). Molecular blueprint of uropathogenic Escherichia coli virulence provides clues toward the development of anti-virulence therapeutics. *Virulence* 3 (7), 592–594. doi: 10.4161/viru.22364

Koulaouzidis, A., Bhat, S., Moschos, J., Tan, C., and De Ramon, A. (2007). Nitrofurantoin-induced lung-and hepatotoxicity. *Ann. Hepatol.* 6 (2), 119–121. doi: 10.1016/S1665-2681(19)31944-1

Lavelle, E. S., Alam, P., Meister, M., Florian-Rodriguez, M., Elmer-Lyon, C., Kowalski, J., et al. (2019). Antibiotic prophylaxis during catheter-managed postoperative urinary retention after pelvic reconstructive surgery: a randomized controlled trial. *Obstet. Gynecol.* 134 (4), 727–735. doi: 10.1097/AOG.0000000000003462

Lawson, P. A., Citron, D. M., Tyrrell, K. L., and Finegold, S. M. (2016). Reclassification of clostridium difficile as clostridioides difficile (Hall and O'Toole 1935) Prévot 1938. *Anaerobe* 40, 95–99. doi: 10.1016/j.anaerobe.2016.06.008

Le, V. V. H., and Rakonjac, J. (2021). Nitrofurans: revival of an "old" drug class in the fight against antibiotic resistance. *PloS Pathog.* 17 (7), e1009663. doi: 10.1371/journal.ppat.1009663

Long, B., and Koyfman, A. (2018). The emergency department diagnosis and management of urinary tract infection. *Emerg. Med. Clin. North Am.* 36 (4), 685–710. doi: 10.1016/j.emc.2018.06.003

Luk, T., Edwards, B. D., Bates, D., Evernden, C., and Edwards, J. (2021). Nitrofurantoin-induced liver failure: a fatal yet forgotten complication. *Can. Family Physician* 67 (5), 342–344. doi: 10.46747/cfp.6705342

Mala, R., Annie Aglin, A., Ruby Celsia, A. S., Geerthika, S., Kiruthika, N., VazagaPriya, C., et al. (2017). Foley Catheters functionalised with a synergistic combination of antibiotics and silver nanoparticles resist biofilm formation. *IET Nanobiotechnol.* 11 (5), 612–620. doi: 10.1049/iet-nbt.2016.0148

Matthews, P. C., Barrett, L. K., Warren, S., Stoesser, N., Snelling, M., Scarborough, M., et al. (2016). Oral fosfomycin for treatment of urinary tract infection: a retrospective cohort study. *BMC Infect. Dis.* 16 (1), 556. doi: 10.1186/s12879-016-1888-1

Mazzulli, T., Skulnick, M., Small, G., Marshall, W., Hoban, D., Zhanel, G., et al. (2001). Susceptibility of community gram-negative urinary tract isolates to mecillinam and other oral agents. *Can. J. Infect. Dis. = J. canadien Des. maladies infectieuses* 12, 289–292.

McKinnell, J. A., Stollenwerk, N. S., Jung, C. W., and Miller, L. G. (2011). Nitrofurantoin compares favorably to recommended agents as empirical treatment of uncomplicated urinary tract infections in a decision and cost analysis. *Mayo Clin. Proc.* 86 (6), 480–488. doi: 10.4065/mcp.2010.0800

McOsker, C. C., and Fitzpatrick, P. M. (1994). Nitrofurantoin: mechanism of action and implications for resistance development in common uropathogens. *J. Antimicrob. Chemother.* 33 Suppl A, 23–30. doi: 10.1093/jac/33.suppl_A.23

Mohebi, S., Hossieni Nave, H., Norouzi, A., Kandehkar Gharaman, M., Taati Moghadam, M., et al. (2016). Detection of extended spectrum beta lactamases on class I integron in Escherichia coli isolated from clinical samples. *J. Mazandaran Univ. Med. Sci.* 26 (138), 66–76.

Moussaoui, F., and Alaoui, T. (2016). Evaluation of antibacterial activity and synergistic effect between antibiotic and the essential oils of some medicinal plants. *Asian Pacif. J. Trop. Biomed.* 6 (1), 32–37. doi: 10.1016/j.apjtb.2015.09.024

Muller, A. E., Verhaegh, E. M., Harbarth, S., Mouton, J. W., and Huttner, A. (2017). Nitrofurantoin's efficacy and safety as prophylaxis for urinary tract infections: a systematic review of the literature and meta-analysis of controlled trials. *Clin. Microbiol. Infect.* 23 (6), 355–362. doi: 10.1016/j.cmi.2016.08.003

Munoz-Davila, M. J. (2014). Role of old antibiotics in the era of antibiotic resistance. highlighted nitrofurantoin for the treatment of lower urinary tract infections. *Antibiotics (Basel)* 3 (1), 39–48.

Naber, K. G., Schito, G., Botto, H., Palou, J., and Mazzei, T. (2008). Surveillance study in Europe and Brazil on clinical aspects and antimicrobial resistance epidemiology in females with cystitis (ARESC): implications for empiric therapy. *Eur. Urol.* 54 (5), 1164–1175. doi: 10.1016/j.eururo.2008.05.010

Naggar, V. F., and Khalil, S. A. (1979). Effect of magnesium trisilicate on nitrofurantoin absorption. *Clin. Pharmacol. Ther.* 25 (6), 857–863. doi: 10.1002/cpt1979256857

Nakagawa, S., Kurimoto, Y., Ezumi, M., Nakatani, K., Mizunaga, S., and Yamagishi, Y. (2021). In vitro and in vivo antibacterial activity of nitrofurantoin against clinical isolates of e. coli in Japan and evaluation of biological cost of nitrofurantoin resistant strains using a mouse urinary tract infection model. *J. Infect. Chemother.* 27 (2), 250–255.

National Institute of Diabetes and Digestive and Kidney Diseases (US) (2017). *LiverTox: clinical and research information on drug-induced liver injury* (Nih). (National Institute of Diabetes and Digestive and Kidney Diseases). Available at: https://livertox.nih.gov.

Paralikar, P., Ingle, A. P., Tiwari, V., Golinska, P., Dahm, H., Rai, M., et al. (2019). *Evaluation of antibacterial efficacy of sulfur nanoparticles alone and in combination with antibiotics against multidrug-resistant uropathogenic bacteria. J. Environ. Sci. Health Part A* 54 (5), 381–390. doi: 10.1080/10934529.2018.1558892

Patel, R. A., and Gallagher, J. C. (2010). Drug fever. *Pharmacotherapy* 30 (1), 57–69. doi: 10.1592/phco.30.1.57

Pickard, R., Oluboyede, Y., Brennand, C., von Wilamowitz-Moellendorff, A., and McClurg, D. (2018). Continuous low-dose antibiotic prophylaxis to prevent urinary tract infection in adults who perform clean intermittent self-catheterisation: the AnTIC RCT. *Health Technol. Assess.* 22 (24), 1–102. doi: 10.3310/hta22240

Procop, G. W., Tuohy, M. J., Wilson, D. A., Williams, D., and Hadziyannis, E. (2003). Cross-class resistance to non-beta-lactam antimicrobials in extended-spectrum beta-lactamase-producing klebsiella pneumoniae. *Am. J. Clin. Pathol.* 120 (2), 265–267. doi: 10.1309/BWQKWB2WN6W5X1CC

Race, P. R., Lovering, A. L., Green, R. M., Ossor, A., White, S. A., Searle, P. F., et al. (2005). Structural and mechanistic studies of Escherichia coli nitroreductase with the antibiotic nitrofurazone. reversed binding orientations in different redox states of the enzyme. *J. Biol. Chem.* 280 (14), 13256–13264. doi: 10.1074/jbc.M409652200

Rego, L., Glazer, C., and Zimmern, P. (2016). Risks of long-term use of nitrofurantoin for urinary tract prophylaxis in the older patient. *Urol. Sci.* 27(4), 193-8. doi: 10.1016/j.urols.2016.07.004

Roth, A. R., and Basello, G. M. (2003). Approach to the adult patient with fever of unknown origin. *Am. Fam Physician* 68 (11), 2223–2228.

Sakaan, S. A., Twilla, S. A., Usery, J. D., Winton, J. B., Self, J. C., and , T. H. (2014). *Nitrofurantoin-induced hepatotoxicity: a rare yet serious complication. South Med. J.* 107 (2), 107–113. doi: 10.1097/SMJ.0000000000000059

Sanchez, G. V., Babiker, A., Master, R. N., Luu, T., Mathur, A., and Bordon, J. (2016). Antibiotic resistance among urinary isolates from female outpatients in the united states in 2003 and 2012. *Antimicrob. Agents Chemother.* 60 (5), 2680–2683. doi: 10.1128/AAC.02897-15

Sandegren, L., Lindqvist, A., Kahlmeter, G., and Andersson, D. (2008). Nitrofurantoin resistance mechanism and fitness cost in Escherichia coli. *J. Antimicrob. Chemother.* 62, 495–503. doi: 10.1093/jac/dkn222

Sanhueza, L., Melo, R., Montero, R., Maisey, K., Mendoza, L., and Wilkens, M. (2017). Synergistic interactions between phenolic compounds identified in grape pomace extract with antibiotics of different classes against Staphylococcus aureus and Escherichia coli. *PloS One* 12 (2), e0172273. doi: 10.1371/journal.pone.0172273

Sastry, S. S., and Jayaraman, R. (1984). Nitrofurantoin-resistant mutants of Escherichia coli: isolation and mapping. *Mol. Gen. Genet.* 196 (2), 379–380. doi: 10.1007/BF00328076

Sekyere, J. O., and Asante, J. (2018). Emerging mechanisms of antimicrobial resistance in bacteria and fungi: advances in the era of genomics. *Future Microbiol.* 13, 241–262. doi: 10.2217/fmb-2017-0172

Shakti, L., and Veeraraghavan, B. (2015). Advantage and limitations of nitrofurantoin in multi-drug resistant Indian scenario. *Indian J. Med. Microbiol.* 33 (4), 477–481. doi: 10.4103/0255-0857.167350

Sherigar, J. M., Fazio, R., Zuang, M., and Arsura, E. (2012). Autoimmune hepatitis induced by nitrofurantoin. the importance of the autoantibodies for an early diagnosis of immune disease. *Clin. Pract.* 2 (4), e83.

Slekovec, C., Leroy, J., Huttner, A., Ruyer, O., Talon, D., Hocquet, D., et al. (2014). When the precautionary principle disrupts 3 years of antibiotic stewardship: nitrofurantoin in the treatment of urinary tract infections. *J. Antimicrob. Chemother.* 69 (1), 282–284. doi: 10.1093/jac/dkt328

Stein, G. E. (1999). Comparison of single-dose fosfomycin and a 7-day course of nitrofurantoin in female patients with uncomplicated urinary tract infection. *Clin. Ther.* 21 (11), 1864–1872. doi: 10.1016/S0149-2918(00)86734-X

Stine, J. G., and Northup, P. G. (2016). Autoimmune-like drug-induced liver injury: a review and update for the clinician. *Expert Opin. Drug Metab. Toxicol.* 12 (11), 1291–1301. doi: 10.1080/17425255.2016.1211110

Tan, I. L., Polydefkis, MJ., Ebenezer, G. J., Hauer, P., and McArthur, J. C. (2012). Peripheral nerve toxic effects of nitrofurantoin. *Arch. Neurol.* 69 (2), 265–268. doi: 10.1001/archneurol.2011.1120

Tulara, N. K. (2018). Nitrofurantoin and fosfomycin for extended spectrum beta-lactamases producing Escherichia coli and Klebsiella pneumoniae. *J. Glob. Infect. Dis.* 10 (1), 19–21. doi: 10.4103/jgid.jgid_72_17

Vahid, B., and Wildemore, B. M. (2006). Nitrofurantoin pulmonary toxicity: a brief review. *Curr. Respir. Med. Rev.* 2 (4), 439–442. doi: 10.2174/157339806778777276

Vazquez-Muñoz, R., Meza-Villezcas, A., Fournier, P., Soria-Castro, E., Juarez-Moreno, K., Gallego-Hernández, A., et al. (2019). Enhancement of antibiotics antimicrobial activity due to the silver nanoparticles impact on the cell membrane. *PloS One* 14 (11), e0224904. doi: 10.1371/journal.pone.0224904

Vervoort, J., Xavier, B. B., Stewardson, A., Coenen, S., Godycki-Cwirko, M., Adriaenssens, N., et al. (2014). An in vitro deletion in ribE encoding lumazine synthase contributes to nitrofurantoin resistance in escherichia coli. *Antimicrob. Agents Chemother.* 58 (12), 7225–7233. doi: 10.1128/AAC.03952-14

Vickery, S. B., Burch, A. D., and Vickery, P. B. (2022). Differentiating probable nitrofurantoin-induced drug fever from antipsychotic-induced hyperthermia in a patient receiving clozapine. *Ment. Health Clin.* 12 (3), 205–209. doi: 10.9740/mhc.2022.06.205

Wang, Y., Gray, J. P., Mishin, V., Heck, D. E., Laskin, D. L., Laskin, J. D., et al. (2008). Role of cytochrome P450 reductase in nitrofurantoin-induced redox cycling and cytotoxicity. *Free Radic. Biol. Med.* 44 (6), 1169–1179. doi: 10.1016/j.freeradbiomed.2007.12.013

Wijma, R. A., Huttner, A., Koch, B. C., Mouton, J. W., and Muller, A. E. (2018). Review of the pharmacokinetic properties of nitrofurantoin and nitroxoline. *J. Antimicrob. Chemother.* 73 (11), 2916–2926. doi: 10.1093/jac/dky255

Wonnacott, S., Gala, D., Shah, M., Kaul, D., and Kumar, V. (2022). An unusual case of drug-induced liver injury secondary to nitrofurantoin use. *Cureus* 14 (7), e26882. doi: 10.7759/cureus.26882

Zhanel, G. G., Hisanaga T. L., Laing, N. M., DeCorby, M. R., Nichol, K. A., Palatnik, L. P., et al. (2005). Antibiotic resistance in outpatient urinary isolates: final results from the north American urinary tract infection collaborative alliance (NAUTICA). *Int. J. Antimicrob. Agents* 26 (5), 380–388. doi: 10.1016/j.ijantimicag.2005.08.003

Zhong, Z.-X., Cui, Z.-H., Li, X.-J., Tang, T., Zheng, Z.-J., Ni, W.-N., et al. (2020). Nitrofurantoin combined with amikacin: a promising alternative strategy for combating MDR uropathogenic Escherichia coli. *Front. Cell. Infect. Microbiol.* 10, 608547. doi: 10.3389/fcimb.2020.608547

Zhou, A., Kang, T. M., Yuan, J., Beppler, C., Nguyen, C., Mao, Z., et al. (2015). Synergistic interactions of vancomycin with different antibiotics against Escherichia coli: trimethoprim and nitrofurantoin display strong synergies with vancomycin against wild-type e. coli. *Antimicrob. Agents Chemother.* 59 (1), 276–281. doi: 10.1128/AAC.03502-14

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 17 August 2023
DOI 10.3389/fcimb.2023.1223696

OPEN ACCESS

EDITED BY
Sanket Kaushik,
Amity University Jaipur, India

REVIEWED BY
Weihui Wu,
Nankai University, China
Laura Fernandez-Garcia,
Institute of Biomedical Research of A Coruña (INIBIC), Spain

*CORRESPONDENCE
Guangtao Huang
haitao3140@sina.com

†These authors have contributed equally to this work

RECEIVED 16 May 2023
ACCEPTED 24 July 2023
PUBLISHED 17 August 2023

CITATION
Qian Y, Zhou D, Li M, Zhao Y, Liu H, Yang L, Ying Z and Huang G (2023) Application of CRISPR-Cas system in the diagnosis and therapy of ESKAPE infections.
Front. Cell. Infect. Microbiol. 13:1223696.
doi: 10.3389/fcimb.2023.1223696

Application of CRISPR-Cas system in the diagnosis and therapy of ESKAPE infections

Yizheng Qian[1,2†], Dapeng Zhou[1,2,3†], Min Li[1,2], Yongxiang Zhao[1,2], Huanhuan Liu[1,2], Li Yang[1,2], Zhiqin Ying[1,2] and Guangtao Huang[1,2,4*]

[1]Department of Burns and Plastic Surgery, Affiliated Hospital of Zunyi Medical University, Zunyi, China, [2]The Collaborative Innovation Center of Tissue Damage Repair and Regeneration Medicine of Zunyi Medical University, Zunyi, China, [3]Department of Burn Plastic and Wound Repair Surgery, The Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, China, [4]Department of Burn and Plastic Surgery, Department of Wound Repair, Shenzhen Institute of Translational Medicine, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen, China

Antimicrobial-resistant ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens represent a global threat to human health. ESKAPE pathogens are the most common opportunistic pathogens in nosocomial infections, and a considerable number of their clinical isolates are not susceptible to conventional antimicrobial therapy. Therefore, innovative therapeutic strategies that can effectively deal with ESKAPE pathogens will bring huge social and economic benefits and ease the suffering of tens of thousands of patients. Among these strategies, CRISPR (clustered regularly interspaced short palindromic repeats) system has received extra attention due to its high specificity. Regrettably, there is currently no direct CRISPR-system-based anti-infective treatment. This paper reviews the applications of CRISPR-Cas system in the study of ESKAPE pathogens, aiming to provide directions for the research of ideal new drugs and provide a reference for solving a series of problems caused by multidrug-resistant bacteria (MDR) in the post-antibiotic era. However, most research is still far from clinical application.

KEYWORDS

CRISPR-Cas, ESKAPE, pathogen, infection, diagnosis, therapy

1 Introduction

ESKAPE pathogen infection often leads to high mortality, and expensive treatment fee, which often brings a heavier financial burden. The U.S. Centers for Disease Control and Prevention (CDC) estimates that ESKAPE pathogens cause more than 2 million infections and at least 29,000 deaths annually in the United States (Sivalingam et al., 2019; De Oliveira et al., 2020; Sutradhar et al., 2021). At the same time, the cost of the five bacteria in the ESKAPE pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*,

Acinetobacter baumannii, and *Pseudomonas aeruginosa*) in the United States is approximately $2.9 billion per annum (Shrestha et al., 2018). For some middle- and low-income developing countries, the situation is a lot more severe. Global antibiotic consumption increased by 65% between 2000 and 2015, mainly inspired by low- and middle-income countries (Klein et al., 2018).

Mastering some genetic characteristics of ESKAPE pathogens is one of the important strategies to avoid this series of problems. *A. baumannii* can produce carbapenemase, which is a class of β-lactamases that can hydrolyze carbapenem antibiotics (Wu et al., 2020), while the drug resistance of *P. aeruginosa* is more complicated, which may be related to the production of the enzyme, membrane permeability and changes in target sites, biofilm synthesis, and the generation of adaptive resistance (Du et al., 2020). *Enterobacter* spp., multidrug-resistant *P. aeruginosa*, and multidrug-resistant *A. baumannii* have the ability to produce β-lactamase, which hydrolyzes β-lactam antibiotics (such as penicillins, cephalosporins, and carbapenems), thus making the drugs lose their antibacterial properties. Acquired resistance is more destructive, which means that bacteria acquire resistance-related genes by means of mobile elements (such as zygotic plasmids, transposons, insertion sequences, and integration) (Wieland et al., 2018). ESKAPE pathogenic bacteria have strong mutation ability and fast reproduction speed, and the adaptive gene mutations generated by them can spread rapidly through plasmids. Among them, clinical isolates of *K. pneumoniae* usually carry diverse conjugative plasmids (including fertility plasmid and resistant plasmid), allowing drug resistance to disseminate among strains or even between different strains through conjugation (Sun et al., 2019).

In addition, some tangible reasons play an important role. Among them, the irregular use of antibiotics has traditionally been part of the more recognized and difficult problems in the world. Improper selection of antibiotics, insufficient doses, and poor patient compliance with antibacterial therapy will increase antibiotic resistance. The reason may be that the academic community's incomplete understanding of the mechanism of ESKAPE pathogens resistance and unbalanced economic factors restricts the progress of recent drug research and development. Being dependent on statistics, from 2007 to 2009, a total of 40 million antibiotic prescriptions were issued in outpatient clinics in the United States, of which 27 million (67.5%) were proved to be unnecessary (Shapiro et al., 2014).

Antibiotics research and development investment costs a fortune, along with the long development cycle, the high failure rate, and the low return on investment. On a global scale, each antibiotic takes an average of 11.8 years to develop at a cost of USD$ 1.5 billion (Towse et al., 2017). This has led to the reluctance of most pharmaceutical companies to develop antibiotics. As an oxazolidinone antibiotic that can effectively deal with ESKAPE pathogens, linezolid has limited its promotion due to the high production cost. In addition to the cost of product development, environmental pollution caused by the production of antibiotics has also become another obstacle on the road to antibacterial. Most of the pharmaceutical wastewater are antibiotic production wastewater, and the Dichromate Index (CODcr, that is, the chemical oxygen consumption measured in accordance with using potassium dichromate as an oxidant) in such wastewater is high, so are the biological toxicity and chromaticity. In addition to that, there are extremely fluctuating pH and complex governance processes (Qin et al., 2016). Traditional methods are time consuming, consumable, and inefficient to deal with drug-resistant bacterial infections. In recent years, some innovative strategies to deal with ESKAPE pathogens have emerged and have gradually grown up to become a hot spot in the field of clinical trials and antibacterial therapy. The scholarly community is trying to alleviate the pressure of nosocomial infections brought about by drug-resistant bacteria to some extent through these potential emerging antibacterial strategies (Gonzalez de Aledo et al., 2021). The CRISPR-Cas system, consisting of CRISPR sequences and CRISPR-associated proteins (Cas proteins), is an adaptive immune system restricted to bacteria and archaic, which can protect the host from the invasion of foreign nucleic acids. According to the composition and function of Cas proteins. CRISPR-Cas systems can be classified into two main classes, and each one divided into several types (Figure 1) (Strich and Chertow, 2019). At present, research on CRISPR-Cas system is mainly focused on the molecular mechanism of its immune function and its application in the field of gene editing. The research on the influence of CRISPR-Cas system on the stability of the host genome is quite rare. A related study found that among the 4,500 spacers derived from the NCBI database, 35% of the spacers had homology to the microbial genome, and some of these spacers targeted sequences on mobile genetic elements. The above results indicate that these genome-targeting spacers are not accidental, and they may play some important roles in the evolution of bacteria. Bacteria's intrinsic CRISPR system allows bacteria to be immune to foreign DNA such as phage. The spacer sequence in the CRISPR structure plays an important part in the immune process. Research revealed that the spacer sequence has the phenomenon of insertion and selective deletion during bacterial evolution, which makes the CRISPR structure polymorphic, and there are differences between different strains of the same species. Therefore, the CRISPR structure can be employed as an ideal site for bacterial typing and evolutionary research (Mohanraju et al., 2016).

In addition, CRISPR is a gene-editing tool that can also be used for pathogen detection. It involves designing a specific CRISPR system that includes Cas proteins and guide RNA (gRNA) that matches the target pathogen's DNA sequence. When the Cas protein binds to the target DNA, it can cut the DNA or bind to it, depending on the specific system used. In pathogen detection, Cas proteins are used to target the pathogen's DNA. If the target DNA is present in a sample, the Cas protein will either cut it or bind to it, producing detectable signals such as specific DNA fragments or color changes. By detecting these signals, we can determine the presence of the target pathogen. For example, Janice's team found that Lachnospiraceae bacterium ND2006 Cas12a (LbCas12a) has an extraordinary ability to non-specifically cleave ssDNA after binding to RNA-guided DNA (Janice S. Chen et al., 2018). Using this feature, combined with fluorescently labeled ssDNA signal reporter molecules, the DNA endonuclease-targeted CRISPR trans reporter (DETECTR) biosensing platform was developed, which can rapidly and accurately detect HPV and can distinguish HPV

10.3389/fcimb.2023.1223696

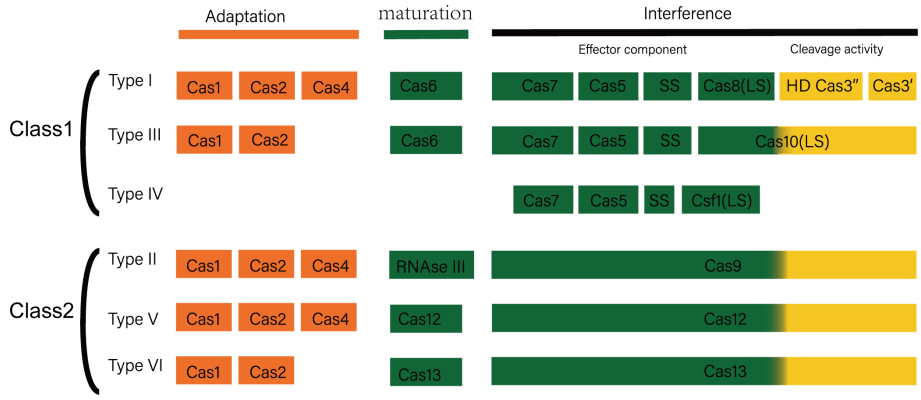


FIGURE 1
Composition and function of CRISPR-Cas system. CRISPR/Cas system is an adaptive immune defense formed by bacteria and archaea in the long-term evolution process, which fights against invading viruses and foreign DNA through three different but continuous stages: adaptation, crRNA maturation, and interference. CRISPR-Cas system mainly includes two categories: category 1 and category 2. Type 1 CRISPR-Cas systems include types I, III, and IV, and type 2 systems include types II, V, and VI. The first type of CRISPR-Cas system uses the cooperation of multiple Cas proteins to interfere, while the second type of system is interfered by a single protein.

subtypes with very similar sequences. Combined with loop-mediated isothermal amplification (LAMP) technology, a highly sensitive detection of dispassionate human papillomavirus (HPV) was achieved. Utilizing various Cas12 effector proteins, researchers have developed a variety of new CRISPR/Cas12 biosensing platforms for pathogen detection, including HOLMES, HUDSON, and other CRISPR/Cas biosensing systems (Li et al., 2018). By combining RPA, LAMP, and other technologies, an ultra-detection of pathogenic bacteria has been achieved (Wu et al., 2017; Myhrvold et al., 2018; Li et al., 2019a). In order to shorten the detection time, Wang et al. attached Cas12a to the tube wall and added it to the system by centrifugation after RPA amplification to realize integrated detection, which could shorten the detection time to 30 min (Wang et al., 2019a). Finally, CRISPR can also be applied to the treatment of ESKAPE infections. Potential applications of CRISPR in ESKAPE treatment include gene editing to weaken drug resistance, gene regulation to control resistance-related gene expression, pathogen inactivation by cutting specific DNA sequences, and introducing antagonistic genes to combat drug-resistant bacteria. These applications show promise in developing new therapeutic strategies against ESKAPE infections, but further research and clinical studies are needed to validate their safety and effectiveness.

2 Application of CRISPR-Cas system in the diagnosis and therapy of ESKAPE infections

2.1 Application of CRISPR technology in the detection and diagnosis of ESKAPE infections

Most studies on pathogen detection using CRISPR have involved the following mechanisms. The CRISPR/Cas12 system is an RNA-guided DNA-targeting CRISPR system. Taking Cas12a as an example, the effector protein can catalyze the maturation of its own crRNA without the involvement of tracer RNA. The Cas12a–crRNA binary complex can achieve precise aiming at the target DNA sequence by targeting a PAM site rich in T nucleotides. CrRNA is usually composed of 19nt repeat sequence (repeat) and 23-25nt spacer sequence (spacer). The repeats contain palindrome sequences, which can form a stable stem-loop structure through base pairing, and its conformations stability is affected by the intramolecular interaction of hydrated magnesium ions (Mg (H2O)6)2+ and Cas12a. Cas12a recognizes and binds crRNAs being dependent on the length and sequence specificity of the stem-loop structure. Spacer sequences, which make up of RNA sequences transcribed from exogenous DNA, are bacterial antigens against the same invaders. After bacterial recognition of foreign DNA, Cas12a utilizes the RuvC domain of cis-cleave target DNA adjacent to the PAM sequence. After the dissociation and release of the DNA fragment at the distal end of the PAM, the cleavage activity of Cas12a trans-nuclease is activated, the signal reporter molecule can be cut in large quantities, and the specificity of the target nucleic acid can be achieved by detecting the corresponding signal (such as fluorescence) (Dong et al., 2016; Watters et al., 2018).

2.1.1 *Staphylococcus aureus*

New advancements have also been made in the application of the CRISPR-Cas system for the detection of *S. aureus*. Zhou et al. developed a bacterial sensing strategy with the name of CCB detection (CRISPR-Cas13a based bacterial detection), taking advantages of CRISPR-Cas13a system, namely, the crRNA programmability and Cas13a "collateral effect" of promiscuous RNase activity upon target RNA recognition. *S. aureus* was selected as the model bacterium to validate the performance of CCB detection. The validation process involved four steps, namely, 1) straightforward extraction of genomic DNA; 2) specific gene amplification through PCR; 3) *in vitro* transcription; and 4) cleavage of reporter RNA, known as the "collateral effect," to

indicate the presence of the target analyte. Remarkably, CCB detection demonstrated successful detection of the target genomic DNA (gDNA) at concentrations as low as 100 aM. The limit of detection (LOD) was determined to be 1 CFU/mL, with a dynamic detection range spanning from 100 to 107 CFU/mL for *S. aureus*. The entire sample-to-answer process for this biosensor required <4 h. CCB detection exhibited excellent selectivity for *S. aureus*, with no interference from other bacterial species. Moreover, the application of CCB detection in real food samples, including those with both known and unknown levels of bacteria (whether spiked or non-spiked), yielded results comparable to the conventional culture-based counting method. Notably, CCB detection offered advantages such as reduced assay time and increased sensitivity. With its reliability, sensitivity, specificity, and simplicity, the proposed CCB-detection technique can be expanded and applied to the detection of other bacteria. It holds significant potential for a wide range of applications, including food safety inspections, disease diagnosis, and environmental monitoring (Zhou et al., 2020).

Meanwhile, Li et al. developed a new platform for MRSA (methicillin-resistant *S. aureus*) detection. Although the current diagnostic methods used in clinical practice, such as PCR and culture-based techniques, are commonly employed, they are not suitable for rapid point-of-care testing (POCT). With the recent advancements in CRISPR/Cas technology, new opportunities for rapid point-of-care detection have emerged. In the study undertaken by Yanan Li et al., a platform for the rapid, precise, and contamination-free detection of MRSA was developed by integrating the Cas12 system with recombinase polymerase amplification (RPA) in a single tube. By employing this approach, visual detection of MRSA could be achieved within a mere 20 min. The assay results, obtained using the one-tube RPA-CRISPR/Cas12a platform, can be visualized through lateral flow test strips (LFS) and fluorescence-based methods, including real-time and end-point fluorescence. This versatile platform allows for the specific detection of MRSA, with a sensitivity of 10 copies for the fluorescence method and a range of 10–100 copies for LFS. The results obtained from 23 samples of clinical MRSA isolates demonstrated a coincidence rate of 100% for the fluorescence method and 95.7% for LFS, as compared to qPCR. In conclusion, the one-tube RPA-CRISPR/Cas12a platform proves to be an efficient method for MRSA detection, holding significant potential for practical applications in future point-of-care testing (Li et al., 2022).

Su designed and synthesized specific primers based on the sequence of the conserved region of *S. aureus* thermostable nuclease gene (nyc) and established the isothermal amplification technology mediated by *S. aureus* recombinase by optimizing the reaction conditions, expressing and purifying CRISPR-Cas13a protein, designing specific crRNA, and using crRNA to guide CRISPR-Cas13a protein to detect RAA products; the sensitivity and specificity of the optimized method were evaluated, and this method and real-time PCR method were used to detect the golden yellow color in food samples. *Staphylococcus* was observed, and the consistency of the method was evaluated. The sensitivity of CRISPR-Cas13a-assisted RAA detection of *S. aureus* was 101 CFU/mL, which was higher than that of real-time PCR, approximately 102 CFU/mL; the detection time was only 30 min, and there was no cross-reaction with other food-borne pathogens. The positive rates of 80 food samples detected by this method and real-time PCR were both 8.75%, with high consistency (kappa=1, p>0.05). In the end, the conclusion is that the established CRISPR-Cas13a-assisted RAA method has the advantages of simplicity, rapidity, sensitivity, and specificity, and provides a modern technical means for the detection of *S. aureus* (Su et al., 2020).

2.1.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a prevalent culprit in hospital-acquired infections. An urgent need exists for a prompt, precise, and convenient detection approach to facilitate early diagnosis and targeted treatment of *K. pneumoniae* infections. To address this, Qiu et al. devised a novel assay called CRISPR-top (CRISPR-mediated testing in a single vessel). It combines the power of LAMP (loop-mediated isothermal amplification) with CRISPR/Cas12b-based detection, enabling a streamlined process performed at a constant temperature. Their optimized *K. pneumoniae* CRISPR-top assay accurately identifies the presence of *K. pneumoniae* strains within 60 min at a temperature of 56°C. By fine-tuning the reaction mixture composition, they achieved optimal results with 0.53 mM (each) FIP and BIP primers, 0.27 mM LF primer, 0.13 mM (each) F3 and B3 primers, and a 2 mM ssDNA fluorescence probe. Remarkably, their assay exhibits a detection limit of 1 pg genomic DNA per reaction, equivalent to 160 K. *pneumoniae* cells or 1.6×10^5 CFU/mL in samples, surpassing the sensitivity of traditional LAMP methods by a factor of 10. Validation studies using a diverse panel of 105 strains, including *K. pneumoniae* clinical isolates and non-*K. pneumoniae* strains, demonstrated accurate identification rates. Additionally, they conducted a comprehensive evaluation of the *K. pneumoniae* CRISPR-top assay using 58 respiratory symptomatic sputum samples. Impressively, their assay achieved a specificity of 100% (33/33) and a sensitivity of 96% (24/25), yielding a positive predictive value of 100% (24/24) and a negative predictive value of 97.1% (33/34), surpassing the performance of conventional LAMP detection methods. In summary, the study conducted by Qiu and colleagues introduces the *K. pneumoniae* CRISPR-top assay as a rapid, straightforward, and highly specific means of detecting *K. pneumoniae* infections (Qiu et al., 2022).

2.1.3 *Acinetobacter baumannii*

In 2020, Li developed a versatile CRISPR-Cas12a platform for detecting a wide range of analytes (Li et al., 2020), including *A. baumannii*, at ultralow concentrations. The platform utilizes LbaCas12a, which acts as a signal amplifier by recognizing single-stranded DNA intermediates generated by non-DNA targets. With the help of functional nucleotides, such as DNAzyme and aptamer, ultrasensitive bioassays for Pb2+ and *A. baumannii* were designed, achieving a limit of detection of approximately 0.053 nM and 3 CFU/mL, respectively. Additionally, the platform allows simultaneous detection of four microRNAs (miRNAs) without significant interference, indicating its potential for high-throughput analysis of miRNA expression profiles. Afterwards, in

the year 2021, Wang developed a rapid platform that integrates multiplex PCR with CRISPR-Cas array for the detection of multidrug-resistant *A. baumannii* (MDRAB) (Wang et al., 2021). The platform utilizes a multiplex PCR amplification strategy to simultaneously amplify the housekeeping gene and four β-lactamase genes of *A. baumannii*. The platform also utilizes the indiscriminate single-stranded DNase activity of LbaCas12a to generate a single fluorescent signal for the multiplex PCR products. This platform enables genotypic antibiotic susceptibility testing (g-AST) of *A. baumannii* within 2 h, with a detection limit down to 50 CFU/mL. Their studies demonstrate the potential of the CRISPR-Cas12a method for rapid and specific detection of multiple genes, promoting its application in the diagnosis and treatment of multidrug-resistant bacteria.

2.1.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a significant bacterial pathogen responsible for hospital-acquired infections and poses a threat to patients with cystic fibrosis. In 2020, Mukama et al. introduced a novel approach called CIA (CRISPR/Cas and LAMP-based lateral flow biosensor) for the detection of *P. aeruginosa* (Mukama et al., 2020). By combining CRISPR/Cas systems (LbaCas12a and AaCas12b) with loop-mediated isothermal amplification (LAMP), the method achieves high sensitivity and specificity. It utilizes a lateral flow biosensor (LFB) and collateral cleavage of a biotinylated DNA reporter to detect the target gene. The CIA-based LFB demonstrates the ability to accurately distinguish *P. aeruginosa* from other bacteria in complex samples. Their cost-effective and efficient method shows great potential for clinical diagnosis of infectious diseases. Finally, Qiu et al. introduced another novel detection method called CRISPR-top assay (Qiu et al. 2023, which is based on CRISPR-Cas12b and allows the rapid and accurate identification of *P. aeruginosa*. The assay is performed within a single tube, requiring only one fluid-handling step and no specialized instruments. Under optimized conditions, including a temperature of 55°C and specific primer concentrations, the assay demonstrated high specificity, inclusivity, and exclusivity. With a limit of detection of 10 copies per reaction, the CRISPR-top assay exhibited promising results in the analysis of 46 respiratory specimens, showing a sensitivity of 85.3% and a specificity of 100%. Overall, their study suggests that the *P. aeruginosa* CRISPR-top assay can serve as an efficient and practical tool for rapid detection, particularly in resource-limited settings.

2.1.5 Enterobacter species

A total of 135 strains with complete sequence and 203 strains with whole genome shotgun sequence of *E. coli* were identified in GenBank by Liang Wenjuan et al. using BLAST. Additionally, 361 strains of *E. coli*, including 38 strains of *E. coli* O157:H7, were identified in the laboratory using PCR. The identified strains were then analyzed using the CRISPR Finder tool. To compare the spacers, DANMAN was employed, and phylogenetic trees of the cas gene were constructed using Clustal X and Mega 5.1.Then, they obtained the following results. A new descriptive method was developed to investigate the positioning of CRISPR/cas in *E. coli*. The presence of CRISPR1 was detected in 77.04%, 100.00%, and 75.62% of the 135 strains with complete sequence, 203 strains with whole genome shotgun sequence, and 361 laboratory strains, respectively. Similarly, CRISPR2 was found in 74.81%, 100.00%, and 92.24% of the respective strains. However, CRISPR3 and CRISPR4 were only detected in 11.85%, 0%, and 1.39% of the mentioned strains. Among the strains analyzed, one strain from GenBank and two laboratory strains contained four CRISPR loci. Additionally, one *E. coli* strain in our dataset exhibited an insertion sequence downstream of the non-cas CRISPR1. A unique CRISPR was identified in eight strains of O55:H7, 180 strains of O157:H7, eight strains of O157: HNM, 40 strains of O104:H4, four strains of O145:H28, and across all 699 *E. coli* strains. The phylogenetic tree revealed two distinct groups based on the cas type, either I-E or I-F. The authors thus concluded that CRISPR/Cas has the potential to serve as a valuable molecular biomarker in epidemiological surveillance studies, enabling the identification of highly virulent or novel strains of *E. coli* (Liang et al., 2016).

2.2 Application of CRISPR-Cas system in the therapy of ESKAPE infections

Although CRISPR-Cas technology has shown its great efficiency in gene editing, it is hitherto not considered as a possible antimicrobial treatment because of the delivery issue. Most studies introduce the CRISPR-Cas system into their experimental bacterial cells using plasmid electroporation; however, this method apparently cannot be successfully performed *in vivo*. Thus, another method is to be considered such as phage delivery (Fage et al., 2021). As an accurate gene editing technology, CRISPR-Cas system can accurately edit the target gene at a fixed point. Based on this effect and combined with phage delivery, CRISPR-Cas system is theoretically enabled to exert an antimicrobial effect *in vivo*. The basic principle is that after infecting drug-resistant bacteria with phage carrying genes related to CRISPR-Cas system, the cleavage of drug-resistant genes by CRISPR-Cas system targeting bacteria will lead to the loss of drug resistance, and finally, the drug-resistant bacteria will recover their sensitivity to antibiotics (Figure 2) (Bikard et al., 2012; Bikard et al., 2014; Citorik et al., 2014; Fage et al., 2021). Direct use of CRISPR-Cas system can eliminate bacteria by targeting drug-resistant genes or virulence genes and at the same time limit the transfer and prevalence of harmful genes among microorganisms, which provides a new direction for the study of preventing and treating bacterial multidrug resistance at the gene level.

2.2.1 *Enterococcus faecium* and *Enterococcus faecalis*

Enterococcus spp., a group of cocci that stain Gram-positive and typically arrange themselves in pairs or short chains, represents a genus of bacteria. While they are commonly found in the gastrointestinal microbiota, two specific species, namely, *E. faecalis* and *E. faecium*, are known to frequently cause infections. Their remarkable ability to endure on inert surfaces for extended periods has elevated their significance in the context of hospital-

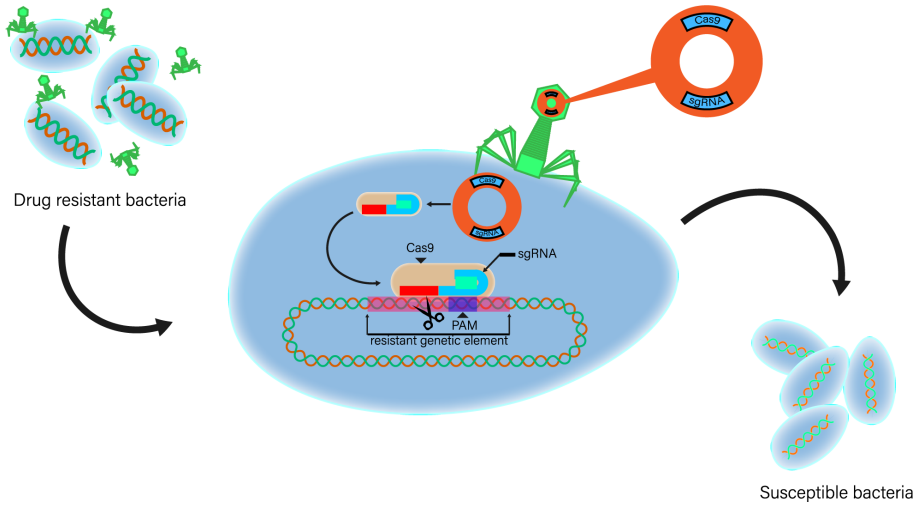


FIGURE 2
The second type of system is interfered by a single protein. Basic strategy of using exogenous CRISPR-Cas as antibacterial agent. CRISPR-Cas phage vector is a plasmid construct cloned with CRISPR-Cas element and phage packaging sequence. Phage carrier particles are adsorbed on bacteria, and plasmids carrying genes related to CRISPR-Cas system are transferred to drug-resistant bacteria. Exogenous Cas gene and sgRNA expression sequence were expressed in host cells. The cleavage of targeted drug-resistant genes by CRISPR-Cas system will lead to the loss of drug-resistant plasmids or DNA double-strand breaks. When bacteria cannot re-cyclize plasmids or repair their genomes, drug-resistant bacteria will die. Phage continues to enter the replication cycle and synthesize offspring and lyse cells, further optimizing the therapeutic effect.

acquired infections. Mutations and/or excessive production of a penicillin-binding protein (PBP) called PBP5, belonging to the class B category, endow these bacteria with inherent resistance to a wide range of β-lactam antibiotics. However, it is noteworthy that ampicillin remains effective against the majority of *E. faecalis* strains. Moreover, enterococci possess intrinsic resistance to aminoglycosides, thereby limiting the available therapeutic options.

The horizontal transfer of antibiotic resistance genes limits the treatment options of infection (Liu et al., 2013). Price and his colleagues used *E. faecalis* T11 as a model organism to evaluate the role of CRISPR-Cas system in genome defense against common conjugated plasmids (Price et al., 2016). They proved that CRISPR-Cas, together with restriction modification, reduced plasmid acquisition by bacteria in biofilm by four times. This study highlights the importance of CRISPR-cas system of *E. faecalis* in regulating horizontal gene transfer. Understanding the basic biology and molecular basis of pathogenicity of *E. faecalis* is helpful to effectively improve and improve the ability to formulate treatment strategies. A basic part of the biological research of *E. faecium* depends on the ability to produce mutants, but this process is often time consuming and ineffective (Zhang et al., 2012). Maat described a method based on CRISPR-Cas9 to improve the current genetic toolbox of *E. faecium* (de Maat et al., 2019). In short, it is a mutant that uses CRISPR-Cas9 as an anti-selection strategy, relies on the high internal recombination rate of *E. faecalis* for allele exchange, and combines CRISPR-Cas9 to produce specific gene mutations in the chromosome of *E. faecium* more effectively.

So far, there have been almost no direct research results on using CRISPR-Cas for the treatment of *E. faecium*. In 2019, Rodrigues and colleagues presented a potential approach for treating *E. faecalis* using the CRISPR-Cas system (Rodrigues et al., 2019). They conducted a study where they successfully integrated a CRISPR system targeting the tetM and ermB genes, known for conferring resistance to tetracycline and erythromycin, respectively, into a pheromone-responsive plasmid (PRP) called pKH88[sp-tetM] and pKH88[sp-ermB]. The transmission of these plasmids was achieved through conjugation, using *E. faecalis* CK135 as the donor strain and *E. faecalis* OG1SSp as the recipient strain. *In vitro* experiments demonstrated the effective elimination of antibiotic resistance. Subsequently, using an *in vivo* C57BL6/J mouse model, the researchers observed that although the recombination rate was low, the transconjugants that successfully acquired the PRPs were unable to acquire erythromycin-resistance genes. This finding led the authors to propose the potential utilization of probiotic *E. faecalis* strains carrying these PRPs to impede the colonization of patients by resistant *E. faecalis* strains.

2.2.2 *Staphylococcus aureus*

Staphylococcus aureus is a pathogenic bacterium that can provoke a variety of infectious diseases, which seriously threaten human life and health. It has been reported that antibiotic-resistant strains were found on the surface of public devices such as ATM keyboards (Akinola et al., 2022). Clinical overuse of antibiotics to treat *S. aureus* infections have led to the prevalence of drug-resistant *S. aureus*. Methicillin-resistant *S. aureus* (MRSA) is a class of *S. aureus* resistant to β-lactam antibiotics (Souza-Silva et al., 2022). The global spread of MRSA causes severe persistent infection. Methicillin resistance in MRSA is taken from the resistance gene mecA on the movable genetic element SCCmec, which encodes the low-affinity penicillin-binding protein PBP2a. SCCmec elements can spread in *Staphylococcus* by horizontal transfer, resulting in epidemics of drug resistance.

Guan mainly considered the effects of type III-A CRISPR-Cas system self-targeted attack on the resistance gene mecA on the

movable genetic element SCCmec in MRSA clinical strains on host genome stability and drug resistance. Through analyzing the genome sequence of transformants, it was found that the CRISPR-Cas system targeting the genome is under a lethal effect on the host, but a small number of bacteria can escape the attack of CRISPR-Cas through genome remodeling. Most of the escapers are under a large-scale loss of genome sequence, which is a DNA fragment of approximately 16 kb including the target gene within the SCCmec. The deletion of the anti-mecA spacers or loss-of-function mutation in Cas genes required for targeting allowed the remaining escapers surviving. The transcription efficiency of different length leader sequences was analyzed by RT-qPCR, the result indicating that the effective length of the naive leader sequence in the AHI strain was 252 bp. Thus, the 252-bp leader was selected as the promoter for the construction of the CRISPR plasmid. Studies have shown that the targeted attack of the type III-A CRISPR-Cas system is transcription dependent, and the attack effect can only be exerted when the spacer targets the coding strand of the target. By changing the length of the spacer sequence, it was found that the length of the spacer did not affect the processing of crRNA, and the length of the mature crRNA was fixed, both 43 or 37 nt. However, the length of the spacer does influence the targeted attack efficiency of the CRISPR-Cas system. This research not only shows that the targeted attack of the CRISPR-Cas system on the genome can promote the remodeling of the genome but also further elucidates the molecular mechanism of action of the type III-A CRISPR-Cas system (Guan, 2019).

In the reported strains and the five newly discovered MRSA strains, the CRISPR-Cas system is bordering on SCCmec, which suggests that the integration site of the CRISPR-Cas system may be near the insertion position of SCCmec in the genome. Analysis of CRISPR sequences found that the repeats of the -a system in *Staphylococcus* were highly conserved, while most of the homologous sequences of spacers were consulted on the template strand of the cleavage region in phage. Furthermore, a comparison of six strains found that the AH1 strain with fewer spacers contained more resistance and virulence genes than the other five strains. They found that the target recognition is dependent on sequence complementarity between crRNA and target RNA and that Cas6 and CSM complexes are necessary for immune function. For functions other than immunity, they found that CRISPR can be associated with governing the expression of virulence-related genes in *S. aureus*, but the mechanism still needs further research to determine. An author supposed that these findings may provide an innovative insight into the application of CRISPR-Cas systems in the therapy of MRSA infection (Cao, 2017).

Yang used the sgRNAcas9 v2.0 software to design the oligos sequence targeting the mecA gene and lighted it with the vector pCas9, which was digested with endonuclease and picked up by gel, and successfully constructed the pCas9:mecA-C-terminal plasmid (Yang, 2016). The research results show that, first, among the 32 *S. araneus* strains with 45 confirmed CRISPR loci involved in the database, 19 strains (59.4%) all contained one confirmed CRISPR locus, and 40.6% of the strains' genomes contained two confirmed CRISPR loci. The 45 repeat sequences included in the study can form a conservative dumbbell-shaped RNA secondary structure and can be split into 3 and 15 groups according to sequence similarity. In group 1, the RNA secondary structure with 5 base pairs in the stem is under a lower minimum free energy (MFE) than the structure with 3 base pairs. In group 2, the secondary structure with the longer stem holds the smallest MFE. In group 3, the number of base pairs in the stem is the same, and the resultant structure with superior "GC" content in the stem has a smaller MFE. Second, a total of five spacer sequences in strains 08BA02176 and MSHR1132 were like exogenous plasmid or phage DNA sequences, and one of the spacer sequences was completely consistent with a sequence in the plasmid encoding Panton–Valentine leukocidin. Third, CRISPR sites are separated into 17 types and are classified into three major groups. In group 1, the vast majority (75.0%) of the strains with the same CRISPR type also had the same MLST type. In the second group, the two CRISPR A strains were both MLST398. In group 3, strains with different CRISPR types correspond to different MLST types. Among the six protein-coding sites adjacent to the CRISPR site, more than three are similar in sequence to the protein-coding sites at the same position around other CRISPR sites, and most of the CRISPR sites adjacent to these similar protein-coding sites have the same CRISPR type (Kumari et al., 2022). Fourth, pCas9:mecA could successfully eliminate pET-21a(+)-mecA in *E. coli* BL21(D3) or significantly reduce its replication. The control plasmid pCas9 had no such effect. Neither pCas9:mecA nor the control PCas9 had obvious elimination effect on the pET-21a(+) control plasmid. It can be observed that the more bases in the stem of the resultant structure of the repeat RNA, the higher the "GC" content, the greater the possibility that CRISPR can function. The mecA plasmid can specifically target the mecA gene, thereby causing the elimination of the drug-resistant plasmid pET-21a(+)-mecA or greatly reducing its replication (to the extent that it is not visible by gel electrophoresis) to limit the drug-resistant gene mecA spread between strains, thereby immunizing susceptible strains (Folliero et al., 2022).

Jiang used CRISPR/Cas9 technology to construct srtA gene deletion strains and complemented strains of methicillin-resistant *S. aureus* USA300 and tested their effects on the virulence of the strains. Jiang designed three pairs of srtA gene sgRNAs and constructed pCasSA-sgRNA plasmids with pCasSA as the vector. After being modified by the defective *S. aureus* RN4220 strain, it was transferred into USA300, and the cleavage efficiency of pCasSA-sgRNA plasmid was tested (Jiang et al., 2020). The left and right homology arms of the srtA gene were amplified and fused and inserted into the pCasSA-sgRNA plasmid to construct the knockout plasmid pCasSA-sgRNA-srtA. The knockout plasmid was modified and transferred into USA300, and the srtA gene deletion strain was obtained by screening and identification. Jiang compared and assessed the differences in the growth of the USA300 strain, the survival rate of mice, the microbial load in organs, and the histopathological changes in the kidneys after the deletion of the srtA gene. At the same time, Jiang used the pLI50 plasmid as a vector to construct a complementing plasmid pLI50-srtA and a complementing strain to verify whether there are different in the phenotype of distinct strains. After chloramphenicol screening, Jiang obtained two gene deletion strains. Report to the wild-type

strain, the deletion of the srtA gene did not affect the growth of the USA300 strain but significantly reduced the mortality of infected mice, the bacterial load of the heart and kidney, and the degree of renal tissue lesions. Its function was returned after complementation by the srtA gene. The gene editing method of methicillin-resistant *S. aureus* USA300 strain with srtA gene deletion and complementation was satisfactorily established.

Zhang used bioinformatics methods to examine the distribution of CRISPR, repeat sequences, spacer sequences, cps genes, and the relationship between the presence of CRISPR and the bacterial mecA gene (Zhang et al., 2019). The results showed that 196 confirmed CRISPR structures and 1,757 suspected CRISPR structures were found in the genomes of 325 *Staphylococcus* strains; 25 strains contained gas gene clusters, which could be divided into III-A (48.1%) and II-C (51.9%) %) two types; it is determined that most of the repeat sequences in the CRISPR array are 2, and there are 14 repeat sequences that can form a stem-loop structure. Chromosomal evolution analysis shows that they can be roughly classified into three categories. It was determined that 53 of the 437 spacer sequences in the CRISPR array matched plasmids or phages, and some sequences matched multiple plasmids or phages at the same time. The carrying rates of mecA in the presence and absence of gas gene clusters were 28.00% and 54.15%, respectively, and the difference was statistically significant ($\chi 2 = 6.37$, $p<0.05$), and the two were negatively correlated. From this, it can be concluded that the CRISPR-Cas system carrying rate in the *Staphylococcus* genome is low, and the structure and function of the locus and sans gene are not perfect. Only a few strains contain a complete CRISPR-Cas system. There are more suspicious structures, and the number of DRs and spacers is lower than that of other bacteria. An intact staphylococcal CRISPR-Cas system may limit horizontal transfer of the mecA gene.

2.2.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae, a facultative anaerobe, encapsulated, and Gram-negative rod, belongs to the Enterobacterales order and is commonly found in the gastrointestinal tract of humans. In its wild-type form, this bacterium exhibits intrinsic resistance to aminopenicillins (such as ampicillin and amoxicillin) and carboxypenicillins (like ticarcillin and piperacillin) due to the presence of the chromosomal β-lactamase SHV-1. However, the worrisome aspect lies in the ability of *K. pneumoniae* to acquire resistance to nearly all approved antimicrobials. This resistance is achieved through a combination of plasmid-mediated carbapenemases and other resistance mechanisms.

Yao's research (Yao et al., 2022) focuses on the treatment potential of Carbapenem-resistant *K. pneumoniae* (CRKP). CRKP is a significant health threat due to its resistance to multiple drugs. The resistance in CRKP is primarily attributed to large plasmids containing multiple resistance genes. Understanding the function of these genes is crucial for combating CRKP infections. However, there is a lack of efficient genetic manipulation tools for studying plasmid-borne genes in clinical *K. pneumoniae*. Traditional gene knockout methods are time consuming and laborious, prompting researchers to explore the use of CRISPR systems. While CRISPR-Cas9 has been successful in deleting chromosomal genes in *K. pneumoniae*, it resulted in plasmid loss when targeting plasmid-borne genes. The low homologous recombination efficiency further hindered gene manipulation. To address these challenges, Yao's research introduces a CRISPR interference (CRISPRi) system that utilizes a catalytically inactive Cas9 (dCas9) nuclease and a single-guide RNA (sgRNA). The CRISPRi system allows for the inhibition of gene expression without causing double-stranded breaks or relying on homology-directed repair. It has been widely used to study gene function in various organisms. The study establishes an all-in-one CRISPRi tool specifically designed for CRKP. This system effectively shuts down the expression of individual and multiple resistance genes on large multidrug-resistant plasmids. It also facilitates the exploration of potential operons. The CRISPRi tool enables the easy manipulation of multicopy genes on plasmids in clinically pathogenic bacteria, including *K. pneumoniae* and *E. coli*. In general, their research presents a new CRISPRi tool that offers a rapid and efficient approach for investigating the function of plasmid-borne genes in complex clinical isolates, particularly in the context of Carbapenem-resistant *K. pneumoniae*.

2.2.4 *Acinetobacter baumannii*

Acinetobacter baumannii, a Gram-negative, belongs to the family Moraxellaceae in the class Proeo-bacteria of Eubacteria (Eze et al., 2018). *Acinetobacter baumannii* is currently one of the leading pathogens that cause nosocomial infections, including ventilator-associated and bloodstream infections (Harding et al., 2018). Feng utilized PCR product cloning and sequencing and CRISPR system bioinformatics analysis on 89 clinical isolates to study the regulation of the *A. baumannii* CRISPR system on target genes (Feng et al., 2019). The results showed that the two isolate (AB43 and ATCC19606) possessed the CRISPR system, and the genes targeted by their inter-regional regions included type IV secretory protein Virb5, Zona toxin protein, phage protein, and DNA polymerase. The CRISPR system inhibits the coding and expression of Virb5 and zona toxin protein genes, which may have a certain regulatory effect on the biofilm formation of *A. baumannii*.

In response to the problem that "although phage therapy can solve the drug resistance of bacteria, the specific recognition of phage and host bacteria limits its application," He tried to use CRISPR-Cas9 technology to perform gene editing on the filament protein sequence of the phage genome. Therefore, the scope of its bactericidal spectrum can be broadened, the modification of the binding specificity of phage host can be realized, or it can provide certain ideas and operational paths for clinical treatment.

Wang and colleagues developed a CRISPR-Cas9-based, rapid genomic editing platform to analyze the mechanisms involved in oxidative stress in *A. baumannii* by introducing deletions, insertions, and point mutations. The platform works by coupling a Cas9 nuclease-mediated genome cleavage system with the RecAb recombination system. The authors also developed a cytidine base-editing system to allow programmed C to T conversions. Then, they took the advantages of these techniques to comprehensively analyze the possible resistance genes in a clinically isolated multidrug-

resistant strain by generating premature stop codons close to the genes to unravel each of their distinct effect on drug resistance (Wang et al., 2019b). The research may provide certain guidance for clinical antibiotic selection.

2.2.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a versatile Gram-negative bacterium widely known for its opportunistic pathogenicity. It is commonly found in diverse environments, including soil, water, and hospital settings. With its impressive metabolic capabilities, *P. aeruginosa* can utilize a wide range of organic compounds as energy sources. This bacterium poses a significant threat in healthcare settings, particularly among immunocompromised individuals and patients with cystic fibrosis. *Pseudomonas aeruginosa* exhibits intrinsic resistance to many antibiotics and can acquire additional resistance mechanisms through genetic adaptations. Its ability to form biofilms further contributes to its persistence and chronic infections.

In 2020, Xiang' study (Xiang et al., 2020) employs the CRISPR interference (CRISPRi) technology using a catalytically deactivated Cas9 (dCas9) to study gene regulation in *P. aeruginosa*. Unlike the wild-type Cas9, dCas9 does not cut DNA but can bind to target sites using guide RNAs (gRNAs) and inhibit the transcription of target genes. This approach allows for interference with the expression or function of essential genes without completely eliminating them. In this research, a CRISPRi platform was constructed for *P. aeruginosa* using an arabinose-inducible pBAD vector. The expression of the prtR gene, which cannot be knocked out by conventional methods, was downregulated using this system. prtR and prtN form a regulatory cascade controlling the production of pyocins, bacteriocins that mediate bacterial cell lysis. The PrtR protein inhibits the expression of pyocin genes by binding to the promoter region. By controlling gene expression in an arabinose concentration-dependent manner, the study demonstrated the suppression of pyocin expression, leading to bacterial cell death. RNAseq analysis of the prtR-knockdown strain provided insights into downstream genes regulated by prtR, including both known and previously unknown targets. The results highlight the valuable application of CRISPRi as a tool for studying essential genes in *P. aeruginosa*.

2.2.6 *Enterobacter* species

Enterobacter spp. comprises a diverse group of Gram-negative bacteria that are widely distributed in various ecological niches. They belong to the family Enterobacteriaceae and are characterized by their rod-shaped morphology. *Enterobacter* spp. includes several clinically relevant species, such as *Enterobacter cloacae* and *Enterobacter aerogenes*. These bacteria are opportunistic pathogens and can cause a range of infections, including urinary tract infections, respiratory tract infections, and bloodstream infections, particularly in hospitalized patients and those with compromised immune systems. One of the concerning aspects of *Enterobacter* spp. is their ability to develop resistance to multiple antibiotics, including extended-spectrum β-lactams and carbapenems. This acquired resistance poses a significant challenge in clinical settings and underscores the importance of effective infection control measures and appropriate antibiotic stewardship.

Li utilized the disk diffusion method to detect its drug sensitivity and the PCR method to detect and compare the phylogenetic groups, drug resistance genes, and CRISPR system of *E. coli* cutoff from fecal samples of diarrhea patients and healthy people (Li et al., 2021). A total of 142 strains of *E. coli* were collected from 63 chronic diarrhea patients (disease group) and 79 healthy individuals (healthy group). The drug susceptibility results showed that the ampicillin resistance rate was 48.0%, and other antimicrobial drug sensitivity rates were 73.1%–100.0%. The detection rate of the CRISPR system in 63 strains of *E. coli* extracted from fecal samples from patients with chronic diarrhea was significantly lower than that of 79 strains from healthy people. The detection rate of CRISPR system in 43 high-virulence strains (group B2 and group D) was significantly greater than that in 99 low-virulence strains (group an and B1). The results showed that the fecal isolates of *E. coli* remained highly susceptible to frequently used antibiotics. The CRISPR system may play a significant role in the spread of virulence and drug resistance genes in *E. coli* isolated from feces.

Fan used CRISPR/Cas9 gene editing technology to inhibit the expression of O157:H7Stx gene in enterohemorrhagic *E. coli* (EHEC) and assessed its effect on bacterial growth and cytotoxicity. Fan Huan designed primers for the EHEC O157: H7Stx gene, constructed a CRISPR/Cas9 expression plasmid pdCas9-Stx, and transformed it into EHEC O157:H7 competent cells. RT-PCR and colloidal gold methods were utilized for detect the expression of the Stx gene, and the strain growth curve was drawn. The culture supernatant of the strain was inoculated with Vero cells to observe the cytoplastic condition. The results showed that the successfully constructed pdCas9-Stx expression plasmid could specifically inhibit EHEC O157:H7Stx gene expression and reduce cytotoxicity (Fan et al., 2016).

A toll-like receptor 5 (TLR5) is an influential member of the toll-like receptor family and plays an important regulatory role in the inflammatory response caused by Gram-negative bacteria. Xu knocked out the TLR5 gene of porcine alveolar macrophages (PAMS) by CRISPR/Cas9 gene knockout technology and then counted the colonies to discover the adhesion ability of *E. coli* F18ab and F18ac and *Salmonella* after TLR5 gene knockout (Xu et al., 2021). The results showed that the two designed sgRNAs were successfully inserted into the CRISPR/Cas9 vector with correct sequences, and they could be stably expressed after transfection into porcine alveolar macrophages, and the PCR sequencing peaks showed nested peaks, indicating that the knockout vector had cutting activity. Xu finally obtained a monoclonal cell line TLR5-sg1 with a deletion of 15 bases using public limiting dilution method. The results showed that the transcription level of TLR5 gene was significantly downregulated, and its protein level was also significantly reduced. The results of bacterial adhesion test showed that the number of adherent *E. coli* F18ab, F18ac, and *Salmonella* in TLR5 knockout cells was significantly lower than those in the control group. This study successfully established the porcine TLR5 gene knockout 3D4/21 cell line, and the knockout of TLR5 gene would reduce the adhesion of *E. coli* and *Salmonella* to 3D4/21 cells, which is to further explore the biological function of TLR5

gene. A cell model was established to provide experimental material for in-depth study of the immune regulation role of TLR5 gene in inflammatory response.

3 Discussion

Since the discovery of antibiotics, the game between humans and drug-resistant bacteria has never stopped (Hutchings et al., 2019). Antibiotics are widely used in the prevention and treatment of bacterial infections in healthcare system, food production, animal husbandry, and agriculture, bringing convenience to human beings (Manyi-Loh et al., 2018; Hutchings et al., 2019; Roth et al., 2019; Tyrrell et al., 2019). However, the abuse of antibiotics contributed to the occurrence of antibiotic-resistant strains and accelerated the evolution of antibiotic resistance (Laxminarayan et al., 2016; Nadeem et al., 2020). Horizontal transmission of ESKAPE resistance gene accelerated the prevalence of drug-resistant bacteria worldwide (Das et al., 2022). This grim reality requires real-time and rapid diagnosis methods and efficient and specific treatment methods. Since the discovery of CRISPR repeats in bacteria, CRISPR-Cas system has been widely explored in the field of genome editing from being initially considered unimportant outside the field of microbiology. CRISPR-Cas technology has become a milestone discovery in the field of genetic engineering, which has created a brand-new research method for the research in the fields of cell biology and molecular biology (Doudna and Charpentier, 2014; Wang and Doudna, 2023). The application of CRISPR-Cas in fields other than genetic engineering has also developed rapidly, especially in biosensing and infectious disease treatment, which has aroused great interest (Wu et al., 2021; Kostyusheva et al., 2022). In this review, we outline the potential application of CRISPR-Cas technology in ESKAPE, including the development of rapid and instant diagnosis methods and treatments to solve drug-resistant bacterial infections.

Diagnosis based on CRISPR-Cas has developed from an experimental nucleic acid sensing tool to a diagnostic technique for rapid, economical, and ultra-sensitive detection of clinical pathogens. CRISPR-Cas diagnosis is rapidly entering clinical application. For example, the first CRISPR-Cas diagnostic system for SARS-CoV-2-"SHERLOCK"-has been authorized by FDA for emergency use (Gupta et al., 2021). The widespread distribution of "SHERLOCK" enables clinicians and disease control departments to identify pathogens without passing a large number of complex and time-consuming ultra-sensitive tests, which not only reduces the cost of field deployment but also provides opportunities for better controlling the outbreak of infectious diseases (Mustafa and Makhawi, 2021). However, the diagnostic method of CRISPR-Cas is still in the development stage, and many obstacles may still hinder its further development. This technology must overcome many challenges before it can become an exciting new pathogen monitoring method. At present, the main disadvantage of common CRISPR-Cas diagnostic methods is that the nucleic acid of the target pathogen needs to be pre-amplified before detection to achieve sensitivity below the fmol range (Xu et al., 2020). The pre-amplification process not only increases the detection cost but also prolongs the reaction time, and also increases the complexity of the detection process. Modifying Cas protein or crRNA, detecting specific protein markers on the surface of pathogens, and adopting non-primer signal amplification strategy may be potential improvement schemes. Another major drawback is the off-target effect, which will lead to misunderstanding of the results, including false positives. Using bioinformatics methods to carefully design and select the guide RNA with the minimum potential off-target effect and to develop new Cas proteins with the least tolerance to mismatched sgRNA sequences can reduce the unexpected impact of off-target effect (Lee et al., 2016; Li et al., 2018; Li et al., 2019b). A fatal weakness of various diagnostic methods based on CRISPR-Cas is that they cannot quantify the pathogen load and can only provide relatively positive or negative results (van Dongen et al., 2020). How to standardize the results of different patients is another potential bottleneck of CRISPR-Cas diagnosis, which needs complete innovation of detection methods. In addition, the accurate information of genomes of newly emerging or clinically uncommon pathogens has not been fully explored. It is difficult to detect such pathogens on the CRISPR-Cas diagnostic platform, and our failure to retrieve relevant reports on the application of CRISPR-Cas in *E. faecalis* and *E. faecalis* proves this again.

CRISPR-Cas system has become the most efficient and convenient gene editing tool found so far. CRISPR-Cas gene editing technology has broad application prospects, especially in the treatment of many diseases caused by gene mutation or pathogen infection (Strich and Chertow, 2019; Sharma et al., 2021; Katti et al., 2022; Yeh et al., 2022). At present, the common methods of treating drug-resistant bacteria by CRISPR-Cas therapy can be divided into two categories. Targeting specific regions of bacterial chromosomes is a pathogen-centered method, while targeting plasmids carrying drug-resistant genes is another gene-centered method (Shabbir et al., 2019; Ekwebelem et al., 2021). According to different infection situations, a reasonable targeting scheme can eliminate specific strains and reduce the abundance of drug-resistant strains or genes in the host microbial community. The development of new CRISPR-Cas technology will achieve unprecedented control to eliminate drug-resistant bacteria without damaging beneficial bacteria. In addition, we suggest that the treatment of ESKAPE-resistant bacteria may be realized in the following two ways in the future: knocking down the essential host promoter of drug-resistant bacteria during infection, and enhancing the expression of host restriction factors by CRISPRa to improve the host's resistance to drug-resistant bacteria. Although CRISPR-Cas system has shown impeccable superiority in the treatment of drug-resistant bacteria, it is still only the first step in the clinical treatment of drug-resistant bacteria with CRISPR-Cas. This therapy faces various challenges in terms of safety, delivery, efficiency, and regulatory approval. The first is the most important safety in clinical treatment, which is a matter of priority before any kind of therapy is widely used. The off-target effect of CRISPR-Cas leads to nonspecific cleavage and cytotoxicity (Lin et al., 2014; Ortinski et al., 2017). The off-target effect can be predicted by various computer design tools, such as DeepCRISPR and CRISPRitz. Some strategies

have been adopted to reduce the mis-effect (Chuai et al., 2018; Cancellieri et al., 2020). It should also be noted that the natural inhibitor of CRISPR-Cas system—the anti-CRISPR-Cas system—has been widely used to minimize off-target effect and improve target specificity (Aschenbrenner et al., 2020; Dolgin, 2020). The lack of efficient *in vivo* delivery system is another major bottleneck of the CRISPR-Cas system. So far, several potential strategies for transient *in vivo* expression of CRISPR-Cas components have been introduced, including viruses, plasmids, lipid nanoparticles, and extracellular vesicles (Elaswad et al., 2018; Campbell et al., 2019; Chen et al., 2019). Considering that it may be more difficult for the CRISPR-Cas system to enter bacteria than host cells, it is necessary to find safer delivery and powerful gene editing methods to apply it to ESKAPE therapy.

The CRISPR-Cas system is a powerful tool for gene editing. There are still many discussions about CRISPR-Cas technology, especially on the molecular level to fight against several pathogens that seriously endanger patients' lives and health. Although CRISPR-Cas technology has outstanding advantages in the diagnosis and treatment of ESKAPE infection, more research is still in the laboratory stage, and there is still a long way to go from emerging technology to commercialization. Although it is still in its infancy and even needs a new biotechnology revolution to achieve a technological leap, the diagnosis and treatment methods based on CRISPR-Cas technology are the dominant players of the potential rules of the game. Using the method based on CRISPR-Cas to diagnose and treat pathogens including ESKAPE is a new reality in the field of clinical response to drug-resistant bacteria. In a word, solving the challenges and obstacles including effectiveness, specificity, and safety, and releasing the full potential of CRISPR-Cas system will bring exciting hope for preventing and fighting antibiotic resistance.

Author contributions

YQ and DZ performed literature search, led the review of the literature, and were involved in the visualization of concepts and in writing the first draft. ML provided revisions and additional conceptual input to the manuscript. YZ and HL prepared the figures. LY and ZY provided assistance for data acquisition. GH conceived, supervised, and finalized the work for submission. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (Nos. 81960353 and 82172238).

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

Akinola, O. T., Okedigba, I., and Elutade, O. O. (2022). Occurrence, antibiotic susceptibility and resistance genes among Staphylococcus aureus isolated from keypads of automated teller machines (ATM) in a private university, Nigeria. *Sci. Afr.* 15, 1–8. doi: 10.1016/j.sciaf.2022.e01111

Aschenbrenner, S., Kallenberger, S. M., Hoffmann, M. D., Hoffmann, M.D., Huck, A., Eils, R., et al. (2020). Coupling Cas9 to artificial inhibitory domains enhances CRISPR-Cas9 target specificity. *Sci. Adv.* 6, eaay0187. doi: 10.1126/sciadv.aay0187

Bikard, D., Euler, C. W., Jiang, W., Nussenzweig, P.M., Goldberg, G.W., Duportet, X., et al. (2014). Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nat. Biotechnol.* 32, 1146–1150. doi: 10.1038/nbt.3043

Bikard, D., Hatoum-Aslan, A., Mucida, D., and Marraffini, L. A. (2012). CRISPR interference can prevent natural transformation and virulence acquisition during in *vivo* bacterial infection. *Cell Host Microbe* 12, 177–186. doi: 10.1016/j.chom.2012.06.003

Campbell, L. A., Richie, C. T., Maggirwar, N. S., and Harvey, B. K. (2019). Cas9 ribonucleoprotein complex delivery: methods and applications for neuroinflammation. *J. Neuroimmune Pharmacol.* 14, 565–577. doi: 10.1007/s11481-019-09856-z

Cancellieri, S., Canver, M. C., Bombieri, N., Giugno, R., and Pinello, L. (2020). CRISPRitz: rapid, high-throughput and variant-aware in silico off-target site identification for CRISPR genome editing. *Bioinformatics* 36, 2001–2008. doi: 10.1093/bioinformatics/btz867

Cao, L. (2017). Identification and functional study of type III-A CRISPR-Cas system in clinical isolates of Staphylococcus aureus. *University of Science and Technology of China A dissertation for doctor's degree 2017* 306, 686–696. doi: 10.1016/j.ijmm.2016.08.005

Chen, G., Abdeen, A. A., Wang, Y., Shahi, P.K., Robertson, S., Xie, R., et al. (2019). A biodegradable nanocapsule delivers a Cas9 ribonucleoprotein complex for in *vivo* genome editing. *Nat. Nanotechnol* 14, 974–980. doi: 10.1038/s41565-019-0539-2

Chen, J. S., Ma, E., Harrington, L. B., da Costa, M., Tian, X., Palefsky, J.M., et al. (2018). CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science* 360, 436–439. doi: 10.1126/science.aar6245

Chuai, G., Ma, H., Yan, J., Chen, M., Hong, N., Xue, D., et al. (2018). DeepCRISPR: optimized CRISPR guide RNA design by deep learning. *Genome Biol.* 19, 80. doi: 10.1186/s13059-018-1459-4

Citorik, R. J., Mimee, M., and Lu, T. K. (2014). Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nat. Biotechnol.* 32, 1141–1145. doi: 10.1038/nbt.3011

Das, S., Bombaywala, S., Srivastava, S., Kapley, A., Dhodapkar, R., Dafale, N.A., et al. (2022). Genome plasticity as a paradigm of antibiotic resistance spread in ESKAPE pathogens. *Environ. Sci. pollut. Res. Int.* 29, 40507–40519. doi: 10.1007/s11356-022-19840-5

de Maat, V., Stege, P. B., Dedden, M., Hamer, M., Pijkeren van, J.-P., Willems, R.J.L., et al. (2019). CRISPR-Cas9-mediated genome editing in vancomycin-resistant Enterococcus faecium. *FEMS Microbiol. Lett.* 366, 1–22. doi: 10.1093/femsle/fnz256

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, 1–49. doi: 10.1128/CMR.00181-19

Dolgin, E. (2020). The kill-switch for CRISPR that could make gene-editing safer. *Nature* 577, 308–310. doi: 10.1038/d41586-020-00053-0

Dong, D., Ren, K., Qiu, X., Zheng, J., Guo, M., Guan, X., et al. (2016). The crystal structure of Cpf1 in complex with CRISPR RNA. *Nature* 532, 522–526. doi: 10.1038/nature17944

Doudna, J. A., and Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346, 1258096. doi: 10.1126/science.1258096

Du, X., Zhou, H., Fu, Y., Qing, Y., and Jianying, Z. (2020). Antibiotic resistance and prognostic factors of Pseudomonas aeruginosa bloodstream infection. *Chin. J. Infect. Chemother.* 20, 118-124. doi: CNKI:SUN:KGHL.0.2020-02-003

Ekwebelem, O. C., Aleke, J., Ofielu, E., and Nnorom-Dike, O. (2021). CRISPR-cas9 system: A revolutionary tool in the fight against antimicrobial resistance: retracted. *Infect. Microbes Dis.* 3, 51–56. doi: 10.1097/IM9.0000000000000049

Elaswad, A., Khalil, K., Cline, D., Page-McCaw, P., Chen, W., Michel, M., et al. (2018). Microinjection of CRISPR/Cas9 Protein into Channel Catfish, Ictalurus punctatus, Embryos for Gene Editing. *J. Vis. Exp*, e56275. doi: 10.3791/56275

Eze, E. C., Chenia, H. Y., and El Zowalaty, M. E. (2018). Acinetobacter baumannii biofilms: effects of physicochemical factors, virulence, antibiotic resistance determinants, gene regulation, and future antimicrobial treatments. *Infect. Drug Resist.* 11, 2277–2299. doi: 10.2147/IDR.S169894

Fage, C., Lemire, N., and Moineau, S. (2021). Delivery of CRISPR-Cas systems using phage-based vectors. *Curr. Opin. Biotechnol.* 68, 174–180. doi: 10.1016/j.copbio.2020.11.012

Fan, H., Wu, T., Zhu, X., Yiyue, G., Xiaoyan, Z., Yongjun, J., et al. (2016). Inhibition of StxII gene expression of Enterohemorrhagic Escherichia coli O157:H7 by CRISPR/Cas9 genome editing technology. *Jiangsu J. Prev. Med.* 27, 520–523.

Feng, H., Li, J., Wang, Y., Mengying, L., Wenhao, Z., Duanyang, L., et al. (2019). Screening of CRIPSPR system in Acinetobacter baumannii and analysis of its regulation on target genes. *J. Yangzhou Univ. (Agricultural Life Sci. Edition)* 40, 54–57;64.

Folliero, V., Lama, S., Franci, G., Giugliano, R., D'Auria, G., Ferranti, P., et al. (2022). Casein-derived peptides from the dairy product kashk exhibit wound healing properties and antibacterial activity against Staphylococcus aureus: Structural and functional characterization. *Food Res. Int.* 153, 1–11. doi: 10.1016/j.foodres.2022.110949

Gonzalez de Aledo, M., Gonzalez-Bardanca, M., Blasco, L., Pacios, O., Bleriot, I., Fernández-García, L., et al. (2021). CRISPR-cas, a revolution in the treatment and study of ESKAPE infections: pre-clinical studies. *Antibiotics (Basel)* 10, 1–23. doi: 10.3390/antibiotics10070756

Guan, J. (2019). Molecular mechanism of genomic remodeling mediated by type III-A CRISPR-Cas system in Staphylococcus aureus. *University of Science and Technology of China A dissertation for doctor's degree 2019*.

Gupta, R., Kazi, T. A., Dey, D., Ghosh, A., Ravichandiran, V., Swarnakar, S., et al. (2021). CRISPR detectives against SARS-CoV-2: a major setback against COVID-19 blowout. *Appl. Microbiol. Biotechnol.* 105, 7593–7605. doi: 10.1007/s00253-021-11583-6

Harding, C. M., Hennon, S. W., and Feldman, M. F. (2018). Uncovering the mechanisms of Acinetobacter baumannii virulence. *Nat. Rev. Microbiol.* 16, 91–102. doi: 10.1038/nrmicro.2017.148

Hutchings, M. I., Truman, A. W., and Wilkinson, B. (2019). Antibiotics: past, present and future. *Curr. Opin. Microbiol.* 51, 72–80. doi: 10.1016/j.mib.2019.10.008

Jiang, C. Z., Qiaoying,, Wang, M., Pan, Y., Liu, X., et al. (2020). Construction of srtA-Knockout Strain in Staphylococcus aureus by CRISPR/Cas9 Technology. *Biotechnol. Bull.* 36, 253-265. doi: 10.13560/j.cnki.biotech.bull.1985.2020-0195

Katti, A., Diaz, B. J., Caragine, C. M., Sanjana, N. E., and Dow, L. E. (2022). CRISPR in cancer biology and therapy. *Nat. Rev. Cancer* 22, 259–279. doi: 10.1038/s41568-022-00441-w

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., et al. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. U.S.A.* 115, E3463–e3470. doi: 10.1073/pnas.1717295115

Kostyusheva, A., Brezgin, S., Babin, Y., Vasilyeva, I., Glebe, D., Kostyushev, D., et al. (2022). CRISPR-Cas systems for diagnosing infectious diseases. *Methods* 203, 431–446. doi: 10.1016/j.ymeth.2021.04.007

Kumari, R., Rathi, R., Pathak, S. R., and Dalal, V.. (2022). Structural-based virtual screening and identification of novel potent antimicrobial compounds against YsxC of Staphylococcus aureus. *J. Mol. Structure* 1255, 132476. doi: 10.1016/j.molstruc.2022.132476

Laxminarayan, R., Matsoso, P., Pant, S., Brower, C., Røttingen, J.-A., Klugman, K., et al. (2016). Access to effective antimicrobials: a worldwide challenge. *Lancet* 387, 168–175. doi: 10.1016/S0140-6736(15)00474-2

Lee, C. M., Cradick, T. J., and Bao, G. (2016). The neisseria meningitidis CRISPR-cas9 system enables specific genome editing in mamMalian cells. *Mol. Ther.* 24, 645–654. doi: 10.1038/mt.2016.8

Li, S., Cheng, Q., Wang, J., Li, X., Zhang, Z., Gao, S., et al. (2018). CRISPR-Cas12a-assisted nucleic acid detection. *Cell Discovery* 4, 20. doi: 10.1038/s41421-018-0028-z

Li, Y., Li, S., Wang, J., and Liu, G. (2019b). CRISPR/cas systems towards next-generation biosensing. *Trends Biotechnol.* 37, 730–743. doi: 10.1016/j.tibtech.2018.12.005

Li, L. X., Li, S. Y., Wu, N., Wu, J., Wang, G., Zhao, G., et al. (2019a). HOLMESv2: A CRISPR-cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation. *ACS Synthetic Biol.* 8, 2228–2237. doi: 10.1021/acssynbio.9b00209

Li, Y., Shi, Z., Hu, A., Cui, J., Yang, K., Liu, Y., et al (2022). Rapid one-tube RPA-CRISPR/cas12 detection platform for methicillin-resistant staphylococcus aureus. *LID* 12, 829. doi: 10.3390/diagnostics12040829

Li, J., Yang, L., Yu, T., Xiaoyan, T., Yongmei, H., Haichen, W., et al. (2021). Effect of CRISPR system on drug resistance and virulence of Escherichia coli isolated from faecal specimen. *Chin. J. Microecol* 33, 394–265. doi: 10.13381/j.cnki.cjm.202104004

Li, J., Yang, S., Zuo, C., Zuo, C., Dai, L., Guo, Y., et al. (2020). Applying CRISPR-cas12a as a signal amplifier to construct biosensors for non-DNA targets in ultralow concentrations. *ACS Sens* 5, 970–977. doi: 10.1021/acssensors.9b02305

Liang, W. J., Zhang, R. G., Duan, G. C., Hong, L., Zhang, B., Xi, Y., et al. (2016). [A surveillance study on CRISPR/Cas molecular biomarker in Escherichia coli]. *Zhonghua Liu Xing Bing Xue Za Zhi* 37, 1080–1086. doi: 10.3760/cma.j.issn.0254-6450.2016.08.005

Lin, Y., Cradick, T. J., Brown, M. T., Deshmukh, H., Ranjan, P., Sarode, N., et al. (2014). CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. *Nucleic Acids Res.* 42, 7473–7485. doi: 10.1093/nar/gku402

Liu, Y., Wang, Y., Schwarz, S., Li, Y., Shen, Z., Zhang, Q., et al. (2013). Transferable multiresistance plasmids carrying cfr in Enterococcus spp. from swine and farm environment. *Antimicrob. Agents Chemother.* 57, 42–48. doi: 10.1128/AAC.01605-12

Manyi-Loh, C., Mamphweli, S., Meyer, E., and Okoh, A. (2018). Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules* 23, 1–48. doi: 10.3390/molecules23040795

Mohanraju, P., Makarova, K. S., Zetsche, B., Zhang, F., Koonin, E.V., and Oost Der Van, J. (2016). Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. *Science* 353, aad5147. doi: 10.1126/science.aad5147

Mukama, O., Wu, J., Li, Z., Liang, Q., Yi, Z., Lu, X., et al. (2020). An ultrasensitive and specific point-of-care CRISPR/Cas12 based lateral flow biosensor for the rapid detection of nucleic acids. *Biosens Bioelectron* 159, 112143. doi: 10.1016/j.bios.2020.112143

Mustafa, M. I., and Makhawi, A. M. (2021). SHERLOCK and DETECTR: CRISPR-cas systems as potential rapid diagnostic tools for emerging infectious diseases. *J. Clin. Microbiol.* 59, 1–30. doi: 10.1128/JCM.00745-20

Myhrvold, C., Freije, C. A., Gootenberg, J. S., Abudayyeh, O.O., Metsky, H. C., Durbin, A.F., et al. (2018). Field-deployable viral diagnostics using CRISPR-Cas13. *Science* 360, 444–448. doi: 10.1126/science.aas8836

Nadeem, S. F., Gohar, U. F., Tahir, S. F., Mukhtar, H., Pornpukdeewattana, S., Nukthamna, P., et al. (2020). Antimicrobial resistance: more than 70 years of war between humans and bacteria. *Crit. Rev. Microbiol.* 46, 578–599. doi: 10.1080/1040841X.2020.1813687

Ortinski, P. I., O'Donovan, B., Dong, X., and Kantor, B. (2017). Integrase-deficient lentiviral vector as an all-in-one platform for highly efficient CRISPR/cas9-mediated gene editing. *Mol. Ther. Methods Clin. Dev.* 5, 153–164. doi: 10.1016/j.omtm.2017.04.002

Price, V. J., Huo, W., Sharifi, A., and Palmer, K. L. (2016). CRISPR-Cas and Restriction-Modification Act Additively against Conjugative Antibiotic Resistance Plasmid Transfer in Enterococcus faecalis. *mSphere* 1, 1–13. doi: 10.1128/mSphere.00064-16

Qin, S., Li, H., Shan, D., Qin, Z., and Yonglei, X. (2016). The present situation and research progress in the treatment of tetracycline antibiotic manufacturing wastewater. *J. Of Tianjin Univ. Of Technol.* 32 (02), 50–54;60. doi: 10.3969/j.issn.1673-095X.2016.002.012

Qiu, X., Liu, X., Ma, X., Wang, R., Chen, S., Li, F., et al. (2022). One-pot isothermal LAMP-CRISPR-based assay for klebsiella pneumoniae detection. *Microbiol. Spectr.* 10, e01545-22. doi: 10.1128/spectrum.01545-22

Qiu, X., Liu, X., Wang, R., Ma, X., Han, L., Yao, J., et al. (2023). Accurate, sensitive, and rapid detection of pseudomonas aeruginosa based on CRISPR/cas12b with one fluid-handling step. *Microbiol. Spectr.* 11, e03523-22. doi: 10.1128/spectrum.03523-22

Rodrigues, M., McBride, S. W., Hullahalli, K., Palmer, K. L., and Duerkop, B. A. (2019). Conjugative delivery of CRISPR-cas9 for the selective depletion of antibiotic-resistant enterococci. *Antimicrob. Agents Chemother.* 63, 1–35. doi: 10.1128/AAC.01454-19

Roth, N., Kasbohrer, A., Mayrhofer, S., Zitz, U., Hofacre, C., and Domig, K. J. (2019). The application of antibiotics in broiler production and the resulting antibiotic resistance in Escherichia coli: A global overview. *Poult Sci.* 98, 1791–1804. doi: 10.3382/ps/pey539

Shabbir, M. A. B., Shabbir, M. Z., Wu, Q., Mahmood, S., Sajid, A., Maan, M. K., et al. (2019). CRISPR-cas system: biological function in microbes and its use to treat antimicrobial resistant pathogens. *Ann. Clin. Microbiol. Antimicrob.* 18, 21. doi: 10.1186/s12941-019-0317-x

Shapiro, D. J., Hicks, L. A., Pavia, A. T., and Hersh, A. L. (2014). Antibiotic prescribing for adults in ambulatory care in the USA 2007-09. *J. Antimicrob. Chemother.* 69, 234–240. doi: 10.1093/jac/dkt301

Sharma, G., Sharma, A. R., Bhattacharya, M., Lee, S.-S., and Chakraborty, C. (2021). CRISPR-cas9: A preclinical and clinical perspective for the treatment of human diseases. *Mol. Ther.* 29, 571–586. doi: 10.1016/j.ymthe.2020.09.028

Shrestha, P., Cooper, B. S., Coast, J., Oppong, R., Do Thi Thuy, N., Phodha, T., et al. (2018). Enumerating the economic cost of antimicrobial resistance per antibiotic consumed to inform the evaluation of interventions affecting their use. *Antimicrob. Resist. Infect. Control* 7, 98. doi: 10.1186/s13756-018-0384-3

Sivalingam, P., Poté, J., and Prabakar, K. (2019). Environmental prevalence of carbapenem resistance enterobacteriaceae (CRE) in a tropical ecosystem in India: human health perspectives and future directives. *Pathogens* 8, 1–15. doi: 10.3390/pathogens8040174

Souza-Silva, T., Rossi, C. C., Andrade-Oliveira, A. L., Vilar Cecílio, L., Pereira Fábia, M., Araújo, B., et al. (2022). Interspecies transfer of plasmid-borne gentamicin resistance between Staphylococcus isolated from domestic dogs to Staphylococcus aureus. *Infect. Genet. Evol.* 98, 105230. doi: 10.1016/j.meegid.2022.105230

Strich, J. R., and Chertow, D. S. (2019). CRISPR-cas biology and its application to infectious diseases. *J. Clin. Microbiol.* 57, 1–35. doi: 10.1128/JCM.01307-18

Su, X., Ge, Y., Zheng, Q., Xiaojuan, Z., Yin, C., Tao, W., et al. (2020). Rapid detection of Staphylococcus aureus by CRISPR-Cas13a combined with recombinase aided amplification(RAA). *J. Pathogen Biol.* 15, 253–258. doi: CNKI:SUN:ZISC.0.2020-03-002

Sun, Y., Zhang, Z., and Qi, R. (2019). Study on plasmids-mediated quinolone resistance genes of Klebsiella pneumoniae in a hospital. *J. Of Taishan Med. Coll.* 40, 536–538. doi: 10. 3969 /j. issn. 1004-7115. 2019. 07. 015

Sutradhar, I., Ching, C., Desai, D., Suprenant, M., Briars, E., Heins, Z., et al. (2021). Computational model to quantify the growth of antibiotic-resistant bacteria in wastewater. *mSystems* 6, e0036021. doi: 10.1128/mSystems.00360-21

Towse, A., Hoyle, C. K., Goodall, J., Hirsch, M., Mestre-Ferrandiz, J., Rex, J. H., et al. (2017). Time for a change in how new antibiotics are reimbursed: Development of an insurance framework for funding new antibiotics based on a policy of risk mitigation. *Health Policy* 121, 1025–1030. doi: 10.1016/j.healthpol.2017.07.011

Tyrrell, C., Burgess, C. M., Brennan, F. P., and Walsh, F. (2019). Antibiotic resistance in grass and soil. *Biochem. Soc. Trans.* 47, 477–486. doi: 10.1042/BST20180552

van Dongen, J. E., Berendsen, J. T. W., Steenbergen, R. D. M., Wolthuis, R. M. F., Eijkel, J. C. T., Segerink, L. I., et al. (2020). Point-of-care CRISPR/Cas nucleic acid detection: Recent advances, challenges and opportunities. *Biosens Bioelectron* 166, 112445. doi: 10.1016/j.bios.2020.112445

Wang, J. Y., and Doudna, J. A. (2023). CRISPR technology: A decade of genome editing is only the beginning. *Science* 379, eadd8643. doi: 10.1126/science.add8643

Wang, Y., Guo, Y., Zhang, L., Yang, Y., Yang, S., Yang, L., et al. (2021). Integration of multiplex PCR and CRISPR-Cas allows highly specific detection of multidrug-resistant Acinetobacter Baumannii. *Sensors Actuators B: Chem.* 334, 1–8. doi: 10.1016/j.snb.2021.129600

Wang, Y., Wang, Z., Chen, Y., Hua, X., Yu, Y., Ji, Q., et al. (2019b). A highly efficient CRISPR-cas9-based genome engineering platform in acinetobacter baumannii to understand the H2O2-sensing mechanism of oxyR. *Cell Chem. Biol.* 26, 1732–1742 e1735. doi: 10.1016/j.chembiol.2019.09.003

Wang, B., Wang, R., Wang, D., Wu, J., Li, J., Wang, J, et al. (2019a). Cas12aVDet: A CRISPR/cas12a-based platform for rapid and visual nucleic acid detection. *Anal. Chem.* 91, 12156–12161. doi: 10.1021/acs.analchem.9b01526

Watters, K. E., Fellman, C., Bai, H. B., Ren, S. M., and Doudna, J. A. (2018). Systematic discovery of natural CRISPR-Cas12a inhibitors. *Science* 362, 236-239. doi: 10.1126/science.aau5138

Wieland, K., Chhatwal, P., and Vonberg, R. P. (2018). Nosocomial outbreaks caused by Acinetobacter baumannii and Pseudomonas aeruginosa: Results of a systematic review. *Am. J. Infect. Control* 46, 643–648. doi: 10.1016/j.ajic.2017.12.014

Wu, Y., Battalapalli, D., Hakeem, M. J., Selamneni, V., Zhang, P., Draz, M. S., et al. (2021). Engineered CRISPR-Cas systems for the detection and control of antibiotic-resistant infections. *J. Nanobiotechnology* 19, 401. doi: 10.1186/s12951-021-01132-8

Wu, D., Guan, X. Y., Zhu, Y. W., Ren, K., and Huang, Z.. (2017). Structural basis of stringent PAM recognition by CRISPR-C2c1 in complex with sgRNA. *Cell Res.* 27, 705–708. doi: 10.1038/cr.2017.46

Wu, Z., Wang, S., Zhao, J., Zhao, J., and Zhou, X. (2020). Drug resistance mechanism of carbapenem-resistant acinetobacter baumannii and selection of antibacterial drugs. *Med. Recapitulate* 26, 138–147.

Xiang, L., Qi, F., Jiang, L., Tan, J., Deng, C., Wei, Z., et al. (2020). CRISPR-dCas9-mediated knockdown of prtR, an essential gene in Pseudomonas aeruginosa. *Lett. Appl. Microbiol.* 71, 386–393. doi: 10.1111/lam.13337

Xu, C., Gao, Z., Bi, Z., Wu, S., and Bao, W. (2021). Establishment of TLR5 gene knockout porcine alveolar macrophage cell line mediated by CRISPR/Cas9 and its effect on Gram-negative bacterial adhesion. *J. Yangzhou Univ.* 42, 32-39. doi: 10.16872/j.cnki.1671-4652.2021.02.005

Xu, H., Zhang, X., Cai, Z., Dong, X., Chen, G., Li, Z., et al. (2020). An isothermal method for sensitive detection of mycobacterium tuberculosis complex using clustered regularly interspaced short palindromic repeats/cas12a cis and trans cleavage. *J. Mol. Diagn.* 22, 1020–1029. doi: 10.1016/j.jmoldx.2020.04.212

Yang, S. (2016). Analyzing the CRISPR structures in staphylococcus aureus and using CRISPR/Cas9 technic to target the mecA gene. *Zhengzhou Univ. degree Master*.

Yao, S., Wei, D., Tang, N., Song, Y., Wang, C., Feng, J., et al. (2022). Efficient suppression of natural plasmid-borne gene expression in carbapenem-resistant klebsiella pneumoniae using a compact CRISPR interference system. *Antimicrob. Agents Chemother.* 66, e0089022. doi: 10.1128/aac.00890-22

Yeh, T. K., Jean, S. S., Lee, Y. L., Lu, M.-C., Ko, W.-C., Lin, H.-J., et al. (2022). Bacteriophages and phage-delivered CRISPR-Cas system as antibacterial therapy. *Int. J. Antimicrob. Agents* 59, 106475. doi: 10.1016/j.ijantimicag.2021.106475

Zhang, M., Bi, C., Wang, M., Fu, H., Mu, Z., Zhu, Y., et al. (2019). Analysis of the structures of confirmed and questionable CRISPR loci in 325 Staphylococcus genomes. *J. Basic Microbiol.* 59, 901–913. doi: 10.1002/jobm.201900124

Zhang, X., Paganelli, F. L., Bierschenk, D., Kuipers, A., Bonten, M. J. M., Willems, R. J. L., et al. (2012). Genome-wide identification of ampicillin resistance determinants in Enterococcus faecium. *PloS Genet.* 8, e1002804. doi: 10.1371/journal.pgen.1002804

Zhou, J., Yin, L., Dong, Y., Peng, L., Liu, G., Man, S., et al. (2020). CRISPR-Cas13a based bacterial detection platform: Sensing pathogen Staphylococcus aureus in food samples. *Anal. Chim. Acta* 1127, 225–233. doi: 10.1016/j.aca.2020.06.041

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 27 October 2023
DOI 10.3389/fcimb.2023.1274175

OPEN ACCESS

EDITED BY
Sanket Kaushik,
Amity University Jaipur, India

REVIEWED BY
Brian Weinrick,
Trudeau Institute, United States
Yasu S. Morita,
University of Massachusetts Amherst, United States
Ian Sparks,
University of Massachusetts Amherst, United States, in collaboration with reviewer YSM

*CORRESPONDENCE
Alyssa Schami
aschami@txbiomed.org
Jordi B. Torrelles
jtorrelles@txbiomed.org

RECEIVED 07 August 2023
ACCEPTED 02 October 2023
PUBLISHED 27 October 2023

CITATION
Schami A, Islam MN, Belisle JT and Torrelles JB (2023) Drug-resistant strains of *Mycobacterium tuberculosis*: cell envelope profiles and interactions with the host. *Front. Cell. Infect. Microbiol.* 13:1274175. doi: 10.3389/fcimb.2023.1274175

Drug-resistant strains of *Mycobacterium tuberculosis*: cell envelope profiles and interactions with the host

Alyssa Schami[1,2]*, M. Nurul Islam[3], John T. Belisle[3] and Jordi B. Torrelles[1,4]*

[1]Population Health Program, Texas Biomedical Research Institute, San Antonio, TX, United States, [2]Integrated Biomedical Sciences Program, University of Texas Health Science Center at San Antonio, San Antonio, TX, United States, [3]Mycobacteria Research Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, United States, [4]International Center for the Advancement of Research & Education, International Center for the Advancement of Research & Education, Texas Biomedical Research Institute, San Antonio, TX, United States

In the past few decades, drug-resistant (DR) strains of *Mycobacterium tuberculosis* (*M.tb*), the causative agent of tuberculosis (TB), have become increasingly prevalent and pose a threat to worldwide public health. These strains range from multi (MDR) to extensively (XDR) drug-resistant, making them very difficult to treat. Further, the current and future impact of the Coronavirus Disease 2019 (COVID-19) pandemic on the development of DR-TB is still unknown. Although exhaustive studies have been conducted depicting the uniqueness of the *M.tb* cell envelope, little is known about how its composition changes in relation to drug resistance acquisition. This knowledge is critical to understanding the capacity of DR-*M.tb* strains to resist anti-TB drugs, and to inform us on the future design of anti-TB drugs to combat these difficult-to-treat strains. In this review, we discuss the complexities of the *M.tb* cell envelope along with recent studies investigating how *M.tb* structurally and biochemically changes in relation to drug resistance. Further, we will describe what is currently known about the influence of *M.tb* drug resistance on infection outcomes, focusing on its impact on fitness, persister-bacteria, and subclinical TB.

KEYWORDS

Mycobacterium tuberculosis, tuberculosis, drug resistance, cell envelope lipids, M.tb-host interactions

1 Introduction

Tuberculosis (TB), the disease caused by the pathogen *Mycobacterium tuberculosis* (*M.tb*), is currently estimated to cause ~1.6 million deaths annually worldwide and is considered a leading cause of death due to a single infectious organism (WHO, 2022a). Recently, the World Health Organization (WHO) updated their definitions of drug-resistant (DR)-TB along with the newest approved treatment regimens being implemented. As defined, multi drug-resistant (MDR)-TB is caused by a *M.tb* strain with resistance to isoniazid (INH, H) and rifampicin (RIF, R). Pre-extensively drug-resistant (pre-XDR)-TB is caused by a *M.tb* strain resistant to RIF (may also be resistant to INH) and to at least one fluoroquinolone [levofloxacin (LEV) or moxifloxacin (MFX, M)]. Lastly, *M.tb* strains that cause extensively drug-resistant (XDR)-TB are resistant to RIF (may also be resistant to INH), at least one fluoroquinolone (LEV or MFX), and one "Group A" drug [bedaquiline (BDQ, B) or linezolid (LZD, L)] (Table 1) (WHO, 2022c). From this point on, the one letter drug abbreviation will only be used when referring to a combined treatment.

Standard anti-TB treatment regimens for both drug-susceptible (DS)- and DR-TB were also recently updated by the WHO in May 2022. Previously, treatment for DS-TB included a 2-month intensive phase of treatment with INH, RIF, pyrazinamide (PZA, Z) and ethambutol (EMB, E), followed by a 4-month continuation phase with just INH and RIF (2HRZE/4HR) (WHO, 2022b). The new WHO recommendation for DS-TB treatment also includes implementation of a newer, 4-month daily treatment regimen consisting of rifapentine (RPT), INH, PZA, and MFX that is non-inferior to the 6-month DS-TB treatment regimens (Table 2) (Carr et al., 2022, WHO, 2022b). The WHO updated recommendations for DR-TB treatment includes three separate treatment options for diagnosed patients. Choice of DR-TB treatment regimen is based on factors such as the *M.tb* drug-resistance profile, prior exposure to anti-TB drugs, and the extent of pulmonary TB disease among others (WHO, 2022c). Of the three possible treatment regimens, the newest and preferred DR-TB regimen for RIF-resistant (RR)/MDR-TB cases consists of BDQ, pretomanid (Pa), LZD, and MFX (BPaL+ M) as an all-oral regimen that lasts 6 months. For pre-XDR-TB cases, the same treatment regimen is recommended without MFX (Nyang'wa et al., 2022, WHO, 2022c). An additional recommended treatment for MDR/RR-TB is a 9-month all-oral regimen consisting of BDQ for 4-6 months in combination with a fluoroquinolone, ethionamide (ETH), EMB, INH (high dose), PZA, and clofazimine (CFZ), and treatment with a fluoroquinolone, CFZ, EMB, and PZA for the remaining 5 months (WHO, 2022c). Finally, a longer individualized treatment regimen is recommended for patients with DR-TB that have not had desirable outcomes with other regimens, are intolerant to specific components of the other regimens, or have XDR-TB (WHO, 2022c). These individualized regimens last at least 18 months and have poor adherence to due to the long duration and the increased likelihood of adverse side effects; thus, one of the other two regimens is preferred if possible.

Many anti-TB drugs target *M.tb* cell envelope biogenesis (Table 3). However, the mechanisms used by *M.tb* to regulate its cell envelope composition and the outcomes in relation to drug resistance (and, consequently, how this regulation influences TB pathogenesis) are poorly understood. Various studies have examined the complex cell envelope of *M.tb* and show that there are differences in lipid composition that are strain specific (Torrelles et al., 2004; Torrelles et al., 2008; Torrelles et al., 2011; Howard et al., 2018). Thus, in order to advance the TB field, it is required that we increase our understanding of similarities and/or differences in the cell envelope composition of DR-*M.tb* strains and how these changes influence bacteria-host cell interactions and infection outcomes. In this review, we will present what is currently known about how the *M.tb* cell envelope changes in relation to drug resistance, examine the influence that drug resistance has on *M.tb* infection outcomes, and address some of the gaps in knowledge that remain to be elucidated.

2 The *M.tb* cell envelope

The *M.tb* cell envelope is a thick and complex structure that provides a unique barrier of protection to the bacterium in various environments (Garcia-Vilanova et al., 2019). It is comprised of multiple layers that each contribute to its complex composition (detailed in Figure 1, adapted from (Jackson, 2014). The innermost layer is a plasma membrane similar to that of many other bacterial species (Jackson, 2014). Outside of the plasma membrane, a layer of peptidoglycan (PG) is covalently attached to the arabinogalactan

TABLE 1 Updated drug resistance categories based on the new WHO definitions (WHO, 2022c).

Drug Resistance Category	Resistant to:			
	Rifampicin	Isoniazid	Levofloxacin or Moxifloxacin*	Bedaquiline or Linezolid**
Rifampicin resistant (RR)	+	-	-	-
Multi drug-resistant (MDR)	+	+	-	-
Pre-extensively drug-resistant (Pre-XDR)	+	Possibly	+	-
Extensively drug-resistant (XDR)	+	Possibly	+	+

*At least one fluoroquinolone drug.
**At least one "Group A" drug.
"+" signifies resistance, "-" signifies susceptibility.

TABLE 2 Updated treatment regimens for each disease classification based on the new WHO recommendations (Carr et al., 2022; Nyang'wa et al., 2022, WHO, 2022b, WHO, 2022c).

Disease Classification	Treatment Regimen	Length of Treatment	All-Oral	Age Recommendation
DS-TB	2HRZE/4HR	2 months of HRZE; 4 months of HR (6 months total)	Yes	All ages
	2HPMZ/2HPM	2 months of HPMZ; 2 months of HPM (4 months total)	Yes	People ≥ 12 years old
MDR/RR-TB	BPaLM*	6 months	Yes	People ≥ 14 years old
	9-month all-oral	9 months	Yes	All ages
Pre-XDR-TB	BPaL	6 months	Yes	People ≥ 14 years old
MDR/RR-TB, pre-XDR-TB,** or XDR-TB**	Longer individualized	18 months	No	All ages

*Preferred method of treatment for MDR/RR-TB.
**Longer individualized treatment regimen only suggested if other treatment regimens cannot be used.
H, Isoniazid; R, Rifampicin; Z, Pyrazinamide; E, Ethambutol; M, Moxifloxacin; P, Rifapentine; B, Bedaquiline; Pa, Pretomanid; L, Linezolid.

TABLE 3 Mechanisms of action of Anti-TB Drugs.

Drug Target	Drug	Mechanism(s) of Action	Treatment Regimen*	References
DNA/RNA/ Protein Synthesis	RIF	Inhibits *M.tb* DNA-dependent RNA polymerase to suppress RNA synthesis	DS-TB	Campbell et al., 2001; Tupin et al., 2010
	RPT	Inhibits *M.tb* DNA-dependent RNA polymerase to suppress RNA synthesis (similar to RIF); Longer half-life, lower MIC, and higher protein binding than RIF	DS-TB	Alfarisi et al., 2017; Zheng et al., 2017
	MOX	Inhibits *M.tb* topoisomerase II (DNA gyrase)	DS-TB or MDR/RR-TB	Naidoo et al., 2017
	LNZ	Prevents the formation of functional bacterial 70S ribosomal initiation complex to inhibit bacterial protein translation	MDR/RR-TB or Pre-XDR-TB	Diekema and Jones, 2000
Cell Envelope Permeability	INH	Competitively inhibits InhA through the covalent formation of the INH-NAD adduct to prevent MAc biosynthesis	DS-TB	Wilson et al., 1995; Chen et al., 2002; Broussy et al., 2003; Schroeder et al., 2005; Vilchèze and Jacobs, 2007
	PZA	Pyrazinoic acid (active form of PZA) inhibits fatty acid synthesis by interfering with FAS I, blocks ATP production, and inhibits *M.tb* protein synthesis	DS-TB	Ngo et al., 2007; Zimhony et al., 2007
	EMB	Inhibits *M.tb* arabinosyltransferases to interfere with the biosynthesis of arabinose-containing cell envelope components and prevent bacterial division	DS-TB	Escuyer et al., 2001; Zhang et al., 2003; Amin et al., 2008; Goude et al., 2009; Zhang et al., 2020; Lee and Nguyen, 2023
	Pa	Inhibits MAc biosynthesis in aerobic conditions; Causes respiratory poisoning of *M.tb* via the nitrosylating effects of nitroimidazole, which release reactive nitrogen species under anaerobic conditions	MDR/RR-TB or Pre-XDR-TB	Singh et al., 2008; Thompson et al., 2017
	ETH	Competitively inhibits InhA (similar to INH) through the covalent formation of the ETH-NAD adduct to prevent mycolic acid biosynthesis	MDR/RR-TB	Banerjee et al., 1994
	DLM	Inhibits methoxy- and keto-mycolic acid synthesis via the F420 coenzyme system and generates nitrous oxide via the nitrosylating effects of nitroimidazole	MDR/RR-TB	Singh et al., 2008; Khoshnood et al., 2021
Energy Production	BDQ	Inhibits the activity of mycobacterial ATP synthase	MDR/RR-TB or Pre-XDR-TB	Sarathy et al., 2019
	CFZ	Interferes with K^+ uptake and ATP production by interacting with *M.tb* membrane phospholipids to destabilize the membrane	MDR/RR-TB	Cholo et al., 2012

*Longer treatment regimens for DR-TB may use an individualized combination of drugs listed based on previous outcomes with specific drugs, intolerance, or the diagnosis of XDR-TB.
RIF, Rifampicin; RPT, Rifapentine; MFX, Moxifloxacin; LZD, Linezolid; INH, Isoniazid; PZA, Pyrazinamide; EMB, Ethambutol; Pa, Pretomanid; DLM, Delamanid; ETH, Ethionamide; BDQ, Bedaquiline; CFZ, Clofazimine.

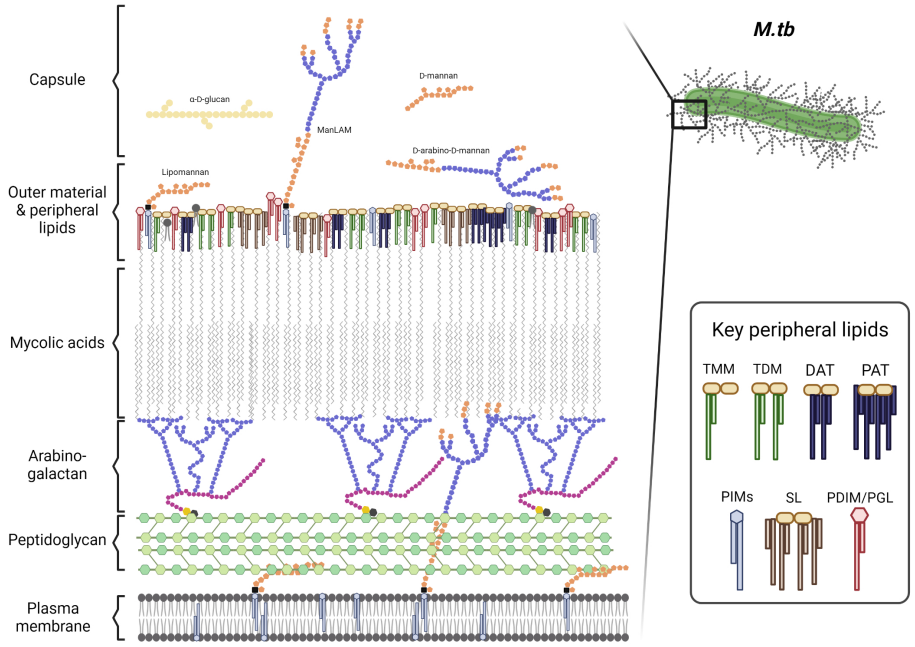


FIGURE 1
The *M.tb* cell envelope. Adapted from Mary Jackson (Jackson, 2014). TMM, trehalose monomycolate; TDM, trehalose dimycolate; DAT, diacyl-trehalose; PAT, pentaacyl-trehalose; PIMs, phosphatidyl-*myo*-inositol mannosides; SL, sulfolipids; PDIM, phthiocerol dimycocerosates; and PGL, phenolic glycolipids. Cell envelope components size are not depicted at a proportional scale. Figure created in BioRender.

(AG) polysaccharide. The non-reducing ends of AG are esterified to the long-chain (C_{60}-C_{90}) mycolic acids (MAcs) that are unique in nature to *Mycobacterium spp* and complete the 'cell wall core' of the cell envelope (Jackson, 2014; Garcia-Vilanova et al., 2019). Non-covalently bound lipoglycans, glycolipids, and lipids span the MAcs and create a peripheral lipid layer. On the top of this peripheral lipid layer the outer material '*capsule*' is composed of α-glucan, arabinomannan, mannan, and several proteins among others. The peripheral lipid layer includes key lipid classes known to influence infection outcomes such as phthiocerol dimycoserosates (PDIMs), phenolic glycolipids (PGLs, present in some *M.tb* strains), trehalose monomycolate (TMM) and dimycolate (TDM), diacyl/triacyl/pentaacyl-trehaloses (DAT/TAT/PAT), sulfolipids (SL-1), and phosphatidyl-*myo*-inositol mannosides (PIMs) and their associated lipoglycans such as lipomannan (LM) and mannose-capped lipoarabinomannan (ManLAM) (Figure 1) (Daffe and Etienne, 1999; Constant et al., 2002; Reed et al., 2004; Tsenova et al., 2005; Reed et al., 2007; Sinsimer et al., 2008; Jackson, 2014; Garcia-Vilanova et al., 2019). The importance of the peripheral lipid layer is underscored by the fact that it accounts for 40% of the *M.tb* cell envelope composition (Garcia-Vilanova et al., 2019).

The *M.tb* cell envelope is an evolving and adaptive component of the bacterium and is critical to its protection against adverse environmental conditions during infection (Garcia-Vilanova et al., 2019). Many anti-TB drugs such as INH and EMB target cell envelope biogenesis to break down this barrier and weaken the bacterium. Anti-TB drugs targeting the cell envelope can be bactericidal, allowing other drugs to go through. Importantly, many mutations that confer resistance to anti-TB drugs arise within these cell envelope biogenesis pathways, making the generation of new anti-TB drugs critical to combating the recent increase in DR-TB (Batt et al., 2020).

Overall, the *M.tb* cell envelope has many distinct layers that come together to give protection to *M.tb* in various environments and resistance to many different drugs (Jarlier and Nikaido, 1994; Jackson, 2014). These key lipids are described briefly below in the context of drug resistance, while a detailed description of the peripheral layer of the cell envelope is provided elsewhere (Garcia-Vilanova et al., 2019).

3 *M.tb* cell envelope targets for anti-TB drugs

Understanding the *M.tb* cell envelope is critical to recognizing the complexity of the anti-TB drug treatment regimens required to kill DS- and DR-*M.tb* (Table 3). For 2HRZE/4HR treatment regimen, INH is a prodrug and its activation is associated with reduction of *M.tb* ferric catalase-peroxidase KatG by hydrazine to form ferrous KatG. Then, ferrous KatG reacts with oxygen to form oxyferrous KatG to activate INH (Wang et al., 1998). Once activated, INH inhibits InhA, the *M.tb* enoyl reductase, by forming a covalent adduct with the NAD cofactor. This INH-NAD adduct acts as a slow, tight-binding competitive inhibitor of InhA to interfere with MAc biosynthesis (Wilson et al., 1995; Chen et al., 2002; Broussy et al., 2003; Schroeder et al., 2005; Vilchèze and Jacobs, 2007). RIF inhibits *M.tb* DNA-dependent RNA polymerase, leading to a suppression of RNA synthesis and bacterial death (Campbell et al., 2001; Tupin et al., 2010). PZA diffuses into active *M.tb* expressing the pyrazinamidase enzyme that converts PZA to

the active form pyrazinoic acid, where it interferes with fatty acid synthase (FAS) I (Ngo et al., 2007; Zimhony et al., 2007). This hinders *M.tb*'s ability to synthesize new fatty acids required for bacterial growth and replication (Ngo et al., 2007). Accumulation of pyrazinoic acid also disrupts membrane potential by interfering with ATP production as well as binding to the ribosomal protein S1 (RpsA), inhibiting trans-translation and blocking *M.tb* proteins being synthesized with high fidelity. (Shi et al., 2011) EMB directly inhibits *M.tb* arabinosyltransferases (EmbA, EmbB, and EmbC), interfering with the biosynthesis of arabinose-containing *M.tb* cell envelope components such as AG and LAM, as well as preventing bacterial division (Escuyer et al., 2001; Zhang et al., 2003; Amin et al., 2008; Goude et al., 2009; Zhang et al., 2020; Lee and Nguyen, 2023). EMB is thought to have a major impact on the cell envelope structure and permeability, as the arabinan domain of AG sustains the MAc barrier (Goude et al., 2009). Thus, less or smaller/truncated arabinan domain in AG could decrease the binding sites for MAcs, leading to a thinner MAc barrier and the accumulation of free MAcs, TMM, and TDM (Takayama et al., 1979). Further, *M.tb* surface exposed LAM interacts with host cells; thus, EMB-derived truncated or reduced levels of LAM on the *M.tb* cell envelope may decrease *M.tb* interactions with phagocytes (Zhang et al., 2003; Torrelles et al., 2004; Kang et al., 2005; Torrelles et al., 2008). Overall, the 2HRZE/4HR treatment for DS-TB is designed to increase permeability by dismantling the *M.tb* cell envelope (INH, EMB, PZA), followed by a direct inhibition of RNA-polymerases (RIF) driving bacterial death.

In the case of the new WHO treatment regimen recommendation for DS-TB of 4-month daily treatment regimen including RPT, INH, PZA, and MFX, the substitution of RIF for RPT adds value in multiple ways. Although RPT also inhibits *M.tb* DNA-dependent RNA polymerase activity, it has lower minimum inhibitory concentration (MIC), a longer half-life, and higher protein binding affinity, which altogether results in shorter therapies with reduced cytotoxicity (Alfarisi et al., 2017; Zheng et al., 2017). Further, replacing EMB with MFX is motivated by the fact that MFX inhibits *M.tb* topoisomerase II (DNA gyrase) (Naidoo et al., 2017). This enzyme is essential, as DNA gyrase is involved in the replication, transcription and repair of *M.tb* DNA. Thus, adding RPT and MFX while keeping the bactericidal effects of INH and PZA on the *M.tb* cell envelope is intended to maximize the effects of RPT and MFX on the bacterium.

For DR-TB, the newest oral treatment regimen approved is BPaL + MFX (for MDR/RR-TB) or BPaL alone for pre-XDR TB. The overall effects of the combination of these drugs on the cell envelope is unknown. However, BDQ is a diarylquinoline that inhibits the activity of mycobacterial ATP synthase that is essential for the generation of energy in *M.tb* (Sarathy et al., 2019). Pretomanid (Pa) inhibits *M.tb* MAc biosynthesis and causes respiratory poisoning of *M.tb* through the nitrosylating effects of nitroimidazole, causing the release of reactive nitrogen species under anaerobic conditions (Singh et al., 2008; Thompson et al., 2017, 2019). Pa molecular mechanisms of action may also involve effects on *fasI* and *fasII* (involved in mycobacterial fatty acid synthesis), as well as *efpA* (encoding the efflux pump EfpA implicated in the efflux of INH), the *cyd* operon (encoding cytochrome *bd* oxidase involved in the mycobacterial electron transport chain), and the *iniBAC* operon (INH induced operon involved in mycobacterial cell envelope stress responses) (Manjunatha et al., 2009). Finally, LZD interferes with bacterial protein translation by preventing the formation of the functional bacterial 70S ribosomal initiation complex, which is essential for *M.tb* proliferation (Diekema and Jones, 2000). Thus, the BPaL treatment regimen alters the *M.tb* MAc layer (biosynthesis), ATP production, and bacterial proliferation. These effects, together with the effects of MFX on *M.tb* DNA replication, transcription, and repair, initially makes BPaL + MFX a suitable treatment regimen for MDR/RR-TB. In this regard, other drugs such as ETH, delamanid (DLM), CFZ, and high doses of INH could also be used for the treatment of MDR/RR-TB. Increasing INH critical concentration aids in maintaining disruption of MAc biosynthesis. Further, ETH, like PZA, is a nicotinic acid derivative related to INH and thus, it acts similarly to INH where the covalent ETH-NAD adduct formed can act as a slow, tight-binding competitive inhibitor of InhA (Banerjee et al., 1994). DLM similarly inhibits the synthesis of methoxy- and keto-MAcs by disrupting the mycobacteria coenzyme F420 system and generating nitrous oxide through the nitrosylating effects of its nitroimidazole metabolites (Singh et al., 2008; Khoshnood et al., 2021). In the case of CFZ, its activity appears to be membrane-directed, interacting with *M.tb* membrane phospholipids to generate antimicrobial lysophospholipids which destabilizes the membrane, interfering with K^+ uptake and ultimately ATP production (Cholo et al., 2012). However, the use of CFZ could be a double-edged sword, as it is also linked to local inhibition of host memory and effector T cell responses.

In summary, all anti-TB drug treatment regimens are developed to target *M.tb* cell envelope permeability, energy production, and DNA/RNA/protein synthesis (Table 3). The use of a combination of drugs in treatment regimens increases the permeability of the *M.tb* cell envelope, while simultaneously targeting key processes for survival. These are also necessary to prevent the emergence of drug resistance and shorten treatments. Treatment length and drug cytotoxicity drives drug misuse and provides *M.tb* with an ideal scenario to evolve mechanisms of drug resistance, potentially changing its cell envelope properties to its own benefit.

4 Specific *M.tb* cell envelope components and their relationship with drug resistance

The *M.tb* cell envelope has evolved over time to increase *M.tb* hydrophobicity, transmissibility, and pathogenesis during infection (Jankute et al., 2017). Indeed, some reports suggest that modern *M.tb* strains evolved from *M. canetti*, increasing their hydrophobicity and ability to be transmitted by increasing the amount of nonpolar lipids in comparison to polar lipids on the cell envelope (Fabre et al., 2004; Jankute et al., 2017; Briquet et al., 2019). However, while studies have examined the evolution of the *M.tb* cell envelope over time, there is still a large gap in knowledge related to how the *M.tb* cell envelope changes in relation to drug

resistance. Here we summarize what is known about DS *vs*. DR-*M.tb* cell envelope changes and how they influence *M.tb*-host interactions.

4.1 Cell envelope thickness

As aforementioned, the *M.tb* cell envelope is a critical barrier of protection for the bacterium. It has also been shown to vary drastically between strains (Torrelles et al., 2004; Torrelles et al., 2008; Torrelles et al., 2011); therefore, it is logical to question whether such variation extends to *M.tb* strains in relation to drug resistance patterns. Some groups have examined the morphological similarities and differences between *M.tb* clinical isolates that range in drug resistance and found significant changes in morphology across strains (Velayati et al., 2018). Initial studies using transmission electron microscopy (TEM) showed significant increases in cell envelope thickness of both MDR- and XDR-*M.tb* strains compared to DS strains (Velayati et al., 2009a). Notably, MDR-*M.tb* strains are thicker in the electron-transparent and electron-opaque outer layer, while XDR-*M.tb* strains showed a denser peptidoglycan layer (Velayati et al., 2009a). In a subsequent study, the same group examined what they denote as totally drug-resistant (TDR)-*M.tb* strains using TEM, showing that these strains produce a thicker cell envelope and '*round/oval shaped*' bacilli compared to both DS- and MDR-*M.tb* strains' *rod shape* (Velayati et al., 2009b). Atomic force microscopy (AFM) produced similar findings of increased cell envelope thickness and surface roughness of *M.tb* strains denoted as extremely drug-resistant (XXDR) when compared to DS-*M.tb* strains (Velayati et al., 2010). Both TEM and AFM studies were able to reproduce XDR- and XXDR-*M.tb* strains' round bacilli as well as a fourth "*adaptive*" type of cell division not found in DS- or MDR-*M.tb* clinical isolates (Farnia et al., 2010; Velayati et al., 2010). These studies suggest that in contrast to the three other types of cell division found in all *M.tb* clinical isolates (symmetrical, asymmetrical, and branching), this fourth adaptive type resulted in the '*round shaped*' bacilli and may be due to the bacteria's efforts to protect itself and survive within a drug-driven hostile environment (Farnia et al., 2010). Indeed, these changes in *M.tb* cell envelope structure and shape were later associated with dormancy, where in the first 18 months of using an anaerobic culture to model latency, *M.tb* showed a thickened cell envelope and formation of the '*round shaped spore-like*' bacilli (Velayati et al., 2011). This suggests that the shape, size, and thickness of the *M.tb* cell envelope may also be related to the amount of time that the bacteria remain latent during infection, and not solely in relation to drug resistance (Velayati et al., 2011). Importantly, many of these studies predate the most recent WHO definitions of TB drug resistance, so *M.tb* strains previously denoted as XXDR or TDR are now categorized as pre-XDR or XDR under the new definitions (WHO, 2022c). This is critical to note for future studies that will examine the structural characteristics of DR-*M.tb* strains.

4.2 Cell envelope lipid composition

While the modern *M.tb* strains cell envelope has increased hydrophobicity compared to more ancient mycobacterial strains such as *M. canetti* and *M. kansasii* (Jankute et al., 2017), not much is known about whether a further increase in hydrophobic lipids on the *M.tb* cell envelope in relation to drug resistance occurs. A few studies examined the prevalence of glycolipids and lipoglycans that range in polarity and have found variations between *M.tb* strains that are DS *vs.* DR. For instance, one study showed that LAM structural variations (*e.g.* highly succinylated and truncated arabinan domain with lesser mannose caps) in an EMB-resistant strain of *M.tb* was responsible for influencing host responses (Torrelles et al., 2004). Further, another group compared MDR- *vs.* DS-*M.tb* strains and found that there were critical differences between three major classes of lipids, namely on fatty acyls (e.g, MAc), glycerolipids (e.g., TAGs), and glycerophospholipids (e.g., PIMs) (Rahul Pal et al., 2015; Pal et al., 2017). A separate study looking at the lipid composition of INH-resistant *M.tb* clinical isolates found that these had notably lower α-MAc, PDIMs, TMM, and PIMs when compared to laboratory DS-*M.tb* strains (Nieto et al., 2018). However, changes to cell wall composition is not specific to the cell wall targeting anti-*M.tb* drugs, as one group revealed an increase in expression of genes responsible for PDIM biosynthesis and alteration in PDIM structures in strains with *rpoB* mutations resulting in RIF resistance (Bisson et al., 2012). While these studies suggest that the lipid composition of DR-*M.tb* strains is altered, a consensus of how the cell envelope of DR-*M.tb* strains change in comparison to DS-*M.tb* strains remains to be established. Below we will discuss MAcs along with key subsets of *M.tb* peripheral lipids and what is known about each lipid subset in relation to drug resistance.

4.2.1 Mycolic acids

MAcs are long-chain ($C_{60\text{-}90}$) fatty acids that are covalently bonded via esterification to the non-reducing ends of AG to complete the *M.tb* cell wall 'core' (Jackson, 2014; Garcia-Vilanova et al., 2019). They are a key component of the mycobacterial membrane and critically contribute to the hydrophobicity of the cell envelope which protects the *M.tb* bacterium during infection and from anti-TB drugs. Studies comparing the cell wall structure of *in vivo* and *in vitro* derived *M.tb* have shown that *in vivo* grown cells have a more condensed AG-PG structure, but with increased esterification by mycolic acids. *M.tb* produces three subclasses of MAcs (α-, methoxy-, and keto-MAcs) (Nataraj et al., 2015). In addition to being covalently bound, MAcs can be found free and esterified to peripheral lipids defined as virulence factors such as TMM and TDM (Brennan and Nikaido, 1995; Belisle et al., 1997; Grzegorzewicz et al., 2012). TMM can also serve as a means to transport MAcs through the *M.tb* cell envelope (Varela et al., 2012), where enzymes from the Ag85 complex called mycolyl transferases covalently link free MAcs to the AG to form the MAc layer as well as transfer an additional MAc to TMM to form TDM (Belisle et al., 1997; Puech et al., 2002).

Multiple anti-TB drugs target MAc biosynthesis to increase permeability of the *M.tb* cell envelope, allowing for other anti-TB drugs to effectively target DNA/RNA/protein synthesis and energy production. Interestingly, most genes involved in MAc synthesis are considered essential, suggesting that the MAc biosynthetic pathway should be further targeted in the design of new anti-TB drugs (Nataraj et al., 2015). It is thought that increased MAcs – and thus, increased hydrophobicity of the *M.tb* cell envelope – may offer additional resistance for DR-*M.tb* strains. However, it is plausible that the MAc structure rather than their abundance may vary in DR-*M.tb* strains. Increases in MAc carbon chain length would not only explain earlier reports in cell envelope thickness of DR-*M.tb* strains (Velayati et al., 2009a, Velayati et al., 2009b, Velayati et al., 2010), but may also contribute to increased hydrophobicity of the DR-*M.tb* cell envelope, thus allowing for greater protection against more hydrophilic molecules including many anti-TB drugs. Because of this, it is critical to elucidate the role(s) that MAcs play in *M.tb* drug resistance and how changes in MAc abundance and/or structure ultimately influences DR-*M.tb* hydrophobicity, transmissibility, and infection outcomes.

4.2.2 Phthiocerol dimycocerosates and phenolic glycolipids

The biosynthetically related PDIMs and PGLs are predominantly nonpolar lipids that contribute to the hydrophobicity of the *M.tb* cell envelope. Both PDIMs and PGLs are considered important *M.tb* complex virulence factors, with PDIMs only being found in pathogenic mycobacterial species (except for *M. gastri*) (Onwueme et al., 2005; Samanta et al., 2017). In this regard, PDIMs are linked to suppression of the early immune response by controlling TNF and IL-6 production, as well as being potentially involved in phagosomal maturation arrest (Rousseau et al., 2004; Howard et al., 2018; Garcia-Vilanova et al., 2019). Many studies revealed that PDIM-deficient *M.tb* and *M. bovis* mutants have a high degree of attenuation *in vitro* and *in vivo* (Camacho et al., 1999; Cox et al., 1999; Rousseau et al., 2004; Murry et al., 2009; Kirksey et al., 2011; Day et al., 2014), with PDIM-deficient mutants also showing greater susceptibility to anti-TB drugs when compared to the wild-type or complemented strains (Chavadi et al., 2011). PGLs are also produced by pathogenic mycobacteria such as *M.tb* (some strains), *M. bovis*, *M. leprae*, and *M. marinum*. However, the importance of PGLs in *M.tb* virulence is still debatable. Many *M.tb* strains – including laboratory strains $H_{37}R_v$ and Erdman, as well as the highly transmissible clinical isolate CDC1551 – harbor a mutation in the *Pks15/1* gene disabling PGL production (Reed et al., 2004; Samanta et al., 2017). Conversely, genetic lineage 2 (termed '*W-Beijing*' lineage) *M.tb* strains have an intact *Pks15/1* gene and thus contain PGLs that may contribute to their hypervirulence phenotype described in animal models (Reed et al., 2004; Samanta et al., 2017).

Studies have begun examining the relationship between PDIMs on the mycobacterial cell envelope and drug resistance. An initial study using multiple *M.tb* strains showed that the presence of PDIMs is critical for reducing the cell envelope permeability of hydrophobic probes and detergents (Camacho et al., 2001). This study examined *M.tb* strains with deficiencies in genes related to PDIM biosynthesis and transport (*fadD26*, *mmpl7*, and *drrC*) and found that these presented higher permeability to hydrophobic probes, with *M.tb* Δ*fadD26* being the most sensitive to detergents (Camacho et al., 2001; Rens et al., 2021). These results notably did not show any differences in the MIC of multiple hydrophilic anti-TB drugs (INH, PZA, and EMB), indicating that the PDIM-deficient mutants' susceptibilities did not extend to anti-TB drug effects (Camacho et al., 2001). However, a later study using *M. marinum* Δ*tesA* mutants, a gene encoding a thioesterase that hydrolyses long chain fatty acyl esters involved in PDIMs biosynthesis, showed that PDIM and/or PGL deficiencies are directly associated with hyper-susceptibility to multiple drugs including cefuroxime (CXM), doxycycline (DOX), RIF, ciprofloxacin, and ampicillin (Chavadi et al., 2011). Not only was this finding reproducible in a subsequent study, but it was also shown that another *M. marinum* Δ*papA5* mutant, a gene encoding a polyketide-associated protein with acyl transferase activity involved in PDIMs biosynthesis, had hyper-susceptibility to CXM, DOX, RIF, and erythromycin (Mohandas et al., 2016). Similar findings were shown for both PDIM-deficient *M. bovis* BCG and *M.tb* strains having higher degree of susceptibility to vancomycin (Soetaert et al., 2015). Importantly, RIF-acquired resistance in MDR-*M.tb* strains (particularly through mutation of *rpoB*) is associated with upregulation of the PDIMs biosynthetic pathway (Bisson et al., 2012), increased PDIMs levels (Howard et al., 2018), and changes in over 100 cell envelope lipids (Lahiri et al., 2016). Overall, these data indicate that PDIMs play an important role in the hydrophobicity and permeability of the *M.tb* cell envelope, and that their biosynthesis could be a critical pathway to target when designing novel anti-TB drugs as well as developing a point of care diagnostic tool to distinguish DS- *vs.* DR-TB (Figure 2).

4.2.3 Trehalose-containing glycolipids

M.tb uses trehalose as both a carbon source and a core component of several cell surface glycolipids known as virulence factors of *M.tb*. The mycolyl-containing trehalose lipids are the most abundant subset composed namely by TDM (6,6'-dimycoloyl-α-D-trehalose) and TMM (6-monomycoloyl-α-D-trehalose). These lipids are comprised of long acyl chains, and it is widely assumed that the mycolates face towards the plasma membrane of the bacterium and trehalose groups face towards the cell envelope surface. Other important trehalose-containing lipids are the acyltrehalose lipids, mainly diacyl- (DAT), triacyl- (TAT), and pentaacyl-trehalose (PAT) (Gautier et al., 1992). These acyltrehalose lipids are valuable components of the *M.tb* cell envelope in that they aid in maintaining the structure of the cell envelope, providing anchors for the outer material layer that thickens the cell envelope (Rousseau et al., 2003; Bailo et al., 2015). Sulfolipids (SL) make up yet another subset of trehalose-containing lipids found on the cell envelope of virulent strains of *M.tb* (Middlebrook et al., 1959; Goren, 1970).

Overall, the role of trehalose-containing lipids in *M.tb* development of drug resistance remains unknown, although trehalose acts as a reactive oxygen species scavenger. Recent studies indicate that trehalose metabolism is associated with both

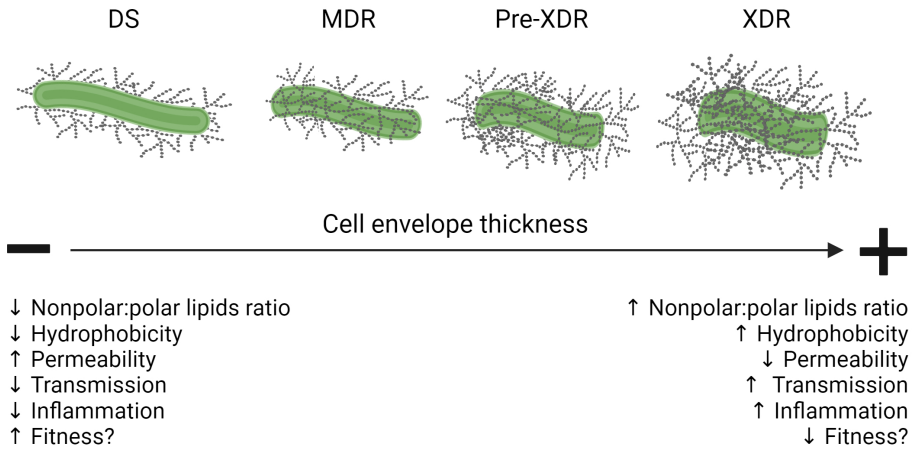


FIGURE 2
Summary of cell envelope changes in DS- *vs.* DR-*M.tb* and their influence on infection outcomes. Schematic showing that as *M.tb* increases its drug resistance pattern, its cell envelope thickness and hydrophobicity increases, driving reduced permeability and resulting in higher transmission rates while at the same time compromising its fitness within the host by triggering damaging inflammatory responses. Figure created in BioRender. Down black arrows signify a decrease. Up black arrows signify an increase.

M.tb drug tolerance and permanent drug-resistance (Lee et al., 2019). In the presence of INH and RIF, *M.tb* is found to bypass the production of trehalose-containing lipids (Lee et al., 2019). *M.tb* also activates isocitrate lyases involved in the tricarboxylic acid (TCA) cycle to tolerate INH, RIF, and streptomycin after sublethal exposure by circumventing reactive ROS production (Nandakumar et al., 2014). These data suggest that *M.tb* may shift its metabolism (including trehalose metabolism) to circumvent anti-TB drug effects directly linked to the harmful production of ROS (Dwyer et al., 2014; Dong et al., 2015). This is supported by multiple studies indicating that as *M.tb* becomes tolerant to anti-TB drugs in response to a hypoxic environment (e.g. granuloma), it simultaneously downregulates the production of TDM/TMM (Galagan et al., 2013; Eoh et al., 2017). Indeed, *M.tb* drug-tolerant persisters seem to have the capacity to alter trehalose metabolism to survive in a non-replicating state under hypoxic conditions (Eoh et al., 2017). Herein, under anti-TB drug-free environmental pressure, both drug-tolerant persisters and DR-*M.tb* strains can undergo a trehalose-catalytic shift that is absent in DS-*M.tb* strains (Lee et al., 2019). This shift ultimately allows for bacterial survival even when anti-TB drugs cause energy depletion due to the ATP-producing electron transport chain becoming dysregulated (Lee et al., 2019). Alternatively, several studies address targeting trehalose-containing glycolipid transport proteins, such as the mycobacterial membrane protein large 3 (Mmpl3), in the design of new anti-TB drugs (Su et al., 2019; Zhang et al., 2019). Targeting the transport of glycolipids such as TMM to the cell envelope may aid in weakening the cell envelope and possibly increase the efficacy of other anti-TB drugs. Further, the acetylation of *M.tb* proteins plays a critical mechanism of bacterial adaptation to changing environments, being implicated in virulence, drug resistance, regulation of metabolism, and host anti-TB immune responses (Sun et al., 2022; Huang et al., 2023). It is plausible that increased acylation of *M.tb* cell envelope trehalose-containing lipids could be play a similar role (Sun et al., 2022).

4.2.4 Mannose-containing glycolipids and lipoglycans

A predominant family of cell envelope lipids are the mannose-containing glycolipids that include PIMs – glycosylated derivatives of phosphatidyl-*myo*-inositol (PI) – and their associated lipoglycans LM and ManLAM (described in detail elsewhere) (Chatterjee et al., 1992; Chatterjee et al., 1993; Besra and Brennan, 1997; Besra et al., 1997; Chatterjee and Khoo, 1998; Nigou et al., 2003; Torrelles and Schlesinger, 2010; Turner and Torrelles, 2018). Not only are these molecules found in the periphery, but they are also interspersed throughout the plasma membrane and cell wall core (Venisse et al., 1995; Crick et al., 2003; Torrelles and Schlesinger, 2010). Mannose-containing molecules such as arabinomannan, mannan, and manno-glycoproteins are also present on the cell envelope (Torrelles and Schlesinger, 2010), along with mannosyl-β-1-phosphomycoketides (MPM), a mannose phosphate suggested to activate CD1c-restricted T cells (Matsunaga et al., 2004).

The biosynthesis of PIMs is complex, as these are largely heterogeneous molecules that vary in their mannose content, as well as the number and structure of their acyl groups (Garcia-Vilanova et al., 2019). As such, PIMs can be separated into lower- and higher-order structures that coincide with the number of mannose residues (Torrelles et al., 2006). Of the many PIMs found, the most common in the *M.tb* complex cell envelope are PIM_2 and Ac_1PIM_2 (di- and tri-acylated PIM_2, respectively) as well as PIM_6 and Ac_1PIM_6 (di- and tri-acylated PIM_6) (Khoo et al., 1995; Garcia-Vilanova et al., 2019). LM is a lipoglycan that is biochemically related to PIMs and thought to be synthesized from PIM_4 (Gilleron et al., 1999; Guerardel et al., 2002; Guérardel et al., 2003). Along with PIMs, LM is shown to regulate cytokine, oxidant, and T cell responses (Barnes et al., 1992; Chan et al., 2001; Gilleron et al., 2001; Torrelles and Schlesinger, 2010). ManLAM is an abundant mannose-containing macromolecules on the *M.tb* cell envelope and is biochemically related to PIMs and LM (Brennan and Nikaido, 1995; Besra et al., 1997). The heterogeneous structure

of ManLAM (described in detail elsewhere) (Chatterjee et al., 1992; Chatterjee et al., 1993; Besra and Brennan, 1997; Besra et al., 1997; Chatterjee and Khoo, 1998; Nigou et al., 2003; Turner and Torrelles, 2018) is defined as containing a mannosyl-phosphatidyl-*myo*-inositol (MPI)-anchor, a carbohydrate core (D-mannan and D-arabinan), and mannose-capping motifs (Torrelles and Schlesinger, 2010). ManLAM biological properties, including its capacity to interact with the macrophage mannose receptor driving phagosome maturation arrest (Fratti et al., 2003; Kang et al., 2005), are described in detail elsewhere (Turner and Torrelles, 2018). Previous studies showed not only that ManLAM is a virulence factor of *M.tb*, but that it varies in size and structure, with certain *M.tb* clinical isolates having truncated forms of ManLAM that is ultimately associated with decreased phagocytosis and lower association with host cell recognition receptors [e.g. mannose receptor (MR), DC-SIGN in phagocytes] (Torrelles et al., 2004; Torrelles et al., 2008).

Efforts have begun to elucidate what role(s) molecules involved in the biosynthesis and transport of mannose-containing glycolipids may play in driving *M.tb* drug resistance. For ManLAM, the ratio of Ara : Man (considered normal at approximately 1.0) (Khoo et al., 2001) varies by *M.tb* strain, with DR- and/or hypervirulent *M.tb* strains showing a greater amount of variability in LAM size and branching (Khoo et al., 2001; Torrelles et al., 2004; Torrelles et al., 2008; Torrelles et al., 2012). Further, a study examining drug resistance of *M. abscessus* showed that mutation of arabinosyltransferase C (an ortholog of *M.tb embC*) resulted in increased permeability of the cell envelope due to the abolishment of LAM biosynthesis driving hypersensitivity to multiple anti-TB drugs (Wang et al., 2022). In a separate study, treatment of *M. bovis* BCG with INH and EMB resulted in increased surface exposure of LAM, which could be linked to the reduction in MAc abundance by the action of INH, as well as an attempt from the bacteria to maintain cell envelope integrity after exposure to anti-TB drugs (Alsteens et al., 2008). This is supported by the upregulation of genes related to LAM biosynthesis following INH treatment (Yimcharoen et al., 2022). That said, this needs to be carefully considered as for this study, the authors used a MDR *M.tb* strain with a *katG* mutation, which should render it insensitive to INH.

Overall, hydrophilic mannose-containing lipoglycans may have low friction within the *M.tb* cell envelope, increasing its hydrophilicity while at the same time reducing its permeability; thus, the biosynthesis pathways of mannose-containing lipoglycans could be important targets for future anti-TB drugs. Indeed, ManLAM/LM structural changes could have a significant impact on the *M.tb* cell envelope integrity, increasing sensitivity to drugs, as well as faster killing by host cells, opening the avenue for combined drug and host-directed therapies.

5 The influence of drug resistance on infection outcomes

Fitness has been defined as a comprehensive measure of the bacteria's ability to survive, reproduce, and undergo transmission, and could represent virulence as well (Zhan et al., 2020). It is true that many mechanistically related forms of drug resistance are associated with reduced bacterial growth (Gagneux et al., 2006b; Andersson and Hughes, 2010; Bhatter and Mistry, 2013; Zhan et al., 2020). This fact in the TB field is debatable; indeed, studies show that INH-resistant *M.tb* strains with mutations in *katG* have decreased virulence, pathology, and fitness in the mouse model (Nieto et al., 2016). However, other studies report that DR-*M.tb* strains range in virulence and that certain drug resistance conferring mutations are at "*non-cost*" and do not decrease fitness (Ordway et al., 1995). This could be explained by the existence of secondary mutations compensating for fitness cost. These secondary mutations could be an indirect, subsequent result of the primary mutations driving drug resistance. For example, an epidemiological study found that 47% of *M.tb* clinical isolates with a *rpoB* mutation conferring RIF resistance also had fitness-compensatory mutations in *rpoA* or *rpoC* (Casali et al., 2014).

Additional factors that can influence infection outcomes include *M.tb* lineage and host genetics and epigenetics that can cause variabilities in the immune response during infection (Saelens et al., 2019; Carey et al., 2022). Previous epidemiological studies suggest that lineage 2 "Beijing" strains are associated with increased virulence, transmissibility, and drug resistance compared to other lineages (Kong et al., 2007; Cowley et al., 2008; de Jong et al., 2008; Thwaites et al., 2008; Niemann et al., 2010; Casali et al., 2014; Carey et al., 2022). Recent reports on DR-TB patients also show cytokine signatures associated with increased TB disease severity and hyper-inflammation, indicating that some DR-*M.tb* strains may have increased virulence (Sampath et al., 2023). However, this conclusion needs to be handled with caution, as it seems possible that DR-*M.tb* appears more virulent in patients simply because it is drug resistant.

Another study assessed the adaptation of *M.tb* and found that strains from multiple lineages have adapted to specific human populations, and that these adaptations are consistent with the geographic spread of each lineage in various parts of the world (Gagneux et al., 2006a). Thus, it is plausible that DR-*M.tb* strains' primary and secondary mutations, lineage, and host specific genetic and epigenetic factors may account for the vast amount of variability in disease severity and the immune response seen in DR-TB cases. These factors may allow for a small subset of DR-*M.tb* strains with little to no fitness cost to be preferentially transmitted to new hosts, ultimately favoring an increase in DR-TB over time (Figure 2) (Zhan et al., 2020).

M.tb persisters also complicate our understanding of DR-TB and infection outcomes. These persisters are a subpopulation of *M.tb* that are genotypically the same as the rest of the population, but phenotypically drug tolerant and do not replicate in the presence of drugs (Torrey et al., 2016). Thus, bactericidal drugs do not kill these. Indeed, studies show that clinical isolates with a high-persister phenotype have greater *ex vivo* survival than those with a low-persister phenotype, with specific mutations found in genes related to PDIM biosynthesis in the high persisters (Torrey et al., 2016). However, while persisters can act as a source for drug resistant mutations (Prabowo et al., 2013), the association between drug resistance (not simply drug tolerance) and persister *M.tb* needs to be studied further.

Aside from active TB and latent *M.tb* infection (LTBI), incipient TB (likely to progress to active TB without causing detectable

abnormalities) and subclinical TB (does not cause clinical TB-related symptoms, but does cause abnormalities that are detectable using radiologic or microbiologic assays) are also forms of the disease that need to be considered (Drain et al., 2018). In this regard, in 2021 it was estimated that approximately 7 million people were living with subclinical TB, and while these people do not exhibit classic symptoms of active TB disease, they are still able to transmit the infection to others (Kendall et al., 2021). Unfortunately, little is known about incipient and subclinical cases of TB, and even less is known about how drug resistance influences the likelihood of developing these TB stages in comparison to LTBI or active TB. What is clear, however, is that the prevalence of DR-TB is likely to increase if patients presenting subclinical TB are indeed infected with DR-*M.tb* and able to transmit it to others. Thus, understanding how drug resistance influences *M.tb* infection stages and outcomes is an important avenue for further exploration as we work to end TB.

Most studies examining DR-TB are in the context of pulmonary TB. However, there is also evidence of extrapulmonary TB resistance to both first- and second-line anti-TB drugs. Several studies involving extrapulmonary TB indicate that the rate of DR-TB in these patients goes between 4.4% to 20% (Dusthackeer et al., 2015). Similar to extrapulmonary TB, cases of disseminated DR-TB are reported around the world and can also complicate the already difficult-to-manage problem of DR-TB (Jung et al., 2016; Kaplan et al., 2018).

6 Concluding remarks

The *M.tb* cell envelope is a unique and complex structure that can adapt to its environment. Key peripheral lipids on the *M.tb* cell envelope contribute to the hydrophobicity of the bacterium and act as virulence factors to influence *M.tb*-host interactions. Although MDR/RR-, pre-XDR-, and XDR-TB have emerged in recent decades, how the cell envelope of DR-*M.tb* changes in comparison to DS-*M.tb* remains poorly understood. Recent studies have started to address genotypic changes naturally occurring in DR-*M.tb* strains that influence the efficacy of anti-TB drugs (Bellerose et al., 2020), and what is currently known about these evolutionary processes and adaptation of DR-*M.tb* are reviewed in detail elsewhere (Allué-Guardia et al., 2021). Examination of other superbugs such as methicillin-resistant *Staphylococcus aureus* (MRSA) showed that MRSA strains can alter their cell envelope to strategically evade the immune response (Gerlach et al., 2018), and that the glycosylation of cell envelope teichoic acids is required for methicillin resistance (Brown et al., 2012). It is possible that we can apply knowledge gained from other superbugs to DR-TB with respect to changes in the cell envelope composition of MDR/RR-, pre-XDR-, and XDR-*M.tb* strains and how these changes impact TB clinical presentation, host responses, and subsequent pathogenesis.

Studies in recent years suggest that the cell envelope of DR-*M.tb* strains thicken, and PDIMs may be playing a determinant role in controlling permeability to hydrophobic drugs. If this is the case, infection with a DR-*M.tb* strain may result in increased virulence, as the host immune system could skew towards a more pro-inflammatory response and increased necrosis of infected cells due to the greater amount of hydrophobic lipids on the cell envelope (Figure 3). Another possibility is that, due to the increase in the amount of hydrophobic *vs.* hydrophilic lipids and their associated lipoglycans (*e.g.* PIMs/LM/ManLAM), there is missed opportunity for host cells to recognize hydrophilic lipids that favorably modulate the host immune response towards decreasing inflammation. One outstanding question is whether host-signaling patterns are altered by DR-*M.tb* due to cell envelope changes and, if so, do these alterations influence immune cell recruitment to and granuloma formation at the site of infection? Recent studies have begun to address the genotypic changes in DS- *vs.* MDR-*M.tb*, focusing on mutations that alter the efficacy of anti-TB drugs *in vivo* (Bellerose et al., 2020). However, it will be critical in future studies to also examine pre-XDR- and XDR-*M.tb* strains to assess their natural genetic variations that may influence drug efficacy, if any of these genetic mutations are associated with changes in the *M.tb* cell envelope composition, and how these changes ultimately influence TB pathogenesis.

Another outstanding question related to DR-TB is whether increased amounts of nonpolar lipids (and thus, increased hydrophobicity) on the cell envelope of DR-*M.tb* strains contributes to enhanced aerosolization and transmissibility of DR-*M.tb*, which was previously demonstrated in a study showing that increased hydrophobicity enhances aerosol transmissibility in

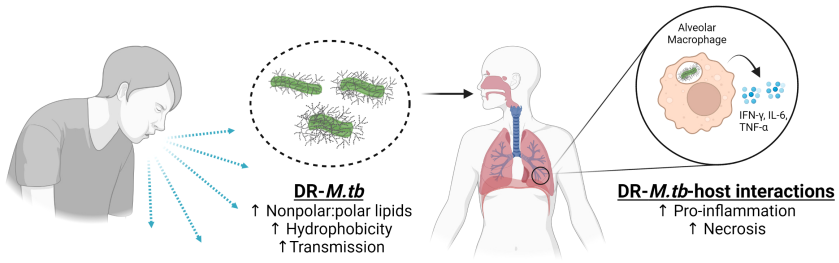


FIGURE 3
Summary of DR-*M.tb*-host interactions. Schematic showing that DR-*M.tb* strains with increased levels of nonpolar lipids have increased hydrophobicity and transmission leading to the increase in the host cell pro-inflammatory responses in the lung environment, favoring cell death by necrosis rather than apoptosis and driving the progression of the infection. Figure created in BioRender. Up and black arrows signify an increase.

non-tuberculous mycobacteria (Falkinham, 2003; Jankute et al., 2017). If so, it is plausible that increased hydrophobicity would ultimately allow these strains to be easily transmissible and increase their prevalence over time, thus associating drug resistance with an overall increase in fitness, TB pathogenesis, and impaired host immune responses. Addressing these questions will ultimately aid the TB field in designing novel anti-TB drugs in response to these changes and improving diagnosis of DR-TB in POC settings to reduce transmission and improve public health worldwide.

Author contributions

AS: Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. MI: Conceptualization, Writing – review & editing. JB: Conceptualization, Writing – review & editing. JT: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work has been partially supported by the Texas Biomed Douglass Graduate Student Fellowship to AS, as well as by NIH/NIAID (R01 AI-146340) to JT. AS and JT are part of The Interdisciplinary NexGen TB Research Advancement Center (IN-TRAC) at Texas Biomedical Research Institute, a NIH/NIAID funded program (P30-AI168439).

Acknowledgments

We thank Ms. Angelica Olmo-Fontanez for her time and advice in editing figures. In memory of Mrs. Rose Caroline Lopez, who assisted many of us to integrate and be successful in the department of Microbiology, Immunology and Pathology at Colorado State University.

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

Author disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

References

Allué-Guardia, A., García, J. I., and Torrelles, J. B. (2021). Evolution of drug-resistant mycobacterium tuberculosis strains and their adaptation to the human lung environment. *Front. Microbiol.* 12, 612675. doi: 10.3389/fmicb.2021.612675

Alfarisi, O., Alghamdi, W. A., Al-Shaer, M. H., Dooley, K. E., and Peloquin, C. A. (2017). Rifampin vs. rifapentine: what is the preferred rifamycin for tuberculosis? *Expert Rev. Clin. Pharmacol.* 10 (10), 1027–1036. doi: 10.1080/17512433.2017.1366311

Alsteens, D., Verbelen, C., Dague, E., Raze, D., Baulard, A. R., and Dufrêne, Y. F. (2008). Organization of the mycobacterial cell wall: a nanoscale view. *Pflugers Arch.* 456 (1), 117–125. doi: 10.1007/s00424-007-0386-0

Amin, A. G., Goude, R., Shi, L., Zhang, J., Chatterjee, D., and Parish, T. (2008). EmbA is an essential arabinosyltransferase in Mycobacterium tuberculosis. *Microbiol. (Reading)* 154 (Pt 1), 240–248. doi: 10.1099/mic.0.2007/012153-0

Andersson, D. I., and Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* 8 (4), 260–271. doi: 10.1038/nrmicro2319

Members of the Advisory Committee. (2019). FDA briefing document: pretomanid tablet, 200mg. Meeting of the antimicrobial drugs advisory committee (AMDAC). Available at: https://www.fda.gov/media/127592/download

Bailo, R., Bhatt, A., and Ainsa, J. A. (2015). Lipid transport in Mycobacterium tuberculosis and its implications in virulence and drug development. *Biochem. Pharmacol.* 96 (3), 159–167. doi: 10.1016/j.bcp.2015.05.001

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., et al. (1994). *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* 263, 227–230. doi: 10.1126/science.8284673

Barnes, P. F., Chatterjee, D., Abrams, J. S., Lu, S., Wang, E., Yamamura, M., et al. (1992). Cytokine production induced by Mycobacterium tuberculosis lipoarabinomannan. Relationship to chemical structure. *J. Immunol.* 149 (2), 541–547.

Batt, S. M., Burke, C. E., Moorey, A. R., and Besra, G. S. (2020). Antibiotics and resistance: the two-sided coin of the mycobacterial cell wall. *Cell Surf* 6, 100044. doi: 10.1016/j.tcsw.2020.100044

Belisle, J. T., Vissa, V. D., Sievert, T., Takayama, K., Brennan, P. J., and Besra, G. S. (1997). Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* 276, 1420–1422. doi: 10.1126/science.276.5317.1420

Bellerose, M. M., Proulx, M. K., Smith, C. M., Baker, R. E., Ioerger, T. R., and Sassetti, C. M. (2020). Distinct Bacterial Pathways Influence the Efficacy of Antibiotics against Mycobacterium tuberculosis. *mSystems* 5 (4). doi: 10.1128/mSystems.00396-20

Besra, G. S., and Brennan, P. J. (1997). The mycobacterial cell wall: biosynthesis of arabinogalactan and lipoarabinomannan. *Biochem. Soc Trans.* 25 (3), 845–850. doi: 10.1042/bst0250845

Besra, G. S., Morehouse, C. B., Rittner, C. M., Waechter, C. J., and Brennan, P. J. (1997). Biosynthesis of mycobacterial lipoarabinomannan. *J. Biol. Chem.* 272, 18460–18466. doi: 10.1074/jbc.272.29.18460

Bhatter, P., and Mistry, N. (2013). Fitness of acquired drug resistant Mycobacterium tuberculosis isolates from DOTS compliant patients. *Tuberculosis (Edinb)* 93 (4), 418–424. doi: 10.1016/j.tube.2013.03.006

Bisson, G. P., Mehaffy, C., Broeckling, C., Prenni, J., Rifat, D., Lun, D. S., et al. (2012). Upregulation of the phthiocerol dimycocerosate biosynthetic pathway by rifampin-resistant, rpoB mutant Mycobacterium tuberculosis. *J. Bacteriol* 194 (23), 6441–6452. doi: 10.1128/JB.01013-12

Brennan, P. J., and Nikaido, H. (1995). The envelope of mycobacteria. *Annu. Rev. Biochem.* 64, 29–63. doi: 10.1146/annurev.bi.64.070195.000333

Briquet, A., Vong, R., Roseau, J.-B., Javelle, E., Cazes, N., Riviere, F., et al. (2019). Clinical features of Mycobacterium canettii infection: a retrospective study of 20 cases among French soldiers and relatives. *Clin. Infect. Dis. an Off. Publ. Infect. Dis. Soc. America* 69 (11), 2003–2010. doi: 10.1093/cid/ciz107

Broussy, S., Coppel, Y., Nguyen, M., Bernadou, J., and Meunier, B. (2003). 1H and 13C NMR characterization of hemiamidal isoniazid-NAD(H) adducts as possible inhibitors of InhA reductase of Mycobacterium tuberculosis. *Chemistry* 9 (9), 2034–2038. doi: 10.1002/chem.200204637

Brown, S., Xia, G., Luhachack, L. G., Campbell, J., Meredith, T. C., Chen, C., et al. (2012). Methicillin resistance in Staphylococcus aureus requires glycosylated wall teichoic acids. *Proc. Natl. Acad. Sci. U.S.A.* 109 (46), 18909–18914. doi: 10.1073/pnas.1209126109

Camacho, L. R., Constant, P., Raynaud, C., Lanéelle, M.-A., Triccas, J. A., Gicquel, B., et al. (2001). Analysis of the phthiocerol dimycocerosate locus of *Mycobacterium tuberculosis*. Evidence that this lipid is involved in the cell wall permeability barrier. *J. Biol. Chem.* 276 (23), 19845–19854. doi: 10.1074/jbc.M100662200

Camacho, L. R., Ensergueix, D., Perez, E., Gicquel, B., and Guilhot, C. (1999). Identification of a virulence gene cluster of Mycobacterium tuberculosis by signature-tagged transposon mutagenesis. *Mol. Microbiol.* 34 (2), 257–267. doi: 10.1046/j.1365-2958.1999.01593.x

Campbell, E. A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A., et al. (2001). Structural mechanism for rifampicin inhibition of bacterial rna polymerase. *Cell* 104 (6), 901–912. doi: 10.1016/S0092-8674(01)00286-0

Carey, A. F., Wang, X., Cicchetti, N., Spaulding, C. N., Liu, Q., Hopkins, F., et al. (2022). Multiplexed strain phenotyping defines consequences of genetic diversity in mycobacterium tuberculosis for infection and vaccination outcomes. *mSystems* 7 (3), e0011022. doi: 10.1128/msystems.00110-22

Carr, W., Kurbatova, E., Starks, A., Goswami, N., Allen, L., and Winston, C. (2022). Interim guidance: 4-month rifapentine-moxifloxacin regimen for the treatment of drug-susceptible pulmonary tuberculosis - United States 2022. *MMWR Morb Mortal Wkly Rep.* 71 (8), 285–289. doi: 10.15585/mmwr.mm7108a1

Casali, N., Nikolayevskyy, V., Balabanova, Y., Harris, S. R., Ignatyeva, O., Kontsevaya, I., et al. (2014). Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nat. Genet.* 46 (3), 279–286. doi: 10.1038/ng.2878

Chan, E. D., Morris, K. R., Belisle, J. T., Hill, P., Remigio, L. K., Brennan, P. J., et al. (2001). Induction of inducible nitric oxide synthase-NO• by lipoarabinomannan of *Mycobacterium tuberculosis* is mediated by MEK1-ERK, MKK7-JNK, and NF-kB signaling pathways. *Infect. Immun.* 69 (4), 2001–2010. doi: 10.1128/IAI.69.4.2001-2010.2001

Chatterjee, D., and Khoo, K.-H. (1998). Mycobcterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. *Glycobiology* 8 (2), 113–120. doi: 10.1093/glycob/8.2.113

Chatterjee, D., Khoo, K.-H., McNeil, M. R., Dell, A., Morris, H. R., and Brennan, P. J. (1993). Structural definition of the non-reducing termini of mannose- capped LAM from *Mycobacterium tuberculosis* through selective enzymatic degradation and fast atom bombardment-mass spectrometry. *Glycobiology* 3, 497–506. doi: 10.1093/glycob/3.5.497

Chatterjee, D., Lowell, K., Rivoire, B., Mcneil, M. R., and Brennan, P. J. (1992). Lipoarabinomannan of mycobacterium-tuberculosis - capping with mannosyl residues in some strains. *J. Biol. Chem.* 267 (9), 6234–6239. doi: 10.1016/S0021-9258(18)42686-5

Chavadi, S. S., Edupuganti, U. R., Vergnolle, O., Fatima, I., Singh, S. M., Soll, C. E., et al. (2011). Inactivation of tesA reduces cell wall lipid production and increases drug susceptibility in mycobacteria. *J. Biol. Chem.* 286 (28), 24616–24625. doi: 10.1074/jbc.M111.247601

Chen, X., Ji, Z. L., and Chen, Y. Z. (2002). TTD: therapeutic target database. *Nucleic Acids Res.* 30 (1), 412–415. doi: 10.1093/nar/30.1.412

Cholo, M. C., Steel, H. C., Fourie, P. B., Germishuizen, W. A., and Anderson, R. (2012). Clofazimine: current status and future prospects. *J. Antimicrob. Chemother.* 67 (2), 290–298. doi: 10.1093/jac/dkr444

Constant, P., Perez, E., Malaga, W., Laneelle, M. A., Saurel, O., Daffe, M., et al. (2002). Role of the pks15/1 gene in the biosynthesis of phenolglycolipids in the Mycobacterium tuberculosis complex. Evidence that all strains synthesize glycosylated p-hydroxybenzoic methyl esters and that strains devoid of phenolglycolipids harbor a frameshift mutation in the pks15/1 gene. *J. Biol. Chem.* 277 (41), 38148–38158. doi: 10.1074/jbc.M206538200

Cowley, D., Govender, D., February, B., Wolfe, M., Steyn, L., Evans, J., et al. (2008). Recent and rapid emergence of W-Beijing strains of Mycobacterium tuberculosis in Cape Town, South Africa. *Clin. Infect. Dis.* 47 (10), 1252–1259. doi: 10.1086/592575

Cox, J. S., Chen, B., McNeil, M., and Jacobs, W. R. Jr. (1999). Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. *Nature* 402 (6757), 79–83. doi: 10.1038/47042

Crick, D. C., Brennan, P. J., and McNeil, M. R. (2003). "The cell wall of *Mycobacterium tuberculosis*," in *Tuberculosis*. Eds. W. M. Rom and S. M. Garay (Philadelphia: Lippincott Williams and Wilkins).

Daffe, M., and Etienne, G. (1999). The capsule of Mycobacterium tuberculosis and its implications for pathogenicity. *Tuber. Lung Dis.* 79 (3), 153–169. doi: 10.1054/tuld.1998.0200

Day, T. A., Mittler, J. E., Nixon, M. R., Thompson, C., Miner, M. D., Hickey, M. J., et al. (2014). *Mycobacterium tuberculosis* strains lacking surface lipid phthiocerol dimycocerosate are susceptible to killing by an early innate host response. *Infect. Immun.* 82 (12), 5214–5222. doi: 10.1128/IAI.01340-13

de Jong, B. C., Hill, P. C., Aiken, A., Awine, T., Antonio, M., Adetifa, I. M., et al. (2008). Progression to active tuberculosis, but not transmission, varies by Mycobacterium tuberculosis lineage in The Gambia. *J. Infect. Dis.* 198 (7), 1037–1043. doi: 10.1086/591504

Diekema, D. I., and Jones, R. N. (2000). Oxazolidinones: a review. *Drugs* 59 (1), 7–16. doi: 10.2165/00003495-200059010-00002

Dong, T. G., Dong, S., Catalano, C., Moore, R., Liang, X., and Mekalanos, J. J. (2015). Generation of reactive oxygen species by lethal attacks from competing microbes. *Proc. Natl. Acad. Sci. U.S.A.* 112 (7), 2181–2186. doi: 10.1073/pnas.1425007112

Drain, P. K., Bajema, K. L., Dowdy, D., Dheda, K., Naidoo, K., Schumacher, S. G., et al. (2018). Incipient and subclinical tuberculosis: a clinical review of early stages and progression of infection. *Clin. Microbiol. Rev.* 31 (4). doi: 10.1128/CMR.00021-18

Dusthackeer, A., Sekar, G., Chidambaram, S., Kumar, V., Mehta, P., and Swaminathan, S. (2015). Drug resistance among extrapulmonary TB patients: Six years experience from a supranational reference laboratory. *Indian J. Med. Res.* 142 (5), 568–574. doi: 10.4103/0971-5916.171284

Dwyer, D. J., Belenky, P. A., Yang, J. H., MacDonald, I. C., Martell, J. D., Takahashi, N., et al. (2014). Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc. Natl. Acad. Sci. U.S.A.* 111 (20), E2100–E2109. doi: 10.1073/pnas.1401876111

Eoh, H., Wang, Z., Layre, E., Rath, P., Morris, R., Branch Moody, D., et al. (2017). Metabolic anticipation in Mycobacterium tuberculosis. *Nat. Microbiol.* 2, 17084. doi: 10.1038/nmicrobiol.2017.84

Escuyer, V. E., Lety, M. A., Torrelles, J. B., Khoo, K. H., Tang, J. B., Rithner, C. D., et al. (2001). The role of the embA and embB gene products in the biosynthesis of the terminal hexaarabinofuranosyl motif of *Mycobacterium smegmatis* arabinogalactan. *J. Biol. Chem.* 276 (52), 48854–48862. doi: 10.1074/jbc.M102272200

Fabre, M., Koeck, J.-L., Le Fleche, P., Simon, F., Herve, V., Vergnaud, G., et al. (2004). High genetic diversity revealed by variable-number tandem repeat genotyping and analysis of hsp65 gene polymorphism in a large collection of "Mycobacterium canettii" strains indicates that the M. tuberculosis complex is a recently emerged clone of "M. canettii". *J. Clin. Microbiol.* 42 (7), 3248–3255. doi: 10.1128/JCM.42.7.3248-3255.2004

Falkinham, J. O. 3rd (2003). Mycobacterial aerosols and respiratory disease. *Emerging Infect. Dis.* 9 (7), 763–767. doi: 10.3201/eid0907.02-0415

Farnia, P., Mohammad, R. M., Merza, M. A., Tabarsi, P., Zhavnerko, G. K., Ibrahim, T. A., et al. (2010). Growth and cell-division in extensive (XDR) and extremely drug resistant (XXDR) tuberculosis strains: transmission and atomic force observation. *Int. J. Clin. Exp. Med.* 3 (4), 308–314.

Fratti, R. A., Chua, J., Vergne, I., and Deretic, V. (2003). Mycobacterium tuberculosis glycosylated phosphatidylinositol causes phagosome maturation arrest. *Proc. Natl. Acad. Sci. U. S. A* 100 (9), 5437–5442. doi: 10.1073/pnas.0737613100

Gagneux, S., Deriemer, K., Van, T., Kato-Maeda, M., de Jong, B. C., Narayanan, S., et al. (2006a). Variable host-pathogen compatibility in Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U. S. A* 103 (8), 2869–2873. doi: 10.1073/pnas.0511240103

Gagneux, S., Long, C. D., Small, P. M., Van, T., Schoolnik, G. K., and Bohannan, B. J. (2006b). The competitive cost of antibiotic resistance in Mycobacterium tuberculosis. *Science* 312 (5782), 1944–1946. doi: 10.1126/science.1124410

Galagan, J. E., Minch, K., Peterson, M., Lyubetskaya, A., Azizi, E., Sweet, L., et al. (2013). The Mycobacterium tuberculosis regulatory network and hypoxia. *Nature* 499 (7457), 178–183. doi: 10.1038/nature12337

Garcia-Vilanova, A., Chan, J., and Torrelles, J. B. (2019). Underestimated manipulative roles of mycobacterium tuberculosis cell envelope glycolipids during infection. *Front. Immunol.* 10, 2909. doi: 10.3389/fimmu.2019.02909

Gautier, N., Lopez Marin, L. M., Laneelle, M. A., and Daffe, M. (1992). Structure of mycoside F, a family of trehalose-containing glycolipids of *Mycobacterium fortuitum*. *FEMS Microbiol. Lett.* 77 (1-3), 81–87. doi: 10.1111/j.1574-6968.1992.tb05494.x

Gerlach, D., Guo, Y., De Castro, C., Kim, S. H., Schlatterer, K., Xu, F. F., et al. (2018). Methicillin-resistant Staphylococcus aureus alters cell wall glycosylation to evade immunity. *Nature* 563 (7733), 705–709. doi: 10.1038/s41586-018-0730-x

Gilleron, M., Nigou, J., Cahuzac, B., and Puzo, G. (1999). Structural study of the lipomannans from *Mycobacterium bovis* BCG: characterisation of multiacylated forms of the phosphatidyl-*myo*-inositol anchor. *J. Mol. Biol.* 285 (5), 2147–2160. doi: 10.1006/jmbi.1998.2438

Gilleron, M., Ronet, C., Mempel, M., Monsarrat, B., Gachelin, G., and Puzo, G. (2001). Acylation state of the phosphatidylinositol mannosides from *Mycobcterium bovus* bacillus calmette guerin and ability to induce granuloma and recruit natural killer T cells. *J. Biol. Chem.* 276 (37), 34896–34904. doi: 10.1074/jbc.M103908200

Goren, M. B. (1970). Sulfolipid I of Mycobacterium tuberculosis, strain H37Rv. II. Structural studies. *Biochim. Biophys. Acta* 210 (1), 127–138. doi: 10.1016/0005-2760(70)90068-8

Goude, R., Amin, A. G., Chatterjee, D., and Parish, T. (2009). The arabinosyltransferase EmbC is inhibited by ethambutol in Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 53 (10), 4138–4146. doi: 10.1128/AAC.00162-09

Grzegorzewicz, A. E., Pham, H., Gundi, V. A., Scherman, M. S., North, E. J., Hess, T., et al. (2012). Inhibition of mycolic acid transport across the Mycobacterium tuberculosis plasma membrane. *Nat. Chem. Biol.* 8 (4), 334–341. doi: 10.1038/nchembio.794

Guérardel, Y., Maes, E., Briken, V., Chirat, F., Leroy, Y., Locht, C., et al. (2003). Lipomannan and lipoarabinomannan from a clinical isolate of Mycobacterium

kansasii: novel structural features and apoptosis-inducing properties. *J. Biol. Chem.* 278 (38), 36637–36651. doi: 10.1074/jbc.M305427200

Guerardel, Y., Maes, E., Elass, E., Leroy, Y., Timmerman, P., Besra, G. S., et al. (2002). Structural study of lipomannan and lipoarabinomannan from *Mycobacterium chelonae*. Presence of unusual components with alpha 1,3-mannopyranose side chains. *J. Biol. Chem.* 277 (34), 30635–30648. doi: 10.1074/jbc.M204398200

Howard, N. C., Marin, N. D., Ahmed, M., Rosa, B. A., Martin, J., Bambouskova, M., et al. (2018). Mycobacterium tuberculosis carrying a rifampicin drug resistance mutation reprograms macrophage metabolism through cell wall lipid changes. *Nat. Microbiol.* 3 (10), 1099–1108. doi: 10.1038/s41564-018-0245-0

Huang, Y., Zhu, C., Pan, L., and Zhang, Z. (2023). The role of Mycobacterium tuberculosis acetyltransferase and protein acetylation modifications in tuberculosis. *Front. Cell Infect. Microbiol.* 13, 1218583. doi: 10.3389/fcimb.2023.1218583

Jackson, M. (2014). The mycobacterial cell envelope-lipids. *Cold Spring Harb. Perspect. Med.* 4 (10). doi: 10.1101/cshperspect.a021105

Jankute, M., Nataraj, V., Lee, O. Y. C., Wu, H. H. T., Ridell, M., Garton, N. J., et al. (2017). The role of hydrophobicity in tuberculosis evolution and pathogenicity. *Sci. Rep.* 7 (1), 1315. doi: 10.1038/s41598-017-01501-0

Jarlier, V., and Nikaido, H. (1994). Mycobacterial cell wall: Structure and role in natural resistance to antibiotics. *FEMS Microbiol. Lett* 123, 11–18. doi: 10.1111/j.1574-6968.1994.tb07194.x

Jung, E. K., Chang, J. Y., Lee, Y. P., Chung, M. K., Seo, E. K., Koo, H. S., et al. (2016). A case of disseminated multidrug-resistant tuberculosis involving the brain. *Infect. Chemother.* 48 (1), 41–46. doi: 10.3947/ic.2016.48.1.41

Kang, P. B., Azad, A. K., Torrelles, J. B., Kaufman, T. M., Beharka, A., Tibesar, E., et al. (2005). The human macrophage mannose receptor directs *Mycobacterium tuberculosis* lipoarabinomannan-mediated phagosome biogenesis. *J. Exp. Med.* 202 (7), 987–999. doi: 10.1084/jem.20051239

Kaplan, S. R., Topal, J., Sosa, L., Malinis, M., Huttner, A., Malhotra, A., et al. (2018). A patient with central nervous system tuberculomas and a history of disseminated multi-drug-resistant tuberculosis. *J. Clin. Tuberc Other Mycobact Dis.* 10, 9–16. doi: 10.1016/j.jctube.2017.12.004

Kendall, E. A., Shrestha, S., and Dowdy, D. W. (2021). The epidemiological importance of subclinical tuberculosis. A critical reappraisal. *Am. J. Respir. Crit. Care Med.* 203 (2), 168–174. doi: 10.1164/rccm.202006-2394PP

Khoo, K. H., Suzuki, R., Morris, H. R., Dell, A., Brennan, P. J., and Besra, G. S. (1995). Structural definition of the glycopeptidolipids and the pyruvylated, glycosylated acyltrehalose from Mycobacterium butyricum. *Carbohydr. Res.* 276, 449–455. doi: 10.1016/0008-6215(95)00184-u

Khoo, K.-H., Tang, J.-B., and Chatterjee, D. (2001). Variation in mannose-capped terminal arabinan motifs of lipoarabinomannans from clinical isolates of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. *J. Biol. Chem.* 276 (6), 3863–3871. doi: 10.1074/jbc.M004010200

Khoshnood, S., Taki, E., Sadeghifard, N., Kaviar, V. H., Haddadi, M. H., Farshadzadeh, Z., et al. (2021). Mechanism of action, resistance, synergism, and clinical implications of delamanid against multidrug-resistant mycobacterium tuberculosis. *Front. Microbiol.* 12, 717045. doi: 10.3389/fmicb.2021.717045

Kirksey, M. A., Tischler, A. D., Siméone, R., Hisert, K. B., Uplekar, S., Guilhot, C., et al. (2011). Spontaneous phthiocerol dimycocerosate-deficient variants of Mycobacterium tuberculosis are susceptible to gamma interferon-mediated immunity. *Infect. Immun.* 79 (7), 2829–2838. doi: 10.1128/iai.00097-11

Kong, Y., Cave, M. D., Zhang, L., Foxman, B., Marrs, C. F., Bates, J. H., et al. (2007). Association between Mycobacterium tuberculosis Beijing/W lineage strain infection and extrathoracic tuberculosis: Insights from epidemiologic and clinical characterization of the three principal genetic groups of M. tuberculosis clinical isolates. *J. Clin. Microbiol.* 45 (2), 409–414. doi: 10.1128/JCM.01459-06

Lahiri, N., Shah, R. R., Layre, E., Young, D., Ford, C., Murray, M. B., et al. (2016). Rifampin resistance mutations are associated with broad chemical remodeling of mycobacterium tuberculosis. *J. Biol. Chem.* 291 (27), 14248–14256. doi: 10.1074/jbc.M116.716704

Lee, J. J., Lee, S. K., Song, N., Nathan, T. O., Swarts, B. M., Eum, S. Y., et al. (2019). Transient drug-tolerance and permanent drug-resistance rely on the trehalose-catalytic shift in Mycobacterium tuberculosis. *Nat. Commun.* 10 (1), 2928. doi: 10.1038/s41467-019-10975-7

Lee, N., and Nguyen, H. (2023). *Ethambutol* (StatPearls. Treasure Island (FL): StatPearls Publishing. StatPearls Publishing Copyright © 2023.

Manjunatha, U., Boshoff, H. I., and Barry, C. E. (2009). The mechanism of action of PA-824: Novel insights from transcriptional profiling. *Commun. Integr. Biol.* 2 (3), 215–218. doi: 10.4161/cib.2.3.7926

Matsunaga, I., Bhatt, A., Young, D. C., Cheng, T. Y., Eyles, S. J., Besra, G. S., et al. (2004). Mycobacterium tuberculosis pks12 produces a novel polyketide presented by CD1c to T cells. *J. Exp. Med.* 200 (12), 1559–1569. doi: 10.1084/jem.20041429

Middlebrook, G., Coleman, C. M., and Schaefer, W. B. (1959). Sulfolipid from virulent tubercle bacilli. *Proc. Natl. Acad. Sci. U.S.A.* 45 (12), 1801–1804. doi: 10.1073/pnas.45.12.1801

Mohandas, P., Budell, W. C., Mueller, E., Au, A., Bythrow, G. V., and Quadri, L. E. (2016). Pleiotropic consequences of gene knockouts in the phthiocerol dimycocerosate and phenolic glycolipid biosynthetic gene cluster of the opportunistic human pathogen Mycobacterium marinum. *FEMS Microbiol. Lett.* 363 (5), fnw016. doi: 10.1093/femsle/fnw016

Murry, J. P., Pandey, A. K., Sassetti, C. M., and Rubin, E. J. (2009). Phthiocerol dimycocerosate transport is required for resisting interferon-gamma-independent immunity. *J. Infect. Dis.* 200 (5), 774–782. doi: 10.1086/605128

Naidoo, A., Naidoo, K., McIlleron, H., Essack, S., and Padayatchi, N. (2017). A review of moxifloxacin for the treatment of drug-susceptible tuberculosis. *J. Clin. Pharmacol.* 57 (11), 1369–1386. doi: 10.1002/jcph.968

Nandakumar, M., Nathan, C., and Rhee, K. Y. (2014). Isocitrate lyase mediates broad antibiotic tolerance in Mycobacterium tuberculosis. *Nat. Commun.* 5, 4306. doi: 10.1038/ncomms5306

Nataraj, V., Varela, C., Javid, A., Singh, A., Besra, G. S., and Bhatt, A. (2015). Mycolic acids: deciphering and targeting the Achilles' heel of the tubercle bacillus. *Mol. Microbiol.* 98 (1), 7–16. doi: 10.1111/mmi.13101

Ngo, S. C., Zimhony, O., Chung, W. J., Sayahi, H., Jacobs, W. R. Jr., and Welch, J. T. (2007). Inhibition of isolated Mycobacterium tuberculosis fatty acid synthase I by pyrazinamide analogs. *Antimicrob. Agents Chemother.* 51 (7), 2430–2435. doi: 10.1128/AAC.01458-06

Niemann, S., Diel, R., KheChinashvili, G., Gegia, M., Mdivani, N., and Tang, Y. W. (2010). Mycobacterium tuberculosis Beijing lineage favors the spread of multidrug-resistant tuberculosis in the Republic of Georgia. *J. Clin. Microbiol.* 48 (10), 3544–3550. doi: 10.1128/JCM.00715-10

Nieto, R. L., Mehaffy, C., Creissen, E., Troudt, J., Troy, A., Bielefeldt-Ohmann, H., et al. (2016). Virulence of Mycobacterium tuberculosis after Acquisition of Isoniazid Resistance: Individual Nature of katG Mutants and the Possible Role of AhpC. *PloS One* 11 (11), e0166807. doi: 10.1371/journal.pone.0166807

Nieto, R. L., Mehaffy, C., Islam, M. N., Fitzgerald, B., Belisle, J., Prenni, J., et al. (2018). Biochemical characterization of isoniazid-resistant mycobacterium tuberculosis: can the analysis of clonal strains reveal novel targetable pathways? *Mol. Cell Proteomics* 17 (9), 1685–1701. doi: 10.1074/mcp.RA118.000821

Nigou, J., Gilleron, M., and Puzo, G. (2003). Lipoarabinomannans: from structure to biosynthesis. *Biochimie* 85 (1-2), 153–166. doi: 10.1016/S0300-9084(03)00048-8

Nyang'wa, B. T., Berry, C., Kazounis, E., Motta, I., Parpieva, N., Tigay, Z., et al. (2022). A 24-week, all-oral regimen for rifampin-resistant tuberculosis. *N Engl. J. Med.* 387 (25), 2331–2343. doi: 10.1056/NEJMoa2117166

Onwueme, K. C., Vos, C. J., Zurita, J., Ferreras, J. A., and Quadri, L. E. (2005). The dimycocerosate ester polyketide virulence factors of mycobacteria. *Prog. Lipid Res.* 44 (5), 259–302. doi: 10.1016/j.plipres.2005.07.001

Ordway, D. J., Sonnenberg, M. G., Donahue, S. A., Belisle, J. T., and Orme, I. M. (1995). Drug-resistant strains of *Mycobacterium tuberculosis* exhibit a range of virulence for mice. *Infect. Immun.* 63, 741–743. doi: 10.1128/iai.63.2.741-743.1995

Pal, R., Hameed, S., Kumar, P., Singh, S., and Fatima, Z. (2017). Comparative lipidomics of drug sensitive and resistant Mycobacterium tuberculosis reveals altered lipid imprints. *3 Biotech.* 7 (5), 325. doi: 10.1007/s13205-017-0972-6

Prabowo, S. A., Gröschel, M. I., Schmidt, E. D., Skrahina, A., Mihaescu, T., Hastürk, S., et al. (2013). Targeting multidrug-resistant tuberculosis (MDR-TB) by therapeutic vaccines. *Med. Microbiol. Immunol.* 202 (2), 95–104. doi: 10.1007/s00430-012-0278-6

Puech, V., Guilhot, C., Perez, E., Tropis, M., Armitige, L. Y., Gicquel, B., et al. (2002). Evidence for a partial redundancy of the fibronectin-binding proteins for the transfer of mycoloyl residues onto the cell wall arabinogalactan termini of Mycobacterium tuberculosis. *Mol. Microbiol.* 44 (4), 1109–1122. doi: 10.1046/j.1365-2958.2002.02953.x

Rahul Pal, S. H., Kumar, P., Singh, S., and Fatima, Z. (2015). Comparative lipidome profile of sensitive and resistant *Mycobacterium tuberculosis* strain. *Int. J. Curr. Microbiol. Appl. Sci.* (Special Issue 1), 189–197.

Reed, M. B., Domenech, P., Manca, C., Su, H., Barczak, A. K., Kreiswirth, B. N., et al. (2004). A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature* 431 (7004), 84–87. doi: 10.1038/nature02837

Reed, M. B., Gagneux, S., Deriemer, K., Small, P. M., and Barry, C. E. III (2007). The W-Beijing lineage of Mycobacterium tuberculosis overproduces triglycerides and has the DosR dormancy regulon constitutively upregulated. *J. Bacteriol* 189 (7), 2583–2589. doi: 10.1128/JB.01670-06

Rens, C., Chao, J. D., Sexton, D. L., Tocheva, E. I., and Av-Gay, Y. (2021). Roles for phthiocerol dimycocerosate lipids in Mycobacterium tuberculosis pathogenesis. *Microbiol. (Reading)* 167 (3). doi: 10.1099/mic.0.001042

Rousseau, C., Neyrolles, O., Bordat, Y., Giroux, S., Sirakova, T. D., Prevost, M. C., et al. (2003). Deficiency in mycolipenate- and mycosanoate-derived acyltrehaloses enhances early interactions of Mycobacterium tuberculosis with host cells. *Cell. Microbiol.* 5 (6), 405–415. doi: 10.1046/j.1462-5822.2003.00289.x

Rousseau, C., Winter, N., Pivert, E., Bordat, Y., Neyrolles, O., Ave, P., et al. (2004). Production of phthiocerol dimycocerosates protects Mycobacterium tuberculosis from the cidal activity of reactive nitrogen intermediates produced by macrophages and modulates the early immune response to infection. *Cell Microbiol.* 6 (3), 277–287. doi: 10.1046/j.1462-5822.2004.00368.x

Saelens, J. W., Viswanathan, G., and Tobin, D. M. (2019). Mycobacterial evolution intersects with host tolerance. *Front. Immunol.* 10, 528. doi: 10.3389/fimmu.2019.00528

Samanta, S., Singh, A., Biswas, P., Bhatt, A., and Visweswariah, S. S. (2017). Mycobacterial phenolic glycolipid synthesis is regulated by cAMP-dependent lysine acylation of FadD22. *Microbiol. (Reading)* 163 (3), 373–382. doi: 10.1099/mic.0.000440

Sampath, P., Rajamanickam, A., Thiruvengadam, K., Natarajan, A. P., Hissar, S., Dhanapal, M., et al. (2023). Cytokine upsurge among drug-resistant tuberculosis endorse the signatures of hyper inflammation and disease severity. *Sci. Rep.* 13 (1), 785. doi: 10.1038/s41598-023-27895-8

Sarathy, J. P., Gruber, G., and Dick, T. (2019). Re-understanding the mechanisms of action of the anti-mycobacterial drug bedaquiline. *Antibiotics (Basel)* 8 (4). doi: 10.3390/antibiotics8040261

Schroeder, E. K., Basso, L. A., Santos, D. S., and de Souza, O. N. (2005). Molecular dynamics simulation studies of the wild-type, I21V, and I16T mutants of isoniazid-resistant Mycobacterium tuberculosis enoyl reductase (InhA) in complex with NADH: toward the understanding of NADH-InhA different affinities. *Biophys. J.* 89 (2), 876–884. doi: 10.1529/biophysj.104.053512

Shi, W., Zhang, X., Jiang, X., Yuan, H., Lee, J. S., Barry, C. E. 3rd, et al. (2011). Pyrazinamide inhibits trans-translation in Mycobacterium tuberculosis. *Science* 333 (6049), 1630–1632. doi: 10.1126/science.1208813

Singh, R., Manjunatha, U., Boshoff, H. I., Ha, Y. H., Niyomrattanakit, P., Ledwidge, R., et al. (2008). PA-824 kills nonreplicating Mycobacterium tuberculosis by intracellular NO release. *Science* 322 (5906), 1392–1395. doi: 10.1126/science.1164571

Sinsimer, D., Huet, G., Manca, C., Tsenova, L., Koo, M. S., Kurepina, N., et al. (2008). The phenolic glycolipid of *Mycobacterium tuberculosis* differentially modulates the early host cytokine response but does not in itself confer hypervirulence. *Infect. Immun.* 76 (7), 3027–3036. doi: 10.1128/IAI.01663-07

Soetaert, K., Rens, C., Wang, X. M., De Bruyn, J., Lanéelle, M. A., Laval, F., et al. (2015). Increased vancomycin susceptibility in mycobacteria: a new approach to identify synergistic activity against multidrug-resistant mycobacteria. *Antimicrob. Agents Chemother.* 59 (8), 5057–5060. doi: 10.1128/AAC.04856-14

Su, C.-C., Klenotic, P. A., Bolla, J. R., Purdy, G. E., Robinson, C. V., and Yu, E. W. (2019). MmpL3 is a lipid transporter that binds trehalose monomycolate and phosphatidylethanolamine. *Proc. Natl. Acad. Sci. United States America* 116 (23), 11241–11246. doi: 10.1073/pnas.1901346116

Sun, M., Ge, S., and Li, Z. (2022). The role of phosphorylation and acylation in the regulation of drug resistance in mycobacterium tuberculosis. *Biomedicines* 10 (10). doi: 10.3390/biomedicines10102592

Takayama, K., Armstrong, E. L., Kunugi, K. A., and Kilburn, J. O. (1979). Inhibition by ethambutol of mycolic acid transfer into the cell wall of Mycobacterium smegmatis. *Antimicrob. Agents Chemother.* 16 (2), 240–242. doi: 10.1128/AAC.16.2.240

Thompson, A. M., Bonnet, M., Lee, H. H., Franzblau, S. G., Wan, B., Wong, G. S., et al. (2017). Antitubercular nitroimidazoles revisited: synthesis and activity of the authentic 3-nitro isomer of pretomanid. *ACS Med. Chem. Lett.* 8 (12), 1275–1280. doi: 10.1021/acsmedchemlett.7b00356

Thwaites, G., Caws, M., Chau, T. T., D'Sa, A., Lan, N. T., Huyen, M. N., et al. (2008). Relationship between Mycobacterium tuberculosis genotype and the clinical phenotype of pulmonary and meningeal tuberculosis. *J. Clin. Microbiol.* 46 (4), 1363–1368. doi: 10.1128/JCM.02180-07

Torrelles, J. B., Azad, A. K., and Schlesinger, L. S. (2006). Fine discrimination in the recognition of individual species of phosphatidyl-*myo*-inositol mannosides from *Mycobacterium tuberculosis* by C-type lectin pattern recognition receptors. *J. Immunol.* 177 (3), 1805–1816. doi: 10.4049/jimmunol.177.3.1805

Torrelles, J. B., Khoo, K. H., Sieling, P. A., Modlin, R. L., Zhang, N., Marques, A. M., et al. (2004). Truncated structural variants of lipoarabinomannan in *Mycobacterium leprae* and an ethambutol-resistant strain of *Mycobacterium tuberculosis*. *J. Biol. Chem.* 279 (39), 41227–41239. doi: 10.1074/jbc.M405180200

Torrelles, J. B., Knaup, R., Kolareth, A., Slepushkina, T., Kaufman, T. M., Kang, P. B., et al. (2008). Identification of *Mycobacterium tuberculosis* clinical isolates with altered phagocytosis by human macrophages due to a truncated lipoarabinomannan. *J. Biol. Chem.* 283, 31417–31428. doi: 10.1074/jbc.M806350200

Torrelles, J. B., and Schlesinger, L. S. (2010). Diversity in *Mycobacterium tuberculosis* mannosylated cell wall determinants impacts adaptation to the host. *Tuberculosis* 90 (2), 84–93. doi: 10.1016/j.tube.2010.02.003

Torrelles, J. B., Sieling, P. A., Arcos, J., Knaup, R., Bartling, C., Rajaram, M. V., et al. (2011). Structural differences in lipomannans from pathogenic and nonpathogenic mycobacteria that impact CD1b-restricted T cell responses. *J. Biol. Chem.* 286 (41), 35438–35446. doi: 10.1074/jbc.M111.232587

Torrelles, J. B., Sieling, P. A., Zhang, N., Keen, M. A., McNeil, M. R., Belisle, J. T., et al. (2012). Isolation of a distinct Mycobacterium tuberculosis mannose-capped lipoarabinomannan isoform responsible for recognition by CD1b-restricted T cells. *Glycobiology* 22 (8), 1118–1127. doi: 10.1093/glycob/cws078

Torrey, H. L., Keren, I., Via, L. E., Lee, J. S., and Lewis, K. (2016). High persister mutants in mycobacterium tuberculosis. *PloS One* 11 (5), e0155127. doi: 10.1371/journal.pone.0155127

Tsenova, L., Ellison, E., Harbacheuski, R., Moreira, A. L., Kurepina, N., Reed, M. B., et al. (2005). Virulence of selected Mycobacterium tuberculosis clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli. *J infect. Dis* 192 (1), 98–106. doi: 10.1086/430614

Tupin, A., Gualtieri, M., Roquet-Banères, F., Morichaud, Z., Brodolin, K., and Leonetti, J. P. (2010). Resistance to rifampicin: at the crossroads between ecological, genomic and medical concerns. *Int. J. Antimicrob. Agents* 35 (6), 519–523. doi: 10.1016/j.ijantimicag.2009.12.017

Turner, J., and Torrelles, J. B. (2018). Mannose-capped lipoarabinomannan in Mycobacterium tuberculosis pathogenesis. *Pathog. Dis.* 76 (4). doi: 10.1093/femspd/fty026

Varela, C., Rittmann, D., Singh, A., Krumbach, K., Bhatt, K., Eggeling, L., et al. (2012). MmpL genes are associated with mycolic acid metabolism in mycobacteria and corynebacteria. *Chem. Biol.* 19 (4), 498–506. doi: 10.1016/j.chembiol.2012.03.006

Velayati, A. A., Farnia, P., and Hoffner, S. (2018). Drug-resistant Mycobacterium tuberculosis: Epidemiology and role of morphological alterations. *J. Glob Antimicrob. Resist.* 12, 192–196. doi: 10.1016/j.jgar.2017.10.006

Velayati, A. A., Farnia, P., Ibrahim, T. A., Haroun, R. Z., Kuan, H. O., Ghanavi, J., et al. (2009a). Differences in cell wall thickness between resistant and nonresistant strains of Mycobacterium tuberculosis: using transmission electron microscopy. *Chemotherapy* 55 (5), 303–307. doi: 10.1159/000226425

Velayati, A. A., Farnia, P., Masjedi, M. R., Ibrahim, T. A., Tabarsi, P., Haroun, R. Z., et al. (2009b). Totally drug-resistant tuberculosis strains: evidence of adaptation at the cellular level. *Eur. Respir. J.* 34 (5), 1202–1203. doi: 10.1183/09031936.00081909

Velayati, A. A., Farnia, P., Masjedi, M. R., Zhavnerko, G. K., Merza, M. A., Ghanavi, J., et al. (2011). Sequential adaptation in latent tuberculosis bacilli: observation by atomic force microscopy (AFM). *Int. J. Clin. Exp. Med.* 4 (3), 193–199.

Velayati, A. A., Farnia, P., Merza, M. A., Zhavnerko, G. K., Tabarsi, P., Titov, L. P., et al. (2010). New insight into extremely drug-resistant tuberculosis: using atomic force microscopy. *Eur. Respir. J.* 36 (6), 1490–1493. doi: 10.1183/09031936.00064510

Venisse, A., Rivière, M., Vercauteren, J., and Puzo, G. (1995). Structural analysis of the mannan region of lipoarabinomannan from *Mycobacterium bovis* BCG. Heterogeneity in phosphorylation state. *J. Biol. Chem.* 270, 15012–15021. doi: 10.1074/jbc.270.25.15012

Vilchèze, C., and Jacobs, W. R. Jr (2007). The mechanism of isoniazid killing: clarity through the scope of genetics. *Annu. Rev. Microbiol.* 61, 35–50. doi: 10.1146/annurev.micro.61.111606.122346

Wang, J. Y., Burger, R. M., and Drlica, K. (1998). Role of superoxide in catalase-peroxidase-mediated isoniazid action against mycobacteria. *Antimicrob. Agents Chemother.* 42 (3), 709–711. doi: 10.1128/AAC.42.3.709

Wang, S., Cai, X., Yu, W., Zeng, S., Zhang, J., Guo, L., et al. (2022). Arabinosyltransferase C mediates multiple drugs intrinsic resistance by altering cell envelope permeability in mycobacterium abscessus. *Microbiol. Spectr.* 10 (4), e0276321. doi: 10.1128/spectrum.02763-21

WHO (2022a). *Global tuberculosis report 2022* (Geneva: World Health Organization).

WHO (2022b). *WHO consolidated guidelines on tuberculosis. Module 4: treatment - drug-susceptible tuberculosis treatment* (Geneva: World Health Organization).

WHO (2022c). *WHO operational handbook on tuberculosis. Module 4: Treatment - drug-resistant tuberculosis treatment 2022 update* (Geneva: World Health Organization).

Wilson, T. M., De Lisle, G. W., and Collins, D. M. (1995). Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium bovis*. *Mol. Microbiol.* 15, 1009–1015. doi: 10.1111/j.1365-2958.1995.tb02276.x

Yimcharoen, M., Saikaew, S., Wattanananandkul, U., Phunpae, P., Intorasoot, S., Kasinrerk, W., et al. (2022). The regulation of manLAM-related gene expression in mycobacterium tuberculosis with different drug resistance profiles following isoniazid treatment. *Infect. Drug Resist.* 15, 399–412. doi: 10.2147/IDR.S346869

Zhan, L., Wang, J., Wang, L., and Qin, C. (2020). The correlation of drug resistance and virulence in *Mycobacterium tuberculosis*. *Biosafety Health* 2, 18–24. doi: 10.1016/j.bsheal.2020.02.004

Zhang, B., Li, J., Yang, X., Wu, L., Zhang, J., Yang, Y., et al. (2019). Crystal structures of membrane transporter mmpL3, an anti-TB drug target. *Cell* 176 (3), 636–648.e613. doi: 10.1016/j.cell.2019.01.003

Zhang, N., Torrelles, J. B., McNeil, M. R., Escuyer, V. E., Khoo, K. H., Brennan, P. J., et al. (2003). The Emb proteins of mycobacteria direct arabinosylation of lipoarabinomannan and arabinogalactan via an N-terminal recognition region and a C-terminal synthetic region. *Mol. Microbiol.* 50 (1), 69–76. doi: 10.1046/j.1365-2958.2003.03681.x

Zhang, L., Zhao, Y., Gao, Y., Wu, L., Gao, R., Zhang, Q., et al. (2020). Structures of cell wall arabinosyltransferases with the anti-tuberculosis drug ethambutol. *Science* 368 (6496), 1211–1219. doi: 10.1126/science.aba9102

Zheng, C., Hu, X., Zhao, L., Hu, M., and Gao, F. (2017). Clinical and pharmacological hallmarks of rifapentine's use in diabetes patients with active and latent tuberculosis: do we know enough? *Drug Des. Devel Ther.* 11, 2957–2968. doi: 10.2147/DDDT.S146506

Zimhony, O., Vilchèze, C., Arai, M., Welch, J. T., and Jacobs., W. R. Jr (2007). Pyrazinoic acid and its n-propyl ester inhibit fatty acid synthase type I in replicating tubercle bacilli. *Antimicrob. Agents Chemother.* 51 (2), 752–754. doi: 10.1128/AAC.01369-06

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 31 October 2023
DOI 10.3389/fcimb.2023.1282902

OPEN ACCESS

EDITED BY
Sanket Kaushik,
Amity University Jaipur, India

REVIEWED BY
Anna Malgorzata Brzostek,
Polish Academy of Sciences, Poland
Xuejiao Hu,
Guangdong Provincial People's Hospital, China

*CORRESPONDENCE
Yi Liao
1191063186@qq.com

RECEIVED 25 August 2023
ACCEPTED 17 October 2023
PUBLISHED 31 October 2023

CITATION
Wang D-M, Liu H, Zheng Y-L, Xu Y-H and Liao Y (2023) Epidemiology of Nontuberculous Mycobacteria in Tuberculosis suspects, Southwest of China, 2017-2022.
Front. Cell. Infect. Microbiol. 13:1282902.
doi: 10.3389/fcimb.2023.1282902

Epidemiology of Nontuberculous Mycobacteria in Tuberculosis suspects, Southwest of China, 2017-2022

Dong-Mei Wang[1], Hong Liu[1], Yong-Li Zheng[1], Yuan-Hong Xu[1] and Yi Liao[2]*

[1]Department of Science and Education Division, Public Health Clinical Center of Chengdu, Chengdu, Sichuan, China, [2]Department of Clinical Laboratory Medicine, Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, China

Objectives: This study summarizes the epidemiological characteristics, species distribution, and drug sensitivity of clinical nontuberculous mycobacteria (NTM) isolates at the Public Health Clinical Center of Chengdu, China, from January 2017 to December 2022.

Methods: We retrospectively analyzed data from patients with clinically isolated NTM strains. Chi-square analysis assessed the rate of *Mycobacterium* strain isolation over 6 years.

Results: The number of samples tested for *Mycobacterium tuberculosis* (MTB) and/or NTM increased each year, while MTB detection decreased and NTM detection rose significantly each year (P=0.03). The average age of NTM patients was 51 ± 17.53 years, with a 14.1% HIV infection rate. The predominant isolates were *Mycobacterium avium-intracellulare* (MAC) and *M. chelonae*/*M. abscessus*, with 96.4% of cases being of Han ethnicity. Amikacin, moxifloxacin, and clarithromycin were effective against *M. avium* and *M. intracellulare*; linezolid, amikacin, and cefoxitin were effective against *M. chelonae*/*M. abscessus*. Over 90% of NTM cases originated from the respiratory tract.

Conclusion: The NTM isolation rate in Southwest China has risen in recent years, primarily among elderly patients with a high HIV co-infection rate. The main NTM isolates were MAC and *M. chelonae*/*M. abscessus*. Amikacin, moxifloxacin, clarithromycin, and linezolid exhibited strong antibacterial activity against SGM, while amikacin and linezolid displayed relatively better antibacterial activity against RGM. The prevalence of NTM infection may be positively associated with regional economic development and health conditions.

KEYWORDS

epidemiology, TB, drug resistance, NTM, clinical characteristics

Introduction

Nontuberculous mycobacteria (NTM) are part of the *Mycobacterium* species, distinct from those in the *M. tuberculosis* complex or *M. leprae*, and typically act as opportunistic pathogens (Falkinham, 1996; Falkinham, 2002). This term encompasses around 200 distinct *Mycobacterium* species, with new species continually emerging (Zhou et al., 2020). In recent years, both in China and globally, NTM infections have been on the rise (Wang et al., 2019; Liu et al., 2021; Thornton et al., 2021). Among clinical laboratories in China, the *Mycobacterium* avium-intracellulare complex (MAC) and the *M. chelonae*/*M. abscessus* complex are the two most commonly encountered NTM complexes, and they are among the most drug-resistant species (Wang et al., 2016; Wang et al., 2019; Guo et al., 2021). However, limited studies have explored NTM drug susceptibility due to small sample sizes or restricted antibiotic types being tested. Therefore, this study aims to summarize NTM identification and drug sensitivity data from the largest sample size in a major central city in Southwest China. This information can provide valuable insights for the prevention and treatment of NTM diseases.

Methods

Study population and diagnostic criteria

This study included all patients with culture-positive NTM infections treated at the Public Health Clinical Center of Chengdu (PHCC) in Sichuan Province, China, from January 2017 to December 2022. During the 6-year study period, a total of 126,368 suspected mycobacterial infections were cultured using the BACTEC™ MGIT 960 System (Becton Dickinson & Co., NJ, USA), resulting in 26,510 *Mycobacterium tuberculosis* (MTB) culture-positive cases and 587 NTM culture-positive cases. Diagnosis and categorization of NTM-infected patients were based on the 2012 and 2020 NTM Diagnosis and Treatment Expert Consensus (Editorial Board of CSTB, 2016; Tuberculosis Branch of Chinese Medical Association, 2020), the Clinical Diagnosis and Treatment Guidelines for Tuberculosis in China (Chinese Medical Association, 2005), and the updated guidelines from the World Health Organization. Diagnosis of human immunodeficiency virus (HIV) followed the Chinese HIV and HIV Infection Diagnostic Criteria (WS293–2008) (From the Centers for Disease Control and prevention, 1993).

Bacterial strains culture, identification, and drug sensitivity

We employed the BACTEC™ MGIT 960 System for culturing mycobacteria. Extrapulmonary samples (such as pleural fluid, spinal fluid, and lymph nodes) were obtained through lumbar puncture, pleural tap, fine needle aspiration, lymph node biopsy, and other procedures (Wang et al., 2017). Initial identification of NTM bacteria primarily relied on the MPT 64 antigen detection (Colloidal Gold immunochromatography) or polymerase chain reaction (PCR) methods. Subsequently, P-nitrobenzoic acid (PNB) and thiophene-2-carboxylic acid hydrazide (TCH) were employed for NTM revalidation using the MicroDST ™ (Yinke AUTOBIO Diagnostics Co., Ltd, Zhuhai, China) approach (Cao et al., 2021). Further identification of NTM species/complexes was conducted using Genechip, following the manufacturer's instructions (CapitalBio Corp., Chengdu, China). Strains that couldn't be identified via genechip were subjected to analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Microflex LT; Bruker Daltonics, Bremen, Germany) or 16S rDNA sequencing.

The Clinical and Laboratory Standards Institute (CLSI) recommends the microplate dilution method for *in vitro* drug sensitivity testing of some Slow-Growing Mycobacteria (SGMs) and Rapid-Growing Mycobacteria (RGMs). However, the exact Minimum Inhibitory Concentration (MIC) values for the strains require comprehensive evaluation and adjustment for clinical practice (Clinical and Laboratory Standards Institute (CLSI), [[NoYear]]). In this study, drug resistance tests (DST) for culture-positive NTM isolates were conducted using MicroDST ™ (Yinke AUTOBIO Diagnostics Co., Ltd, Zhuhai, China). The MIC was defined as the lowest drug concentration inhibiting visible growth of the tested isolates. MIC breakpoints and sensitivity/resistance determinations were interpreted following reagent instructions, and the protocol was executed in accordance with the manufacturer's recommendations (Wang et al., 2020). We employed a total of 13 antimicrobial agents in this study, including rifampicin (1, ***4***, 6, 16 μg/mL), clarithromycin (0.5, 4, ***16***, 64 μg/mL), imipenem/cilastatin (IPM/CS) (0.5, 4, 16, ***64*** μg/mL), linezolid (0.5, 2, 8, ***32*** μg/mL), amikacin (1, 4, 16, ***64*** μg/mL), ethambutol (2.5, ***5***, 10, 20 μg/mL), and rifabutin (0.5, ***2***, 8, 32–10 μg/mL), at four concentrations. Cefoxitin (4, 16, 32, 40, 64, ***80***, 128, 160 μg/mL), tobramycin (0.5, 1, 2, 4, 8, ***16***, 32, 64 μg/mL), moxifloxacin (0.125, 0.25, 0.5, 1, 2, 4, ***8***, 16 μg/mL), doxycycline (0.5, 1, 4, ***8***, 16, 32, 64, 128 μg/mL), minocycline (0.5, 1, 4, ***8***, 16, 32, 64, 128 μg/mL), and sulfamethoxazole (8, 16, 32, 64, ***80***, 128, 160, 256 μg/mL) were employed at eight concentrations. Bold and italicized values in the aforementioned drug concentrations represent the resistance breakpoint for each drug. For NTM from the same case, site, and type, the DST results from the initial culture were considered. Monitoring was conducted using control strains H37Rv (ATCC 25618) and *M. smegmatis* (CGMCC 1.2621).

Laboratory quality control

External quality assessment (EQA) was carried out for smear, culture, and DST at the Innovation Alliance for TB Diagnosis and Treatment in Beijing, China. Additionally, a blinded retesting of approximately 10% of isolates from the study laboratory was conducted by a specialized Centers for Disease Control and Prevention.

Statistical analysis

Data were analyzed using SPSS Statistics Client 19.0 (SPSS Inc., IL, USA). Normally distributed measurement data were presented

as means, while categorical variables were expressed as numbers and percentages. Chi-square (χ^2) analysis was employed to assess variations in the *Mycobacterium* strain rate, age, and sex ratio over five years. Statistical significance was set at $P < 0.05$.

Ethics approval and consent to participate

This study received approval from the Ethics Committee of PHCC (Approval No. 2017Y025). All patient information used in this study was routinely collected through the mandatory notification system. The requirement for informed consent was waived by the ethics committee.

Results

Demographic and clinical characteristics

From January 2017 to December 2022, a total of 126,368 non-repeated clinical specimens with suspected mycobacterial infections were cultured at PHCC. Among these specimens, 26,510 (21.0%) tested positive in culture. MTB was detected in 25,923 (97.8%) positive samples, while NTM was found in 587 (2.2%) samples. Among all NTM cases, 13 were co-infected with both MTB and NTM (Figure 1; Table 1). Over the 6-year study period, a significant increase was observed in the number of samples tested for MTB and/or NTM each year, corresponding to a significant annual rise in NTM detection ($\chi^2 = 18.01$, P=0.03; Figure 1). The mean age of the 587 NTM patients was 51 ± 17.53 years (range: 13–88 years). Within this subgroup, 349/587 (59.5%) were males, and 238/587 (40.5%) were females, with a male-to-female ratio of 1.5. There was no significant difference in the male-to-female ratio over the 6-year period ($\chi^2 = 0.71$, P>0.05; Figure 2). The majority of NTM-infected cases were middle-aged and elderly patients, and the proportion of each age group showed no significant difference over the 6 years ($\chi 2 = 11.16$, P>0.05; as shown in Figure 2; Table 1).

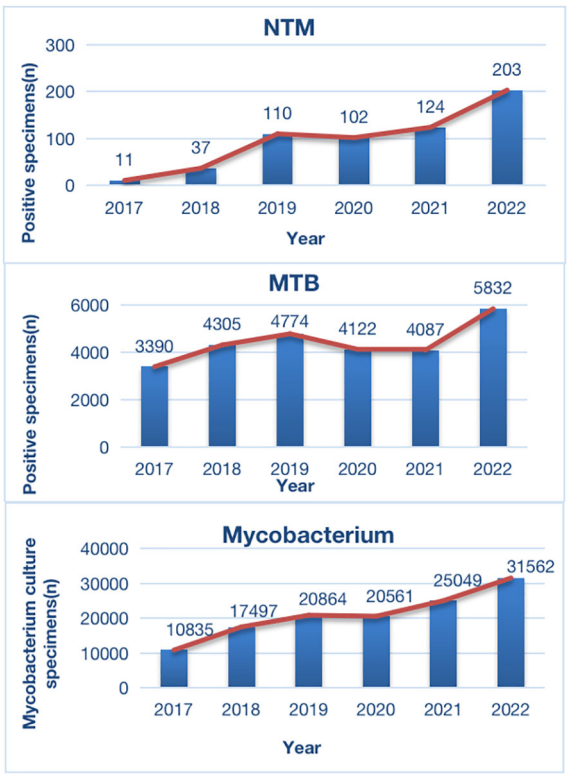


FIGURE 1
Distribution of Positive Specimens in *Mycobacterium* Culture During 2017-2021. MTB, *Mycobacterium* tuberculosis; NTM, non-tuberculous mycobacteria.

Twenty-one (3.6%) patients belonged to ethnic minorities. Among the 587 patients, 93 (15.8%) had at least one co-infectious disease: HIV infection in 83 (14.1%), diabetes mellitus in 29 (4.9%), hepatitis B virus in 12 (2.0%), and hypertension in 9 (1.5%), syphilis in 3 (0.5%), and hepatitis E virus in 1 (0.2%). Additionally, 99 (16.8%) patients experienced at least one adverse drug reaction: liver dysfunction in 82 (14.0%), hyperuricemia in 24 (4.1%), leucopenia in 19 (3.2%), thrombocytopenia in 8 (1.4%), and drug eruption in 7 (1.2%) patients.

NTM species identification

The DNA microarray chip identified NTM species as follows: *M. avium* in 154 (26.2%), *M. chelonae*/*M. abscessus* in 151 (25.7%), *M. intracellulare* in 145 (24.7%), *M. kansasii* in 37 (6.3%), *M. fortuitum* in 19 (3.2%), *M. scrofulaceum* in 15 (2.6%), *M. gordonae* in 13 (2.2%), *M. lentiflavum* in 10 (1.7%), and mixed infections of MTB and NTM in 13 (2.2%). Additionally, 30 specimens initially identified as other *Mycobacterium* spp. by the Genechip (CapitalBio Corporation) included *M. szulgai, M. malmoense, M. terrae, M. peregrinum, M. margueri, M. phlei, M. septicum, M. marseillense, M. shigaense, M. xenopi, M. simiae*, and *M. lentil*, identified through 16S rDNA sequencing or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry systems. Moreover, a significant rise in NTM detection was observed from 2017 to 2022 (Table 2; Figure 3).

Drug resistance of the NTM species

Out of the 587 patients with NTM disease, antimicrobial susceptibility testing was conducted for 113, as depicted in Table 3. Among the Slowly Growing Mycobacteria (SGM), amikacin (high-level resistance ≥ 64 μg/mL) demonstrated the highest activity against *M. avium*, with a resistance rate of 2/29 (6.9%). Moxifloxacin (high-level resistance ≥ 8 μg/mL) exhibited the highest activity against *M. intracellulare*, with a resistance rate of 2/27 (7.4%). Clarithromycin (high-level resistance ≥ 16 μg/mL) displayed effective antibacterial effects on both *M. avium* and *M. intracellulare*, with drug resistance rates of 5/29 (17.2%) and 6/27 (22.2%), respectively. In this study, all six strains of *M. kansasii* were completely sensitive to clarithromycin, linezolid (high-level resistance ≥ 32 μg/mL), amikacin, and moxifloxacin. Eight strains of *M. gordonae* showed complete sensitivity to rifampicin (high-

10.3389/fcimb.2023.1282902

TABLE 1 General characteristics among patients with NTM disease (n=587).

Category		No. of subjects (NTM, n=574)	No. of subjects (NTM/MTB*, n=13)	No. of subjects (%) Total (n=587)
Sex				
	Male	341	8	349 (59.5)
	Female	233	5	238 (40.5)
Age				
	Mean ± SD; years (range)	52 ± 18.47 (13-88)	43 ± 21.26 (26-80)	51 ±17.53
	<14	2	0	2 (0.3)
	14–24	45	0	45 (7.7)
	25–44	149	7	156 (26.6)
	45–65	250	4	254 (43.3)
	>65	128	2	130 (22.2)
Chinese Ethnic minorities	Han	554	12	566 (96.4)
	Tibetan	17	1	18 (3.1)
	Others	3	0	3 (0.5)
Co-infectious disease				
	AIDS	82	1	83 (14.1)
	Syphilis	3	0	3 (0.5)
	Combined with Diabetes	28	1	29 (4.9)
	Combined with hypertension	9	0	9 (1.5)
	Hepatitis E	1	0	1 (0.2)
	Hepatitis B	12	0	12 (2.0)
Adverse drug reaction				
	Liver dysfunction	80	2	82 (14.0)
	Hyperuricemia	24	0	24 (4.1)
	Leucopenia	19	0	19 (3.2)
	Thrombocytopenia	8	0	8 (1.4)
	Drug eruption	7	0	7 (1.2)

* NTM/MTB, among 587 cases of NTM infection, 13 cases of MTB and NTM co-infection.

level resistance ≥ 4 μg/mL), clarithromycin, linezolid, amikacin, cefoxitin (high-level resistance ≥ 80 μg/mL), and moxifloxacin. Linezolid, amikacin, tobramycin (high-level resistance ≥ 16 μg/mL), and moxifloxacin also exhibited strong antibacterial activity against *M. scrofulaceum*.

Regarding the Rapidly Growing Mycobacteria (RGM), linezolid, amikacin, and cefoxitin were the most effective agents against *M. chelonae*/*M. abscessus*. Rifampicin, clarithromycin, linezolid, amikacin, and moxifloxacin showed complete sensitivity in two strains of *M. lentiflavum*, while linezolid, amikacin, cefoxitin, and moxifloxacin demonstrated complete sensitivity in three strains of *M. fortuitum*.

NTM species distribution from different specimen types

Among the 113 NTM strains tested for drug sensitivity in this study, over 90% were isolated from respiratory tract samples [sputum 90/113 (79.6%), bronchoalveolar lavage fluid 12/113 (10.6%)]. Cerebrospinal fluid contributed 7/113 (6.2%) samples, while lymph nodes, the digestive tract, and pleural effusion each provided 2/113 (1.8%), 1/113 (0.9%), and 1/113 (0.9%) samples, respectively. The primary NTM strains isolated from these six tissue sources were *M. chelonae*/*M. abscessus* and MAC.

10.3389/fcimb.2023.1282902

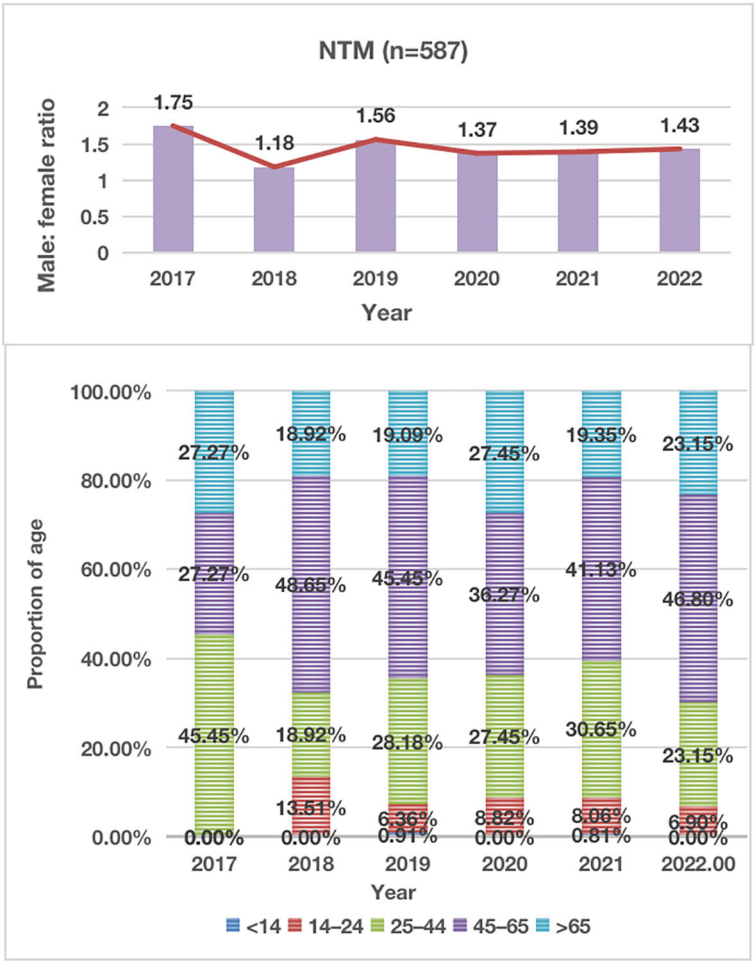


FIGURE 2
Distribution of Male-to-Female Ratio and Proportion of Age in NTM Patients During 2017-2021. NTM, nontuberculous mycobacteria.

TABLE 2 Identification of Nontuberculous Mycobacteria among patients with NTM disease (n=587).

Species	2017	2018	2019	2020	2021	2022	Total (%)
M. intracellulare	4	13	33	17	27	51	145 (24.7)
M. avium	0	9	29	31	32	53	154 (26.2)
M. scrofulaceum	0	0	1	2	2	10	15 (2.6)
M. chelonae / M. abscessus	5	11	29	23	31	52	151 (25.7)
M. Kansasii	1	1	8	5	10	12	37 (6.3)
M. fortuitum	0	0	3	4	5	7	19 (3.2)
M. gordonae	0	0	2	5	4	2	13 (2.2)
M. lentiflavum	0	0	0	3	3	4	10 (1.7)
M. tuberculosis / NTM co-infection	0	0	1	7	2	3	13 (2.2)
Other species*	1	3	4	7	6	9	30 (5.1)
Total (%)	11	37	110	104	122	203	587

Other species* include *M. szulgai, M. malmoense, M. terrae, M.peregrinum, M. margueri, M. phlei, M. septicum,M. marseillense,M. shigaense, M. xenopi, M. simiae* and *M. lentil.*

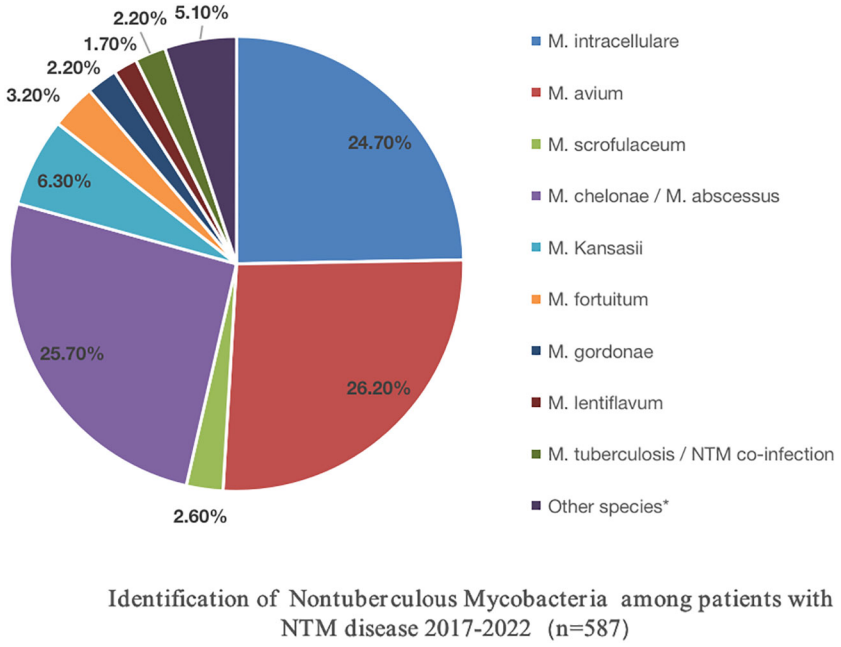


FIGURE 3
Identification of Nontuberculous Mycobacteria Among Patients with NTM Disease (n=587).

Discussion

According to recent reports from China and other countries, there has been a gradual increase in the incidence of NTM infection and laboratory isolation rates (Wang et al., 2016; Wang et al., 2019; Guo et al., 2021; Liu et al., 2021; Thornton et al., 2021). NTMs are inherently resistant to many anti-TB drugs (ATDs), and treatment plans depend on factors such as the NTM species, infection site, and the severity of the infection (Lee et al., 2015; Daley et al., 2020; Gopalaswamy et al., 2020). The prevalence of NTM strains varies among different regions and populations. For instance, the most common NTM isolates reported in various countries include *M. kansasii* and MAC in Poland (Przybylski et al., 2023), MAC and *M. gordonae* in Italy (Giannoni et al., 2023), *M. fortuitum*, and M. simiae in Iran (Tarashi et al., 2023), and MAC, *M. abscessus*, and *M. kansasii* in Turkey (Babalik et al., 2023). In Switzerland, common NTM isolates include *M. avium* and *M. gordonae* (Vongthilath-Moeung et al., 2022). However, information regarding NTM isolates and drug resistance profiles in southwest China has been scarce. Therefore, accurately understanding the NTM epidemic and drug resistance situation is crucial for early differential diagnosis and treatment of TB and NTM diseases.

In southwest China, there has been limited data on NTM infections. Our preliminary study discussed only a small sample of NTM-infected individuals in Southwest China, while a few studies have reported on large samples of clinically NTM-infected individuals and the dynamic sensitivity of NTM to multiple antibiotics in China and worldwide (Jeong et al., 2017; Zhou et al., 2020; Maya et al., 2022). In this study, we conducted a systematic analysis of NTM clinical infection cases in Southwest China over the past six years. The results indicated a rising trend in the number of patients visiting PHCC for mycobacteria culture evaluation each year from 2017 to 2022. This trend may be attributed to increased public awareness of healthcare in recent years and the expanding reach of PHCC in Southwest China. However, in 2020, there was a slight decline in this trend, likely due to the initial COVID-19 pandemic control measures, which resulted in reduced public mobility and hospital visits. Nevertheless, the trend resumed its upward trajectory after 2021. Moreover, among mycobacteria-positive cultures, the count of MTB cases increased from 2017 to 2019 and then gradually decreased until 2020. This trend is consistent with the recent control measures for TB, which have led to decreasing incidence and mortality rates each year (World Health Organization, 2021). Notably, the number of isolated clinical NTM cases showed a continuous upward trend from 2017 to 2022, rising from 11 cases in 2017 to 203 cases in 2022. This finding aligns with reports of increasing NTM infection cases worldwide and the observation that laboratory isolation rates have been on the rise each year (López-Roa et al., 2020).

In this study, NTM infections were primarily observed in middle-aged and elderly individuals, with those aged over 45 accounting for 65.5% of the cases. This is in contrast to our previous studies, where the majority of MTB infections were in middle-aged and young individuals (Wang et al., 2017; López-Roa et al., 2020; Wang et al., 2020). The co-infection rate of acquired immune deficiency syndrome (AIDS) with NTM cases was 14.1%, slightly higher than the 11.5% reported in our previous study (Wang et al., 2019). This increase may be linked to the rising incidence of AIDS cases in southwest China in recent years

TABLE 3 Number of Nontuberculous Mycobacteria clinical strains resistant to drugs in vitro experiments.

Antimicrobial agents	No of NTM species/complex (%)									
	SGM (n=73)						RGM (n=40)			
	M. avium (n=29)	*M. intracellulare* (n=27)	*M. kansasii* (n=6)	*M. gordonae* (n=8)	*M. scrofulaceum* (n=3)	Total (%)	*M. chelonae/ M. abscessus*(n=35)	*M. lentiflavum* (n=2)	*M. fortuitum* (n=3)	Total (%)
Rifampicin	14 (48.3)	8 (29.6)	3 (50.0)	0 (0)	2 (66.7)	27 (37.0)	33 (94.3)	0 (0.0)	3 (100.0)	36 (90.0)
Clarithromycin	5 (17.2)	6 (22.2)	0 (0)	0 (0)	2 (66.7)	13 (17.8)	22 (62.9)	0 (0.0)	3 (100.0)	25 (62.5)
Imipenem/ cilastatin (IPM/ CS)	29 (100.0)	26 (96.3)	6 (100.0)	8 (100.0)	3 (100.0)	72 (98.6)	35 (100.0)	2 (100.0)	3 (100.0)	40 (100.0)
linezolid	11 (37.9)	11 (40.7)	0 (0)	0 (0)	1 (33.3)	23 (31.5)	15 (42.9)	0 (0.0)	0 (0.0)	15 (37.5)
Amikacin	2 (6.9)	5 (18.5)	0 (0)	0 (0)	1 (33.3)	8 (11.0)	18 (51.4)	0 (0.0)	0 (0.0)	18 (45.0)
Ethambutol	19 (65.5)	10 (37.0)	1 (16.7)	2 (25.0)	2 (66.7)	34 (46.6)	33 (94.3)	1 (50.0)	2 (66.7)	36 (90.0)
Rifabutin	19 (65.5)	15 (55.6)	2 (33.3)	1 (12.5)	2 (66.7)	39 (53.4)	33 (94.3)	1 (50.0)	3 (100.0)	37 (92.5)
Cefoxitin	19 (65.5)	17 (63.0)	6 (100.0)	0 (0)	2 (66.7)	44 (60.3)	20 (57.1)	1 (50.0)	0 (0.0)	21 (52.5)
Tobramycin	7 (24.1)	8 (29.6)	4 (66.7)	7 (87.5)	1 (33.3)	27 (37.0)	33 (94.3)	2 (100.0)	1 (33.3)	36 (90.0)
Moxifloxacin	9 (31.0)	2 (7.4)	0 (0)	0 (0)	1 (33.3)	12 (16.4)	29 (82.9)	0 (0.0)	0 (0.0)	29 (72.5)
Doxycycline	29 (100.0)	25 (92.6)	6 (100.0)	6 (75.0)	3 (100.0)	69 (94.5)	35 (100.0)	1 (50.0)	2 (66.7)	38 (95.0)
Minocycline	28 (96.6)	25 (92.6)	2 (33.3)	5 (62.5)	3 (100.0)	63 (86.3)	35 (100.0)	2 (100.0)	2 (66.7)	39 (97.5)
Sulfamethoxazole	27 (93.1)	25 (92.6)	4 (66.7)	7 (87.5)	3 (100.0)	66 (90.4)	34 (97.1)	2 (100.0)	3 (100.0)	39 (97.5)

RGM, rapidly growing non-tuberculous mycobacteria; SGM, slowly growing non-tuberculous mycobacteria. rifampicin (1, 4, 6, 16 μg/mL), clarithromycin (0.5, 4, 16, 64 μg/mL), imipenem/cilastatin (IPM/CS) (0.5, 4, 16, 64 μg/mL), linezolid (0.5, 2, 8, 32 μg/mL), amikacin (1, 4, 16, 64 μg/mL), ethambutol (2.5, 5, 10, 20 μg/mL), and rifabutin (0.5, 2, 8, 32–10 μg/mL), at four concentrations, while cefoxitin (4, 16, 32, 40, 64, 80, 128, 160 μg/mL), tobramycin (0.5, 1, 2, 4, 8, 16, 32, 64 μg/mL), moxifloxacin (0.125, 0.25, 0.5, 1, 2, 4, 8, 16 μg/mL), doxycycline (0.5, 1, 4, 8, 16, 32, 64, 128 μg/mL), minocycline (0.5, 1, 4, 8, 16, 32, 64, 128 μg/mL), and sulfamethoxazole (8, 16, 32, 64, 80, 128, 160, 256 μg/mL) was used at eight concentrations.The bold and italic markings in the above drug concentrations indicate the resistance breakpoint of each drug.

10.3389/fcimb.2023.1282902

(Zhang et al., 2015; Wang et al., 2016; Yang et al., 2018). Additionally, some NTM cases were complicated by varying degrees of diabetes and hypertension, indicating that middle-aged and elderly patients with underlying pulmonary diseases, HIV, and compromised immunity are risk factors for NTM infections.

In our previous study, we observed that MTB infections accounted for a significant proportion of ethnic minorities, particularly among children with TBM in Southwest China (Wang et al., 2020). However, in this study, 96.4% of NTM-infected patients were of Han ethnicity from central cities, with minority groups representing a small subset of the infected individuals. This pattern contrasts with the MTB-infected population model in southwest China, suggesting a positive correlation between NTM infection and the regional level of economic development and sanitation conditions.

Among the 587 NTM cases in this study, the top three isolated strains were *M. avium*, *M. chelonae*/*M. abscessus*, and *M. intracellulare*, followed by *M. kansasii*. These clinical strains mirrored the domestic epidemic trend (Jing et al., 2012; Wu et al., 2014; Tan et al., 2018; Liu et al., 2019). Among the 113 cases that underwent *in vitro* drug sensitivity testing, 90.3% of the strains originated from the respiratory tract, followed by cerebrospinal fluid, lymph nodes, the gastrointestinal tract, and pleural effusion. Intriguingly, MAC and *M. chelonae*/*M. abscessus* were the predominant strains in various tissues, followed by *M. kansasii*, *M. fortuitum*, *M. scrofulaceum*, *M. gordoniae*, and *M. lentiflavum*.

NTM naturally exhibits resistance to various anti-TB drugs, and its clinical isolation rate is relatively low. Furthermore, the emergence of NTM resistance poses a significant challenge in clinical treatment. Currently, research on NTM drug resistance is limited, with only a few reports on the sensitivity of NTM to various antibiotics in China, often based on small sample sizes or focused on a single antibiotic (Lan et al., 2011; Li et al., 2017). In this study, we conducted comprehensive *in vitro* sensitivity tests on 113 NTM strains with 13 antibiotics. The results revealed that clarithromycin exhibited significant antibacterial efficacy against *M. avium* and *M. intracellulare*, with drug resistance rates of 17.2% and 22.2%, respectively. The average drug resistance rate among SGM was 17.8%, while for RGM, it was 62.5%. Aminoglycosides are commonly used and effective drugs for NTM treatment. In our study, amikacin demonstrated potent antibacterial effects on *M. avium* and *M. intracellulare*, with low drug resistance rates of 6.9% and 18.5%, respectively. The antibacterial activity of amikacin against MAC was superior to that of clarithromycin. Notably, amikacin also displayed good antibacterial efficacy against both SGM and RGM, with drug resistance rates of 13.7% and 45.0%, respectively. In addition to its effectiveness against MAC, tobramycin's antibacterial activity against *M. chelonae*/*M. abscessus* was notable at 94.3%, although it differed from amikacin at 51.4%. This discrepancy may be attributed to variations in ethnicity, geographical regions, or clinical isolates. While aminoglycosides demonstrate potent antibacterial activity against NTM, their prolonged use can lead to hepatorenal toxicity and ototoxicity. Therefore, these drugs should be used judiciously in clinical practice. Additionally, in this study, the fluoroquinolone moxifloxacin exhibited robust antibacterial activity against SGM but showed weaker effectiveness against RGM.

Conclusions

The isolation rate of NTM in southwest China has shown an increasing trend in recent years. The majority of infected cases involve elderly patients, and there has been an elevated proportion of individuals with HIV infection. The predominant clinical isolates are MAC and *M. chelonae*/*M. abscessus*, followed by *M. kansasii* and *M. fortuitum*. Among the tested antibiotics, amikacin, moxifloxacin, clarithromycin, and linezolid demonstrated effective antibacterial activity against SGM, whereas linezolid and amikacin exhibited relatively better antibacterial activity against RGM. The incidence of NTM infection may be positively correlated with the level of regional economic development and healthcare conditions.

Limitations

One significant limitation of this study is the extended treatment cycle required for NTM infections (Lee et al., 2015; Daley et al., 2020; Gopalaswamy et al., 2020), often exceeding one year or even longer. Consequently, many cases experience issues such as loss to follow-up, poor treatment outcomes, and a high recurrence rate. Although follow-up observations are ongoing for some cases to assess treatment efficacy, the data collection for these cases is incomplete. Future research should aim to provide more comprehensive and valuable reference information for clinical use.

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/s.

Ethics statement

The studies involving humans were approved by This study was approved by the Ethics Committee of PHCC [2017Y] 025. 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

DW: Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review &

editing. HL: Data curation, Investigation, Resources, Writing – review & editing. YZ: Data curation, Investigation, Supervision, Writing – review & editing. YX: Data curation, Investigation, Writing – review & editing. YL: Data curation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by 2023 Chengdu Medical Research Project, Sichuan, China (202311013240) (to DMW).

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

Babalik, A., Koç, E. N., Sekerbey, H. G., Dönmez, G. E., Balikci, A., and Kilicaslan, Z. (2023). Nontuberculous mycobacteria isolation from sputum specimens: A retrospective analysis of 1061 cases. *Int. J. Mycobacteriol.* 12 (1), 55–65. doi: 10.4103/ijmy.ijmy_10_23

Cao, X. J., Li, Y. P., Wang, J. Y., Zhou, J., and Guo, X. G. (2021). MPT64 assays for the rapid detection of *Mycobacterium* tuberculosis. *BMC Infect. Dis.* 21 (1), 336. doi: 10.1186/s12879-021-06022-w

Clinical and Laboratory Standards Institute (CLSI) (2015). *Susceptibility Testing of Mycobacteria, Nocardia spp., and Other Aerobic Actinomycetes. 3rd Edition* (USA: CLSI guideline. M24).

Daley, C. L., Iaccarino, J. M., Lange, C., Cambau, E., Wallace, R. J. Jr, Andrejak, C., et al. (2020). Treatment of nontuberculous mycobacterial pulmonary disease: an official ATS/ERS/ESCMID/IDSA clinical practice guideline. *Clin. Infect. Dis.* 71 (4), e1–e36. doi: 10.1093/cid/ciaa241

Editorial Board of CSTB (2016). Nontuberculous mycosis diagnosis and treatment expert consensus. *Chin. J. Tuberc Respir.* 39, 438–443. doi: 10.3760/cma.j.issn.1001-0939.2012.08.006. Chin J Tuberc Respir Dis.

Falkinham, J. O. 3rd (1996). Epidemiology of infection by nontuberculous mycobacteria. *Clin. Microbiol. Rev.* 9 (2), 177–215. doi: 10.1128/CMR.9.2.177

Falkinham, J. O.3rd (2002). Nontuberculous mycobacteria in the environment. *Clin. Chest Med.* 23 (3), 529–551. doi: 10.1016/s0272-5231(02)00014-x

From the Centers for Disease Control and prevention (1993). 1993 revised classifification system for HIV infection and expanded surveillance case defifinition for AIDS among adolescents and adults. *JAMA* 269, 460.

Giannoni, F., Lanni, A., Iacobino, A., and Fattorini, L. (2023). Italian Multicentre Study on Nontuberculous Mycobacteria (IMS-NTM); members of the IMS-NTM laboratory network. Epidemiology and drug susceptibility of nontuberculous mycobacteria (NTM) in Italy in 2016-2020. *Ann. Ist Super Sanita.* 59 (2), 132–138. doi: 10.4415/ANN_23_02_06

Gopalaswamy, R., Shanmugam, S., Mondal, R., and Subbian, S. (2020). Of tuberculosis and non-tuberculous mycobacterial infections - a comparative analysis of epidemiology, diagnosis and treatment. *J. BioMed. Sci.* 27 (1), 74. doi: 10.1186/s12929-020-00667-6

Guo, Q., Wei, J., Zou, W., Li, Q., Qian, X., and Zhu, Z. (2021). Antimicrobial susceptibility profiles of *Mycobacterium* abscessus complex isolates from respiratory specimens in Shanghai, China. *J. Glob Antimicrob. Resist.* 25, 72–76. doi: 10.1016/j.jgar.2021.02.024

Jeong, S. H., Kim, S. Y., Huh, H. J., Ki, C. S., Lee, N. Y., Kang, C. I., et al. (2017). Mycobacteriological characteristics and treatment outcomes in extrapulmonary *Mycobacterium* abscessus complex infections. *Int. J. Infect. Dis.* 60, 49–56. doi: 10.1016/j.ijid.2017.05.007

Jing, H., Wang, H., Wang, Y., Deng, Y., Li, X., Liu, Z., et al. (2012). Prevalence of nontuberculous mycobacteria infection, China, 2004-2009. *Emerg. Infect. Dis.* 18 (3), 527–528. doi: 10.3201/eid1803.110175

Lan, R., Yang, C., Lan, L., Ou, J., Qiao, K., Liu, F., et al. (2011). *Mycobacterium tuberculosis* and non-tuberculous mycobacteria isolates from HIV-infected patients in Guangxi, China. *Int. J. Tuberc Lung Dis.* 15 (12), 1669–1675. doi: 10.5588/ijtld.11.0036

Lee, M. R., Sheng, W. H., Hung, C. C., Yu, C. J., Lee, L. N., and Hsueh, P. R. (2015). *Mycobacterium* abscessus complex infections in humans. *Emerg. Infect. Dis.* 21 (9), 1638–1646. doi: 10.3201/2109.141634

Li, G., Pang, H., Guo, Q., Huang, M., Tan, Y., Li, C., et al. (2017). Antimicrobial susceptibility and MIC distribution of 41 drugs against clinical isolates from China and reference strains of nontuberculous mycobacteria. *Int. J. Antimicrob. Agents.* 49 (3), 364–374. doi: 10.1016/j.ijantimicag.2016.10.024

Liu, C. F., Song, Y. M., He, W. C., Liu, D. X., He, P., Bao, J. J., et al. (2021). Nontuberculous mycobacteria in China: incidence and antimicrobial resistance spectrum from a nationwide survey. *Infect. Dis. Poverty.* 10 (1), 59. doi: 10.1186/s40249-021-00844-1

Liu, S., Gao, X., Zhu, J., Chen, J., Yang, H., and He, L. (2019). Nontuberculous mycobacteria pulmonary disease: A retrospective analysis. *Zhong Nan Da Xue Xue Bao Yi Xue Ban.* 44 (4), 432–436. doi: 10.11817/j.issn.1672-7347.2019.04.014

López-Roa, P., Aznar, E., Cacho, J., Cogollos-Agruña, R., Domingo, D., García-Arata, M. I., et al. (2020). Epidemiology of Non-Tuberculous Mycobacteria isolated from clinical specimens in Madrid, Spain, from 2013 to 2017. *Eur. J. Clin. Microbiol. Infect. Dis.* 39 (6), 1089–1094. doi: 10.1007/s10096-020-03826-7

Maya, T. G., Komba, E. V., Mensah, G. I., Mbelele, P. M., Mpagama, S. G., Mfinanga, S. G., et al. (2022). Drug susceptibility profiles and factors associated with non-tuberculous mycobacteria species circulating among patients diagnosed with pulmonary tuberculosis in Tanzania. *PloS One* 17 (3), e0265358. doi: 10.1371/journal.pone.0265358

Przybylski, G., Bukowski, J., Kowalska, W., Pilaczyńska-Cemel, M., and Krawiecka, D. (2023). Trends from the last decade with nontuberculous mycobacteria lung disease (NTM-LD): clinicians' Perspectives in regional center of pulmonology in Bydgoszcz, Poland. *Pathogens* 12 (8), 988. doi: 10.3390/pathogens12080988

Tan, Y., Su, B., Shu, W., Cai, X., Kuang, S., Kuang, H., et al. (2018). Epidemiology of pulmonary disease due to nontuberculous mycobacteria in Southern China, 2013-2016. *BMC Pulm Med.* 18 (1), 168. doi: 10.1186/s12890-018-0728-z

Tarashi, S., Sakhaee, F., Masoumi, M., Ghazanfari Jajin, M., Siadat, S. D., and Fateh, A. (2023). Molecular epidemiology of nontuberculous mycobacteria isolated from tuberculosis-suspected patients. *AMB Express.* 13 (1), 49. doi: 10.1186/s13568-023-01557-4

Thornton, C. S., Mellett, M., Jarand, J., Barss, L., Field, S. K., and Fisher, D. A. (2021). The respiratory microbiome and nontuberculous mycobacteria: an emerging concern in human health. *Eur. Respir. Rev.* 30 (160), 200299. doi: 10.1183/16000617.0299-2020

Tuberculosis Branch of Chinese Medical Association (2020). Guidelines for diagnosis and treatment of nontuberculous mycobacteriosis (2020 edition). *Chin. J. Tuberc Respir. Dis.* 43 (11), 918–946. doi: 10.3760/cMA.j.cn112147-20200508-00570

Vongthilath-Moeung, R., Plojoux, J., Poncet, A., Renzi, G., Veziris, N., Schrenzel, J., et al. (2022). Nontuberculous mycobacteria under scrutiny in the Geneva area (2015-2020). *Respiration* 101 (4), 367–375. doi: 10.1159/000520033

Wang, D. M., Li, Q. F., Zhu, M., Wu, G. H., Li, X., Xu, Y. H., et al. (2020). Epidemiological, clinical characteristics and drug resistance situation of culture-confirmed children TBM in southwest of China: a 6-year retrospective study. *BMC Infect. Dis.* 20 (1), 318. doi: 10.1186/s12879-020-05041-3

Wang, D. M., Li, Q. F., Zhu, M., Xu, Y. H., Luo, J., Li, Y. J., et al. (2017). Analysis of infection and drug-resistance in 6 107 cases of extrapulmonary tuberculosis in Chengdu area. *Zhonghua Jie He Hu Xi Za Zhi* 40, 592–595. doi: 10.3760/cma.j.issn.1001-0939.2017.08.010

Wang, D. M., Liao, Y., Li, Q. F., Zhu, M., Wu, G. H., Xu, Y. H., et al. (2019). Drug resistance and pathogenic spectrum of patients coinfected with nontuberculous mycobacteria and human-immunodeficiency virus in Chengdu, China. *Chin. Med. J. (Engl)* 132 (11), 1293–1297. doi: 10.1097/CM9.0000000000000235

Wang, D. M., Zhu, M., Xu, Y. H., Luo, J., Li, Y. J., Liu, X. D., et al. (2016). Clinical relevance and drug resistance of nontuberculous mycobacteria, Sichuan area in Chinese. *Chin. J. Infect. Dis.* 34, 520–523. doi: 10.3760/cma.j.issn.1000-6680.2016.09.002

Wang, Y., Yang, Y., Shi, X., Mao, S., Shi, N., and Hui, X. (2016). The spatial distribution pattern of human immunodefificiency virus/acquired immune defificiency syndrome in China. *Geospat Health* 11, 414. doi: 10.4081/gh.2016.414

World Health Organization (2021). *Global tuberculosis report*. (Geneva: WHO, 2021).

Wu, J., Zhang, Y., Li, J., Lin, S., Wang, L., Jiang, Y., et al. (2014). Increase in nontuberculous mycobacteria isolated in Shanghai, China: results from a population-based study. *PLoS One* 9 (10), e109736. doi: 10.1371/journal.pone.0109736

Yang, S., Chiu, A. P. Y., Lin, Q., Zeng, Z., Li, Y., Zhang, Y., et al. (2018). HIV epidemics in Shenzhen and Chongqing, China. *PLoS One* 13, e192849. doi: 10.1371/journal.pone.0192849

Zhang, L., Chow, E. P., Su, S., Yiu, W. L., Zhang, X., Iu, K. I., et al. (2015). A systematic review and meta-analysis of the prevalence, trends, and geographical distribution of HIV among Chinese female sex workers (2000–2011): implications for preventing sexually transmitted HIV. *Int. J. Infect. Dis.* 39, 76–86. doi: 10.1016/j.ijid.2015.08.014

Zhou, L., Xu, D., Liu, H., Wan, K., Wang, R., and Yang, Z. (2020). Trends in the prevalence and antibiotic resistance of non-tuberculous mycobacteria in mainland China, 2000-2019: systematic review and meta-analysis. *Front. Public Health* 8. doi: 10.3389/fpubh.2020.00295

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 19 December 2023
DOI 10.3389/fcimb.2023.1327069

OPEN ACCESS

EDITED BY
Sanket Kaushik,
Amity University Jaipur, India

REVIEWED BY
Varun Chauhan,
Government Institute of Medical Sciences, India
Hazrat Bilal,
Second Affiliated Hospital of Shantou University Medical College, China

*CORRESPONDENCE
Sandip Patil
sandippatil1309@yahoo.com
Feiqiu Wen
fwen62@163.com

RECEIVED 24 October 2023
ACCEPTED 20 November 2023
PUBLISHED 19 December 2023

CITATION
Pai L, Patil S, Liu S and Wen F (2023) A growing battlefield in the war against biofilm-induced antimicrobial resistance: insights from reviews on antibiotic resistance. *Front. Cell. Infect. Microbiol.* 13:1327069. doi: 10.3389/fcimb.2023.1327069

A growing battlefield in the war against biofilm-induced antimicrobial resistance: insights from reviews on antibiotic resistance

Liu Pai[1,2], Sandip Patil[1,2]*, Sixi Liu[1] and Feiqiu Wen[1,2]*

[1]Department of Hematology and Oncology, Shenzhen Children's Hospital, Shenzhen, China, [2]Pediatric Research Institute, Shenzhen Children's Hospital, Shenzhen, China

Biofilms are a common survival strategy employed by bacteria in healthcare settings, which enhances their resistance to antimicrobial and biocidal agents making infections difficult to treat. Mechanisms of biofilm-induced antimicrobial resistance involve reduced penetration of antimicrobial agents, increased expression of efflux pumps, altered microbial physiology, and genetic changes in the bacterial population. Factors contributing to the formation of biofilms include nutrient availability, temperature, pH, surface properties, and microbial interactions. Biofilm-associated infections can have serious consequences for patient outcomes, and standard antimicrobial therapies are often ineffective against biofilm-associated bacteria, making diagnosis and treatment challenging. Novel strategies, including antibiotics combination therapies (such as daptomycin and vancomycin, colistin and azithromycin), biofilm-targeted agents (such as small molecules (LP3134, LP3145, LP4010, LP1062) target c-di-GMP), and immunomodulatory therapies (such as the anti-PcrV IgY antibodies which target Type IIIsecretion system), are being developed to combat biofilm-induced antimicrobial resistance. A multifaceted approach to diagnosis, treatment, and prevention is necessary to address this emerging problem in healthcare settings.

KEYWORDS

biofilms, diagnosis, antimicrobial resistance, infections, treatment

1 Introduction

Biofilms are complex structures formed by communities of microorganisms that attach to biotic and/or abiotic surfaces. With the continuous influx of new colonizers and outflow of old colonizers, the formation and dispersion occur in a periodical cycle that makes biofilm an open system (Ma et al., 2022; Sauer et al., 2022). This

development cycle contains complex and progressive processes which are divided into four stages: initial adhesion, formation of microcolonies, biofilm maturation, and detachment and dispersion (Figure 1). The process of biofilm formation is reversible in the initial stages and depends on environmental conditions but once the colonies pass the initial attachment and adhesion phase, it can lead to irreversible attachment to different surfaces (Caixeta Magalhães Tibúrcio et al., 2022; Sauer et al., 2022). Later, genetic and phenotypic changes take place within the bacteria encapsulated in the matrix of biofilm and lead to biofilm-induced resistance. We divide these changes into physical mechanisms and biological mechanisms. Physically, bacteria produce a thick biofilm matrix to evade antimicrobial agents (Flemming et al., 2023). Biological mechanisms involve the upregulation of efflux pumps (Lewis et al., 2023), cyclic di-GMP regulation (Yu et al., 2023), quorum sensing system (Oliveira et al., 2023), presence of persister cells (Kouhsari et al., 2023), and horizontal gene transfer (Moura de Sousa et al., 2023). These changes occur in bacteria potentially increasing their ability to survive from antibiotics and other life-threatening conditions, and might also enable them to be multi-drug resistance strains. Unfortunately, biofilms have been identified in various clinical settings (Chakraborty et al., 2021; Jakubovics et al., 2021) and pose significant challenges for biofilm-mediated infection management, including difficulties in diagnosing and treating infections caused by biofilm forming bacteria, increased risk of persistent and recurrent infections, and the potential for the spread of resistant bacteria to other patients and healthcare settings. The objective of this review summarizes mechanisms of biofilm-induced resistance, bacteria involved in biofilm formation and biofilm-related diseases, and provides novel precise target strategies which can be effective in the treatment of biofilms-induced infections.

Recently bacterial biofilm has been proven to be an aggressive strategy employed by bacteria, which can not only provide shelter for bacteria in extreme environments like the immune system but also able to attack them (Bordon, 2023).

According to the study conducted by Lucia Vidakovic et al., the human pathogen *Vibrio cholerae* forms biofilms on the surface of various human immune cells, which encases the immune cells and kill them with a high local concentration of a secreted hemolysin before the biofilms disperse in a c-di-GMP-dependent manner (Vidakovic et al., 2023). Besides, differences in biofilm matrix composition and the mechanism of surface attachment are found between biofilms on macrophages and other surfaces. Although many bacterial pathogens can form biofilms during infections, knowledge of whether other pathogens can have similarities in biofilm formation and extra function on immune cells remains unknown. While multispecies biofilms often have distinct properties from single species biofilms, little is known about modulation and interactions among such commensals. A study by Liju Wang et al. shows the interspecies biofilm matrix between *Staphylococcus aureus* and streptococcus is able to mediate polymicrobial interactions (Wang et al., 2023). Another research by Wucher et al. examined how interactions among bacteria at the start of biofilm growth influence the eventual multispecies biofilm architecture and thereby determine predator access to cells within the community (Wucher et al., 2023). Now is the time for us to understand the complex interactions between microorganisms and their host, which is essential in identifying new therapeutic targets for the prevention and treatment of chronic infections. This review summarizes biofilm formation in biofilm-induced antimicrobial resistance in healthcare and provides possible ways to prevent biofilm formation or eliminate mature biofilms.

2 Mechanisms of antimicrobial resistance in biofilms

Bacteria within a biofilm are embedded in a matrix of extracellular polymeric substances (EPSs), now may also be

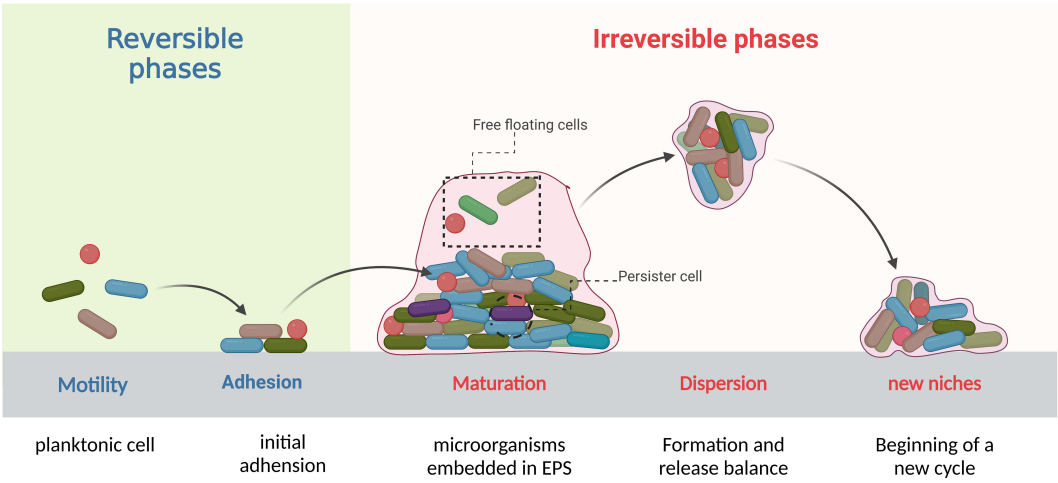


FIGURE 1
Life cycle of biofilm: Expanding the conceptual model of biofilm formation.

presented as "matrixome", which primarily consists of polysaccharides, proteins, water-insoluble compounds, and lipids. Interactions among EPS molecules and the bacteria surrounded by them are complex, but closely related to antimicrobial resistance in biofilms that involves both physical and biological mechanisms (Flemming et al., 2023).

2.1 Physical mechanisms of antimicrobial resistance in biofilms

2.1.1 EPSs and environmental factors

EPSs are dynamic in space and time and the inclusive components interact in involute ways. This enables EPSs to fulfill various functions: (i) stabilize the matrix (ii) acquire nutrients (iii) modulate chemical gradients and (iv) maintain microenvironments within biofilms. As a physical barrier of a biofilm, EPSs prevent antimicrobial agents from reaching the bacterial cells by physically hinder the penetration of antimicrobial agents and decrease their effectiveness. The effects of EPSs on the diffusion of antibiotics have different outcomes based on drug concentration: (i) subinhibitory level of antibiotics may not trigger the defense of bacteria but can be hindered by the formation of EPS (ii) slow growth rate within biofilm weaken the interaction between antibiotics and bacteria, and antibiotics can be removed by efflux pumps,(iii) the antibiotic effect weakens the bacteria that cause absorption, which seems to be a defensive effect of the bacteria, and (iv) slowing down of diffusion decreases (Kosztołowicz and Metzler, 2020). The low penetration rate can lead to inadequate concentrations of antimicrobial agents within the biofilm, and reduce their efficacy. Till now, the information on EPSs is mostly gained from single-species biofilms grown under controlled laboratory conditions. Although we are now short of knowledge in the context of polymicrobial biofilms, the formation, maintenance and transformation of EPSs are identified to be highly regulated by genetic control (cyclic-di-GMP, quorum sensing molecules, small RNAs)and environmental factors (such as oxygen, temperature, nutrients, osmolarity, etc.) (Flemming et al., 2023). Genetic factors we will discuss in the later part of the article.

Environmental factors that affect biofilm formation, include hypoxia, which can create an infectious hypoxic environment that promotes virulence gene expression (André et al., 2022); acidosis can alter the composition of biofilms, with low pH levels that can impair immune system function (Dalwadi and Pearce, 2021; Behbahani et al., 2022). Besides, iron metabolism plays a crucial role in biofilm formation and antibiotic resistance in various pathogenic microorganisms (Zhang et al., 2021; Liu K et al., 2022). A recent study found that iron availability significantly enhances the formation of biofilm structures, suggesting that targeting iron metabolism may disrupt biofilm formation and increase the effectiveness of antibiotic treatment (Liu K et al., 2022).

But different species manage their regulation in different ways, and there is no mutual regulatory pathway for all EPS components in all biofilms of various bacterial strains. Since the research on mixed species biofilms is limited, it is imagined that managing biofilms in technical systems intending to upper-or lower-produce EPS is not feasible. In addition, it is still challenging to identify which member in a given biofilm community produces certain components at what time point and for what function. The existent methods of analysis have merely revealed snapshots of the matrix only. However, what triggers or inhibits the production of specific EPS molecules, and how it interacts with different community members remains unexplored. Besides, bacterial biofilms may display changes in bacterial cell envelop due to the production of a slimy outer layer or the formation of thicker cell walls (Vandana and Das, 2022). These modifications can reduce the permeability of the cell envelope to antimicrobial agents, making it more difficult for them to penetrate the cells.

2.2 Biological mechanisms of antimicrobial resistance in biofilms

2.2.1 Efflux pumps

Biofilm-associated resistance can be attributed to the upregulation of efflux pumps such as *AcrAB-TolC* in *E. coli*, *MexAB-OprM* in *P. aeruginosa*, *AdeFGH* in *A. baumannii*, and *AcrD* in *S. enterica*. They play a key role in the develop of drug resistance in bacteria:(i) efflux drugs to reduce the concentration, (ii) efflux molecules from EPSs to facilitate biofilm matrix formation and (iii) efflux quorum sensing (QS) molecules to regulate QS system. QS is a system that bacteria utilize to synchronize their gene expression and form a biofilm, which we will explain in the later part. Efflux pumps indirectly regulate genes involved in biofilm formation, antibiotic molecules and metabolic intermediates, and influence aggregation by promoting or preventing adhesion to surfaces and other cells (Stephen et al., 2022). Bacteria in the biofilm can upregulate the expression of efflux pumps in response to antimicrobial exposure, thereby reducing the intracellular concentration of the drug (Figure 2). Efflux pumps often have a broad spectrum of activity and can extrude multiple classes of antimicrobial agents, making them important targets to control antibiotic resistance.

To date, eight classes of efflux pump inhibitors (EPIs) have been reported (Table 1). (i) One major class of EPIs display broadspectrum efflux pump inhibitory activity by competitive inhibition (such as Phenylalanine-Arginine-β-Naphthylamide (PaβN)) (Salehi et al., 2021). (ii)Another class of EPIs act directly on the AcrAB-TolC efflux pump by binding to the pocket in the TM region of the L promotor of AcrB (like Pyridylpiperazine-based allosteric inhibitors) (Plé et al., 2022). (iii)EPIs may also function by binding to the "hydrophobic trap" in the T promotor of asymmetric AcrB. This type of inhibitor includes three classes of EPIs, pyridopyrimidine (such as D13-9001 (Kumar Roy and Patra, 2020), the pyranopyridine derivatives (like MBX series (Vargiu et al., 2014), and benzo[h]chromene compounds (Wang et al., 2021). (iv)Also, EPIs can deactivate efflux pumps via binding to site III of AcrA within the AcrAB-TolC complex,like NSC-33353 (D'Cunha et al., 2021). (v)And EPI TXA compounds (such as TXA09155) act by membrane disruption and support efflux inhibition (Zhang et al., 2022b). (vi)Finally, Arylpiperazines can increase the intracellular accumulation of antibiotics (Casalone

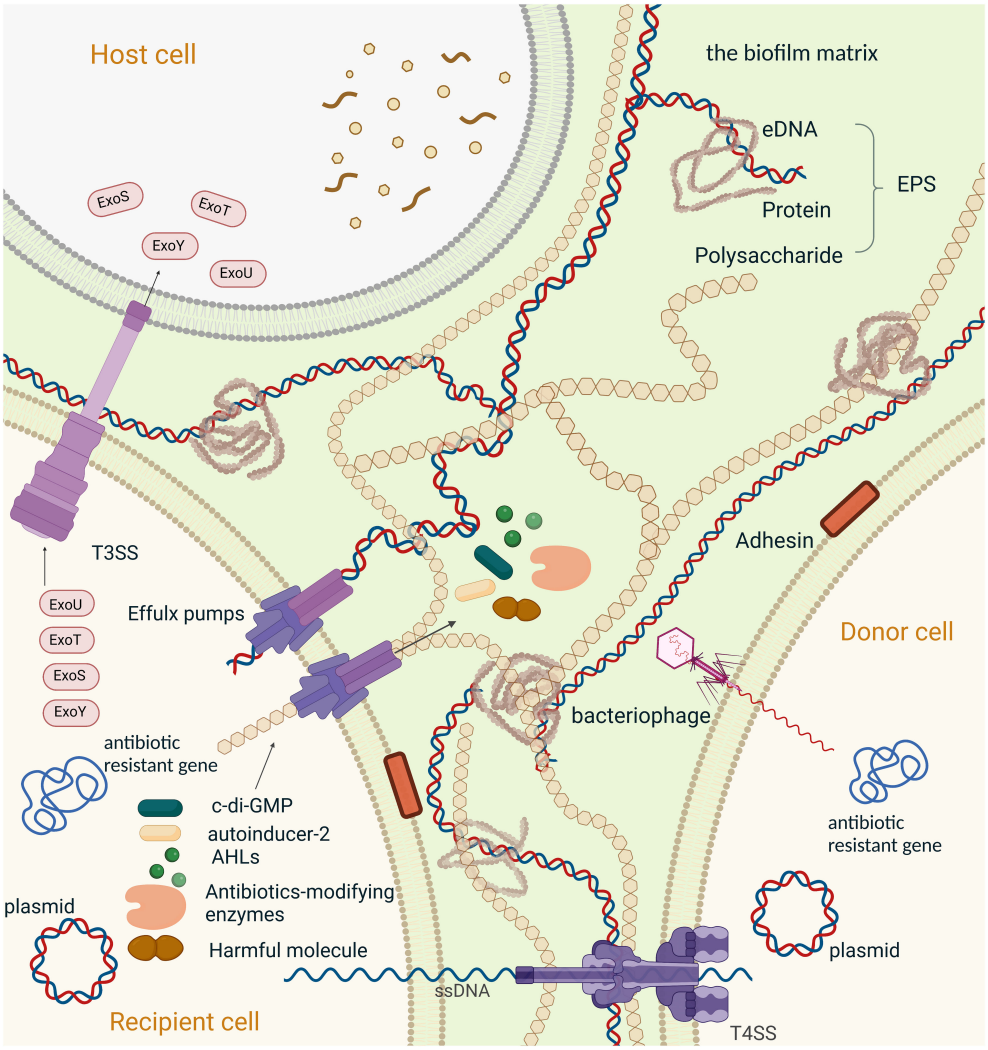


FIGURE 2
Mechanisms of resistance to antimicrobial agents in a biofilm,Created with BioRender.com. Schematic overview of the major mechanisms involved in antimicrobial resistance employed by bacterial biofilms. (1) The second messenger molecule, c-di-GMP, is associated with biofilm formation, virulence, and metabolic activity; Individual bacteria can synchronize their gene expression and alter their behaviour through chemical interactions between organisms in microbial communities, which is generally referred to as quorum sensing (QS)system. (2)N-Acyl homoserine lactones (AHLs) mediated QS system, the main QS systemin Gram-negative bacteria;(3) Autoinducer-2(AI-2) is the most common QS signal that mediated communication between intraspecies and interspecies; (4) Antibiotics-modifying enzymes induce resistance to multiple antibiotics, specially β-lactams, by transforming antimicrobials to a non-toxic form. (5) Efflux pump plays an important role in antibiotic resistance, including the efflux of the functional molecules (c-di-GMP, AHLs, AI-2 and antibiotics-modifying enzymes). extracellular polymeric substances (EPS), also efflux harmful ingredients (such as antibiotics and the metabolic intermediates) out of the bacteria. And is proven to be involved in the regulation of aggregation of the microcolonies and gene expression within biofilm; Horizontal gene transfer (HGT) is the process that passing antibiotic-resistant genes between bacterium. Here we present HGT in two ways: (6) conjugation between bacterium transmitted by plasmids and T4SS, (7) transduction between bacterium transmitted by bacteriophages. (8)T3SS in associated with phagocytic avoidance, cytotoxicity and systemic spread of bacteria. And is function by injecting effectors into the host cell directly, here we take effectors of Pseudomonas aeruginosa (ExoS, ExoT, ExoU, ExoY) as an example. (9) The components of EPS, including extracellular DNA, extracellular proteins and polysaccharides, hinder drug penetration and therefore cause drug resistance in biofilm.

et al., 2020). Each of the EPIs above can boost the efficiency of one or more antibiotics, but the effectiveness of the eight classes of EPIs has not been compared in parallel groups. However, a recent study suggests that the role of the RND efflux pumps *AdeABC*, *AdeFGH*, and *AdeIJK* in biofilm formation may not be crucial, indicating that these pumps may not be promising targets for biofilm inhibition (Abd El-Rahman et al., 2023).

2.2.2 Second messengers

Cyclic di-GMP (c-di-GMP) is a second messenger molecule that has an impact on the biofilm formation and metabolism of bacteria. When the concentration of c-diGMP is high, it promotes biofilm formation, while low levels lead to a planktonic lifestyle, and also influence the antimicrobial resistance of the bacteria (Table 2). High c-di-GMP level in biofilm cause rapid energy spent on the

TABLE 1 Mechanisms of biofilm-induced antibiotic resistance and inhibitors.

Mechanism	Effects	Inhibitors	Inhibitory activity	Target bacteria
c-di-GMP	i)High level of c-di-GMP is associated with biofilm formation, low level of c-di-GMP is associated with a planktonic lifestyle. ii)High level of c-di-GMP forces bacteria to rapidly produce biofilm matrix products with high energy consumption, as a result, bacteria in the biofilm enter a subsequent low metabolic state.	small molecules (LP3134, LP3145, LP4010,LP1062) (Andersen et al., 2021a; Dong et al., 2022; Lichtenberg et al., 2022)	Antagonize DGC enzymes that synthesise c-di-GMP	*Pseudomonas aeruginosa*
		ebselen, ebselen oxide(Lieberman et al., 2014)	Ebselen and ebselen oxide are both inhibitors of c-di-GMP binding to receptors containing an RXXD domain including PelD and diguanylate cyclases (DGC). They both function by covalently modifying cysteine residues.	*Pseudomonas aeruginosa*
T3SS	Inject effectors (ExoS, ExoT, ExoU, ExoY) into host cells	V2L2MD (immunotherapeutic antibodies) (Warrener et al., 2014)	Target PcrV tip protein	*Pseudomonas aeruginosa*
		natural compounds (12(6.4),12(4,6)) (Ngo et al., 2019)	Prevent the binding of PscF to PscE-PscG (two cognate chaperones of the T3SS needle subunit protein PscF)	*Galleria mellonella*
LuxI/R gene (AI-1 QS system)	Primarily mediated by AHL, controls quorum-sensing-controlled gene expression in a tandem regulatory way.	Quercetin (Gopu et al., 2015; Chittasupho et al., 2021)	Act as a competitive inhibitor for signalling compounds in LasR receptor pathway.	*Pseudomonas aeruginosa, Klebsiella pneumoniae*
		azithromcin/PAβN (Phenylalanine-Arginine-β-Naphtylamide,1) (Elsheredy et al., 2021)	inhibit the production of autoinducer C4HSL and four QS-dependent virulence factors (*lasI, lasR, Rh1I and rhiR)*	*Pseudomonas aeruginosa*
Lus/AI-2 QS system	mediate interspecies quorum sensing and intraspecies communication by AI-2	5-Fluorourcil (Sedlmayer et al., 2021)	A quorum-quencher	*Staphylococcus epidermidis, Escherichia coli, Vibrio Harveyi*
Hinder drug penetration	reduce bacterial susceptibility to antibiotics	Nanoparticles combined with laser (Teirlinck et al., 2018)	Increase drug diffusion and lead to biofilm disruption by forming laser-indued vapour nanobubbles (VNBs)	Gram-positive and Gram-negative bacteria
Efflux pumps	i)efflux extracellular polymeric substances (EPSs) to facilitate biofilm matrix formation; ii) efflux quorum sensing (QS) molecules to regulate QS. iii)efflux harmful molecules; iiii) indirectly regulate genes involved in biofilm formation; iiiii) influence aggregation of bacteria	PAβN (Phenylalanine-Arginine-β-Naphtylamide,1) (Lomovskaya et al., 2001; Kaynak Onurdağ et al., 2021)	i)a competitive inhibitor of the three RND pumps of *P. aeruginosa* (MexAB-OprM, MexCD-OprJ, and MexEF-OprN). PAβN is able to potentiate the activity of different families of antibiotics, including fluoroquinolones, macrolides, oxazolidinones, chloramphenicol and rifampin, except aminoglycosides ii) PAβN inhibits the expression of EF system genes (*adeA, adeB, adeR, adeS, adeF, adeG, adeH, adeL*) in *Acinetobacter baumannii*	*Pseudomonas aeruginosa, Acinetobacter baumannii*
		Sodium Malonate (Cauilan and Ruiz, 2022)	An inhibitor of AcrAB-TolC functions by binding to multiple AcrBlocations, including the AcrB proximal binding pocket.	*Escherichia coli*
		D13-9001 (Nakashima et al., 2013; Kumar Roy and Patra, 2020)	A specific inhibitor of MexAB-OprM of *P. aeruginosa* via binding to MexB and AcrB in the deep binding pocket region known as the hydrophobic trap	*pseudomonas aeruginosa*
		MBX2319(Vargiu et al., 2014)	MBX2319 binds to the lower part of the distal pocket in the B protomer of AcrB, impairing the proper binding of substrates	*Enterobacteriaceae*

(Continued)

TABLE 1 Continued

Mechanism	Effects	Inhibitors	Inhibitory activity	Target bacteria
		2H-benzo[h] chromene(Wang et al., 2021; Guo et al., 2023)	C-2 position of 2H-benzo[h]chromene binds to the hydrophobic trap to impair the function of the AcrB-mediated efflux pump	*Escherichia coli*

production of the biofilm matrix components, and results in a subsequent low metabolic state of bacteria in mature biofilms, to some extent, which is a c-di-GMP regulated survival strategy opted by most bacteria (Feng et al., 2020; Lichtenberg et al., 2022).

In *P. aeruginosa*, there are 41 genes responsible for the synthesis and degradation of c-di-GMP, including *PA1120*, which synthesizes c-di-GMP, and *PA2133*, which degrades it (Andersen et al., 2021b). Another important gene involved in this process is *PA5487*, also known as *dgcH*. Which is a diguanylate cyclase that helps to maintain the basal level of c-di-GMP in bacteria (Wei et al., 2019). Interestingly, *dgcH* has been found to boost the fitness of bacteria in the presence of imipenem, a commonly used antibiotic, thus contributing to the development of antimicrobial resistance in *P. aeruginosa* (Figure 2).

Cross-regulating between cAMP and c-di-GMP has been well studied. (Hengge, 2021). For example, cAMP has been reported to regulate the transcription of certain genes that encode c-di-GMP receptors or DGCs/PDEs. Liu et al. described a regulatory pattern in which cAMP and c-di-GMP interact to synergistically regulate biofilm maintenance through their effectors. (Liu C. et al., 2022).

2.2.3 cAMP-Vfr system and T3SS

The Cyclic AMP (cAMP)-Vfr system (CVS) is a global regulator of virulence gene expression in *P. aeruginosa*, including the type III secretion system (T3SS), which is a complex molecular syringe that injects effectors into host cells. The T3SS is a crucial virulence determinant and is composed of three main parts: the basal body, the needle, and the translocon (Ngo et al., 2019). Four effectors have been identified, including ExoS, ExoT, ExoU, and ExoY, which manipulate host cell signaling and/or cause cytotoxicity (Khodayary et al., 2019).

T3SS genes are organized into 10 transcriptional units, each of which is controlled by an *ExsA*-dependent promoter. Vfr, a cAMP-dependent DNA-binding protein, plays a significant role in regulating virulence gene expression, specifically type IV pili and T3SS (Janssen et al., 2020). *ExsA*, a member of the *AraC/XylS* family

TABLE 2 Infections associated with biofilm .

Biofilm infections	Microscopic changes and paraclinical changes	Common pathogens
dental caries and periodontal disease(Mosaddad et al., 2019; Valm, 2019; Moussa et al., 2022)	Microbial community transit from states of health to states of dysbiosis. Which can also be correlated with immune-mediated inflammatory disease in the distant part of the body.	*Streptococcus mutans, Porphyromonas gingivalis, Prevotella* and its species
Chronic rhinosinusitis (Wagner Mackenzie et al., 2017)	Biofilm formation is associated with ciliary destruction and mucus stasis. Persistence of infection and immune provocation may result from superantigens and direct activation of TLR-2 receptors.	*Staphylococcus, Propionibacterium, Corynebacterium, streptococcus*
Pseudomonas aeruginosa in Cystic fibrosis (CF)/COPD (Cantin et al., 2015; Phuengmaung et al., 2022)	Mutation of CFTR gene causes abnormality of chloride channels in mucus and sweating cells which lead to mucosal hyper concentration on the airway surfaces of CF patients. These thick mucus layer hinders the clearance of pathogenic microorganisms and. make the lung of CF patients favorable for biofilm formations	*Pseudomonas aeruginosa, Candida albicans, A. fumigatus*
chronic wounds (Khalid et al., 2023)	Biofilm formation impairs healing in chronic wounds. The spatial distribution of microbials in multi-species biofilms tougher the diagnosis and treatment of wounds.	*Staphylococci aureus, Pseudomonas aeruginosa, E. coli, E. faecalis, C. freundill*
Inflammatory bowel disease(IBD)(Santana et al., 2022)	A group of disorders featured with prolonged inflammation in colon and small intestine, which have been observed with endoscopic mucosal biofilm formation but possess disrupted bile acid metabolism and bacterial dysbiosis.	*enteropathogenic E. coli, Faecalibacterium prausnitzii, Bilophila*
Urinary tracts infections (UTIs) (Zhao et al., 2020)	Biofilms were formed inside the bladder epithelial cells; these intracellular bacterial communities are protected from neutrophil attack and could proliferation. Which properly contribute to chronic and reiterative UTIs.	*uropathogenic E. coli, Staphylococci aureus, Klebsiella, Enterobacter, Proteus mirabilis*
Prosthetic joint infections(Li et al., 2018)	Bacteria colonize the surface of artificial joint by forming biofilm	*Staphylococcus aureus, Staphylococcus epidermidis*

of transcriptional regulators, serves as the central regulator of T3SS gene expression (Janssen et al., 2020). In the latest study, citrus peel extract from Jeju Island (CPEJ) can inhibit bacterial biofilm formation (Kim et al., 2023). The result shows that CPEJ can significantly reduce the c-di-GMP level through increased phosphodiesterase activity, which suggests the potential of CPEJ for applications in clinical.

2.2.4 Quorum sensing

Quorum sensing (QS) is a process by which bacteria can communicate with each other and coordinate their behavior. It can influence the formation and maintenance of biofilms, as well as the expression of genes involved in antimicrobial resistance. For example, some biofilm bacteria can produce QS-dependent enzymes that modify or degrade antimicrobial agents, reducing their effectiveness. The QS system is divided into four types, each using different signal molecules, including N-Acyl homoserine lactones (AHLs), such as autoinducer-2 (AI-2), and diffusible signaling factor (DSF) (Huang et al., 2022).

The LuxI/R signaling pathway mediates quorum sensing using N-Acyl homoserine lactones (AHLs), which tandemly regulate quorum-sensing-controlled gene expression (Yu et al., 2019). In *P. aeruginosa*, *LasI* produces and *LasR* responds to the autoinducer 3OC12–HSL. The LasR:3OC12–HSL complex activates the transcription of many genes including *RhlR*, which encodes a second quorum-sensing receptor. *RhlR* binds to the autoinducer C4–HSL, the product of *RhlI*. RhlR: C4HSL also directs a large regulon of genes, some of which are also members of the *LasR* regulon. This tandem regulatory arrangement allows LasI/R to control the first wave of quorum-sensing–controlled gene expression and RhlI/R to control the second (Yu et al., 2019). The LuxS/AI-2 QS system uses AI-2 to mediate interspecies quorum sensing and controls virulence and biofilm formation in various human pathogens (Sedlmayer et al., 2021). Mutations in different QS systems can impact bacterial fitness and virulence (Jayakumar et al., 2022).

Maryam Alshammari et al. have employed CRISPR/Cas9-HDR system to target quorum sensing and adhesion genes in *Escherichia coli* (Alshammari et al., 2023). The result indicates that the knockout of *luxS*, *fimH*, and *bolA* genes reduced EPS matrix production, which is considered the main factor in adherence, cell aggregation, and biofilm formation.

2.2.5 Persister cells

Another challenge associated with biofilm infections is the presence of persister cells. Persisters are a subpopulation of antibiotic-tolerant bacterial cells that are often slow-growing or growth-arrested (Fisher et al., 2017), but this slow growth rate is reversible under lethal stress. The presence of persister cells contributes to the recalcitrance and relapse of persistent bacterial infections. A recent research has confirmed the ability of persister cells in *Pseudomonas aeruginosa* to evade the innate host response and to contribute to chronic infection (Hastings et al., 2023). Also, it is related to an increase in the risk of antimicrobial resistance in biofilm associated infections.

These phenotypes can occur through several mechanisms, including stress response (RpoS-mediated), toxin-antitoxin (TA) systems, inhibition of ATP production, reactive oxygen species (ROS), efflux pumps, bacterial SOS response, cell-to-cell communication and stringent response (ppGpp- mediated) (Kouhsari et al., 2023). Once formed, persisters can survive exposure to high concentrations of antimicrobial agents, and can remain viable for extended periods of time, even in the absence of nutrients or growth factors (Soares et al., 2019). Fortunately, there is a possible way to wake up these "sleeping bombs" to back to growth. Another study found that reactive nitrogen species (RNS) is produced by the host in response to Salmonella infection after persister formation in macrophages, which intoxicate the TCA cycle of persisters and struck them in the growth arrest stage through lowering cellular respiration and ATP production (Ronneau et al., 2023). This research indicates that inhibition of RNS production can force persister cells to regrow during antibiotic treatment which will boost the effects of antibiotics and facilitate their eradication.

2.2.6 Horizontal gene transfer

Recent studies have highlighted the role of horizontal gene transfer in biofilm-associated antimicrobial resistance (Michaelis and Grohmann, 2023). Horizontal gene transfer can facilitate the spread of resistance genes among bacterial populations within biofilms, leading to the emergence of multidrug resistant strains. Mobile genetic elements, such as plasmids, transposons, and integrons, can transfer antimicrobial resistance genes between bacterial species and strains (Figure 2). A better understanding of the mechanisms of antimicrobial resistance in biofilms is necessary to develop more effective strategies for the prevention and treatment of biofilm-associated infections. New approaches that target the physical and biological barriers to antimicrobial penetration, as well as the mechanisms that contribute to the formation and maintenance of biofilms, may be required to overcome the challenges posed by biofilm-associated antimicrobial resistance. Additionally, the development of new antimicrobial agents or the repurposing of existing drugs may also be necessary to combat biofilm infections, particularly those caused by multidrug-resistant strains.

3 Biofilm-associated infections

Biofilm-associated infections are a significant healthcare challenge, as they are often resistant to conventional antibiotics and can lead to chronic infections. They include different conditions such as oral infection, infection in upper and lower airways, wounds, gastrointestinal infections, urinary tract infections (UTIs) and prosthetic joint infections (PJIs).

3.1 Oral infection

The oral cavity contains various microbial communities living as biofilms, which can lead to dental caries and periodontal disease

(Valm, 2019) (Table 1). The transition from health to dysbiosis is caused by changes in community structure, both *in vivo* and *in vitro* (O'Connell et al., 2020; Moussa et al., 2022).

3.2 Infection in airways

There is strong evidence suggests that bacterial biofilm attached to sinonasal mucosa has played a potential role in the pathogenesis of chronic rhinosinusitis (CRS), an infection in the upper airways (Vickery et al., 2019), where *Staphylococcus aureus* has been implied to be the major pathogen. Besides this, fungal-bacterial interaction has been observed to have affected the virulence of fungi and bacteria and host immune responses (Shin et al., 2023). Although CRS manifests in self in a variety of pathogenic patterns, there is a piece of increasing evidence suggesting that it is associated with sinonasal dysbiosis and the presence of biofilm (Sabino et al., 2021).

As to the lower respiratory tract infections, repeated episodes of infection were caused by *Pseudomonas aeruginosa* in Cystic fibrosis (CF). Which is an autosomal recessive disease characterized by abnormality of chloride channels due to mutations in Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. Recently, rapid synergistic biofilm formation of *Pseudomonas aeruginosa* and *Candida albicans* has been observed *in vitro* and mice (Phuengmaung et al., 2020; Phuengmaung et al., 2022). These biofilms were more prominent than those induced by *Pseudomonas aeruginosa* alone, which supports *Candida*-enhanced *Pseudomonas* growth. Besides, mixedspecies biofilm was attenuated by N-acetyl-1-cysteine (Phuengmaung et al., 2020).

3.3 Wounds

In chronic wound infections, biofilms consisting of *S. aureus* and *P. aeruginosa* are commonly found together leading to delayed healing and persistent infections (Borges et al., 2022). Laboratory models developed to monitor biofilm colonization in chronic wounds and biopsies reveal that bacteria form species specific aggregates in distinct regions of the tissue (Khalid et al., 2023). Real-time detection of volatile metabolites can be used to discriminate between different microbial species in biofilms (Slade et al., 2022).

3.4 Gastrointestinal infections

Biofilm formation is a crucial factor in the development of inflammatory bowel disease and urinary tract infections. The presence of mucosal biofilms disrupted bile acid metabolism and bacterial dysbiosis in the gut microbiome of Inflammatory bowel disease (IBD) patients (Baumgartner et al., 2021). Previous result link the presence of biofilms to a dysbiotic gut microbiome, including the overgrowth of Escherichia coli and Ruminococcus gnavus (Baumgartner et al., 2021).

3.5 Urinary tract infections

In UTIs, biofilm formation is a major mechanism used by bacteria and is significantly correlated with virulence factors and antibiotic resistance (Zhao et al., 2020). Multidrug-resistant uropathogenic bacteria have an increasing impact on UTI management (Mark et al., 2021). Programmable probiotics show promise as a therapeutic method for treating gut-related diseases (Zhou et al., 2022).

3.6 Prosthetic joint infections

PJIs occur when bacteria colonize the surface of an artificial joint, such as a hip or knee replacement, and form a biofilm that makes them more resistant to antibiotics and the body's immune response. PJIs can occur through several routes, including contamination during surgery, hematogenous seeding from another site of infection, or direct extension from adjacent infected tissue (Ahmed et al., 2019). Biofilm formation on prosthetic joints reduces the effectiveness of treatment of PJIs that lead to significant morbidity and mortality. For further therapeutic benefit, new strategies to prevent, diagnose, and treat biofilm-associated infections are needed to be explored.

3.7 Microorganisms involved in biofilm-related infections

3.7.1 *Pseudomonas aeruginosa*

P. aeruginosa displays resistance to multiple antibiotics, including aminoglycosides, quinolones and β-lactams (Jurado-Martín et al., 2021). Biofilm formation is essential for its tremendous ability to adapt to altered environments. The main virulence factors *of P. aeruginosa* consist of: biofilm formation ability, three types of QS(Las, RhI and Pqs), efflux pumps, flagella, type IV pili, T3SS, type VI secretion system, and type II secretion system (Jurado-Martín et al., 2021).

3.7.2 *Acinetobacter baumanni*

A. baumannii can form biofilm on medical surfaces, such as catheters, endotracheal tubes, and ventilators, enables its persistence in hospitals (Roy et al., 2022). The clinical outcome of patients with Carbapenem-resistant *A. baumannii* and *K. pneumoniae* is associated with high mortality rates, particularly in the mid-south region of China, and predominantly belongs to ST457 *A. baumannii* (Li et al., 2020). In addition to biofilm formation, *A. baumannii* acquires antibiotic-resistant genes via horizontal gene transfer (HGT), allowing it to evade the immune system and gain an advantage in hospitals. Antibiotic-resistant genes are transferred via conjugation, transformation, bacteria phage-mediated, nanotube-mediated, or outer membrane vesicles (Roy et al., 2022). *A. baumannii* strong biofilm-forming ability, high efficiency of HGT, and the expression of efflux pumps and antibiotic modifying

enzymes render the pathogen resistant to multiple drugs, including carbapenems, aminoglycosides, and fluoroquinolones.

3.7.3 *Klebsiella pneumoniae*

KP is a Gram-negative facultative anaerobic pathogen with striking virulence factors consisting of adhesive fimbriae, capsule, lipopolysaccharide (LPS), and siderophores or iron carriers. The carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has enzymes such as $bla_{\text{KPC-2}}$ which is one of the most observed carbapenemase genotypes, followed by bla_{NDM}, $bla_{\text{OXA-48-like,}}$ and bla_{IMP} (Wang et al., 2022). The dominant carriage pattern of the antibiotic resistance genotype is the carriage of a single carbapenemase gene. However, the combination of a single genotype results in hypervirulence in bacteria and the convergence of hypervirulence and carbapenem resistance (Roy et al., 2022).

3.7.4 *Escherichia coli*

E. coli has been observed to possess many virulence factors, especially uropathogenic *E. coli* (UPEC), which include: immune suppressors (invasins like the *sisA* and *sisB*), adhesins (*fimH* adhesion, P-fimbrial adhesins) (Bunduki et al., 2021). Tetracycline resistance in *E.coli* is generally very high, followed by quinolones and β-lactams. The results show that *shiA* is the gene mostly associated with virulence, followed by *CSH, fimH/MSHA, traT, sisA, iucD, iutA, kpsMTI and PAI* (Bunduki et al., 2021).

3.7.5 *Enterococcus faecium*

In the case of *Enterococcus faecium* the *efa* gene was the most frequently observed virulence gene, followed by *ace, esp, ebp, cylA, hyl, asa1, gelE, sprE, fsrC, fsrA and fsrB* (Gök et al., 2020). And the highest resistance rate has been observed in ciprofloxacin. Also, *Enterococcus faecium* is highly resistant to ampicillin, streptomycin, gentamicin, vancomycin, teicoplanin and linezolid (Gök et al., 2020).

3.7.6 *Staphylococcus aureus*

In the case of Gram-positive bacteria, *Staphylococcus aureus* is the most commonly isolated pathogen in biofilm-related infections and the leading cause of numerous diseases (such as bacteremia, infectious endocarditis, infections of the skin, soft tissue, pleuropulmonary and the periprosthetic joint infection) (Pietrocola et al., 2022). Which is also the major colonizer of a medical device. An identified mutation in the genome is associated with enhanced biofilm production in *S. aureus in vitro*, that high-level expression of *manA* and *fruB* corrected biofilm deficiencies and enhanced biofilm formation (Long et al., 2023). The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which is the result of the acquisition of *mecA* gene coding for penicillin-binding protein-2a that blocks inhibitory action on peptidoglycan cross-linking, has prompted research into potential anti-virulencetargeted approaches. The strong pathogenicity of *S. aureus* due to its large repertoire of toxins (such as α-hemolysin, exfoliative toxins, and Panton-valentine leucocidin) (Ahmad-Mansour et al., 2021), also results from its facultative anaerobe characteristics (Vudhya Gowrisankar et al., 2021). As one of the main efflux pumps, NorA is an efficient multidrug-resistant system that could enhance drug resistance by increasing drug efflux, thus appearing to be an important target for efflux pump inhibitors such as quino-4-carboxamide derivatives (3a and 3b), which enhanced the bactericidal activities of ciprofloxacin and fluoroquinolone via NorA inhibition *in vitro* (Cannalire et al., 2020). There are also plenty of novel inhibitors that have been proven effective *in vitro* (Cernicchi et al., 2021), but they widen the gap between clinical application and experimental achievement.

4 Discussion

4.1 Detection and monitor of biofilm formation

Recent research has shown that biofilm growth is a developmental process resembling developmental processes in multicellular eukaryotes (Futo et al., 2022). But studying the ontogeny of biofilm *in vivo* is still problematic since it is difficult to develop *in vitro* models of biofilm formation that mimic the internal environment. As a result, the traditional four-step model of biofilm formation is not suitable for biofilm-mediated infections, which has failed to capture many aspects of bacterial biofilm physiology, especially in clinical settings (Sauer et al., 2022). Karin Sauer et al., have presented a new developmental model for biofilm formation that may include all the diverse scenarios and microenvironments where biofilms are formed (Sauer et al., 2022). This expanded model considers biofilm as an open system with a continuous influx of new members in an external/internal environment. Although it is a work in progress based on current knowledge, this new model has interesting insight into the study of biofilm formation.

To define the dynamics and critical transitional phases of the formation process, as well as microbial interactions and colony dynamics, there is an urgent need for non-invasive methods that can provide *in vivo* visual information to record the gross morphological changes of the biofilm. Carlos Molina-Santiago et al. has detailed a novel non-invasive method for tracking bacterial growth and biofilm dynamics, Baclive (Molina-Santiago et al., 2022). However, our ability to follow complex intraspecies and interspecies interactions *in vivo* at the cellular level remained limited.

In addition, early detection and monitoring of biofilms holds significant importance for the diagnosis of biofilm-mediated infections and the prevention of further spread and new lesions. Several methods are currently used for the detection of bacterial communities in biofilm in controlled laboratory settings, including molecular methods (such as quantitative polymerase chain reaction (q-PCR)); microscopy techniques (such as confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM)); spectroscopic method (Raman spectroscopy and surface-enhanced Raman spectroscopy); identification and localization of microorganisms in biofilm (such as fluorescence *in situ* hybridization (FISH)). Various FISH techniques related to biofilm have been developed. For example, FISH can be modified to catalyzed reporter deposition-FISH (CARD-FISH), peptide

nucleic acid (PNA)-FISH, locked nucleic acids (LNA)-FISH that allow multiple bacteria to be identified at the same time (Azeredo et al., 2017). However, these methods are limited in biofilm detection in clinical settings (Xu et al., 2020).

Several new approaches have been recently developed for biofilm studies which can contribute to a more comprehensive understanding of biofilm physiology, structure and composition. One promising approach is optical coherence tomography (OCT), a non-contact method for imaging the topological and internal microstructure of samples in three dimensions (Bouma et al., 2022). In a recent study, OCT has been used to differentiate pathogenic bacteria and biofilms in otitis media (Locke et al., 2022). Another study uses longitudinal catheter-based 3-D OCT to monitor biofilm formation in endotracheal tubes (ETTs) (Dsouza et al., 2021), their results indicate that catheter-based OCT provides a non-invasive way to identify biofilm within the tube and can differentiate biofilm-positive from biofilm negative groups. Moreover, the OCT image-based features obtained can offer possible biomarkers to detect biofilm *in vivo* (Dsouza et al., 2019). Biofilm associated biomarkers are unique molecules produced during biofilm growth (such as specific proteins on bacterial cells, metabolites), or stimulated by biofilm specific host responses (such as antibodies). That can be detected using standard methods (Xu et al., 2020). Elisabeth A. Slade et al. presents a real-time detection of biofilms in a clinical wound infection model by detecting volatile metabolites for species-level discrimination (Slade et al., 2022). The volatile compounds successfully differentiate between the pathogens studied, which can be developed as rapid point-of-care diagnostics for wound infection. Although studies are limited to biomarkers that are unique to bacterial biofilms, especially those present in multiple bacterial species, but detection of biofilm matrix components (such as cellulose (Perumal et al., 2021)) and QS signal profiling might discover potential biomarkers. Other novel approaches, including targeted nanopore sequencing (TNPseq) (Zhang et al., 2022a); metagenomics (Taş et al., 2021); and nanoparticles (Funari and Shen, 2022).

4.2 Treatment strategies

Biofilm targeting therapies can help to prevent and treat bacterial infections by inhibiting the formation of biofilms. One approach is to target the cyclic-di-GMP pathway, which involves inhibiting diguanylate cyclase (DGC) enzymes that synthesize c-di-GMP (Andersen et al., 2021a). Small molecules have been identified that can disperse *P. aeruginosa* and *A. baumannii* biofilms without toxic effects on eukaryotic cells. Another approach targets T3SS which can cause severe tissue damage and inhibit wound repair. Antibodies and natural compounds can interfere with the T3SS structure and/or its function (Ngo et al., 2019; Ranjbar et al., 2019). The cytostatic anti-cancer drug 5fluorouracil (5-FU) has been shown to inhibit AI-2 production and release by MRSA, *S. epidermidis*, *E. coli*, and *V. harveyi*, and could potentially be used as an anti-infective therapy (Sedlmayer et al., 2021). CRISPR/Cas9-HDR system may provide an efficient and site-specific gene editing approach which targets the QS mechanism and adhesion property to suppress biofilm formation (Alshammari et al., 2023).

Novel strategies for biofilm-related infection management include (i) the use of nanoparticles combined with a laser to improve drug diffusion (Teirlinck et al., 2019), (ii)pH-responsive drug delivery systems (Ding et al., 2022), and (iii)targeting the extracellular polymeric substances (EPS) in a mature biofilm to expose the bacteria and permit antibacterial agents to reach the biofilm (Han et al., 2022). Moreover, interfering with iron metabolism can also disrupt iron-dependent biological processes by binding iron-utilizing proteins (Ribeiro et al., 2022). (v) Apart from that, the dispersal of biofilm produced by multi-drug resistant bacteria can be achieved through phage therapy. (Pallavali et al., 2021).

In all, real-time detection and non-invasive biofilm detection of biofilm, as well as biofilm specific labels are to be discovered. With increased sensitivity in signal detection of biofilm formation, localized imaging is for certain to be combined with a therapeutic benefit. In the future, more experiments are required to explore more regulation of biofilm formation and dispersal, in order to induce the dispersal of mature biofilms and restore the bacteria planktonic growth. Besides, ongoing studies show that inhibition of c-di-GMP and cAMP synthesis, as well as interfering QS system can prevent the maintenance of biofilm. And destroying EPS while applying antibiotics would be an effective way to boost antibiotic efficacy. Currently, the combination of nanomaterials and antibiotics is the most feasible way to disrupt biofilms.

5 Conclusion

In conclusion, biofilms represent an emerging battleground in healthcare due to their ability to induce antimicrobial resistance. These microbial communities have developed complex mechanisms that protect them against the action of antibiotics and host defenses, making them challenging to eradicate. The development of biofilm-specific therapies is crucial to overcome these challenges and prevent the emergence of new antibiotic-resistant strains. Several approaches, such as the use of alternative antimicrobial agents, quorum sensing inhibitors, and biofilm disruptors, have shown promising results in laboratory studies. However, the translation of these findings into clinical practice remains a significant challenge. Further research is needed to understand the mechanisms of biofilm formation and antibiotic resistance better, identify novel targets for intervention, and develop more effective strategies for the prevention and treatment of biofilm-related infections. In summary, the battle against biofilm-induced antimicrobial resistance requires a multifaceted approach that involves collaboration between researchers, clinicians, and policymakers to mitigate the public health threat posed by these microbial communities.

Author contributions

LP: Conceptualization, Data curation, Formal analysis, Software, Visualization, Writing – original draft. SP:

Conceptualization, Formal analysis, Investigation, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SL: Conceptualization, Resources, Writing – review & editing. FW: Funding acquisition, Project administration, Resources, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by Shenzhen Fund for Guangdong Provincial High-Level Clinical Key Specialties (No.SZGSP012); Shenzhen Key Medical Discipline Construction Fund (No. SZXK034).

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

Abd El-Rahman, O. A., Rasslan, F., Hassan, S. S., Ashour, H. M., and Wasfi, R. (2023). The RND efflux pump gene expression in the biofilm formation of acinetobacter baumannii. *Antibiot. Basel Switz.* 12, 419. doi: 10.3390/antibiotics12020419

Ahmad-Mansour, N., Loubet, P., Pouget, C., Dunyach-Remy, C., Sotto, A., Lavigne, J.-P., et al. (2021). Staphylococcus aureus toxins: an update on their pathogenic properties and potential treatments. *Toxins* 13, 677. doi: 10.3390/toxins13100677

Ahmed, S. S., Begum, F., Kayani, B., and Haddad, F. S. (2019). Risk factors, diagnosis and management of prosthetic joint infection after total hip arthroplasty. *Expert Rev. Med. Devices* 16, 1063–1070. doi: 10.1080/17434440.2019.1696673

Alshammari, M., Ahmad, A., AlKhulaifi, M., Al Farraj, D., Alsudir, S., Alarawi, M., et al. (2023). Reduction of biofilm formation of Escherichia coli by targeting quorum sensing and adhesion genes using the CRISPR/Cas9-HDR approach, and its clinical application on urinary catheter. *J. Infect. Public Health* 16, 1174–1183. doi: 10.1016/j.jiph.2023.05.026

Andersen, J. B., Hultqvist, L. D., Jansen, C. U., Jakobsen, T. H., Nilsson, M., Rybtke, M., et al. (2021a). Identification of small molecules that interfere with c-di-GMP signaling and induce dispersal of Pseudomonas aeruginosa biofilms. *NPJ Biofilms Microbiomes* 7, 59. doi: 10.1038/s41522-021-00225-4

Andersen, J. B., Kragh, K. N., Hultqvist, L. D., Rybtke, M., Nilsson, M., Jakobsen, T. H., et al. (2021b). Induction of Native c-di-GMP Phosphodiesterases Leads to Dispersal of Pseudomonas aeruginosa Biofilms. *Antimicrob. Agents Chemother.* 65, e02431–e02420. doi: 10.1128/AAC.02431-20

André, A. C., Laborde, M., and Marteyn, B. S. (2022). The battle for oxygen during bacterial and fungal infections. *Trends Microbiol.* 30, 643–653. doi: 10.1016/j.tim.2022.01.002

Azeredo, J., Azevedo, N. F., Briandet, R., Cerca, N., Coenye, T., Costa, A. R., et al. (2017). Critical review on biofilm methods. *Crit. Rev. Microbiol.* 43, 313–351. doi: 10.1080/1040841X.2016.1208146

Baumgartner, M., Lang, M., Holley, H., Crepaz, D., Hausmann, B., Pjevac, P., et al. (2021). Mucosal biofilms are an endoscopic feature of irritable bowel syndrome and ulcerative colitis. *Gastroenterology* 161, 1245–1256.e20. doi: 10.1053/j.gastro.2021.06.024

Behbahani, S. B., Kiridena, S. D., Wijayaratna, U. N., Taylor, C., Anker, J. N., and Tzeng, T.-R. J. (2022). pH variation in medical implant biofilms: Causes, measurements, and its implications for antibiotic resistance. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.1028560

Bordon, Y. (2023). Bacteria form unique biofilms to kill immune cells. *Nat. Rev. Immunol.* 23, 474. doi: 10.1038/s41577-023-00914-5

Borges, E. L., Amorim, G. L., de Miranda, M. B., Martins, F., dos, S., Guedes, A. C. M., et al. (2022). Biofilm model on mice skin wounds. *Acta Cirúrgica Bras.* 37, e370306. doi: 10.1590/acb370306

Bouma, B. E., de Boer, J. F., Huang, D., Jang, I.-K., Yonetsu, T., Leggett, C. L., et al. (2022). Optical coherence tomography. *Nat. Rev. Methods Primer* 2, 1–20. doi: 10.1038/s43586-022-00162-2

Bunduki, G. K., Heinz, E., Phiri, V. S., Noah, P., Feasey, N., and Musaya, J. (2021). Virulence factors and antimicrobial resistance of uropathogenic Escherichia coli (UPEC) isolated from urinary tract infections: a systematic review and metaanalysis. *BMC Infect. Dis.* 21, 753. doi: 10.1186/s12879-021-06435-7

Caixeta Magalhães Tibúrcio, A. A., Paiva, A. D., Pedrosa, A. L., Rodrigues, W. F., Bernardes da Silva, R., and Oliveira, A. G. (2022). Effect of sub-inhibitory concentrations of antibiotics on biofilm formation and expression of virulence genes in penicillin-resistant, ampicillin-susceptible Enterococcus faecalis. *Heliyon* 8, e11154. doi: 10.1016/j.heliyon.2022.e11154

Cannalire, R., Mangiaterra, G., Felicetti, T., Astolfi, A., Cedraro, N., Massari, S., et al. (2020). Structural modifications of the quinolin-4-yloxy core to obtain new staphylococcus aureus norA inhibitors. *Int. J. Mol. Sci.* 21, 7037. doi: 10.3390/ijms21197037

Cantin, A. M., Hartl, D., Konstan, M. W., and Chmiel, J. F. (2015). Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy. *J. Cyst. Fibros.* 14, 419–430. doi: 10.1016/j.jcf.2015.03.003

Casalone, E., Vignolini, T., Braconi, L., Gardini, L., Capitanio, M., Pavone, F. S., et al. (2020). 1-benzyl-1,4-diazepane reduces the efflux of resistance-nodulation-cell division pumps in Escherichia coli. *Future Microbiol.* 15, 987–999. doi: 10.2217/fmb-2019-0296

Cauilan, A., and Ruiz, C. (2022). Sodium malonate inhibits the acrAB-tolC multidrug efflux pump of escherichia coli and increases antibiotic efficacy. *Pathog. Basel Switz.* 11, 1409. doi: 10.3390/pathogens11121409

Cernicchi, G., Felicetti, T., and Sabatini, S. (2021). Microbial efflux pump inhibitors: A journey around quinoline and indole derivatives. *Mol. Basel Switz.* 26, 6996. doi: 10.3390/molecules26226996

Chakraborty, P., Bajeli, S., Kaushal, D., Radotra, B. D., and Kumar, A. (2021). Biofilm formation in the lung contributes to virulence and drug tolerance of Mycobacterium tuberculosis. *Nat. Commun.* 12, 1606. doi: 10.1038/s41467021-21748-6

Chittasupho, C., Manthaisong, A., Okonogi, S., Tadtong, S., and Samee, W. (2021). Effects of quercetin and curcumin combination on antibacterial, antioxidant, *in vitro* wound healing and migration of human dermal fibroblast cells. *Int. J. Mol. Sci.* 23, 142. doi: 10.3390/ijms23010142

Dalwadi, M. P., and Pearce, P. (2021). Emergent robustness of bacterial quorum sensing in fluid flow. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2022312118. doi: 10.1073/pnas.2022312118

D'Cunha, N., Moniruzzaman, M., Haynes, K., Malloci, G., Cooper, C. J., Margiotta, E., et al. (2021). Mechanistic duality of bacterial efflux substrates and inhibitors: example of simple substituted cinnamoyl and naphthyl amides. *ACS Infect. Dis.* 7, 2650–2665. doi: 10.1021/acsinfecdis.1c00100

Ding, H., Tan, P., Fu, S., Tian, X., Zhang, H., Ma, X., et al. (2022). Preparation and application of pH-responsive drug delivery systems. *J. Control. Release Off. J. Control. Release Soc* 348, 206–238. doi: 10.1016/j.jconrel.2022.05.056

Dong, X., Tu, C., Liu, Y., Zhang, R., and Liu, Y. (2022). Identification of the core c-diGMP turnover proteins responsible for root colonization of Bacillus velezensis. *iScience* 25, 105294. doi: 10.1016/j.isci.2022.105294

Dsouza, R., Spillman, D. R., Barkalifa, R., Monroy, G. L., Chaney, E. J., Johnson, M. A., et al. (2021). Efficacy of endotracheal tube suctioning in intubated intensive care unit patients determined by in *vivo* catheter-based optical coherence tomography-a pilot study. *Quant. Imaging Med. Surg.* 11, 1–8. doi: 10.21037/qims-20-549

Dsouza, R., Spillman, D. R., Barkalifa, R., Monroy, G. L., Chaney, E. J., White, K. C., et al. (2019). *In vivo* detection of endotracheal tube biofilms in intubated critical care patients using catheter-based optical coherence tomography. *J. Biophotonics* 12, e201800307. doi: 10.1002/jbio.201800307

Elsheredy, A., El-Soudany, I., Elsherbini, E., Metwally, D., and Ghazal, A. (2021). Effect of azithromycin and phenylalanine-arginine beta-naphthylamide on quorum sensing and virulence factors in clinical isolates of Pseudomonas aeruginosa. *Iran. J. Microbiol.* 13, 37–49. doi: 10.18502/ijm.v13i1.5491

Feng, Q., Ahator, S. D., Zhou, T., Liu, Z., Lin, Q., Liu, Y., et al. (2020). Regulation of exopolysaccharide production by proE, a cyclic-di-GMP phosphodiesterase in pseudomonas aeruginosa PAO1. *Front. Microbiol.* 11. doi: 10.3389/fmicb.2020.01226

Fisher, R. A., Gollan, B., and Helaine, S. (2017). Persistent bacterial infections and persister cells. *Nat. Rev. Microbiol.* 15, 453–464. doi: 10.1038/nrmicro.2017.42

Flemming, H.-C., van Hullebusch, E. D., Neu, T. R., Nielsen, P. H., Seviour, T., Stoodley, P., et al. (2023). The biofilm matrix: multitasking in a shared space. *Nat. Rev. Microbiol.* 21, 70–86. doi: 10.1038/s41579-022-00791-0

Funari, R., and Shen, A. Q. (2022). Detection and characterization of bacterial biofilms and biofilm-based sensors. *ACS Sens.* 7, 347–357. doi: 10.1021/acssensors.1c02722

Futo, M., Široki, T., Koska, S., Čorak, N., Tušar, A., Domazet-Lošo, M., et al. (2022). A novel time-lapse imaging method for studying developing bacterial biofilms. *Sci. Rep.* 12, 21120. doi: 10.1038/s41598-022-24431-y

Gök, Ş.M., Türk Dağı, H., Kara, F., Arslan, U., and Fındık, D. (2020). [Investigation of Antibiotic Resistance and Virulence Factors of Enterococcus faecium and Enterococcus faecalis Strains Isolated from Clinical Samples]. *Mikrobiyol. Bul.* 54, 26–39. doi: 10.5578/mb.68810

Gopu, V., Meena, C. K., and Shetty, P. H. (2015). Quercetin influences quorum sensing in food borne bacteria: *in-vitro* and in-silico evidence. *PloS One* 10, e0134684. doi: 10.1371/journal.pone.0134684

Guo, T., Chen, Y., Chen, W., Semple, S. J., Gu, X., Polyak, S. W., et al. (2023). Design and synthesis of benzochromene derivatives as AcrB inhibitors for the reversal of bacterial multidrug resistance. *Eur. J. Med. Chem.* 249, 115148. doi: 10.1016/j.ejmech.2023.115148

Han, X., Lou, Q., Feng, F., Xu, G., Hong, S., Yao, L., et al. (2022). Spatiotemporal release of reactive oxygen species and NO for overcoming biofilm heterogeneity. *Angew. Chem. Int. Ed Engl.* 61, e202202559. doi: 10.1002/anie.202202559

Hastings, C. J., Himmler, G. E., Patel, A., and Marques, C. N. H. (2023). Immune response modulation by pseudomonas aeruginosa persister cells. *mBio* 14, e0005623. doi: 10.1128/mbio.00056-23

Hengge, R. (2021). Crosstalking second messengers. *Nat. Microbiol.* 6, 9–10. doi: 10.1038/s41564-020-00842-3

Huang, S., Liu, X., Yang, W., Ma, L., Li, H., Liu, R., et al. (2022). Insights into adaptive mechanisms of extreme acidophiles based on quorum sensing/quenchingRelated proteins. *mSystems* 7, e0149121. doi: 10.1128/msystems.01491-21

Jakubovics, N. S., Goodman, S. D., Mashburn-Warren, L., Stafford, G. P., and Cieplik, F. (2021). The dental plaque biofilm matrix. *Periodontol. 2000* 86, 32–56. doi: 10.1111/prd.12361

Janssen, K. H., Corley, J. M., Djapgne, L., Cribbs, J. T., Voelker, D., Slusher, Z., et al. (2020). Hfq and sRNA 179 Inhibit Expression of the Pseudomonas aeruginosa cAMP-Vfr and Type III Secretion Regulons. *mBio* 11, e00363-20. doi: 10.1128/mBio.00363-20

Jayakumar, P., Figueiredo, A. R. T., and Kümmerli, R. (2022). Evolution of Quorum Sensing in Pseudomonas aeruginosa Can Occur via Loss of Function and Regulon Modulation. *mSystems* 7, e0035422. doi: 10.1128/msystems.0035422

Jurado-Martín, I., Sainz-Mejías, M., and McClean, S. (2021). Pseudomonas aeruginosa: an audacious pathogen with an adaptable arsenal of virulence factors. *Int. J. Mol. Sci.* 22, 3128. doi: 10.3390/ijms22063128

Kaynak Onurdağ, F., Kayış, U., and Ökten, S. (2021). [Effect of phenylalaninearginine-beta-naphthylamide to ciprofloxacin minimum inhibitory concentration values and expression of efflux pump system genes in acinetobacter baumannii isolates]. *Mikrobiyol. Bul.* 55, 285–299. doi: 10.5578/mb.20219801

Khalid, A., Cookson, A. R., Whitworth, D. E., Beeton, M. L., Robins, L. I., and Maddocks, S. E. (2023). A synthetic polymicrobial community biofilm model demonstrates spatial partitioning, tolerance to antimicrobial treatment, reduced metabolism, and small colony variants typical of chronic wound biofilms. *Pathogens* 12, 118. doi: 10.3390/pathogens12010118

Khodayary, R., Nikokar, I., Mobayen, M. R., Afrasiabi, F., Araghian, A., Elmi, A., et al. (2019). High incidence of type III secretion system associated virulence factors (exoenzymes) in Pseudomonas aeruginosa isolated from Iranian burn patients. *BMC Res. Notes* 12, 28. doi: 10.1186/s13104-019-4071-0

Kim, H.-S., Ham, S.-Y., Ryoo, H.-S., Kim, D.-H., Yun, E.-T., Park, H.-D., et al. (2023). Inhibiting bacterial biofilm formation by stimulating c-di-GMP regulation using citrus peel extract from Jeju Island. *Sci. Total Environ.* 872, 162180. doi: 10.1016/j.scitotenv.2023.162180

Kosztołowicz, T., and Metzler, R. (2020). Diffusion of antibiotics through a biofilm in the presence of diffusion and absorption barriers. *Phys. Rev. E* 102, 32408. doi: 10.1103/PhysRevE.102.032408

Kouhsari, E., Kaviar, V. H., Asadi, A., Ahmadi, A., Sholeh, M., Mirbalouchzehi, A., et al (2023). Bacterial persister cells: mechanisms of formation, control, and eradication. *Infect. Disord. Drug Targets.* 23 (7), 17–28. doi: 10.2174/1871526523666230511142054

Kumar Roy, R., and Patra, N. (2020). Configuration flipping in distal pocket of multidrug transporter mexB impacts the efflux inhibitory mechanism. *Chemphyschem Eur. J. Chem. Phys. Phys. Chem.* 21, 2516–2524. doi: 10.1002/cphc.202000759

Lewis, B. R., Urddin., M. R., Moniruzzaman, M., Kuo., K., Higgins., A. J., Shah, L. M. N., et al. (2023). Conformational restriction shapes the inhibition of a multidrug efflux adaptor protein. *Nat. Commun.* 14, 3900. doi: 10.1038/s41467-023-39615-x

Li, Z.-L., Hou, Y.-F., Zhang, B.-Q., Chen, Y.-F., Wang, Q., Wang, K., et al. (2018). Identifying common pathogens in periprosthetic joint infection and testing drug-resistance rate for different antibiotics: A prospective, single center study in Beijing. *Orthop. Surg.* 10, 235–240. doi: 10.1111/os.12394

Li, J., Yu, T., Luo, Y., Peng, J.-Y., Li, Y.-J., Tao, X.-Y., et al. (2020). Characterization of carbapenem-resistant hypervirulent Acinetobacter baumannii strains isolated from hospitalized patients in the mid-south region of China. *BMC Microbiol.* 20, 281. doi: 10.1186/s12866-020-01957-7

Lichtenberg, M., Kragh, K. N., Fritz, B., Kirkegaard, J. B., Tolker-Nielsen, T., and Bjarnsholt, T. (2022). Cyclic-di-GMP signaling controls metabolic activity in Pseudomonas aeruginosa. *Cell Rep.* 41, 111515. doi: 10.1016/j.celrep.2022.111515

Lieberman, O. J., Orr, M. W., Wang, Y., and Lee, V. T. (2014). High-throughput screening using the differential radial capillary action of ligand assay identifies ebselen as an inhibitor of diguanylate cyclases. *ACS Chem. Biol.* 9, 183–192. doi: 10.1021/cb400485k

Liu, C., Sun, D., Liu, J., Chen, Y., Zhou, X., Ru, Y., et al. (2022). cAMP and c-di-GMP synergistically support biofilm maintenance through the direct interaction of their effectors. *Nat. Commun.* 13, 1493. doi: 10.1038/s41467-022-29240-5

Liu, K., Tan, S., Ye, W., Hou, L., and Fang, B. (2022). Low-concentration iron promotes Klebsiella pneumoniae biofilm formation by suppressing succinic acid. *BMC Microbiol.* 22, 95. doi: 10.1186/s12866-022-02518-w

Locke, A. K., Zaki, F. R., Fitzgerald, S. T., Sudhir, K., Monroy, G. L., Choi, H., et al. (2022). Differentiation of otitis media-causing bacteria and biofilms via Raman spectroscopy and optical coherence tomography. *Front. Cell. Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.869761

Lomovskaya, O., Warren, M. S., Lee, A., Galazzo, J., Fronko, R., Lee, M., et al. (2001). Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: novel agents for combination therapy. *Antimicrob. Agents Chemother.* 45, 105–116. doi: 10.1128/AAC.45.1.105116.2001

Long, D. R., Penewit, K., Lo, H.-Y., Almazan, J., Holmes, E. A., Bryan, A. B., et al. (2023). *In vitro* selection identifies staphylococcus aureus genes influencing biofilm formation. *Infect. Immun.* 91, e0053822. doi: 10.1128/iai.00538-22

Ma, R., Hu, X., Zhang, X., Wang, W., Sun, J., Su, Z., et al. (2022). Strategies to prevent, curb and eliminate biofilm formation based on the characteristics of various periods in one biofilm life cycle. *Front. Cell. Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.1003033

Mark, D. G., Hung, Y.-Y., Salim, Z., Tarlton, N. J., Torres, E., and Frazee, B. W. (2021). Third-generation cephalosporin resistance and associated discordant antibiotic treatment in emergency department febrile urinary tract infections. *Ann. Emerg. Med.* 78, 357–369. doi: 10.1016/j.annemergmed.2021.01.003

Michaelis, C., and Grohmann, E. (2023). Horizontal gene transfer of antibiotic resistance genes in biofilms. *Antibiot. Basel Switz.* 12, 328. doi: 10.3390/antibiotics12020328

Molina-Santiago, C., Pearson, J. R., Berlanga-Clavero, M. V., Pérez-Lorente, A. I., de Vicente, A., and Romero, D. (2022). A noninvasive method for time-lapse imaging of microbial interactions and colony dynamics. *Microbiol. Spectr.* 10, e0093922. doi: 10.1128/spectrum.00939-22

Mosaddad, S. A., Tahmasebi, E., Yazdanian, A., Rezvani, M. B., Seifalian, A., Yazdanian, M., et al. (2019). Oral microbial biofilms: an update. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 2005–2019. doi: 10.1007/s10096-019-03641-9

Moura de Sousa, J., Lourenço, M., and Gordo, I. (2023). Horizontal gene transfer among host-associated microbes. *Cell Host Microbe* 31, 513–527. doi: 10.1016/j.chom.2023.03.017

Moussa, D. G., Sharma, A. K., Mansour, T. A., Witthuhn, B., Perdigão, J., Rudney, J. D., et al. (2022). Functional signatures of ex-vivo dental caries onset. *J. Oral. Microbiol.* 14, 2123624. doi: 10.1080/20002297.2022.2123624

Nakashima, R., Sakurai, K., Yamasaki, S., Hayashi, K., Nagata, C., Hoshino, K., et al. (2013). Structural basis for the inhibition of bacterial multidrug exporters. *Nature* 500, 102–106. doi: 10.1038/nature12300

Ngo, T.-D., Plé, S., Thomas, A., Barette, C., Fortuné, A., Bouzidi, Y., et al. (2019). Chimeric protein–protein interface inhibitors allow efficient inhibition of type III secretion machinery and pseudomonas aeruginosa virulence. *ACS Infect. Dis.* 5 (11), 1843–1854. doi: 10.1021/acsinfecdis.9b00154

O'Connell, L. M., Santos, R., Springer, G., Burne, R. A., Nascimento, M. M., and Richards, V. P. (2020). Site-specific profiling of the dental mycobiome reveals strong taxonomic shifts during progression of early-childhood caries. *Appl. Environ. Microbiol.* 86, e02825-19. doi: 10.1128/AEM.02825-19

Oliveira, R. A., Cabral, V., Torcato, I., and Xavier, K. B. (2023). Deciphering the quorum-sensing lexicon of the gut microbiota. *Cell Host Microbe* 31, 500–512. doi: 10.1016/j.chom.2023.03.015

Pallavali, R. R., Degati, V. L., Narala, V. R., Velpula, K. K., Yenugu, S., and Durbaka, V. R. P. (2021). Lytic Bacteriophages Against Bacterial Biofilms Formed by Multidrug-Resistant Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, and Staphylococcus aureus Isolated from Burn Wounds. *PHAGE New Rochelle N* 2, 120–130. doi: 10.1089/phage.2021.0004

Perumal, J., Lim, H. Q., Attia, A. B. E., Raziq, R., Leavesley, D. I., Upton, Z., et al. (2021). Novel cellulose fibre-based flexible plasmonic membrane for pointof-care SERS

biomarker detection in chronic wound healing. *Int. J. Nanomedicine* 16, 5869–5878. doi: 10.2147/IJN.S303130

Phuengmaung, P., Mekjaroen, J., Saisorn, W., Chatsuwan, T., Somparn, P., and Leelahavanichkul, A. (2022). Rapid synergistic biofilm production of pseudomonas and candida on the pulmonary cell surface and in mice, a possible cause of chronic mixed organismal lung lesions. *Int. J. Mol. Sci.* 23, 9202. doi: 10.3390/ijms23169202

Phuengmaung, P., Somparn, P., Panpetch, W., Singkham-In, U., Wannigama, D. L., Chatsuwan, T., et al. (2020). Coexistence of Pseudomonas aeruginosa With Candida albicans Enhances Biofilm Thickness Through Alginate-Related Extracellular Matrix but Is Attenuated by N-acetyl-l-cysteine. *Front. Cell. Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.594336

Pietrocola, G., Campoccia, D., Motta, C., Montanaro, L., Arciola, C. R., and Speziale, P. (2022). Colonization and infection of indwelling medical devices by staphylococcus aureus with an emphasis on orthopedic implants. *Int. J. Mol. Sci.* 23, 5958. doi: 10.3390/ijms23115958

Plé, C., Tam, H.-K., Vieira Da Cruz, A., Compagne, N., Jiménez-Castellanos, J.-C., Müller, R. T., et al. (2022). Pyridylpiperazine-based allosteric inhibitors of RNDtype multidrug efflux pumps. *Nat. Commun.* 13, 115. doi: 10.1038/s41467-02127726-2

Ranjbar, M., Behrouz, B., Norouzi, F., and Mousavi Gargari, S. L. (2019). Anti-PcrV IgY antibodies protect against Pseudomonas aeruginosa infection in both acute pneumonia and burn wound models. *Mol. Immunol.* 116, 98–105. doi: 10.1016/j.molimm.2019.10.005

Ribeiro, M., Sousa, C. A., and Simões, M. (2022). Harnessing microbial iron chelators to develop innovative therapeutic agents. *J. Adv. Res.* 39, 89–101. doi: 10.1016/j.jare.2021.10.010

Ronneau, S., Michaux, C., and Helaine, S. (2023). Decline in nitrosative stress drives antibiotic persister regrowth during infection. *Cell Host Microbe* 31, 9931006.e6. doi: 10.1016/j.chom.2023.05.002

Roy, S., Chowdhury, G., Mukhopadhyay, A. K., Dutta, S., and Basu, S. (2022). Convergence of biofilm formation and antibiotic resistance in acinetobacter baumannii infection. *Front. Med.* 9. doi: 10.3389/fmed.2022.793615

Sabino, H. A. C., Valera, F. C. P., Santos, D. V., Fantucci, M. Z., Titoneli, C. C., Martinez, R., et al. (2021). Biofilm and planktonic antibiotic resistance in patients with acute exacerbation of chronic rhinosinusitis. *Front. Cell. Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.813076

Salehi, B., Ghalavand, Z., Yadegar, A., and Eslami, G. (2021). Characteristics and diversity of mutations in regulatory genes of resistance-nodulation-cell division efflux pumps in association with drug-resistant clinical isolates of Acinetobacter baumannii. *Antimicrob. Resist. Infect. Control* 10, 53. doi: 10.1186/s13756-02100924-9

Santana, P. T., Rosas, S. L. B., Ribeiro, B. E., Marinho, Y., and de Souza, H. S. P. (2022). Dysbiosis in inflammatory bowel disease: pathogenic role and potential therapeutic targets. *Int. J.Mol. Sci.* 23, 3464. doi: 10.3390/ijms23073464

Sauer, K., Stoodley, P., Goeres, D. M., Hall-Stoodley, L., Burmølle, M., Stewart, P. S., et al. (2022). The biofilm life cycle: expanding the conceptual model of biofilm formation. *Nat. Rev. Microbiol.* 20, 608–620. doi: 10.1038/s41579-022-00767-0

Sedlmayer, F., Woischnig, A.-K., Unterreiner, V., Fuchs, F., Baeschlin, D., Khanna, N., et al. (2021). 5-Fluorouracil blocks quorum-sensing of biofilm-embedded methicillin-resistant Staphylococcus aureus in mice. *Nucleic Acids Res.* 49, e73. doi: 10.1093/nar/gkab251

Shin, S.-H., Ye, M.-K., Lee, D.-W., and Geum, S.-Y. (2023). Immunopathologic role of fungi in chronic rhinosinusitis. *Int. J. Mol. Sci.* 24, 2366. doi: 10.3390/ijms24032366

Slade, E. A., Thorn, R. M. S., Young, A. E., and Reynolds, D. M. (2022). Real-time detection of volatile metabolites enabling species-level discrimination of bacterial biofilms associated with wound infection. *J. Appl. Microbiol.* 132, 1558–1572. doi: 10.1111/jam.15313

Soares, A., Roussel, V., Pestel-Caron, M., Barreau, M., Caron, F., Bouffartigues, E., et al. (2019). Understanding ciprofloxacin failure in pseudomonas aeruginosa biofilm: persister cells survive matrix disruption. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.02603

Stephen, J., Lekshmi, M., Ammini, P., Kumar, S. H., and Varela, M. F. (2022). Membrane efflux pumps of pathogenic vibrio species: role in antimicrobial resistance and virulence. *Microorganisms* 10, 382. doi: 10.3390/microorganisms10020382

Taş, N., de Jong, A. E., Li, Y., Trubl, G., Xue, Y., and Dove, N. C. (2021). Metagenomic tools in microbial ecology research. *Curr. Opin. Biotechnol.* 67, 184–191. doi: 10.1016/j.copbio.2021.01.019

Teirlinck, E., Barras, A., Liu, J., Fraire, J. C., Lajunen, T., Xiong, R., et al. (2019). Exploring light-sensitive nanocarriers for simultaneous triggered antibiotic release and disruption of biofilms upon generation of laser-induced vapor nanobubbles. *Pharmaceutics* 11, 201. doi: 10.3390/pharmaceutics11050201

Teirlinck, E., Xiong, R., Brans, T., Forier, K., Fraire, J., Van Acker, H., et al. (2018). Laser-induced vapour nanobubbles improve drug diffusion and efficiency in bacterial biofilms. *Nat. Commun.* 9, 4518. doi: 10.1038/s41467-018-06884-w

Valm, A. M. (2019). The structure of dental plaque microbial communities in the transition from health to dental caries and periodontal disease. *J. Mol. Biol.* 431, 2957–2969. doi: 10.1016/j.jmb.2019.05.016

Vandana,, and Das, S. (2022). Genetic regulation, biosynthesis and applications of extracellular polysaccharides of the biofilm matrix of bacteria. *Carbohydr. Polym.* 291, 119536. doi: 10.1016/j.carbpol.2022.119536

Vargiu, A. V., Ruggerone, P., Opperman, T. J., Nguyen, S. T., and Nikaido, H. (2014). Molecular mechanism of MBX2319 inhibition of Escherichia coli AcrB multidrug efflux pump and comparison with other inhibitors. *Antimicrob. Agents Chemother.* 58, 6224–6234. doi: 10.1128/AAC.03283-14

Vickery, T. W., Ramakrishnan, V. R., and Suh, J. D. (2019). The role of staphylococcus aureus in patients with chronic sinusitis and nasal polyposis. *Curr. Allergy Asthma Rep.* 19, 21. doi: 10.1007/s11882-019-0853-7

Vidakovic, L., Mikhaleva, S., Jeckel, H., Nisnevich, V., Strenger, K., Neuhaus, K., et al. (2023). Biofilm formation on human immune cells is a multicellular predation strategy of Vibrio cholerae. *Cell* 186, 2690–2704.e20. doi: 10.1016/j.cell.2023.05.008

Vudhya Gowrisankar, Y., Manne Mudhu, S., Pasupuleti, S. K., Suthi, S., Chaudhury, A., and Sarma, P. V. G. K. (2021). Staphylococcus aureus grown in anaerobic conditions exhibits elevated glutamine biosynthesis and biofilm units. *Can. J. Microbiol.* 67, 323–331. doi: 10.1139/cjm-2020-0434

Wagner Mackenzie, B., Waite, D. W., Hoggard, M., Douglas, R. G., Taylor, M. W., and Biswas, K. (2017). Bacterial community collapse: a meta-analysis of the sinonasal microbiota in chronic rhinosinusitis. *Environ. Microbiol.* 19, 381–392. doi: 10.1111/1462-2920.13632

Wang, Y., Alenazy, R., Gu, X., Polyak, S. W., Zhang, P., Sykes, M. J., et al. (2021). Design and structural optimization of novel 2H-benzo[h]chromene derivatives that target AcrB and reverse bacterial multidrug resistance. *Eur. J. Med. Chem.* 213, 113049. doi: 10.1016/j.ejmech.2020.113049

Wang, Q., Chen, M., Ou, Q., Zheng, L., Chen, X., Mao, G., et al. (2022). Carbapenemresistant hypermucoviscous Klebsiella pneumoniae clinical isolates from a tertiary hospital in China: Antimicrobial susceptibility, resistance phenotype, epidemiological characteristics, microbial virulence, and risk factors. *Front. Cell. Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.1083009

Wang, L., Wang, H., Zhang, H., and Wu, H. (2023). Formation of a biofilm matrix network shapes polymicrobial interactions. *ISME J.* 17, 467–477. doi: 10.1038/s41396-023-01362-8

Warrener, P., Varkey, R., Bonnell, J. C., DiGiandomenico, A., Camara, M., Cook, K., et al. (2014). A novel anti-PcrV antibody providing enhanced protection against Pseudomonas aeruginosa in multiple animal infection models. *Antimicrob. Agents Chemother.* 58, 4384–4391. doi: 10.1128/AAC.02643-14

Wei, Q., Leclercq, S., Bhasme, P., Xu, A., Zhu, B., Zhang, Y., et al. (2019). Diguanylate Cyclases and Phosphodiesterases Required for Basal-Level c-di-GMP in Pseudomonas aeruginosa as Revealed by Systematic Phylogenetic and Transcriptomic Analyses. *Appl. Environ. Microbiol.* 85, e01194-19. doi: 10.1128/AEM.01194-19

Wucher, B. R., Winans, J. B., Elsayed., M., Kadouri., D. E., and Nadell, C. D. (2023). Breakdown of clonal cooperative architecture in multispecies biofilms and the spatial ecology of predation. *Proc. Natl. Acad. Sci. U. S. A.* 120 (6), e2212650120. doi: 10.1073/pnas.2212650120

Xu, Y., Dhaouadi, Y., Stoodley, P., and Ren, D. (2020). Sensing the unreachable: challenges and opportunities in biofilm detection. *Curr. Opin. Biotechnol.* 64, 79–84. doi: 10.1016/j.copbio.2019.10.009

Yu, Z., Yu, D., Mao, Y., Zhang, M., Ding, M., Zhang, J., et al. (2019). Identification and characterization of a LuxI/R-type quorum sensing system in Pseudoalteromonas. *Res. Microbiol.* 170, 243–255. doi: 10.1016/j.resmic.2019.07.001

Yu, Z., Zhang, W., Yang, H., Chou, S. H., Galperin, M. Y., and He, J. (2023). Gas and light: triggers of c-di-GMPmediated regulation. *FEMS Microbiol. Rev.* 47 (4), fuad034. doi: 10.1093/femsre/fuad034

Zhang, Y., Pan, X., Wang, L., and Chen, L. (2021). Iron metabolism in Pseudomonas aeruginosa biofilm and the involved iron-targeted anti-biofilm strategies. *J. Drug Targeting* 29, 249–258. doi: 10.1080/1061186X.2020.1824235

Zhang, Y., Rosado-Lugo, J. D., Datta, P., Sun, Y., Cao, Y., Banerjee, A., et al. (2022b). Evaluation of a conformationally constrained indole carboxamide as a potential efflux pump inhibitor in pseudomonas aeruginosa. *Antibiot. Basel Switz.* 11, 716. doi: 10.3390/antibiotics11060716

Zhang, H., Wang, M., Han, X., Wang, T., Lei, Y., Rao, Y., et al. (2022a). The application of targeted nanopore sequencing for the identification of pathogens and resistance genes in lower respiratory tract infections. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.1065159

Zhao, F., Yang, H., Bi, D., Khaledi, A., and Qiao, M. (2020). A systematic review and meta-analysis of antibiotic resistance patterns, and the correlation between biofilm formation with virulence factors in uropathogenic E. coli isolated from urinary tract infections. *Microb. Pathog.* 144, 104196. doi: 10.1016/j.micpath.2020.104196

Zhou, J., Li, M., Chen, Q., Li, X., Chen, L., Dong, Z., et al. (2022). Programmable probiotics modulate inflammation and gut microbiota for inflammatory bowel disease treatment after effective oral delivery. *Nat. Commun.* 13, 3432. doi: 10.1038/s41467-022-31171-0

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 10 January 2024
DOI 10.3389/fcimb.2023.1332472

OPEN ACCESS

EDITED BY
Sanket Kaushik,
Amity University Jaipur, India

REVIEWED BY
Nubwa Medugu,
Nile University of Nigeria, Nigeria
Mateusz Hasso-Agopsowicz,
World Health Organization, Switzerland
Namdev Shivaji Togre,
Temple University, United States
Naseer Iqbal,
University of Colorado Anschutz Medical Campus, United States

*CORRESPONDENCE
Yanmin Bao
baoyanming1978@163.com
Shaowei Dong
michael.dong.85@gmail.com

†These authors have contributed equally to this work

RECEIVED 03 November 2023
ACCEPTED 19 December 2023
PUBLISHED 10 January 2024

CITATION
Shi X, Patil S, Wang Q, Liu Z, Zhu C, Wang H, Chen Y, Li L, Yang L, Zheng Y, Dong S and Bao Y (2024) Prevalence and resistance characteristics of multidrug-resistant *Streptococcus pneumoniae* isolated from the respiratory tracts of hospitalized children in Shenzhen, China.
Front. Cell. Infect. Microbiol. 13:1332472.
doi: 10.3389/fcimb.2023.1332472

Prevalence and resistance characteristics of multidrug-resistant *Streptococcus pneumoniae* isolated from the respiratory tracts of hospitalized children in Shenzhen, China

Xing Shi[1†], Sandip Patil[2†], Qing Wang[3], Zihao Liu[4], Chunqin Zhu[4], Heping Wang[4], Yunshen Chen[5], Liqiang Li[6], Liang Yang[6], Yuejie Zheng[4], Shaowei Dong[2*] and Yanmin Bao[4*]

[1]Department of Respiratory and Critical Care Medicine, Institute of Shenzhen Respiratory Diseases, The First Affiliated Hospital (Shenzhen People's Hospital), School of Medicine, Southern University of Science and Technology, Shenzhen, China, [2]Department of Haematology and Oncology, Shenzhen Children's Hospital, Shenzhen, Guangdong, China, [3]School of Medicine, Southern University of Science and Technology, Shenzhen, Guangdong, China, [4]Department of Respiratory Medicine, Shenzhen Children's Hospital, Shenzhen, Guangdong, China, [5]Department of Clinical Microbiology Laboratory, Shenzhen Children's Hospital, Shenzhen, Guangdong, China, [6]Shenzhen Third People's Hospital, National Clinical Research Centre for Infectious Diseases, The Second Affiliated Hospital of Southern University of Science and Technology, Shenzhen, Guangdong, China

Background: PCV13 introduction in China has led to a significant reduction of vaccine serotype *Streptococcus pneumoniae*. However, non-vaccine serotypes with highly resistance and invasiveness were often reported in the post-pneumococcal conjugate vaccine era and there was regional differences.

Methods: A total of 669 *S. pneumoniae* strains were collected from the respiratory tracts of hospitalized children at Shenzhen Children's Hospital in 2021 and 2022. Antimicrobial resistance (AMR) characteristics were assessed through antibiotic susceptibility testing performed with the VITEK 2 compact system. AMR genes and single nucleotide polymorphisms (SNPs) in pbp1a, pbp2b, and pbp2x were identified via analysis of whole genome sequencing data. Statistical examination of the data was conducted employing chi-square and Fisher's exact tests.

Results: We found that non-vaccine serotypes strains had accounted for 46.6% of all the pneumococcal isolated strains. The most common non-vaccine serotype is 23A, with a prevalence rate of 8.9%, followed by 15A (6.6%), 6E (5.7%), 34 (3.2%), and 15B (2.9%). The multidrug resistance rates (MDR) of vaccine serotypes were 19F (99.36%), 19A (100%), 23F (98.08%), 6B (100%), and 6C (100%). Meanwhile, the MDR of non-vaccine serotypes were 15B (100.00%), 6E (100%), 15C (100%), 34 (95.24%), and 23A (98.31%). Resistance rates of 6E to more than six antibiotic classes reached 89.47%, which is similar to 19F (83.33%) and 19A (90%). Unique resistance profiles were also identified for non-vaccine serotypes, including significantly higher resistance to chloramphenicol in 6E, 15B, and 15C than in 19F and 19A. Furthermore, through genome sequencing, we revealed strong correlation of *cat-TC* with chloramphenicol resistance, *patA/patB* with tetracycline resistance, *ermB* and *pmrA* with erythromycin resistance.

Conclusion: The introduction of PCV13 into China from 2017 has led to a shift in the dominant composition of pneumococcal strains. There has been a notable rise and spread of multidrug-resistant non-vaccine serotypes among children. Specifically, the non-vaccine serotype 6E, which was not widely reported in China previously, has emerged. To comprehend the resistance mechanisms, it is crucial to further investigate the molecular and genetic characteristics of these non-vaccine serotypes.

KEYWORDS

pneumococcus, PCV13, whole genome sequencing, non-vaccine serotype, MDR phenotype

Introduction

Streptococcus pneumoniae (pneumococcus), has a significant threat to children's health due to their underdeveloped immune systems (CDC, 2022). Pneumococcus frequently colonizes the respiratory tract of children (Fu et al., 2021), leading to various infections, such as community-acquired pneumonia, otitis media, bacteremia, and meningitis (Bogaert et al., 2004). Infections caused by pneumococcus is the leading cause of mortality in children under the age of five globally, responsible for an estimated 294, 000 deaths in HIV-negative children aged 1-59 months in 2015 (Wahl et al., 2018). Over the past two decades, the development of pneumococcal conjugate vaccines (PCVs) has led to a decline in the incidence of invasive pneumococcal disease (IPD) caused by vaccine serotypes (VTs) (Briles et al., 2019; Musher et al., 2022). PCVs also help to reduce antimicrobial resistance (AMR) by preventing infections, reducing antibiotic usage, and promoting herd immunity (Jansen et al., 2021).

However, with the rapid decline of vaccine serotypes after vaccination, there has been a relative increase in the proportion of cases caused by NVT compared to the pre-vaccine era (source: https://www.cdc.gov/pneumococcal/surveillance.html). This highlights the need for increased attention to the epidemiology of NVT serotypes. The prevalence of NVT serotypes varies across different regions, which may be attributed to variations in vaccine coverage, the pre-existing pool of serotypes before vaccination, and the use of different types and dosages of antibiotics. For example, in South Africa, NVTs 15B, 8, and 23B are more commonly associated with pneumococcal disease in children under two years old (Gottenberg et al., 2014). In the United States, NVTs such as 22F, 33F, and 15B/C have become more prevalent (Moore et al., 2016; Balsells et al., 2018). In Asia, NVTs such as 35B and 15A are commonly observed in Japan (Miyazaki et al., 2017), while in Korea, serotypes 10A, 34, and 22F have increased in prevalence after the introduction of PCV13(Kim et al., 2023). In Taiwan, NVTs such as 15A, 23A, 23B, and 34 have been frequently observed (Wu et al., 2020). The emergence of non-vaccine serotypes (NVTs) due to serotype replacement and the development of multi-drug resistance in NVTs pose ongoing challenges (Sings et al., 2019; Wu et al., 2022). Pneumococcus possesses high genome mutation capabilities and the ability to acquire resistance genes from other species by horizontal gene transfer (D'Aeth et al., 2021). Although vaccines offer strong protection against highly pathogenic and highly resistant strains, pneumococcus can still develop vaccine-escape strains that carry multiple resistances within the limits of genetic variation (Azarian et al., 2018). Indiscriminate antibiotic usage has also contributed to the selection pressure for highly resistant bacteria, complicating the treatment of pneumococcal infections. In Asian countries from 2008 to 2009, 59.3% of pneumococcus exhibited multidrug resistance (MDR), with the most prevalent serotypes being 19F, 23F, 19A, 14, and 6B, and the highest MDR rates were found in China at 83.3% (Kim et al., 2012). Importantly, the emergence of MDR in non-vaccine serotypes has caused extensive concern as the proportion of infections increases (Kim et al., 2020; Yamba Yamba et al., 2022). Therefore, it is crucial to monitor and comprehend the impact of PCV13 on serotype replacement and antibiotic resistance in children to inform treatment strategies and guide the development of effective pneumococcal vaccines.

In China, PCV13 was introduced in June 2017, and quickly replaced PCV7 to become the primary pneumococcal conjugate vaccine for children due to its broader serotype coverage and better cost-effectiveness (Ma et al., 2013; Li et al., 2021). Shenzhen is the city with the largest children population and the highest vaccination rate of PCV13 in the south of China. In the clinical data of hospitalized children from 2021 to 2022, we found that the vaccination rate has exceeded 80% in Shenzhen. To promptly and efficiently monitor the characteristics of non-vaccine serotypes in the context of rising vaccination rates, we identified and compared the serotype distribution, resistance profiles, and carried resistance genes of 669 strains collected from the children's respiratory tract, who were hospitalized because of different kinds of respiratory infection diseases in 2021 and 2022. Our findings revealed a high prevalence of MDR among NVT serotypes, particularly 6E, which exhibited a non-susceptibility rate of 89.47% to more than six classes of antibiotics, akin to 19F. Given the lack of effective

defense against NVT strains, monitoring the drug resistance of NVT is of paramount importance. Furthermore, molecular detection demonstrated a strong correlation between non-susceptibility to different antibiotics and the presence of detected genetic materials, indicating a more efficient approach to monitoring changes in resistance trends among prevalent serotypes.

Materials and methods

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee, Shenzhen Children's Hospital, reference number: 202200302 which complies with international ethical standards.

Sputum and BALF culture

A total of 669 non-duplicated (one specimen from one patients) *Streptococcus pneumoniae* strains were isolated from sputum and bronchoalveolar lavage fluid (BALF) samples collected from the children's respiratory tract in the largest tertiary children's hospital in south of China, Shenzhen Children's Hospital, between March, 1, 2021 and March, 31, 2022. The clinical information, including age, sex, source of samples, disease types, vaccination status, etc., is collected.

Sputum and bronchoalveolar lavage fluid (BALF) samples were routinely collected for etiological detection from a diverse patient population with various respiratory illnesses, including acute bronchopneumonia, protracted bacterial bronchitis, bronchiolitis, severe pneumonia, acute bronchitis, chronic pneumonia, bacteremia, and asthma. Upon microscopic examination of the smear, Sputum specimens with white blood cells >25 per low magnification field and squamous epithelial cells <10 per low magnification field are considered satisfactory and subsequently included in the study. The specimens were cultured on blood agar plates, and the resulting colonies were examined for morphology and phenotypic characteristics. The identification of *S. pneumoniae* isolates was performed according to the guidelines provided in the Manual of Clinical Microbiology, 11th edition. Furthermore, the isolates were confirmed using two complementary methods: first, by using automated VITEK 2 system (Biomerieux, France) and second by using mass spectrometry (MALDI-TOF MS, Merier, France). MALDI-TOF MS is a rapid and reliable method for microbial identification that involves the analysis of mass spectra generated from ionised microbial proteins. All strains were preserved in 40% glycerol broth medium at -80°C and were re-culture on 5% horse blood agar under conditions of 5% CO2 and 37°C for 12-15 hours before being subjected to further studies.

Antibiotic sensitivity testing

The antimicrobial susceptibility test (AST) was performed for all 669 confirmed *S. pneumoniae* isolates using the VITEK 2 compact system (BioMerieux, France) with AST-GP68 card. AST was tested for commonly used antibiotics includes; clindamycin (CLI), cefotaxime (CTX), ceftriaxone (CRO), penicillin (PEN), chloramphenicol (CHL), ertapenem (ETP), meropenem (MEM), erythromycin (ERY), linezolid (LNZ), levofloxacin (LVX), moxifloxacin (MFX), ofloxacin (OFX), tetracycline (TCY), trimethoprim-sulfamethoxazole (SXT), and vancomycin (VAN). The AST results were accurately interpreted according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints 2021 (CLSI, 2021). The results for penicillin were interpreted using non-meningitis oral administration breakpoints, whereby susceptibility was defined as ≤ 0.06 μg/ml, intermediate as 0.12-1 μg/ml, and resistance as ≥2 μg/ml. The classification of multi-drug resistance (MDR) phenotype was performed as described previously by Magiorakos et al., MDR was defined as resistance to at least one agent in three or more antimicrobial groups (Magiorakos et al., 2012). MDR3, MDR4, MDR5, and MDR6 were used to represent non-susceptibility to at least 3, 4, 5, and 6 classes of antimicrobials, respectively.

Molecular analysis

The whole-genome DNA was extracted using the TianGen Magnetic Bead Soil and Fecal Genomic DNA Extraction Kit from TianGen, China. The extracted DNA was then quantified using the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific). The DNA library was prepared using the TruSeq® DNA PCR-Free Sample Preparation Kit from Illumina, San Diego, CA, USA. Sequencing was performed on an Illumina Novaseq6000 from Illumina, San Diego, CA, USA. To ensure high quality, sequence segments with low quality were removed using Trimmomatic v0.36 (Bolger et al., 2014) with a SLIDINGWINDOW parameter of 4:20 and a MINLEN parameter of 70. The remaining high-quality reads were assembled using SPAdes v3.11 (Prjibelski et al., 2020). For molecular serotyping, raw reads were analyzed using seroBA v1.0.2 (Epping et al., 2018) with the default parameters and a recommended k-mer size of 71. Antimicrobial resistance genes (ARGs) were detected by performing a blast search (Camacho et al., 2009) against the CARD database (https://card.mcmaster.ca). Genes with a nucleotide identity higher than 99% were considered ARGs.

Statistical analysis

Statistical analysis was performed to compare antibiotic-resistant profiles and genes using the chi-square test and Fisher's exact test. $p < 0.05$ was considered statistically significant.

Results

Epidemiology of pneumococcal infections

An overview of demographic information for 669 patients, including age, sex, and sample types provides Table 1. The

distribution of male (n=396, 59.4%) and female (n = 271, 40.6%) patients is nearly even, with a slight male predominance (Figure 1). Sputum samples comprise the majority of samples (n = 613, 91.6%), while bronchoalveolar lavage fluid (BALF) samples account for the remaining 8.4% (n=56). The median age of patients is 24.9 months, with an interquartile range (IQR) of 12.0-43.0 months. All these children were admitted into Shenzhen children's hospital because of acute or chronic lower respiratory tract infection, including acute bronchopneumonia, protracted bacteria bronchitis and so on.

Distribution of serotypes and coverage of PCVs

Overall, this study reveals the NVT group has a high detection rate of 46.61% among all 669 samples, which is close to the VT group's 53.39%. The most prevalent vaccine serotype is 19F, with a prevalence rate of 23.38%, followed by 23F (7.84%), 6B (7.84%), 6A (4.07%), and 14 (3.02%). The most prevalent non-vaccine serotype is 23A, with a prevalence rate of 8.9%, followed by 15A (6.64%), 6E (5.73%), 34 (3.17%), and 15B (2.87%) (Figure 2A). The data also indicates a consequential shift in prevalence due to vaccination application, with a decrease in VT strains and a corresponding increase in NVT strains (Figure 2B).

TABLE 1 Clinical characteristics.

Characteristics	All patients (n=669)
Age, median (IQR), month	24.9 (12.0-43.0)
Sex, n (%)	
Male	396 (59.4)
Female	271 (40.6)
Sources, n (%)	
BALF	56 (8.4)
Sputum	613 (91.6)
Major Diseases	
Acute bronchopneumonia	336
Acute bronchitis	60
Severe pneumonia	36
Bronchiolitis	22
Protracted bacterial bronchitis	21
Acute asthmatic bronchitis	18
Prolonged pneumonia	13
Sepsis	10
Chronic cough	8
Bronchiolitis	8
Others	137

The antimicrobial susceptibility testing

The result of antimicrobial susceptibility analysis of the ten classes of antibiotics was presented in Table 2. Based on the AMR profiles, lincosamides and macrolides have the highest rates of non-susceptibility in the major serotypes tested, with rates ranging from 94.4% to 100%, while oxazolidinones, fluoroquinolones, and glycopeptides have extremely low rates of non-susceptibility (Table 2). For penicillin, cefotaxime and ceftriaxone, which are often used to treat pneumococcal diseases in China, in common VTs (19F, 19A, 23F, 14, 6A and 6B), the non-susceptibility rate of penicillin were between 95.0% and 97.4%, the non-susceptibility rate of cefotaxime were between 0% and 67.7%, the non-susceptibility rate of ceftriaxone were between 0 and 60.6%, respectively. The serotype of 19F and 19A had the highest drug-resistance to penicillin, cefotaxime and ceftriaxone. In common NVTs (23A, 15A/B/C, 6E and 6C), the non-susceptibility rates of penicillin resistance were between 47.4% and 98.3%, the non-susceptibility rate of cefotaxime were between 0% and 23.5%, the cefotaxime rate of ceftriaxone resistance between 0 and 1.7% respectively. The serotype of 6E and 23A had the highest drug-resistance to penicillin in these common NVTs strain. (Table 2). Furthermore, the resistance rate of serotype 6E to chloramphenicol is significantly higher compared to serotype 19F and other serotypes ($p < 0.001$, Supplementary Table 1).

MDR profiling

The MDR combination patterns vary across different serotypes, as demonstrated in Table 3. The combination of CLI|ERY|TCY is the most frequently observed MDR pattern across all serotypes. The specific MDR combinations differ among serotypes due to resistance to other specific antibiotics. For instance, within the 19F serotype, the most common MDR combination is CLI|ERY|TCY|PEN|SXT|MEM|CRO|CTX (83/156), while within the 6E serotype, the most common MDR combination is CLI|ERY|TCY|PEN|SXT|MEM|CHL (20/38). Through comparison of this combination, we found that chloramphenicol replacing ceftriaxone and cefotaxime constitutes the most common MDR combination of 6E.

MDR patterns of NVT serotype 6E compared to VT serotype 6B

As an NVT serotype, 6E has a similar occurrence rate compared to the VT serotype 6B, as shown in Figure 2A However, 6E exhibits significantly higher rates of multidrug resistance (MDR) than 6B, particularly in MDR5, and MDR6, with rates of 92.1%, and 89.5%, respectively, compared to 6B's rates of 84.6%, and 42.3%, respectively (Table 4). Additionally, 6E shows a higher resistance rate to antibiotics such as chloramphenicol and meropenem, where 25/38 and 34/38 bacterial strains are non-susceptible, respectively, compared to 6B with 0/52 and 22/52 non-susceptible strains ($p <$

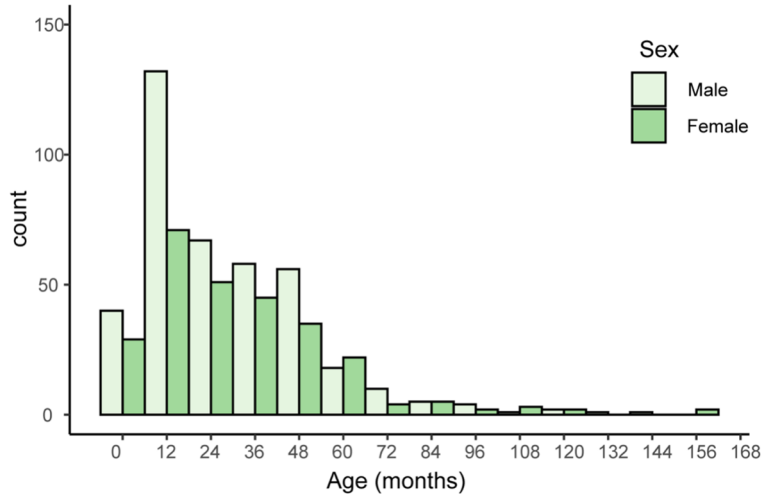


FIGURE 1
Histogram of patients' age and sex. The x-axis represents age in months, with 12-month intervals. The y-axis represents the number of individuals with a given serotype within a particular age range.

0.01, Table 5). These findings suggest that both distinct MDR patterns and vaccine use could play crucial roles in shaping the future prevalence of serotypes 6E and 6B. Therefore, further analysis and monitoring of the spreading of these serotypes are necessary to fully understand their eventual impact.

Characteristics of antibiotic resistance genes

In our study, we observed a strong correlation between the detection of antibiotic resistance genes and the corresponding antibiotic resistance profiles, as illustrated in Figure 3. Specifically, the *cat-TC* gene exhibited the highest consistency of 99.4% with chloramphenicol non-susceptibility. This was followed by the *tetM* gene which showed consistency of 97.3% with tetracycline non-susceptibility. And *ErmB* gene showed consistency of 96.5% with erythromycin non-susceptibility, while the *PatA/PatB* gene showed a consistency of 92.3% with tetracycline non-susceptibility. Lastly, the *pmrA* gene exhibited a correlation of 89.1% with erythromycin non-susceptibility. These findings suggest that the identification of specific antibiotic resistance genes can serve as reliable predictors of the corresponding antibiotic resistance profiles. In addition, we found dozens important polymorphic loci of PBP (Penicillin-binding protein) genes that are strongly associated with penicillin susceptibility, prominently including pbp2b's A1336G (Thr446Ala), T1542C, pbp1a's T1740A and pbp2x's T/G1713A (Supplementary Figure 1; Supplementary Table 2). These polymorphic sites can be used to accurately predict penicillin susceptibility.

Discussion

In Chinese mainland, PCV13 was introduced in 2017. However, it has not been incorporated into the national immunization schedule, resulting in a low nationwide vaccination rate. Shenzhen, the largest city in southern China and a significant economic hub, presents a contrasting picture with an estimated 50% of children receiving the PCV13 vaccine, according to data from the Chinese CDC. However, in the clinical data we gathered from Shenzhen Children's Hospital for the years 2021 to 2022, pertaining to hospitalized children, it was observed that the vaccination rate among these patients has surpassed 80%. Given this, it becomes crucial to investigate the potential changes in the prevalence of NVTs in Shenzhen in the post-PCV13 era.

As the prevalence of NVT serotypes increase, we also found a concurrent shift in the overall drug resistance profile. A study in Taiwan found that the non-susceptibility of pneumococcus to penicillin, cefotaxime and ceftriaxone was decreased along with the decrease of serotypes 19F and 19A (Huang et al., 2022), which is consistent with our data that 19F and 19A provide the majority non-susceptibility to penicillin, cefotaxime and ceftriaxone, while the major NVT serotypes are much more susceptible to these three kinds of antibiotics. It is worth noting that NVT resistance possesses distinctive uniqueness which leads to the emergence of multidrug-resistant serotypes (Lo et al., 2022). Our data reveals that in all airway samples, 65.8% of strains of serotype 6E, 42.1% of strains of serotype 15B, and 41.2% of strains of serotype 15C are non-susceptible to chloramphenicol, while most VT serotypes are susceptible to this antibiotic. Serotype 6E, the third most prevalent NVT serotype, exhibits a comparable level of MDR to serotypes 19F and 19A, with a 89.5% non-susceptibility rate to six classes of antibiotics. With the increasing prevalence of serotypes 6E and 15B/C detected in IPD, it is crucial to closely monitor their MDR profiles (Lo et al., 2022).

The genetic flexibility of Pneumococci allows for rapid adaptation to changing environmental pressures, including vaccine selection pressure (Nj et al., 2013). Resistance to specific antibiotics can result from mutations and genetic recombination in chromosomal genes, such as *pbp2x, pbp2b, and pbp1a*, which confer

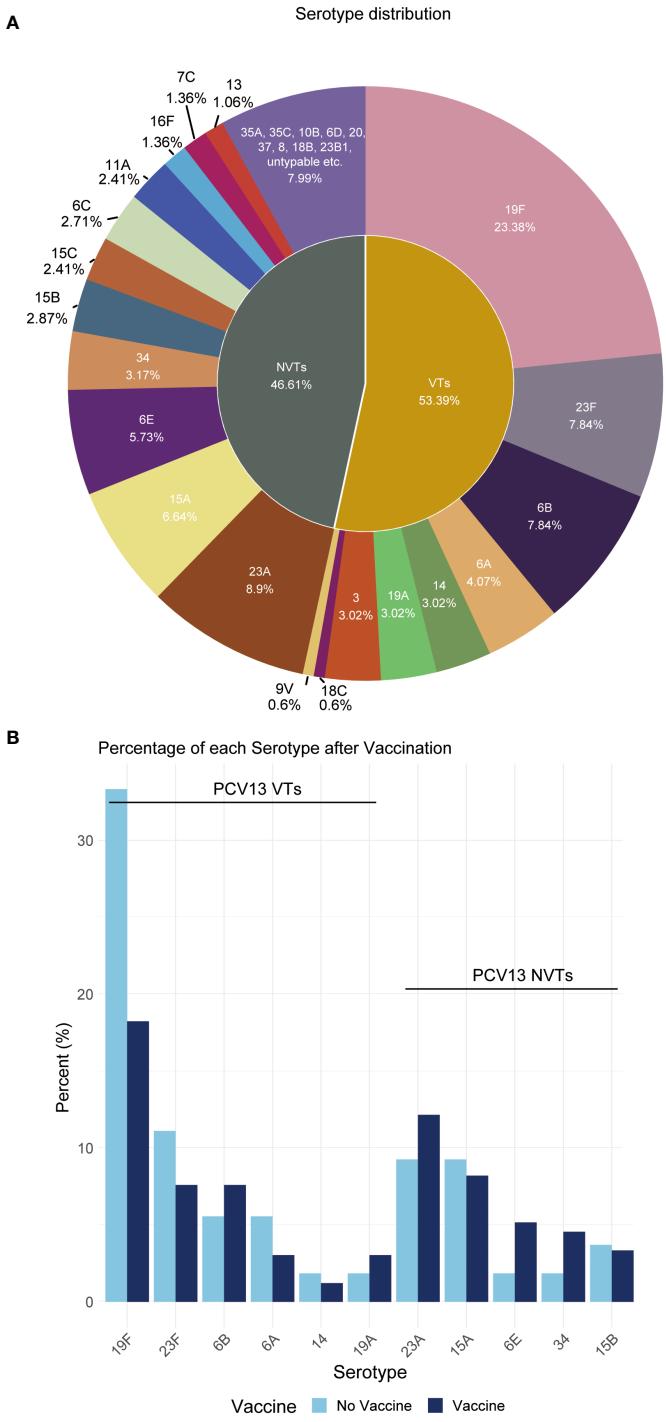


FIGURE 2
Distribution proportion of *S. pneumoniae* serotypes. **(A)** This pie chart shows the distribution of serotypes observed in all children. Each slice represents a different serotype, with the size of the slice proportional to the percent of that serotype. **(B)** Percentage of each serotype pre- and post-vaccination.

resistance to beta-lactam antibiotics (Mosadegh et al., 2022). Additionally, antibiotic resistance genes are often carried on mobile genetic elements (MGEs). For example, Tn916-type integrative and conjugative elements (ICEs) can transfer resistance genes for tetracycline and erythromycin, such as *tetM* and *ermB* (Cochetti et al., 2007; Croucher et al., 2009). The *cat-TC* gene, which confers resistance to chloramphenicol, is often carried on the transposon Tn5253, which serves as an important hotspot for insertion sites (Shoemaker et al., 1979; Ayoubi et al., 1991; Santoro et al., 2019). In our data, we found that the resistant genes *pmrA*, *ermB*, *rlmA*^{II}, *pafA*/*B*, and *tetM* are commonly present in streptococcus, which contribute to the widespread multidrug resistance patterns against macrolides, tetracyclines, and clindamycin. In addition, our study highlights the significance of

TABLE 2 AMR profiles of major serotypes.

	Antibiotics	19F (NS %)	23F (NS %)	6B (NS %)	6A (NS %)	14 (NS %)	19A (NS %)	23A (NS %)	15A (NS %)	6E (NS %)	34 (NS %)	15B (NS %)	15C (NS %)	6C (NS %)
Lincosamides	CLI	152 (100.0)	52 (100.0)	52 (100.0)	27 (100.0)	20 (100.0)	20 (100.0)	59 (100.0)	44 (100.0)	38 (100.0)	21 (100.0)	18 (100.0)	17 (100.0)	18 (100.0)
Beta-Lactam	CTX	155 (67.7)	52 (11.5)	52 (3.8)	27 (0.0)	20 (25.0)	20 (35.0)	59 (6.8)	44 (2.3)	38 (2.6)	21 (0.0)	19 (10.5)	17 (23.5)	18 (0.0)
	CRO	155 (60.6)	52 (5.8)	52 (3.8)	27 (0.0)	20 (20.0)	20 (25.0)	59 (1.7)	44 (0.0)	38 (0.0)	21 (0.0)	19 (0.0)	17 (0.0)	18 (0.0)
	PEN	155 (97.4)	52 (96.2)	52 (90.4)	27 (96.3)	20 (95.0)	20 (95.0)	59 (98.3)	44 (72.7)	38 (97.4)	21 (57.1)	19 (47.4)	17 (52.9)	18 (50.0)
Phenicols	CHL	155 (0.6)	52 (1.9)	52 (0.0)	27 (0.0)	20 (0.0)	20 (0.0)	59 (0.0)	44 (0.0)	38 (65.8)	21 (0.0)	19 (42.1)	17 (41.2)	18 (5.6)
Carbapenem	ETP	155 (1.9)	52 (1.9)	52 (0.0)	27 (0.0)	20 (0.0)	20 (0.0)	59 (0.0)	44 (0.0)	38 (0.0)	21 (0.0)	19 (10.5)	17 (0.0)	18 (0.0)
	MEM	151 (93.4)	51 (66.7)	52 (42.3)	27 (55.6)	20 (95.0)	20 (90.0)	59 (18.6)	44 (40.9)	38 (89.5)	21 (14.3)	19 (36.8)	17 (52.9)	18 (27.8)
Macrolide	ERY	152 (99.3)	51 (98.0)	51 (96.1)	25 (100.0)	20 (100.0)	20 (100.0)	55 (98.2)	41 (97.6)	38 (100.0)	19 (100.0)	18 (94.4)	16 (100.0)	17 (100.0)
Oxazolidinone	LNZ	155 (0.0)	52 (0.0)	52 (0.0)	27 (0.0)	20 (0.0)	20 (0.0)	59 (0.0)	44 (0.0)	38 (0.0)	21 (0.0)	19 (0.0)	17 (0.0)	18 (0.0)
Fluoroquinolone	LVX	155 (0.0)	52 (0.0)	52 (0.0)	27 (0.0)	20 (0.0)	20 (0.0)	59 (0.0)	44 (2.3)	38 (0.0)	21 (0.0)	19 (0.0)	17 (0.0)	18 (0.0)
	MFX	155 (0.0)	52 (0.0)	52 (0.0)	27 (0.0)	20 (0.0)	20 (0.0)	59 (0.0)	44 (0.0)	38 (0.0)	21 (0.0)	19 (0.0)	17 (0.0)	18 (0.0)
	OFX	155 (0.0)	52 (0.0)	52 (0.0)	27 (0.0)	20 (0.0)	20 (0.0)	59 (11.9)	44 (2.3)	38 (0.0)	21 (0.0)	19 (0.0)	17 (0.0)	18 (0.0)
Tetracycline	TCY	155 (96.8)	52 (96.2)	52 (96.2)	27 (74.1)	20 (90.0)	20 (100.0)	58 (96.6)	44 (75.0)	38 (89.5)	21 (85.7)	19 (94.7)	17 (94.1)	18 (94.4)
Folate pathway antagonists	SXT	155 (98.7)	52 (96.2)	52 (100.0)	27 (70.4)	20 (5.0)	20 (100.0)	59 (45.8)	44 (6.8)	38 (92.1)	21 (90.5)	19 (94.7)	17 (94.1)	18 (83.3)
Glycopeptide	VAN	155 (0.0)	52 (0.0)	52 (0.0)	27 (0.0)	20 (0.0)	20 (0.0)	59 (0.0)	44 (0.0)	38 (0.0)	21 (0.0)	19 (0.0)	17 (0.0)	18 (0.0)

CLI, clindamycin; CTX, cefotaxime; CRO, ceftriaxone; PEN, penicillin; CHL, chloramphenicol; ETP, ertapenem; MEM, meropenem; ERY, erythromycin; LNZ, linezolid; LVX, levofloxacin; MFX, moxifloxacin; OFX, ofloxacin; TCY, tetracycline; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin.

10.3389/fcimb.2023.1332472

TABLE 3 The common antimicrobial resistance patterns of pneumococcus.

	Total	19F	23F	6B	6A	14	19A	23A	15A	6E	34	15B	15C	6C
CLI/ERY/TCY	10								6	1		1	1	1
CLI/ERY/TCY/SXT	23	2	1	5	1		1							7
CLI|PEN|ERY	11				7			1	2					1
CLI|PEN|ERY|TCY	38	1	1					27	6	2				1
CLI|PEN|ERY|TCY|SXT	65	6	13	21	2		1	6	3	1	8	2		2
CLI|PEN|ERY|TCY|SXT|MEM	134	34	26	21	14		1	7		9	1	3	4	4
CLI|PEN|ERY|TCY|MEM	32	1			1	12			16		2			
CLI|PEN|ERY|TCY|SXT|MEM|CRO|CTX	91	83	1	1			5	1						
CLI|PEN|ERY|TCY|SXT|MEM|CTX	23	10	3				2	3				1	4	
CLI|PEN|ERY|TCY|SXT|MEM|CHL	21									20				1

CLI, clindamycin; CTX, cefotaxime; CRO, ceftriaxone; PEN, penicillin; CHL, chloramphenicol; ETP, ertapenem; MEM, meropenem; ERY, erythromycin; LNZ, linezolid; LVX, levofloxacin; MFX, moxifloxacin; OFX, ofloxacin; TCY, tetracycline; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin.

TABLE 4 MDR rate of major serotypes.

Serotypes	Total strains	No. of MDR3 strains	No. of MDR4 strains	No. of MDR5 strains	No. of MDR6 strains	MDR3%	MDR4%	MDR5%	MDR6%
6E	38	38	37	35	34	100.00	97.37	92.11	89.47
19F	156	155	154	151	130	99.36	98.72	96.79	83.33
19A	20	20	20	19	18	100.00	100.00	95.00	90.00
23F	52	51	51	48	33	98.08	98.08	92.31	63.46
15C	17	17	16	15	8	100.00	94.12	88.24	47.06
6B	52	52	50	44	22	100.00	96.15	84.62	42.31
15B	19	19	17	15	6	100.00	89.47	78.95	31.58
6A	27	27	20	17	14	100.00	74.07	62.96	51.85
14	20	19	19	19	0	95.00	95.00	95.00	0.00
23A	59	58	54	24	18	98.31	91.53	40.68	30.51
6C	18	18	15	7	5	100.00	83.33	38.89	27.78
34	21	20	18	11	1	95.24	85.71	52.38	4.76
15A	44	37	27	20	0	84.09	61.36	45.45	0.00

CLI, clindamycin; CTX, cefotaxime; CRO, ceftriaxone; PEN, penicillin; CHL, chloramphenicol; ETP, ertapenem; MEM, meropenem; ERY, erythromycin; LNZ, linezolid; LVX, levofloxacin; MFX, moxifloxacin; OFX, ofloxacin; TCY, tetracycline; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin.

TABLE 5 Comparison of antibiotic resistance of 6E and 6B.

	6E			6B			p
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant	
CHL	13	0	25	52	0	0	< 0.001
CLI	0	0	38	0	0	52	1.000
CRO	38	0	0	50	2	0	0.507
CTX	37	1	0	50	1	1	1.000
ERY	0	0	38	2	0	49	0.505

(Continued)

10.3389/fcimb.2023.1332472

TABLE 5 Continued

	6E			6B			p
ETP	38	0	0	52	0	0	1.000
LNZ	38	0	0	52	0	0	1.000
LVX	38	0	0	52	0	0	1.000
MEM	4	31	3	30	21	1	< 0.001
MFX	38	0	0	52	0	0	1.000
OFX	38	0	0	52	0	0	1.000
PEN	1	32	5	5	44	3	0.245
SXT	3	0	35	0	42	10	< 0.001
TCY	4	1	33	2	1	49	0.422
VAN	38	0	0	52	0	0	1.000

CLI, clindamycin; CTX, cefotaxime; CRO, ceftriaxone; PEN, penicillin; CHL, chloramphenicol; ETP, ertapenem; MEM, meropenem; ERY, erythromycin; LNZ, linezolid; LVX, levofloxacin; MFX, moxifloxacin; OFX, ofloxacin; TCY, tetracycline; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin.

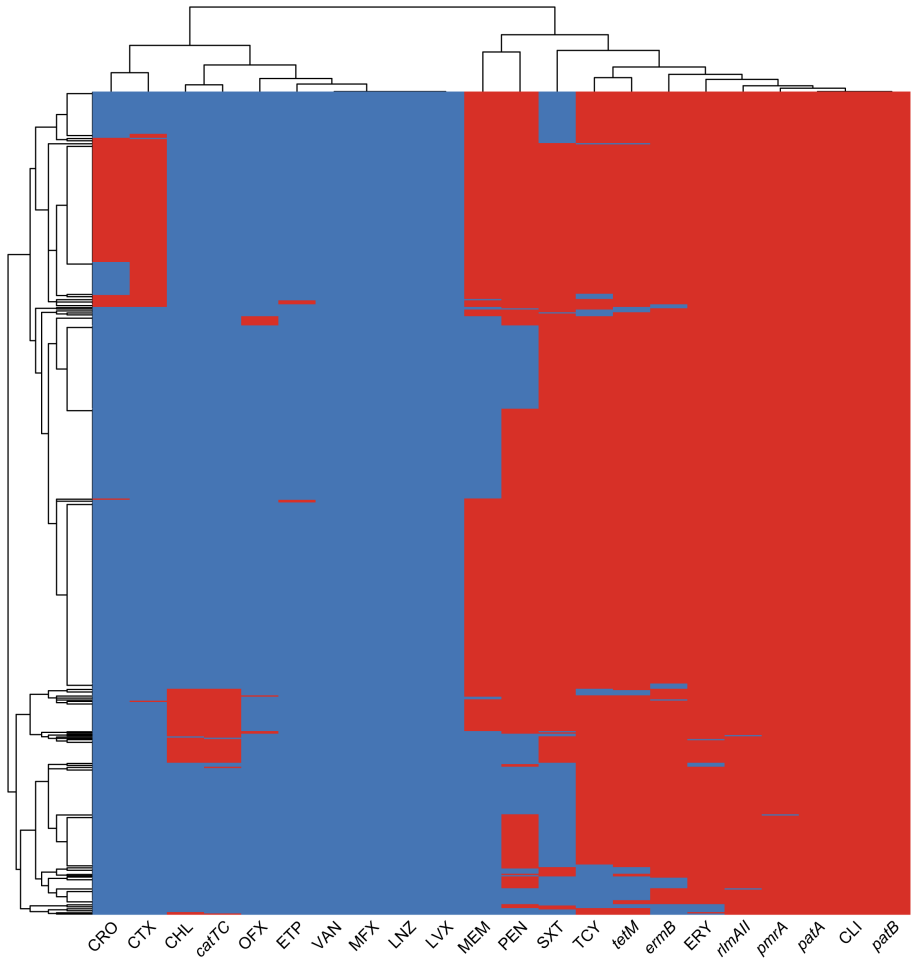


FIGURE 3
The heatmap of antimicrobial resistance phenotypes and resistance genes co-detection. The heatmap displays the clustering results of ARGs and AMR profiles in bacterial isolates. The color of each cell in the heatmap indicates the non-susceptibility (red), and susceptibility (blue) of a particular kind of antibiotic, and the presence (red) or absence (blue) of a particular ARG in a *S. pneumoniae* isolate.

both synonymous and non-synonymous mutation sites in PBP alleles. The non-synonymous mutation, specifically the PBP2b Thr446Ala, which is known to contribute to adaptive resistance to penicillin (Pagliero et al., 2004), holds the highest predictive importance value. However, the results also suggest that synonymous mutation sites could be valuable predictors of penicillin resistance, potentially due to their impact on PBP proteins' expression or function. In summary, to effectively control the spread of antibiotic resistance in *S. pneumoniae*, a multifaceted approach is required. This includes the judicious use of antibiotics, monitoring resistance patterns, and developing new strategies for prevention and treatment of infections caused by resistant strains. The combination of antibiotic resistance genes and chromosomal mutations in different serotypes can result in varying resistance profiles, highlighting the need for continued monitoring of antibiotic resistance in pneumococcal populations. Additionally, due to the limited coverage of vaccines, it is essential to pay attention to multidrug resistance in NVT serotypes of *S. pneumoniae*, as they lack specific preventive measures. Thus, it is necessary to strengthen monitoring and treatment of NVT serotypes of *S. pneumoniae* to reduce their threat to public health, particularly serotype 6E, which exhibits a high level of MDR and is not currently covered by any existing vaccine.

Limitation: It is important to note that this was a retrospective, single-center study and the isolated strains were collected within a year from March 2021 to March 2022. The provided data cannot represent the overall situation of pneumococcal vaccine usage and serotype prevalence in China. However, given the increasing vaccination rates year by year, it can serve as a basis for predicting future trends. Direct comparison data on the drug resistance of pre-vaccine serotypes were not available, and our findings only provide the antimicrobial drug resistance rate for airway colonized serotypes, without inclusion of invasive strains. To address these limitations, we plan to extend our work by including samples from other parts of the country and invasive strains to gain a more comprehensive understanding of the drug resistance patterns. This could potentially provide valuable insights into the emergence of multidrug-resistant non-vaccine type serotypes and guide the development of effective prevention and treatment strategies.

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.

Ethics statement

The studies involving humans were approved by Shenzhen Children's Hospital Institutional Animal Care and Use Committee. 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

XS: Conceptualization, Data curation, Validation, Writing – original draft. SP: Project administration, Writing – review & editing. QW: Data curation, Validation, Writing – original draft. ZL: Formal Analysis, Methodology, Validation, Writing – review & editing. CZ: Formal Analysis, Validation, Writing – review & editing. HW: Methodology, Writing – review & editing. YC: Validation, Writing – review & editing. LL: Data curation, Project administration, Writing – review & editing. LY: Project administration, Writing – review & editing. YZ: Validation, Writing – review & editing. SD: Conceptualization, Methodology, Writing – review & editing. YB: Conceptualization, Funding acquisition, Project administration, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The study was supported by Shenzhen Fundamental Research Program (JCYJ20180228175330567, JCYJ20210324115607021, JCYJ20220530152800001).

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1332472/full#supplementary-material

SUPPLEMENTARY TABLE 1
Comparison of antibiotic resistance of 6E and 19F.

SUPPLEMENTARY TABLE 2
Polymorphic loci of pbp genes associated with penicillin resistance.

SUPPLEMENTARY FIGURE 1
Penicillin-related pbp gene polymorphic sites and their odd ratios.

References

Ayoubi, P., Kilic, A. O., and Vijakumar, M. N. (1991). Tn5253, the pneumococcal omega (cat tet) BM6001 element, is a composite structure of two conjugative transposons, Tn5251 and Tn5252. *J. Bacteriol.* 173 (5), 1617–1622. doi: 10.1128/jb.173.5.1617-1622.1991

Azarian, T., Grant, L. R., Arnold, B. J., Hammitt, L. L., Reid, R., Santosham, M., et al. (2018). The impact of serotype-specific vaccination on phylodynamic parameters of Streptococcus pneumoniae and the pneumococcal pan-genome. *PloS Pathog.* 14, e1006966. doi: 10.1371/journal.ppat.1006966

Balsells, E., Dagan, R., Yildirim, I., Gounder, P. P., Steens, A., Muñoz-Almagro, C., et al. (2018). The relative invasive disease potential of Streptococcus pneumoniae among children after PCV introduction: A systematic review and meta-analysis. *J. Infect.* 77, 368–378. doi: 10.1016/j.jinf.2018.06.004

Bogaert, D., Groot, R., and Hermans, P. W. M. (2004). Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *Lancet Infect. Dis.* 4, 144–154. doi: 10.1016/S1473-3099(04)00938-7

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

Briles, D. E., Paton, J. C., Mukerji, R., Swiatlo, E., and Crain, M. J. (2019). Pneumococcal vaccines. *Microbiol. Spectrum.* doi: 10.1128/microbiolspec.GPP3-0028-2018

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

CDC. (2022). *Types of Pneumococcal Disease*. Available at: https://www.cdc.gov/pneumococcal/about/infection-types.html.

CLSI. (2021). *Performance Standards for Antimicrobial Susceptibility Testing. M100, 31st ed.* (Wayne, PA: Clinical and Laboratory Standards Institute).

Cochetti, I., Tili, E., Vecchi, M., Manzin, A., Mingoia, M., Varaldo, P. E., et al. (2007). New Tn916-related elements causing erm(B)-mediated erythromycin resistance in tetracycline-susceptible pneumococci. *J. Antimicrob. Chemother.* 60, 127–131. doi: 10.1093/jac/dkm120

Croucher, N. J., Finkelstein, J. A., Pelton, S. I., Mitchell, P. K., Lee, G. M., Parkhill, J., et al. (2013). Population genomics of post-vaccine changes in pneumococcal epidemiology. *Nat. Genet.* 45, 656–663. doi: 10.1038/ng.2625

Croucher, N. J., Walker, D., Romero, P., Lennard, N., Paterson, G. K., Bason, N. C., et al. (2009). Role of conjugative elements in the evolution of the multidrug-resistant pandemic clone streptococcus pneumoniaeSpain23F ST81. *J. Bacteriol* 191, 1480. doi: 10.1128/JB.01343-08

D'Aeth, J. C., van der Linden, M. P., McGee, L., de Lencastre, H., Turner, P., Song, J.-H., et al. (2021). The role of interspecies recombination in the evolution of antibiotic-resistant pneumococci. *eLife* 10, e67113. doi: 10.7554/eLife.67113

Epping, L., Tonder, A. J., Gladstone, R. A.The Global Pneumococcal Sequencing Consortium, , Bentley, S. D., Page, A. J., et al. (2018). SeroBA: rapid high-throughput serotyping of Streptococcus pneumoniae from whole genome sequence data. *Microb. Genom.* 4, e000186. doi: 10.1099/mgen.0.000186

Fu, P., Xu, H., Jing, C., Deng, J., Wang, H., Hua, C., et al. (2021). Bacterial epidemiology and antimicrobial resistance profiles in children reported by the ISPED program in China to 2020. *Microbiol. Spectr.* 9, e00283–e00221. doi: 10.1128/Spectrum.00283-21

Gottenberg, A. V., Gouveia, L. de S., Tempia, V., Quan, S., Mollendorf, C. v., Meiring, S., et al. (2014). Effects of vaccination on invasive pneumococcal disease in South Africa. *New Engl. J. Med.* 371, 1889–1899. doi: 10.1056/NEJMoa1401914

Huang, H., Lin, C. Y., Chiu, N. C., Huang, D. T., Huang, C. Y., and Chi, H. (2022). Antimicrobial susceptibility and serotype replacement of Streptococcus pneumoniae in children before and after PCV13 introduction in Taiwan. *J. Microbiol. Immunol. Infect.* 56, 299–310. doi: 10.1016/j.jmii.2022.08.018

Jansen, K. U., Gruber, W. C., Simon, R., Wassil, J., and Anderson, A. S. (2021). The impact of human vaccines on bacterial antimicrobial resistance. A review. *Environ. Chem. Lett.* 19, 4031–4062. doi: 10.1007/s10311-021-01274-z

Kim, S. H., Chung, D. R., Song, J. H., Baek, J. Y., Thamlikitkul, V., Wang, H., et al. (2020). Changes in serotype distribution and antimicrobial resistance of Streptococcus pneumoniae isolates from adult patients in Asia: Emergence of drug-resistant non-vaccine serotypes. *Vaccine* 38, 6065–6073. doi: 10.1016/j.vaccine.2019.09.065

Kim, G. R., Kim, E.-Y., Kim, S. H., Lee, H. K., Lee, J., Shin, J. H., et al. (2023). Serotype distribution and antimicrobial resistance of streptococcus pneumoniae causing invasive pneumococcal disease in korea between 2017 and 2019 after introduction of the 13-valent pneumococcal conjugate vaccine. *Ann. Lab. Med.* 43, 45–54. doi: 10.3343/alm.2023.43.1.45

Kim, S. H., Song, J.-H., Chung, D. R., Thamlikitkul, V., Yang, Y., Wang, H., et al. (2012). Changing trends in antimicrobial resistance and serotypes of streptococcus pneumoniae isolates in asian countries: an asian network for surveillance of resistant pathogens (ANSORP) study. *Antimicrob. Agents Chemother.* 56, 1418. doi: 10.1128/AAC.05658-11

Li, Y., Wang, H., Furnback, W., Wang, B. C. M., Zhu, S., and Dong, P. (2021). The cost-effectiveness of 13-valent pneumococcal conjugate vaccine in seven chinese cities. *Vaccines* 9, 1368. doi: 10.3390/vaccines9111368

Lo, S. W., Mellor, K., Cohen, R., Alonso, A. R., Belman, S., Kumar, N., et al. (2022). Emergence of a multidrug-resistant and virulent Streptococcus pneumoniae lineage mediates serotype replacement after PCV13: an international whole-genome sequencing study. *Lancet Microbe* 3, e735. doi: 10.1016/S2666-5247(22)00158-6

Ma, X., Zhao, R., Ma, Z., Yao, K., Yu, S., Zheng, Y., et al. (2013). Serotype distribution and antimicrobial resistance of streptococcus pneumoniae isolates causing invasive diseases from shenzhen children's hospital. *PloS One* 8, e67507. doi: 10.1371/journal.pone.0067507

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

Miyazaki, H., Shibuya, R., Midorikawa, N., Chang, B., Ohnishi, M., and Matsumoto, T. (2017). Serotype distribution and antimicrobial susceptibility of Streptococcus pneumoniae strains isolated in Japan after introduction of the routine immunization program. *J. infection chemotherapy* 23, 234–240. doi: 10.1016/j.jiac.2016.12.016

Moore, M. R., Link-Gelles, R., Schaffner, W., Lynfield, R., Holtzman, C., Harrison, L. H., et al. (2016). Effectiveness of 13-valent pneumococcal conjugate vaccine for prevention of invasive pneumococcal disease in children in the USA: a matched case-control study. *Lancet Respir. Med.* 4, 399–406. doi: 10.1016/S2213-2600(16)00052-7

Mosadegh, M., Ghahfarokhi, S. H., Ahmadi, A., Pourmand, M. R., Erfani, Y., and Mashhadi, R. (2022). Identification and molecular characterization of penicillin-nonsusceptible Streptococcus pneumoniae isolates recovered from invasive infections in a pre-pneumococcal vaccine era. *J. Clin. Lab. Anal.* 36, e24566. doi: 10.1002/jcla.24566

Musher, D. M., Anderson, R., and Feldman, C. (2022). The remarkable history of pneumococcal vaccination: an ongoing challenge. *Pneumonia* 14, 1–15. doi: 10.1186/s41479-022-00097-y

Pagliero, E., Chesnel, L., Hopkins, J., Croizé, J., Dideberg, O., Vernet, T., et al. (2004). Biochemical characterization of streptococcus pneumoniae penicillin-binding protein 2b and its implication in β-lactam resistance. *Antimicrob. Agents Chemother.* 48, 1848–1855. doi: 10.1128/AAC.48.5.1848-1855.2004

Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., and Korobeynikov, A. (2020). Using SPAdes *de novo* tf assembler. *Curr. Protoc. Bioinf.* 70, e102. doi: 10.1002/cpbi.102

Santoro, F., Iannelli, F., and Pozzi, G. (2019). Genomics and genetics of streptococcus pneumoniae. *Microbiol. Spectrum.* 7. doi: 10.1128/microbiolspec.GPP3-0025-2018

Shoemaker, N. B., Smith, MD, and Guild, W. R. (1979). Organization and transfer of heterologous chloramphenicol and tetracycline resistance genes in pneumococcus. *J. Bacteriol.* 139, 432–441. doi: 10.1128/jb.139.2.432-441.1979

Sings, H. L., De Wals, P., Gessner, B. D., Isturiz, R., Laferriere, C., McLaughlin, J. M., et al. (2019). Effectiveness of 13-valent pneumococcal conjugate vaccine against invasive disease caused by serotype 3 in children: A systematic review and meta-analysis of observational studies. *Clin. Infect. Dis.* 68, 2135–2143. doi: 10.1093/cid/ciy920

Wahl, B., O'Brien, K. L., Greenbaum, A., Majumder, A., Liu, L., Chu, Y., et al. (2018). Burden of Streptococcus pneumoniae and Haemophilus influenzae type b disease in children in the era of conjugate vaccines: global, regional, and national estimates for 2000–15. *Lancet Global Health* 6, e744–e757. doi: 10.1016/S2214-109X(18)30247-X

Wu, C.-J., Lai, J.-F., Huang, I.-W., Shiau, Y.-R., Wang, H.-Y., and Lauderdale, T.-L. (2020). Serotype distribution and antimicrobial susceptibility of streptococcus pneumoniae in pre- and post- PCV7/13 eras, Taiwan 2002–2018. *Front. Microbiol.* 11. doi: 10.3389/fmicb.2020.557404

Wu, X., Zhao, S., Jiang, Y., Xiang, X., Ge, L., Chen, Q., et al. (2022). Effect of pneumococcal conjugate vaccine availability on Streptococcus pneumoniae infections and genetic recombination in Zhejiang, China from 2009 to 2019. *Emerg. Microbes Infect.* 11, 606–615. doi: 10.1080/22221751.2022.2040921

Yamba Yamba, L., Uddén, F., Fuursted, K., Ahl, J., Slotved, H.-C., and Riesbeck, K. (2022). Extensive/multidrug-resistant pneumococci detected in clinical respiratory tract samples in southern Sweden are closely related to international multidrug-resistant lineages. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.824449

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 26 February 2024
DOI 10.3389/fcimb.2024.1296777

OPEN ACCESS

EDITED BY
Sanket Kaushik,
Amity University Jaipur, India

REVIEWED BY
Yingshun Zhou,
Southwest Medical University, China
Samuel J. T. Wardell,
University of Otago, New Zealand

*CORRESPONDENCE
Guanhua Xuan
xuanguanhua@ouc.edu.cn
Jingxue Wang
snow@ouc.edu.cn

†These authors have contributed equally to this work

RECEIVED 19 September 2023
ACCEPTED 17 January 2024
PUBLISHED 26 February 2024

CITATION
Su Q, Lu D, Kong J, Lin H, Xuan G and Wang J (2024) *PqsA* mutation-mediated enhancement of phage-mediated combat against *Pseudomonas aeruginosa*. *Front. Cell. Infect. Microbiol.* 14:1296777. doi: 10.3389/fcimb.2024.1296777

PqsA mutation-mediated enhancement of phage-mediated combat against *Pseudomonas aeruginosa*

Qiao Su†, Di Lu†, Jiuna Kong, Hong Lin, Guanhua Xuan* and Jingxue Wang*

State Key Laboratory of Marine Food Processing & Safety Control, College of Food Science and Engineering, Ocean University of China, Qingdao, China

Phage therapy is a potential approach in the biocontrol of foodborne pathogens. However, the emergence of phage resistance and the narrow host range of most phage isolates continue to limit the antimicrobial efficacy of phages. Here, we investigated the potential of the *pqsA* gene, encoding the anthranilate-CoA ligase enzyme, as an adjuvant for phage therapy. The knockout of the *pqsA* gene significantly enhanced the bactericidal effect of phages vB_Pae_QDWS and vB_Pae_S1 against *Pseudomonas aeruginosa*. Under phage infection pressure, the growth of the PaΔ*pqsA* was significantly inhibited within 8 h compared to the wild-type PAO1. Furthermore, we found that altering phage adsorption is not how PaΔ*pqsA* responds to phage infection. Although *pqsA* represents a promising target for enhancing phage killing, it may not be applicable to all phages, such as types vB_Pae_W3 and vB_Pae_TR. Our findings provide new material reserves for the future design of novel phage-based therapeutic strategies.

KEYWORDS

***P. aeruginosa*, pqsA, resistance, phage, enhancement of phage therapy**

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen that commonly causes spoilage in various vegetables, milk, and meat products (Garedew et al., 2012; Alimi et al., 2022; Gao et al., 2023; Wu et al., 2023). It has the ability to form biofilms, which provide protection against physical and chemical eradication methods, making it a significant concern for foodborne diseases and spoilage (O'Toole et al., 2000; Ma et al., 2022; Li et al., 2023). The global prevalence of multidrug-resistant (MDR) *P. aeruginosa* strains has become a serious threat to public health (Horcajada et al., 2019). In human beings, *P. aeruginosa* is responsible for a wide range of infections with varying levels of severity. *P. aeruginosa* infections can lead to pneumonia, urinary tract infections,

bloodstream infections, wound infections, and respiratory tract infections (Bassetti et al., 2018; De Sousa et al., 2021). Therefore, precise and efficient prevention and control of *P. aeruginosa* infection is of great significance in ensuring food safety and quality, reducing the incidence of foodborne diseases, and protecting public health.

Bacteriophages, which are viruses that specifically infect bacteria, possess significant potential for selectively targeting and combating harmful bacteria (Kortright et al., 2019; Joao et al., 2021; Strathdee et al., 2023). Phages are naturally found in food and their effectiveness as powerful antimicrobials has been extensively documented (Guenther et al., 2009; Goodridge and Bisha, 2011; Sarhan and Azzazy, 2015; Kauppinen et al., 2021). The European Food Safety Authority (EFSA) has evaluated phages and concluded that they are safe for both consumers and the environment, although each phage or phage cocktail intended for use in food must be assessed on a case-by-case basis (Ricci et al., 2017; Rendueles et al., 2022). However, phage therapy can be complicated by the ability of bacteria to defend against phage attacks through various antiviral mechanisms, including spontaneous mutations, DNA restriction-modification, abortive infection systems, and the CRISPR-Cas adaptive immunity system (Labrie et al., 2010; Dupuis et al., 2013; Monteiro et al., 2019; Xuan et al., 2022). Overcoming phage resistance is a key issue that urgently needs to be addressed to enhance the effectiveness of phage therapy.

Several studies have focused on this issue and proposed diverse strategies to enhance the effectiveness of phage therapy. For example, phage genomes can be constructed using synthetic DNA fragments to introduce specific genetic modifications to enhance the efficacy of phage therapy (Lenneman et al., 2021). Researchers have also utilized phages as carriers to deliver biofilm-depolymerases, capsule-depolymerases, quorum-quenching enzymes, and cross-genus cell wall hydrolases with lytic activity, thereby enhancing the antimicrobial activity of phages (Pei et al., 2014; Born et al., 2017; Kilcher et al., 2018). Based on the temperate phage ΦCD24-2, Selle et al. (Selle et al., 2020) engineered a modified version that converted the phage's lifestyle from temperate to virulent using genomic deletions, and simultaneously delivered host-targeting crRNA as a toxin, resulting in significantly enhanced phage-killing efficacy *in vitro* and in a *C. difficile* mouse infection model.

PqsA, an enzyme belonging to the CoA-ligase family, functions as the primary synthase in the biosynthetic pathway of alkyl quinolone (AQ) (Coleman et al., 2008; Witzgall et al., 2017). PqsA plays a pivotal role in regulating the production of virulence factors. Studies have shown that mutations in the *pqsA* gene significantly reduce *P. aeruginosa*'s ability to produce several virulence factors, including pyocyanin and elastase, which are key factors that contribute to *P. aeruginosa* pathogenicity (Bala et al., 2014). Thus, PqsA is often considered as a promising therapeutic target for mitigating or eradicating the virulence of *P. aeruginosa* (Ji et al., 2016; Shaker et al., 2020; Chen et al., 2022). While there is a research foundation for studying the functionality of PqsA protein and screening for inhibitors, its application in combating *P. aeruginosa* infections remains limited, and there is an urgent need to develop more effective treatment options such as phage therapy. However, phage therapy still faces the challenge of bacterial resistance to phage (Labrie et al., 2010; Dupuis et al., 2013; Monteiro et al., 2019; Xuan et al., 2022). Therefore, we initially focus on exploring the potential application of PqsA protein in enhancing phage therapy and identifying essential gene targets for designing phage-antibacterial agents combination therapy to effectively combat *P. aeruginosa* infections. However, there is scarce evidence on the significance of *pqsA* as a crucial gene target in phage therapy for combating *P. aeruginosa*.

In this study, we reported that mutations in the *pqsA* gene make *P. aeruginosa* less likely to develop resistance to phage infection in a short period of time, thereby enhancing phage sterilization. PqsA is expected to act as a new target for designing efficient phage therapy for the control of *P. aeruginosa*.

Materials and methods

Strains, plasmids, and growth conditions

P. aeruginosa strains were cultured under standard conditions at a temperature of 37°C in Luria Bertani broth (LB). Gentamicin and tetracycline were added at concentrations of 30 μg/mL and 50 μg/mL, respectively, for strain construction and plasmid maintenance purposes. Phages specific for *P. aeruginosa* PAO1 were isolated from sewage samples collected in Qingdao, China. The *Pseudomonas* phages, vB_Pae_S1 (accession number OL802210.1), vB_Pae_QDWS (accession number MZ687409.1), vB_Pae_W3 (accession number OK094665.1), and vB_Pae_TR (accession number OL802211.1) have been sequenced and deposited in NCBI GenBank.

Gene knockout

The PaΔ*pqsA* strain was generated following a previously described protocol (Xuan et al., 2021). To construct a *pqsA*-deletion mutant, a 1047-bp fragment and a 1018-bp fragment upstream and downstream of *pqsA* were PCR-amplified from *P. aeruginosa* PAO1 genomic DNA. The two PCR products were fused together and cloned into pK18mobsacBtet plasmid at the EcoRI site. The constructed plasmid was transformed into *E. coli* S17–1 and then transferred via conjugation into *P. aeruginosa* PAO1. Integration into the chromosome of PAO1 was achieved through the first crossover event, followed by selection on tetracycline-containing agar plates with a chemically defined medium that solely utilized sodium gluconate as the carbon source. The double crossover was selected using 12% sucrose, resulting in the *pqsA*-deletion mutant. The colony PCR and DNA sequencing were performed to confirm the correct mutant.

Phage resistance assay

Spot assay. A mixture of 100 μL of bacterial cultures (approximately 1.4 $\times 10^9$ CFU/mL) was added to 5 mL of 0.75%

molten agar and poured onto the prepared LB plate. Subsequently, 3 μL of the phage suspension with serial dilutions was added onto the double-layer agar containing bacterial suspension. The plates were then incubated at 37°C without agitation. After incubation, the plates were examined for the presence of clearing zones.

The bacterial growth reduction assay. *P. aeruginosa* strains were co-cultivated with phage vB_Pae_S1 or vB_Pae_QDWS at a multiplicity of infection (MOI) of 0.1 at 37°C (The initial ratio of phage concentration to bacterial concentration is 1×10^7 PFU/mL: 1.0×10^8 CFU/mL). Bacteria cells were collected at different time points and the absorbance values at OD_{600nm} were measured and recorded using a microplate reader in a 96-well plate.

The efficiency of plating (EOP) assay. 3 μL of the phage suspension at various dilutions (10^5, 10^4, 10^3, and 10^2 PFU/mL) were applied to the surface of *P. aeruginosa* strains. The plates were then incubated at 37°C for 4 h. After incubation, the number of plaque-forming units (PFUs) was counted. The relative efficiency of plating (EOP) was calculated by dividing the average PFU count on the target bacteria by the average PFU count on the control PAO1 bacteria.

One-step growth curve

The one-step growth curve experiment was conducted with some modifications following previously described methods (Xuan et al., 2023b). Briefly, *P. aeruginosa* cells were exposed to isolated phage at an MOI of 0.01 and allowed to adsorb for 3 min (for vB_Pae_QDWS) or 5 min (for vB_Pae_S1) at a temperature of 37°C. The mixture was then centrifuged at 10,000 × g for 1 min, and the resulting pellets were washed three times using LB medium. The supernatant was removed, and the pellets containing the phage-infected bacterial cells were resuspended in 25 mL of fresh LB broth. The suspension was incubated with shaking at 180 rpm and 37°C. Throughout the incubation period, samples were collected at specific time intervals, and the titers of the phage in the aliquots were immediately determined using the double-layer agar method.

Phage killing assay

The overnight cultures of PAO1 and PaΔ*pqsA* were diluted 1:100 and cultured in fresh Casein Soya Bean Digest Broth (TSB) liquid medium to obtain a final bacterial concentration of approximately 0.4-0.6. Then, the bacterial cultures were subjected to a dilution with TSB liquid medium to achieve a final bacterial concentration of about 10^4 CFU/mL. Simultaneously, the different multiplicity of infections (0.001 and 0.01) of phage vB_Pae_QDWS or vB_Pae_S1 were added to the TSB medium. The cultures were incubated at 37°C with shaking, and samples were collected at 0 h, 2 h, 4 h, 8 h, and 12 h time points. The cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS). Subsequently, the cell counts of PAO1 and PaΔ*pqsA* infected with phage vB_Pae_QDWS or vB_Pae_S1 were determined at each time point.

Host range analysis

Thirteen *P. aeruginosa* isolates were used to test the infectivity of phage vB_Pae_S1, vB_Pae_QDWS, vB_Pae_W3, and vB_Pae_TR. To determine the infectivity of the phages on the bacterial strains, a total of 3 μL of phage lysate with a titer of 10^{11} plaque-forming units (PFU) was applied onto an agar plate containing *P. aeruginosa* mixed with 0.5% (w/v) top agar. The plate was then incubated overnight. The infectivity of the phages was assessed by evaluating the turbidity of the plaques formed at the location where the phage lysate was dropped.

Transmission electron microscope (TEM) analysis

Phage adsorption was observed by TEM as described previously (Xuan et al., 2023a). Briefly, *P. aeruginosa* cells were cultivated until reaching an optical density at 600 nm (OD_{600nm}) of 2.5. Subsequently, the cells were mixed with phages vB_Pae_QDWS and vB_Pae_S1 at a multiple infection index (MOI) of approximately 100. After a 5 min (for vB_Pae_S1) or 3 min (for vB_Pae_QDWS) adsorption period, the samples were analyzed using TEM. The specific procedure for TEM analysis involved loading the samples onto a carbon-coated copper grid for 5 min, followed by negative staining with 2% (w/v) phosphotungstic acid (PTA, a common reagent in histological staining, pH 6.8). After drying, the samples were examined using a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan) operating at 100 kV.

Adsorption rate assay

Overnight cultures of *P. aeruginosa* strains PAO1 and PaΔ*pqsA* were diluted 1:100 and cultured in fresh LB medium until reaching an OD_{600nm} of approximately 0.4-0.6. To promote phage adsorption, 0.5 mL of a phage solution (10^5 PFU/mL) was mixed with 0.5 mL of the cell suspension (10^8 PFU/mL) and incubated at 37°C for 5 min (for vB_Pae_S1) or 3 min (for vB_Pae_QDWS). As a control, LB broth mixed with phage vB_Pae_S1 or vB_Pae_QDWS without bacteria was used. Following incubation, the cultures were centrifuged at 7,378 × g for 2 min, and the titer of free phage in the supernatant was determined using the double-layer agar method. The phage adsorption rate was calculated as follows: adsorption rate (%) = [(initial phage titer - phage titer in the supernatant)/(initial phage titer)] × 100.

Bioinformatics analysis

The ViPTree (Nishimura et al., 2017) service (https://www.genome.jp/viptree/) was used to analyze the similarities and relationships between vB_Pae_QDWS and other reported prokaryotic double-stranded DNA viruses. A total of 3080 phage

genomes were used as reference sequences to construct phylogenetic trees using VipTree. Sequence alignment of the whole genomes of four *Pseudomonas* phages was visualized using VipTree software.

Results and discussion

Disruption of *pqsA* of PAO1 could promote phage infection

Here, we evaluated the effect of *pqsA* deletion on the survival of *P. aeruginosa* under the predation pressure of vB_Pae_QDWS. In the bacterial growth reduction assay, we found that phage vB_Pae_QDWS significantly reduced the cell count of *P. aeruginosa*. However, with the passage of time, a slow growth of PAO1 was observed after 240 min. Nevertheless, the growth of PaΔ*pqsA* remained significantly inhibited (Figure 1A). In different time intervals, the spot test findings also indicate that PaΔ*pqsA* is more prone to phage vB_Pae_QDWS infection, leading to the formation of more translucent plaques (Figure 1B). However, there was no significant difference in EOP of vB_Pae_QDWS on PAO1 and PaΔ*pqsA* strains (Figure 1C). Furthermore, growth curve analysis showed similar trends in one-step growth curves of the vB_Pae_QDWS using PAO1 and PaΔ*pqsA* as hosts (Figure 1D).

Similar to the findings with phage vB_Pae_QDWS, we observed that phage vB_Pae_S1 also exhibited significantly higher infectivity towards the knockout strain PaΔ*pqsA* compared to the wild-type PAO1. Stronger growth inhibition after 240 min (Figure 2A) and more translucent plaques (Figure 2B) formed by the phage vB_Pae_S1 on the *pqsA* knockout strain during the phage infection assays were observed. However, there was no significant difference in EOP of vB_Pae_S1 on PAO1 and PaΔ*pqsA* strains (Figure 2C). Furthermore, growth curve analysis showed similar trends in one-step growth curves of the vB_Pae_S1 using PAO1 and PaΔ*pqsA* as hosts (Figure 2D). These results indicated that the knockout of the *pqsA* gene can enhance phage bactericidal efficacy.

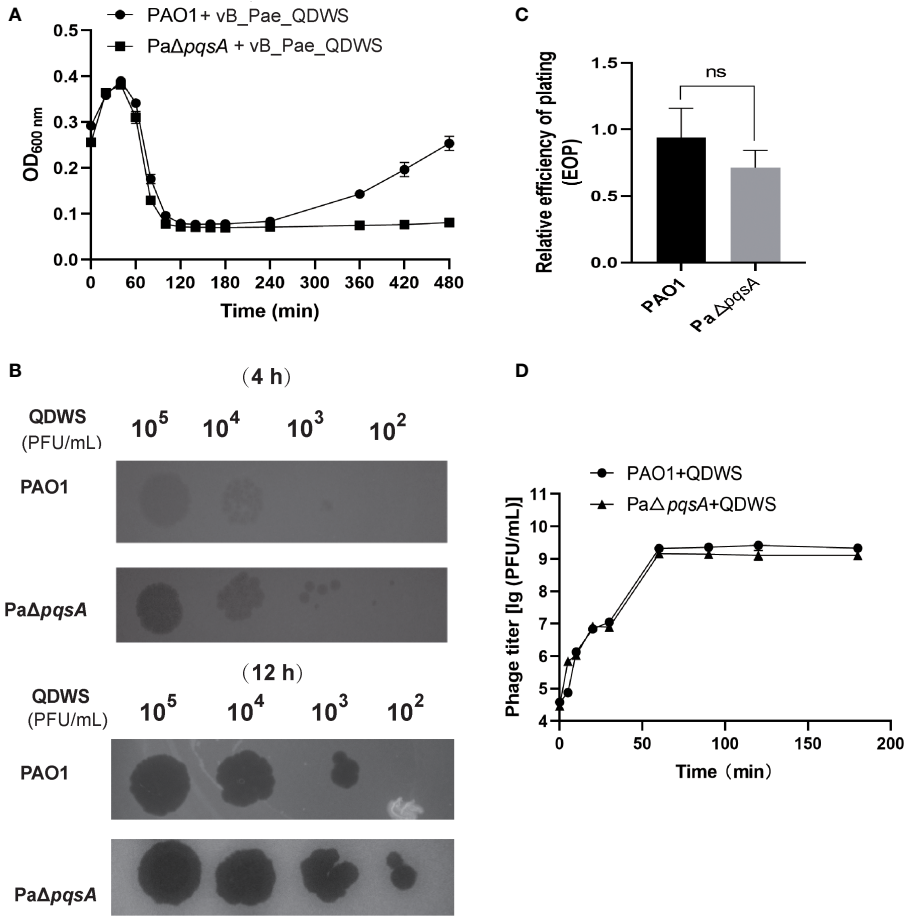


FIGURE 1
Knockout of the *pqsA* gene facilitates phage vB_Pae_QDWS infection of the host *P. aeruginosa*. **(A)** The growth curves of PAO1 and PaΔ*pqsA* strains, when infected with phage vB_Pae_QDWS, were measured at various time points. The samples were monitored by measuring the optical density (OD) at 600 nm using a SynergyH1 microplate reader in a 96-well plate. **(B)** Spot assays were conducted by spotting 3 µL of serial dilutions of phage vB_Pae_QDWS onto PAO1 and PaΔ*pqsA*. **(C)** Relative EOP of phage vB_Pae_QDWS on *P. aeruginosa* strains. Unpaired t tests were performed ('ns', not significant). **(D)** One-step growth curve determination of phage vB_Pae_QDWS on their respective host PAO1 and PaΔ*pqsA*. Mean values ± standard deviation were calculated from three independent experiments.

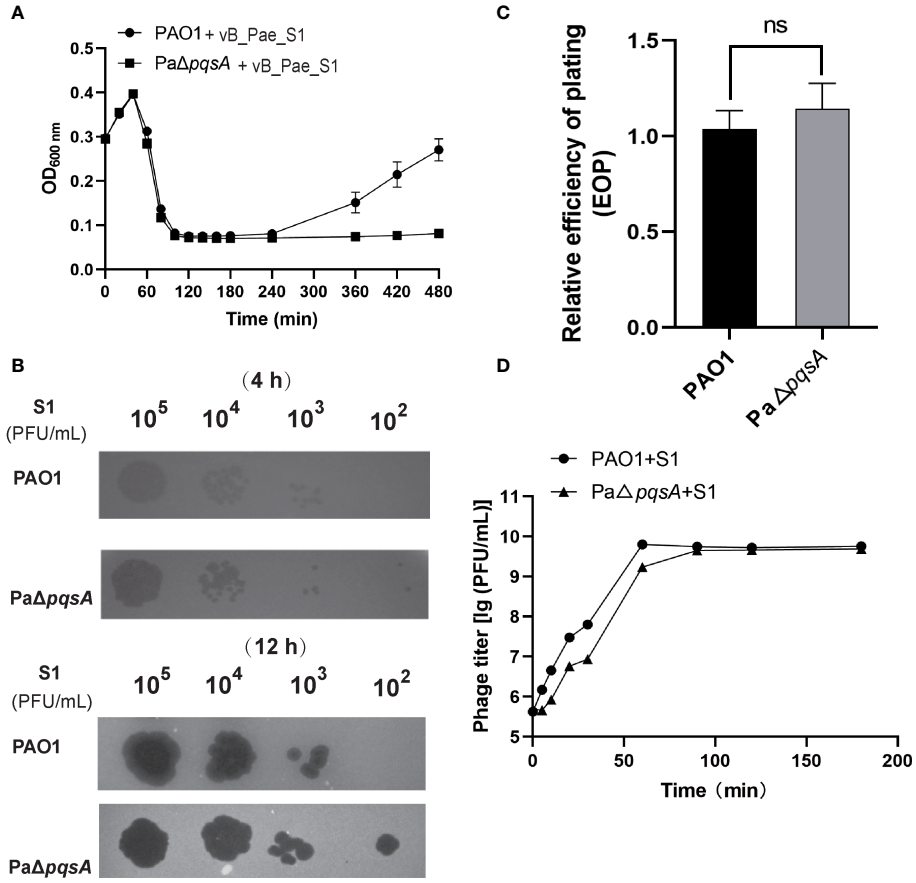


FIGURE 2
Knockout of the *pqsA* gene facilitates phage vB_Pae_S1 infection of the host *P. aeruginosa*. **(A)** The growth curves of the PAO1 and Pa*ΔpqsA* strains were monitored at different time intervals upon infection with phage vB_Pae_S1. The optical density (OD) at 600 nm was measured using a SynergyH1 microplate reader in a 96-well plate. **(B)** Spot assays were performed by spotting 3 µL of serially diluted phage vB_Pae_S1 onto both PAO1 and Pa*ΔpqsA*. **(C)** Relative EOP of phage vB_Pae_S1 on *P. aeruginosa* strains. Unpaired t tests were performed ('ns', not significant). **(D)** One-step growth curve determination of phage vB_Pae_S1 on their respective host PAO1 and Pa*ΔpqsA*. Mean values ± standard deviation were calculated from three independent experiments.

Our findings unveil a paradigmatic phage-bacteria interaction mediated by the *pqsA* target. This discovery provides crucial insight for further research and development of therapeutic strategies harnessing the phage-bacteria interaction, particularly through targeted silencing of the *pqsA* gene to enhance the precision of phage therapy against *P. aeruginosa*. This advancement contributes to filling the research gap in the field of precise pathogen control.

Phages vB_Pae_QDWS and vB_Pae_S1 had genome sizes of 43,170 and 43,058 bp, respectively. Both of them are lytic phages targeting *P. aeruginosa*. The two phages vB_Pae_QDWS and vB_Pae_S1 shared an intergenomic nucleotide identity of 98.39% (Table 1). Here, we explored the lytic abilities of phages vB_Pae_QDWS and vB_Pae_S1 against both PAO1 and Pa*ΔpqsA* at MOI 0.001 and MOI 0.01. Phage vB_Pae_QDWS effectively suppressed the growth of both PAO1 and Pa*ΔpqsA*, as there was no significant increase in bacterial cell numbers within 8 h. However, with prolonged co-cultivation, a noticeable growth acceleration of PAO1 was observed, while Pa*ΔpqsA* maintained a lower cell count (Figure 3A). The results of the lysis assay for phage vB_Pae_S1 were similar to those of vB_Pae_QDWS (Figure 3B). Therefore, our data suggest that the knockout of the *pqsA* gene can enhance the efficacy of phage therapy.

Enhanced infection mediated by *pqsA* mutation is independent of adsorption

Adsorption, as the primary step of phage invasion into the host, often affects the bactericidal efficacy of phages due to changes in their adsorption efficiency (Denes et al., 2015;

TABLE 1 Comparative analysis of the genomic characteristics of vB_Pae_Q+.

	coverage (96%) vB_Pae_QDWS	identity (98.39%) vB_Pae_S1
Genome size (bp)	43,170	43,058
G+C (%)	62.3	62.22
tRNAs	0	0
Predicted ORFs	53	57

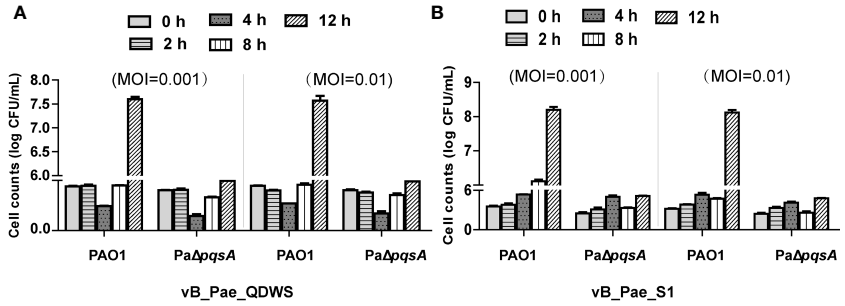


FIGURE 3
The knockout of the *pqsA* gene of *P. aeruginosa* can enhance the efficacy of phage therapy. The ability of phages **(A)** vB_Pae_QDWS or **(B)** vB_Pae_S1 to lyse *P. aeruginosa* PAO1 and PaΔ*pqsA* strains at different multiplicity of infections (0.001 and 0.01) in TSB medium at 37°C. Cell counts of PAO1 and PaΔ*pqsA* infected with phage vB_Pae_QDWS or vB_Pae_S1 were detected at different time points. Mean values ± standard deviation were calculated from three independent experiments.

Harvey et al., 2018). We conducted phage adsorption assays using the PAO1 and PaΔ*pqsA* strains. The results of TEM analysis revealed that a higher number of vB_Pae_QDWS phages were observed surrounding the PAO1 strain compared to the host PaΔ*pqsA* strain (Figure 4A). Additionally, we observed a decrease in the adsorption rate of phages to the PaΔ*pqsA* strain compared to the PAO1 strain (Figure 4B). Similar results were also observed for vB_Pae_S1, with significantly lower adsorption efficiency to PaΔ*pqsA* compared to its efficiency to PAO1 (Figures 4C, D). In theory, a decrease in the adsorption efficiency of phages would significantly reduce their chances of invading the host. However, PaΔ*pqsA* exhibited greater sensitivity to phages compared to PAO1 (Figures 1, 2). These results supported that the enhanced sensitivity of the host to phages mediated by *pqsA* mutation is not attributed to the enhancement of phage adsorption pathway.

Hypothesizing the mechanism of phage sensitivity mediated by *pqsA* mutation

Based on the experimental data mentioned above, we have reasons to speculate that *pqsA* mutation significantly inhibits the development of phage resistance. Literature reports have indicated that phase variation in receptor structure mediates the coexistence of phages and bacteria, maintaining a balance between host sensitivity and phage resistance (Shkoporov et al., 2021; Ramos-Barbero et al., 2023). Growth curve analysis of phage-host cocultures revealed that, after 240 min, the wild-type PAO1 strain exhibited significant growth, while PaΔ*pqsA* remained suppressed (Figures 1A, 2A, 4B), suggesting that the *pqsA* mutant strain might have lost the ability to regulate flexible phase variation in receptor structure in the face of peak phage predation pressure, leading to a deficiency in bacterial immunity against phages compared to the wild-type strain PAO1. The *pqsA* mutation does not affect phage infectivity, as evidenced by the similar phage burst efficiencies (Figures 1C, 2C), as well as comparable growth curve trends of phage infection in the wild-type PAO1 and PaΔ*pqsA* (Figure 1D, 2D). The *pqsA* mutation specifically impairs bacterial immunity against phages, likely mediated by changes in receptor structure phase variation, which is consistent with our observation that knocking out the *pqsA* gene significantly reduces phage adsorption efficiency (Figure 4). Although the *pqsA* gene mutation hinders the rapid development of phage resistance in the host, further research is needed to uncover and elucidate the involvement of PqsA in the regulation of phage-host interactions mediated by receptor phase variation. Future integration of transcriptomic analysis with phenotype association is expected to elucidate important pathways and mechanisms through which the changes in phage-bacteria interactions mediated by *pqsA* mutations.

The multiple effects of silencing the *pqsA* gene on the application of phage therapy

We also compared and investigated multiple *P. aeruginosa* phages (vB_Pae_QDWS, vB_Pae_S1, vB_Pae_W3, and vB_Pae_TR) to analyze the impact of silencing the *pqsA* gene on the bactericidal ability of the phages. As shown in Figure 5A, phage vB_Pae_QDWS and vB_Pae_S1 were classified into different branches from vB_Pae_W3 and vB_Pae_TR, indicating different divergences. Comparative analysis of four *P. aeruginosa* phages in BLASTn is given in Figure 5B. Phage vB_Pae_QDWS and vB_Pae_S1 displayed a close relationship, and vB_Pae_W3 had similarities with vB_Pae_TR. However, except for S1, QDWS shows no similarity to vB_Pae_W3 or vB_Pae_TR. In the bacterial growth reduction assay, we found that deletion of the *pqsA* gene leads to enhanced resistance of the host strain to phage vB_Pae_W3 and vB_Pae_TR. After 180 min, it was observed that the growth rate of

10.3389/fcimb.2024.1296777

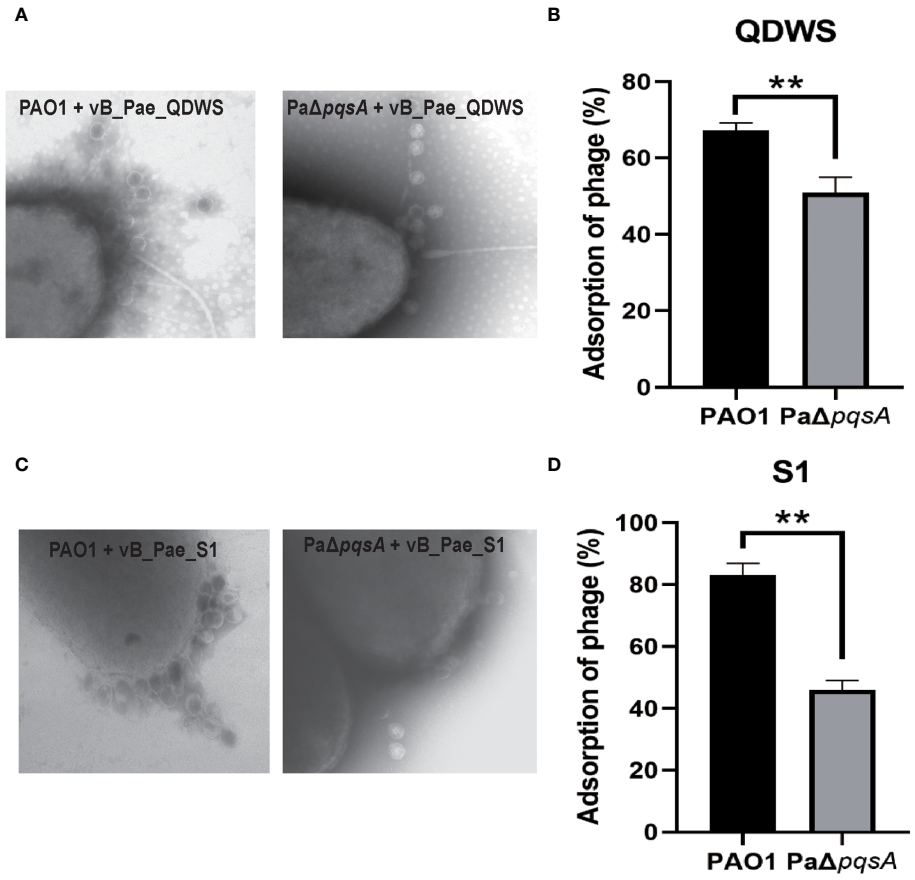


FIGURE 4
The enhanced phage infection mediated by *pqsA* mutation is independent of adsorption. **(A, B)** Adsorption assays of phage vB_Sb_QDWS to *P. aeruginosa* PAO1 and PaΔ*pqsA* strains. **(C, D)** Adsorption assays of phage vB_Sb_S1 to *P. aeruginosa* PAO1 and PaΔ*pqsA* strains. Mean values ± standard deviation were calculated from three independent experiments. A t-test was performed (**, P < 0.01).

PAO1 was significantly delayed compared to that of PaΔ*pqsA* (Figure 5C), which is quite different from the bactericidal effects observed during phage vB_Pae_QDWS and vB_Pae_S1 treatment. Therefore, silencing the *pqsA* gene leads to different outcomes in the interaction between the host and phages, which may be attributed not only to the regulation mediated by changes in the host's genetic and metabolic networks but also to the type of phage involved.

Host range analysis of four *Pseudomonas* phages

The efficiency of the lytic activity of four phages was measured against thirteen *P. aeruginosa* strains using the visual assessment of plaques on the spot test. Seven (53.8%) and six (46.2%) of the *P. aeruginosa* strains tested were lysed by vB_Pae_W3 and vB_Pae_TR, respectively, while phages vB_Pae_QDWS and vB_Pae_S1 exhibit similar lysis spectra, being able to lyse ten (76.9%) *P. aeruginosa* strains. Phages vB_Pae_QDWS and vB_Pae_S1 exhibit stronger lytic activity and a broader range of lysis compared to phages vB_Pae_W3 and vB_Pae_TR, making them appear to have a competitive advantage in combating *P. aeruginosa* (Table 2).

Conclusion

In this study, we show that *pqsA* has the potential to become an important gene target to enhance phage therapy. The deletion of the *pqsA* gene could significantly promote the lysis of phages vB_Pae_QDWS and vB_Pae_S1 on the *P. aeruginosa* PAO1. We speculate that the mechanism may be related to the defect in bacterium-phage immune capability mediated by *pqsA* mutation, as we observed that *pqsA* mutation mainly affects the later stage of phage-host interaction, specifically inhibiting the development of phage-resistant strains. However, the specific regulatory mechanisms still require further research and clarification. Although silencing the *pqsA* gene is expected to enhance the efficacy of phage therapy against *P. aeruginosa*, it also depends on the specific phage type used. For

10.3389/fcimb.2024.1296777

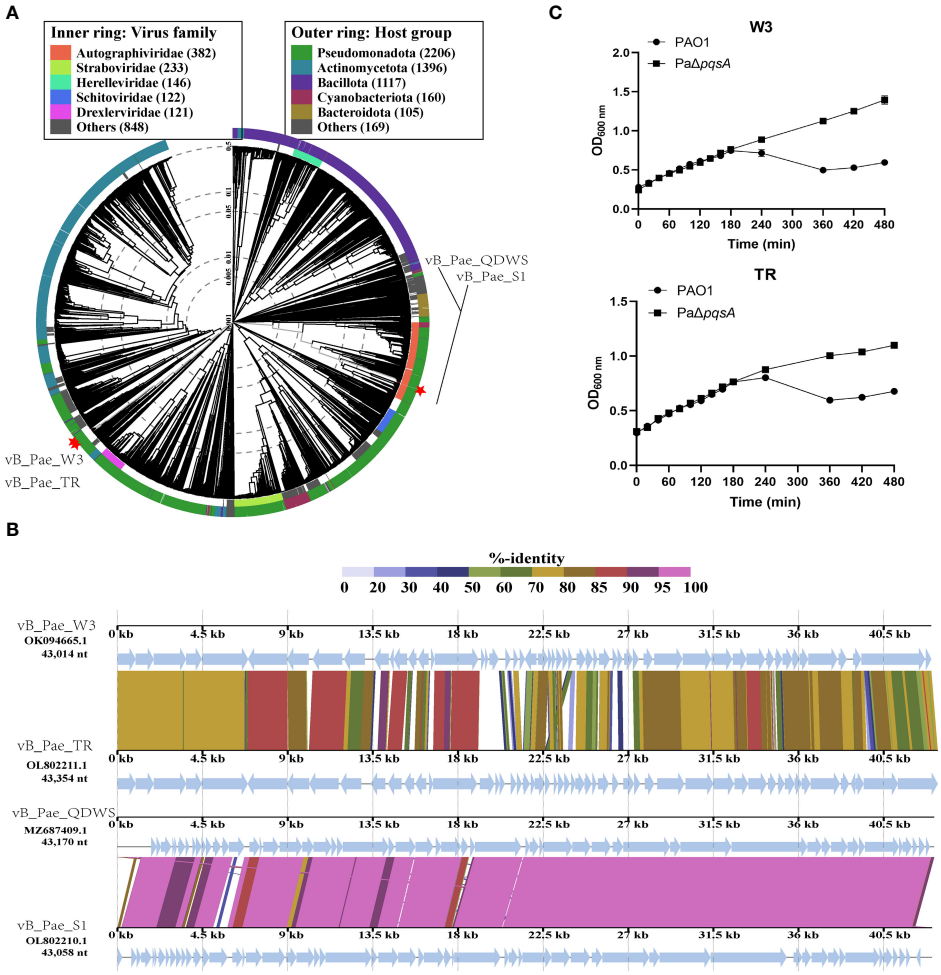


FIGURE 5
The response of PaΔ*pqsA* strain to the infection of phages vB_Pae_W3 and vB_Pae_TR. **(A)** ViPTree analysis of four *Pseudomonas* phages. Phages are identified according to their official ICTV classification, with the outer and inner rings indicating their host group and virus family, respectively. **(B)** Comparative genome alignment of the *Pseudomonas* phages vB_Pae_QDWS, vB_Pae_S1, vB_Pae_W3, and vB_Pae_TR. Analysis was performed using VipTree software. The color indicates the nucleotide sequence identity level (from 0 to 100%). **(C)** The growth curves of PAO1 and PaΔ*pqsA* strains, when infected with phage vB_Pae_W3 and vB_Pae_TR, were measured at various time points.

TABLE 2 Lytic activity of four *Pseudomonas* phages (+, infected; –, uninfected).

Strain	Source	*Pseudomonas* phages			
		vB_Pae_W3	vB_Pae_TR	vB_Pae_S1	vB_Pae_QDWS
P. aeruginosa PAO1	Standard strain	+	+	+	+
P. aeruginosa Y3	this study	-	-	-	-
P. aeruginosa Y4	this study	+	+	+	+
P. aeruginosa Y9	this study	+	-	+	+
P. aeruginosa Y14	this study	+	+	-	-
P. aeruginosa SJ-1	this study	-	-	+	+
P. aeruginosa SJ-2	this study	-	-	+	+
P. aeruginosa SJ-4	this study	+	+	+	+
P. aeruginosa SJ-6	this study	+	+	+	+
P. aeruginosa SJ-8	this study	-	-	+	+
P. aeruginosa SJ-10	this study	-	-	+	+
P. aeruginosa SJ-76	this study	+	+	-	-
P. aeruginosa PA14	Standard strain	-	-	+	+

example, silencing *pqsA* may significantly decrease the therapeutic effectiveness of phages vB_Pae_W3 and vB_Pae_TR. Regardless, we propose that the *pqsA* gene plays a crucial role in mediating the phage-bacteria interaction process. However, in future studies aiming to design novel phage therapies targeting the *pqsA* gene, further validation is required using a broader range of phage 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.

Author contributions

QS: Writing – review & editing, Conceptualization, Data curation, Methodology. DL: Writing – review & editing, Conceptualization, Data curation, Formal analysis, Methodology. JK: Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. HL: Conceptualization, Project administration, Supervision, Writing – review & editing. GX: Writing – original draft, Writing – review & editing. JW: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Natural Science Foundation of Shandong Province of China (ZR2022QC039), the National Natural Science Foundation of China (no. 32201982 and 32270152), and the Earmarked Fund for China Agriculture Research System (CARS-47).

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

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Alimi, B. A., Lawal, R., and Odetunde, O. N. (2022). Food safety and microbiological hazards associated with retail meat at butchery outlets in north-central Nigeria. *Food Control.* 139, 109061. doi: 10.1016/j.foodcont.2022.109061

Bala, A., Chhibber, S., and Harjai, K. (2014). Pseudomonas quinolone signalling system: A component of quorum sensing cascade is a crucial player in the acute urinary tract infection caused by *Pseudomonas aeruginosa*. *Int. J. Med. Microbiol.* 304, 1199–1208. doi: 10.1016/j.ijmm.2014.08.013

Bassetti, M., Vena, A., Croxatto, A., Righi, E., and Guery, B. (2018). How to manage *Pseudomonas aeruginosa* infections. *Drugs Context.* 7, 212527. doi: 10.7573/dic.212527

Born, Y., Fieseler, L., Thöny, V., Leimer, N., Duffy, B., Loessner, M. J., et al. (2017). Engineering of Bacteriophages Y2: dpoL1-C and Y2: luxAB for Efficient Control and Rapid Detection of the Fire Blight Pathogen, Erwinia amylovora. *Appl. Environ. Microb.* 83, e00341–17. doi: 10.1128/AEM.00341-17

Chen, J., Lu, Y., Ye, F., Zhang, H., Zhou, Y., Li, J., et al. (2022). A small-molecule inhibitor of the anthranilyl-coA synthetase pqsA for the treatment of multidrug-resistant *pseudomonas aeruginosa*. *Microbiol. spectrum.* 10, e276421. doi: 10.1128/spectrum.02764-21

Coleman, J. P., Hudson, L. L., Mcknight, S. L., Farrow, J. M., Worth Calfee, M., Lindsey, C. A., et al. (2008). *Pseudomonas aeruginosa* pqsA is an anthranilate-coenzyme A ligase. *J. Bacteriol.* 190, 1247–1255. doi: 10.1128/JB.01140-07

Denes, T., den Bakker, H. C., Tokman, J. I., Guldimann, C., Wiedmann, M., and Wommack, K. E. (2015). Selection and characterization of phage-resistant mutant strains of listeria monocytogenes reveal host genes linked to phage adsorption. *Appl. Environ. Microb.* 81, 4295–4305. doi: 10.1128/AEM.00087-15

De Sousa, T., Hébraud, M., Dapkevicius, M. L. N. E., Maltez, L., Pereira, J. E., Capita, R., et al. (2021). Genomic and metabolic characteristics of the pathogenicity in *pseudomonas aeruginosa*. *Int. J. Mol. Sci.* 22, 12892. doi: 10.3390/ijms222312892

Dupuis, M., Villion, M., Magadán, A. H., and Moineau, S. (2013). CRISPR-Cas and restriction-modification systems are compatible and increase phage resistance. *Nat. Commun.* 4, 2087. doi: 10.1038/ncomms3087

Gao, X., Li, C., He, R., Zhang, Y., Wang, B., Zhang, Z., et al. (2023). Research advances on biogenic amines in traditional fermented foods: Emphasis on formation mechanism, detection and control methods. *Food Chem.* 405, 134911. doi: 10.1016/j.foodchem.2022.134911

Garedew, L., Berhanu, A., Mengesha, D., and Tsegay, G. (2012). Identification of gram-negative bacteria from critical control points of raw and pasteurized cow milk consumed at Gondar town and its suburbs, Ethiopia. *BMC Public Health* 12, 950. doi: 10.1186/1471-2458-12-950

Goodridge, L. D., and Bisha, B. (2011). Phage-based biocontrol strategies to reduce foodborne pathogens in foods. *Bacteriophage* 1, 130–137. doi: 10.4161/bact.1.3.17629

Guenther, S., Huwyler, D., Richard, S., and Loessner, M. J. (2009). Virulent bacteriophage for efficient biocontrol of listeria monocytogenes in ready-to-eat foods. *Appl. Environ. Microb.* 75, 93–100. doi: 10.1128/AEM.01711-08

Harvey, H., Bondy-Denomy, J., Marquis, H., Sztanko, K. M., Davidson, A. R., and Burrows, L. L. (2018). *Pseudomonas aeruginosa* defends against phages through type IV pilus glycosylation. *Nat. Microbiol.* 3, 47–52. doi: 10.1038/s41564-017-0061-y

Horcajada, J. P., Montero, M., Oliver, A., Sorlí, L., Luque, S., Gómez-Zorrilla, S., et al. (2019). Epidemiology and treatment of multidrug-resistant and extensively drug-resistant *pseudomonas aeruginosa* infections. *Clin. Microbiol. Rev.* 32, e00031–19. doi: 10.1128/CMR.00031-19

Ji, C., Sharma, I., Pratihar, D., Hudson, L. L., Maura, D., Guney, T., et al. (2016). Designed small-molecule inhibitors of the anthranilyl-coA synthetase pqsA block quinolone biosynthesis in *pseudomonas aeruginosa*. *ACS Chem. Biol.* 11, 3061–3067. doi: 10.1021/acschembio.6b00575

Joao, J., Lampreia, J., Prazeres, D., and Azevedo, A. M. (2021). Manufacturing of bacteriophages for therapeutic applications. *Biotechnol. Adv.* 49, 107758. doi: 10.1016/j.biotechadv.2021.107758

Kauppinen, A., Siponen, S., Pitkänen, T., Holmfeldt, K., Pursiainen, A., Torvinen, E., et al. (2021). Phage biocontrol of *pseudomonas aeruginosa* in water. *Viruses* 13, 928. doi: 10.3390/v13050928

Kilcher, S., Studer, P., Muessner, C., Klumpp, J., and Loessner, M. J. (2018). Cross-genus rebooting of custom-made, synthetic bacteriophage genomes in L-form bacteria. *Proc. Natl. Acad. Sci. - PNAS.* 115, 567–572. doi: 10.1073/pnas.1714658115

Kortright, K. E., Chan, B. K., Koff, J. L., and Turner, P. E. (2019). Phage therapy: A renewed approach to combat antibiotic-resistant bacteria. *Cell Host Microbe* 25, 219–232. doi: 10.1016/j.chom.2019.01.014

Labrie, S. J., Samson, J. E., and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8, 317–327. doi: 10.1038/nrmicro2315

Lenneman, B. R., Fernbach, J., Loessner, M. J., Lu, T. K., and Kilcher, S. (2021). Enhancing phage therapy through synthetic biology and genome engineering. *Curr. Opin. Biotech.* 68, 151–159. doi: 10.1016/j.copbio.2020.11.003

Li, X., Gu, N., Huang, T. Y., Zhong, F., and Peng, G. (2023). *Pseudomonas aeruginosa*: A typical biofilm forming pathogen and an emerging but underestimated pathogen in food processing. *Front. Microbiol.* 13, 1114199. doi: 10.3389/fmicb.2022.1114199

Ma, L. Z., Wang, D., Liu, Y., Zhang, Z., and Wozniak, D. J. (2022). Regulation of biofilm exopolysaccharide biosynthesis and degradation in *pseudomonas aeruginosa*. *Annu. Rev. Microbiol.* 76, 413–433. doi: 10.1146/annurev-micro-041320-111355

Monteiro, R., Pires, D. P., Costa, A. R., and Azeredo, J. (2019). Phage therapy: going temperate? *Trends Microbiol.* 27, 368–378. doi: 10.1016/j.tim.2018.10.008

Nishimura, Y., Yoshida, T., Kuronishi, M., Uehara, H., Ogata, H., Goto, S., et al. (2017). ViPTree: the viral proteomic tree server. *Bioinf. (Oxford England).* 33, 2379–2380. doi: 10.1093/bioinformatics/btx157

O'Toole, G., Kaplan, H. B., and Kolter, R. (2000). BIOFILM FORMATION AS MICROBIAL DEVELOPMENT. *Annu. Rev. Microbiol.* 54, 49–79. doi: 10.1146/annurev.micro.54.1.49

Pei, R., Lamas-Samanamud, G. R., and Pettinari, M. J. (2014). Inhibition of biofilm formation by T7 bacteriophages producing quorum-quenching enzymes. *Appl. Environ. Microb.* 80, 5340–5348. doi: 10.1128/AEM.01434-14

Ramos-Barbero, M. D., Gómez-Gómez, C., Sala-Comorera, L., Rodríguez-Rubio, L., Morales-Cortes, S., Mendoza-Barberá, E., et al. (2023). Characterization of crAss-like phage isolates highlights Crassvirales genetic heterogeneity and worldwide distribution. *Nat. Commun.* 14, 4295. doi: 10.1038/s41467-023-40098-z

Rendueles, C., Duarte, A. C., Escobedo, S., Fernández, L., Rodríguez, A., García, P., et al. (2022). Combined use of bacteriocins and bacteriophages as food biopreservatives. *A review. Int. J. Food Microbiol.* 368, 109611. doi: 10.1016/j.ijfoodmicro.2022.109611

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Girones, R., et al. (2017). Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. *EFSA J.* 15, e4664. doi: 10.2903/j.efsa.2017.4664

Sarhan, W. A., and Azzazy, H. M. (2015). Phage approved in food, why not as a therapeutic? *Expert Rev. Anti-Infe.* 13, 91–101. doi: 10.1586/14787210.2015.990383

Selle, K., Fletcher, J. R., Tuson, H., Schmitt, D. S., McMillan, L., Vridhambal, G. S., et al. (2020). *In vivo* targeting of clostridioides difficile using phage-delivered CRISPR-cas3 antimicrobials. *MBIO* 11, e00019–20. doi: 10.1128/mBio.00019-20

Shaker, B., Ahmad, S., Thai, T. D., and Eyun, S. (2020). Rational Drug Design for *Pseudomonas aeruginosa* PqsA Enzyme: An *in silico* Guided Study to Block Biofilm Formation. *Front. Mol. biosciences.* 7, 577316. doi: 10.3389/fmolb.2020.577316

Shkoporov, A. N., Khokhlova, E. V., Stephens, N., Hueston, C., Seymour, S., Hryckowian, A. J., et al. (2021). Long-term persistence of crAss-like phage crAss001 is associated with phase variation in Bacteroides intestinalis. *BMC Biol.* 19, 163. doi: 10.1186/s12915-021-01084-3

Strathdee, S. A., Hatfull, G. F., Mutalik, V. K., and Schooley, R. T. (2023). Phage therapy: From biological mechanisms to future directions. *CELL* 186, 17–31. doi: 10.1016/j.cell.2022.11.017

Witzgall, F., Ewert, W., and Blankenfeldt, W. (2017). Structures of the N-terminal domain of pqsA in complex with anthraniloyl- and 6-fluoroanthraniloyl-AMP: substrate activation in pseudomonas quinolone signal (PQS) biosynthesis. *Chembiochem: Eur. J. Chem. Biol.* 18, 2045–2055. doi: 10.1002/cbic.201700374

Wu, X., Yang, L., Wu, Y., Li, H., and Shao, B. (2023). Spread of multidrug-resistant *Pseudomonas aeruginosa* in animal-derived foods in Beijing, China. *Int. J. Food Microbiol.* 403, 110296. doi: 10.1016/j.ijfoodmicro.2023.110296

Xuan, G., Dou, Q., Kong, J., Lin, H., Wang, J., and Kumar, A. (2023a). *Pseudomonas aeruginosa* Resists Phage Infection via Eavesdropping on Indole Signaling. *Microbiol. spectrum.* 11, e391122. doi: 10.1128/spectrum.03911-22

Xuan, G., Kong, J., Wang, Y., Lin, H., and Wang, J. (2023b). Characterization of the newly isolated *Pseudomonas* phage vB_Pae_LC3I3. *Virus Res.* 323, 198978. doi: 10.1016/j.virusres.2022.198978

Xuan, G., Lin, H., Li, X., Kong, J., and Wang, J. (2022). RetS Regulates Phage Infection in Pseudomonas aeruginosa via Modulating the GacS/GacA Two-Component System. *J. Virol.* 96, e19722. doi: 10.1128/jvi.00197-22

Xuan, G., Lv, C., Xu, H., Li, K., Liu, H., Xia, Y., et al. (2021). Sulfane sulfur regulates lasR-mediated quorum sensing and virulence in *pseudomonas aeruginosa* PAO1. *Antioxidants* 10, 1498. doi: 10.3390/antiox10091498

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 16 April 2024
DOI 10.3389/fcimb.2024.1297312

OPEN ACCESS

EDITED BY
Sanket Kaushik,
Amity University Jaipur, India

REVIEWED BY
Giorgia Montrucchio,
University of Turin, Italy
Arta Karruli,
University Medical Center Mother Teresa (QSUT), Albania

*CORRESPONDENCE
Jarosław Janc
jarojanc@gmail.com

RECEIVED 19 September 2023
ACCEPTED 25 March 2024
PUBLISHED 16 April 2024

CITATION
Janc J, Słabisz N, Woźniak A, Łysenko L, Chabowski M and Leśnik P (2024) Infection with the multidrug-resistant Klebsiella pneumoniae New Delhi metallo-B-lactamase strain in patients with COVID-19: *Nec Hercules contra plures?* *Front. Cell. Infect. Microbiol.* 14:1297312. doi: 10.3389/fcimb.2024.1297312

Infection with the multidrug-resistant Klebsiella pneumoniae New Delhi metallo-B-lactamase strain in patients with COVID-19: *Nec Hercules contra plures?*

Jarosław Janc[1]*, Natalia Słabisz[2], Anna Woźniak[3], Lidia Łysenko[4], Mariusz Chabowski[5,6] and Patrycja Leśnik[7]

[1]Department of Anaesthesiology and Intensive Therapy, Hospital of Ministry of the Interior and Administration, Wrocław, Poland, [2]Department of Microbiology, 4th Military Clinical Hospital, Wrocław, Poland, [3]Department of Nursing and Midwifery, Wroclaw Medical University, Wrocław, Poland, [4]Departament of Anaesthesiology and Intensive Care Unit, Wroclaw Medical University, Wrocław, Poland, [5]Department of Surgery, 4th Military Clinical Hospital, Wrocław, Poland, [6]Department of Clinical Surgical Sciences, Faculty of Medicine, Wrocław University of Science and Technology, Wrocław, Poland, [7]Department of Microbiology, Wroclaw Medical University, Wrocław, Poland

Background: During the coronavirus disease 2019 (COVID-19) pandemic, in patients treated for SARS-CoV-2 infection, infections with the *Klebsiella pneumoniae* bacteria producing New Delhi metallo-B-lactamase (NDM) carbapenemase in the USA, Brazil, Mexico, and Italy were observed, especially in intensive care units (ICUs). This study aimed to assess the impact of *Klebsiella pneumoniae* NDM infection and other bacterial infections on mortality in patients treated in ICUs due to COVID-19.

Methods: The 160 patients who qualified for the study were hospitalized in ICUs due to COVID-19. Three groups were distinguished: patients with COVID-19 infection only (N = 72), patients with COVID-19 infection and infection caused by *Klebsiella pneumoniae* NDM (N = 30), and patients with COVID-19 infection and infection of bacterial etiology other than *Klebsiella pneumoniae* NDM (N = 58). Mortality in the groups and chosen demographic data; biochemical parameters analyzed on days 1, 3, 5, and 7; comorbidities; and ICU scores were analyzed.

Results: Bacterial infection, including with *Klebsiella pneumoniae* NDM type, did not elevate mortality rates. In the group of patients who survived the acute phase of COVID-19 the prolonged survival time was demonstrated: the median overall survival time was 13 days in the NDM bacterial infection group, 14 days in the other bacterial infection group, and 7 days in the COVID-19 only group. Comparing the COVID-19 with NDM infection and COVID-19 only groups, the adjusted model estimated a statistically significant hazard ratio of 0.28 ($p = 0.002$). Multivariate analysis revealed that age, APACHE II score, and CRP were predictors of mortality in all the patient groups.

Conclusion: In patients treated for SARS-CoV-2 infection acquiring a bacterial infection due to prolonged hospitalization associated with the treatment of COVID-19 did not elevate mortality rates. The data suggests that in severe

COVID-19 patients who survived beyond the first week of hospitalization, bacterial infections, particularly Klebsiella pneumoniae NDM, do not significantly impact mortality. Multivariate analysis revealed that age, APACHE II score, and CRP were predictors of mortality in all the patient groups.

KEYWORDS

coronavirus disease 2019, Klebsiella pneumoniae, metallo-B-lactamase, hospital-acquired infection, mortality

1 Introduction

After the outbreak of COVID-19 in December 2019 in China, it rapidly spread throughout China and all around the world. Data presented by the World Health Organization show that more than 126 million cases of COVID-19 have been reported worldwide, of which one-fourth have been in the USA with the remainder in other countries, including European nations (Sun et al., 2020; Phannajit et al., 2021).

The SARS-CoV-2 virus infects the body and causes inflammatory changes in the vascular endothelium, activating the immune system and coagulation and causing organ damage, mainly to the lungs, brain, heart, and kidneys (Hekmatnia et al., 2022). Respiratory viruses, such as coronaviruses and influenza viruses, can cause acute damage to lung epithelial cells, allowing other pathogens to infiltrate the affected area (de Wit et al., 2016; Zhong et al., 2021).

This allows the subsequent invasion of other microorganisms, including bacteria and fungi. A hospital environment poses a risk of supra-infection with nosocomial strains. This is particularly true for patients treated in intensive surveillance and intensive care units (ICUs), where they may be exposed to multiple invasive therapeutic procedures. The latter require an integrated, multifaceted approach to preventing multidrug resistance (Pelfrene et al., 2021).

Due to elevated inflammatory parameters, which make it difficult to distinguish COVID-19 from secondary bacterial infections, the empirical use of broad-spectrum antibiotic therapy is recommended (Beović et al., 2020; Langford et al., 2020; Monnet and Harbarth, 2020; Rawson et al., 2020; Wan et al., 2020). This approach has led to the propagation of multidrug-resistant strains. During the COVID-19 pandemic, due to the large numbers of patients with COVID-19 in hospitals, the creation of temporary wards dedicated to the treatment of these patients and the hiring of additional staff often not trained to care for critically ill patients resulted in the spread of multidrug-resistant strains in hospitals.

Klebsiella pneumoniae belongs to the family *Enterobacteriaceae*, which is part of the normal human intestinal flora but can also be responsible for community- and healthcare-associated infections. Due to acquiring resistance genes, a rapid increase in resistance to penicillins and cephalosporins has been observed. Much more dangerous resistance to carbapenems has been reported with increasing frequency and geographical spread since the 1990s (Gupta et al., 2011; Munoz-Price et al., 2013).

Carbapenem-resistant *Enterobacterales* can be resistant to carbapenems as a result of various mechanisms. One of the representatives of this group, *Klebsiella pneumoniae*, produces metallo-beta-lactamases—enzymes that hydrolyze most beta-lactams, including carbapenems (Nordmann et al., 2011).

During the COVID-19 pandemic, in patients treated for SARS-CoV-2 infection, infections with the *Klebsiella pneumoniae* bacteria producing New Delhi metallo-B-lactamase (NDM) carbapenemase in the USA, Brazil, Mexico, and Italy were observed, especially in ICUs (Nori et al., 2020; Porretta et al., 2020; Fernández-García et al., 2022; Monteiro et al., 2023).

The *Klebsiella pneumoniae* NDM strain was first described in 2009, isolated from the urine of a patient in Sweden who had been hospitalized in India (Yong et al., 2009). In Poland, the first NDM infection was reported in 2015 (Sacha et al., 2012). The occurrence of NDM strains is associated with the likelihood of the development of resistance to all available antibiotics and the risk of transmitting resistance genes to various species of microorganisms (Queenan and Bush, 2007). The most significant problem is the production of carbapenemases due to the possibility of horizontal gene transfer between bacteria of the same or different species via plasmids. Currently, there are several types of carbapenemases, including KPC and metallo-B-lactamase enzymes, referred to as NDM (New Delhi metallo-B-lactamase, IMP, VIM) and OXY-48-like carbapenemases. Admission to an ICU and age over 70 years were found to be likely risk factors for infection with CR and ESBL *K. pneumoniae*, with increasing age being correlated with greater risk factors (Wang et al., 2023). Inappropriate antibiotic therapy treatment and extended ICU stays (15 days) were the most significant clinical and epidemiological factors (El Mekes et al., 2020).

Respiratory failure patients tend to have more severe illnesses than those with chronic obstructive pulmonary disease. The ICU is a common place to manage these patients because they frequently require mechanical ventilation and are more likely to become infected or colonized with CR *K. pneumoniae* in the airway.

Between September 2020 and September 2022, a rapid spread of *K. pneumoniae* NDM-type was observed in the wards of the 4th Military Clinical Hospital in Wroclaw, Poland. It concerned

patients treated both in the ICU and wards dedicated to patients with COVID-19.

Staff caring for patients with COVID-19 were protected by required clothing, but contamination of this clothing by microorganisms and errors in hygiene procedures led to the spread of hospital-acquired infections in the hospital environment (Pratt et al., 2001).

The present study aimed to assess the impact of *K. pneumoniae* NDM infection and other bacterial infections on mortality in ICU patients with COVID-19 during the pandemic. In addition, the light sensitivity of the phenotypes occurring in this period and the frequency and types of infection were assessed.

2 Methods

2.1 Study design and settings

The single-center cross-selection study was conducted from September 2020 to September 2022 at the Intensive Care Unit 4 Military Hospital of Wroclaw, Poland. The study was registered in the Australian New Zealand Clinical Trials Registry (ANZCTR) with registration no. ACTRN12621001300864. The stands for The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) were followed and the STROBE checklist was used for enrolment and allocation of patients (von Elm et al., 2007).

2.2 Study participants

The stratification of patients participating in the study is shown in the Flow-chart (Figure 1).

Adult patients with clinical symptoms and lab-confirmed COVID-19 infections (irrespective of gender) who were treated in the ICU of the 4th Military Clinical Hospital in Wroclaw during the period from September 2020 to September 2022 were enrolled in the present study. All of them were treated in an ICU for COVID-19 pneumonia (N = 160). Three groups were distinguished among these patients: patients with COVID-19 infection only (N = 72; COVID-19 only group), patients with COVID-19 infection and an infection caused by Klebsiella pneumoniae NDM (N = 30; COVID-19 with NDM infection group), and patients with COVID-19 infection and infection with bacterial etiology other than *Klebsiella pneumoniae* NDM (N = 58; COVID-19 with other infection group). RT-PCR tests for COVID-19 were used to diagnose SARS-CoV-2 infections. Patients with test-confirmed COVID-19 infections but without clinical symptoms, those with negative SARS-CoV-2 tests, and those aged below 18 years were excluded from the study.

2.3 Intervention

All patients admitted to the intensive care unit underwent a comprehensive set of screening tests (nasal swab, rectal swab) and diagnostic tests appropriate to the site of the ongoing infection (blood, urine, BAL, tissue, wound swab). According to the procedure regarding indications for collecting material for microbiological examinations, samples from the lower respiratory tract should be taken in the case of suspected ventilator-associated pneumonia with concomitant deviations in clinical and radiological findings. Urine cultures were performed in cases of fever with concomitant abnormalities in general urine examination and inflammatory parameter elevation. Immediate blood culture collection was indicated in cases of suspected infection with at least two of the following symptoms and/or parameters: body temperature 38.4–39.4°C, age > 65 years, chills, vomiting, blood pressure drops, leukocyte count >18,000/µl, granulocyte percentage > 80%, platelet count < 15,000/µl, and creatinine > 2 mg/dl. Tissue biopsies or wound

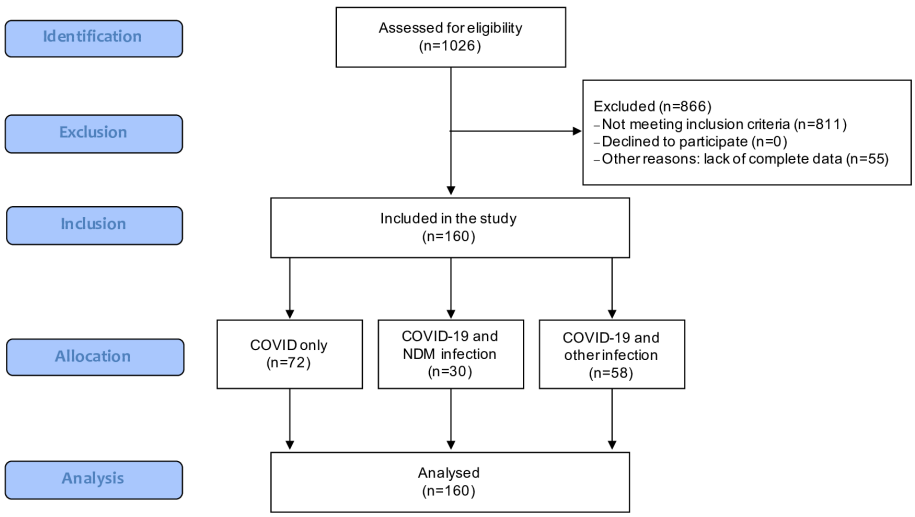


FIGURE 1
Flow-chart of study participants (STROBE).

swabs were taken only when signs of inflammation were present. The diagnosis of systemic infection was always established based on obtaining a positive microbiological result with concurrent clinical symptoms. Tests were repeated during hospitalization in the event of worsening clinical condition or lack of therapeutic success despite the use of targeted antibiotic therapy.

Infections were diagnosed when positive microbiological results were obtained in the presence of concurrent clinical symptoms. Colonization was identified when MDRO microorganisms were cultured from samples collected as part of screening tests, including nasal and rectal swabs.

2.4 Outcomes

The present study aimed to assess the impact of *Klebsiella pneumoniae* NDM infection on mortality in patients with SARS-CoV-2 infection during the pandemic.

2.5 Measures

Demographic data (age, weight, and height) were included in the study data. The procalcitonin (PCT), C-reactive protein (CRP), and white blood cell (WBC) levels were determined in the blood samples collected via venous cannula upon hospital admission and on the third, fifth, and seventh days of hospital stay. In addition, the Acute Physiology and Chronic Health Evaluation (APACHE II) and Simplified Acute Physiology Score (SAPS II) scales were used upon admission, and the Sequential Organ Failure Assessment (SOFA) score was assessed each day. The impact of comorbidities such as hypertension (AH), ischemic heart disease (IHD) and diabetes (DM) was taken into account in the analysis (Table 1).

2.6 Microbiology procedure

2.6.1 Blood culture

The materials for the study included the blood taken after prior disinfection of the skin based on the principles of collection of material for microbiological tests. The BacT/ALERT automatic blood culture monitoring system (bioMérieux, France) was used in the laboratory. Blood (8–10 ml volume) was collected in bottles intended for the cultivation of microorganisms, as follows: BacT/ALERT FA Plus medium (aerobic medium with antibiotic neutralizer; bioMérieux, France) and BacT/ALERT FN Plus medium (anaerobic medium with antibiotic neutralizer; bioMérieux, France). It was collected using a blood culture kit consisting of a sterile "butterfly" needle connected to a holder. Routinely, blood was drawn into a minimum of two blood culture kits (kit = 1 aerobic bottle + 1 anaerobic bottle). The incubation time was 5 days. When information about a positive sample was obtained from the automatic system, inoculation was done on the following solid media: Columbia agar with 5% sheep blood (bioMérieux, France), chocolate agar (bioMérieux, France), and MacConkey agar (bioMérieux, France). Then the plates were incubated at 35–37°C under aerobic conditions and with the addition of CO_2.

2.6.2 Urine culture

Urine samples were obtained either through self-administered clean catch or catheterization. The urine was mixed, and a sterile plastic loop (10 µL) was used to inoculate CHROMID® CPS® Elite/Columbia CNA + 5% sheep blood culture media (bioMérieux, France). All the plates were incubated at 37°C for ≥ 18 h and then examined for evidence of growth. Plates with < 10^3 colony-forming unit (CFU)/µL were reported as normal urogenital flora. Plates with growth (≥ 10^3 CFU/µL) were subcultured based on the standard procedures for bacterial identification and susceptibility.

2.6.3 Quantitative culture of bronchial secretion

Another material for the study was a secretion from the lower respiratory tract using bronchoalveolar lavage (BAL). Quantitative inoculation of BAL was done on the following culture media: Columbia agar with 5% sheep blood (bioMérieux, France), chocolate agar (bioMérieux, France), MacConkey agar (bioMérieux, France), Mannitol salt agar (bioMérieux, France), and Sabouraud medium with chloramphenicol (bioMérieux, France). The plates were incubated at 35–37°C under aerobic conditions and with the addition of CO_2.

2.6.4 Wound swabs/biopsy tissue samples/peritoneal fluid

Wound swabs were taken and placed in Amies transport media (bioMérieux, France), and biopsy tissue samples and peritoneal fluid were stored in thioglycollate broth (bioMérieux, France). A semi-quantitative technique was used to assess bacterial growth from the swabs. The samples were inoculated on Columbia agar (bioMérieux, France), chocolate agar (bioMérieux, France), MacConkey agar (bioMérieux, France), Mannitol salt agar (bioMérieux, France), and Sabouraud agar (bioMérieux, France) for overnight incubation at 37°C + 5% CO_2.

2.6.5 Central catheter tip

Semi-quantitative inoculation of the catheter according to Maki was performed using the following culture media: Columbia agar with 5% sheep blood (bioMérieux, France), MacConkey agar (bioMérieux, France), Mannitol salt agar (bioMérieux, France), and Sabouraud medium with chloramphenicol (bioMérieux, France). The plates were incubated at 35–37°C under aerobic conditions.

2.6.6 Rectal swabs

Rectal swabs for carbapenemase-producing bacilli were taken and placed in Amies transport media (bioMérieux, France); inoculated on CHROMID® CARBA SMART agar (bioMérieux, France), CHROMID® ESBL agar (bioMérieux, France), CHROMID® VRE agar (bioMérieux, France), and CHROMID® MRSA SMART (bioMérieux, France); and incubated aerobically at 35–37°C for 24 h.

10.3389/fcimb.2024.1297312

TABLE 1 Demographic characteristics of study population.

Variable	Total	COVID-19 and NDM infection	COVID-19 and other infection	COVID-19 only	p-value
Population (*N*)	160	30	58	72	
Gender					
Women, *n* (%)	56 (35%)	8 (27%)	17 (29%)	31 (43%)	0.15**
Men, *n* (%)	104 (65%)	22 (73%)	41 (71%)	42 (57%)	
Age (years)					
M ± *SD*	63.6 ± 12.8	65.6 ± 8.7	62.1 ± 15.2	63.9 ± 12.0	0.74*
Me [*Q1*; *Q3*]	67 [57; 72]	67 [61; 73]	66 [51; 73]	67 [55; 71]	
Min - Max	27 – 86	42 - 78	27 – 86	34 – 86	
Body high (cm)					
M ± *SD*	171 ± 10	172 ± 9	172.2 ± 9.0	170.4 ± 10.2	0.55*
Me [*Q1*; *Q3*]	174 [164; 180]	175 [164; 180]	175 [165; 180]	170 [160; 179]	
Min - Max	150 - 194	156 - 187	152 – 190	150 – 194	
Body weight (kg)					
M ± *SD*	88 ± 22	91 ± 22	88.8 ± 19.7	86.4 ± 24.1	0.37*
Me [*Q1*; *Q3*]	85 [73; 100]	90 [76; 101]	88 [74; 100]	83 [70; 97]	
Min - Max	42 – 193	42 - 170	57 – 148	43 – 193	
BMI (kg/m^2)					
M ± *SD*	30.0 ± 7.0	30.7 ± 6.6	29.9 ± 5.8	29.7 ± 7.9	0.38*
Me [*Q1*; *Q3*]	29 [25; 34]	29 [27; 35]	29.2 [25; 35]	28 [24; 33]	
Min - Max	16 - 65	16 - 50	19 – 43	19 – 65	
AH					
0, *n* (%)	67 (42%)	11 (37%)	25 (43%)	31 (43%)	0.81**
1, *n* (%)	93 (58%)	19 (63%)	33 (57%)	41 (57%)	
IHD					
0, *n* (%)	108 (68%)	20 (67%)	38 (66%)	50 (69%)	0.89**
1, *n* (%)	52 (52%)	10 (33%)	20 (34%)	22 (31%)	
DM					
0, *n* (%)	97 (61%)	19 (63%)	35 (60%)	43 (60%)	0.94**
1, *n* (%)	63 (39%)	11 (37%)	23 (40%)	29 (40%)	

n, number of participants; M, mean; Me, median; Min, minimum value; Max, maximum value; Q1, lower quartile; Q3, upper quartile; p, level of statistical significance.
*Kruskal-Wallis test; **Chi-square test.
AH, Arterial Hypertension; IHD, Ischemic Heart Disease; DM, Diabetes mellitus.

2.6.7 Nasal swabs

Nasal swabs for MRSA screening were taken and placed in Amies transport media (bioMérieux, France), inoculated on CHROMID® MRSA SMART (bioMérieux, France), and incubated aerobically at 35–37°C for 24 h.

2.6.8 Identification and drug susceptibility

When a homogeneous bacterial culture had been obtained, the microorganisms were identified, and their drug susceptibility was determined, using the VITEK automated system (bioMérieux, France). If necessary, drug susceptibility could be tested manually using concentration gradient strips (Liofilchem, Italy) and a MuellerHinton II medium (bioMérieux, France). The sensitivity level of the cultured strains to colistin was determined using the commercial ComASP Colistin test (Liofilchem, Italy). The results of the susceptibility tests were interpreted according to the current criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2021, 2022).

2.6.9 Assessment of carbapenemase production

For all bacterial strains resistant to carbapenems, the type of carbapenemase produced was determined using the commercial test RESIST-5 O.O.K.N.V. (CORIS BioConcept, Belgium), and phenotypic methods were used. In addition, strains from infections were sent to the National Reference Center for Microbial Antimicrobial Susceptibility (KORLD) for confirmation.

2.7 Ethical considerations

The study protocol was approved by the Bioethics Committee at The Military Medical Chamber in Warsaw, Jelinka 48 str., 01-646 Warsaw, Poland (approval no.: KB–3/21, approval date: 21.05.2021). The study was carried out in accordance with the guidelines of the Declaration of Helsinki and Good Clinical Practice. Informed and written consent was obtained from all the patients.

2.8 Sample size

Based on the preliminary analysis (COVID-19 only group n=72 vs. COVID-19 and NDM infection group n=30) of the number of events comparisons, the sample size was calculated. The following parameters were used to calculate the sample size: HR = 0.6, alpha = 0.05; power =0.8. In addition, the Bonferroni correction was included. Based on these parameters, the sample size was set at 160 patients (including a 10% dropouts and refusals). The required number of events for the log-rank test are calculated according to Schoenfeld's formula (Schoenfeld, 1983).

2.9 Statistical analysis

The calculations were carried out using STATISTICA v. 13.3 software (TIBCO Software Inc., USA). The qualitative variables were expressed as frequencies (n) and percentages (%). The Fisher's exact test was utilized to assess the association between qualitative variables. The quantitative variables (SOFA, APACHE II, SAPS II, PCT, CRP, WBC, LYMPH, NEUT, NLR) were presented as median values (Me) and quartiles (Q1, Q3) in the tables. The Kruskal-Wallis test was employed to determine the significance of differences in means between the COVID-19 and NDM infection, COVID-19 and other infection, and COVID-19 only groups. The overall survival (OS) was evaluated using the Kaplan-Meier method, and the log-rank test was used to compare the survival curves. The multivariate analysis was performed using the Cox proportional hazard regression model. A p-value less than 0.05 was considered to be statistically significant.

3 Results

3.1 Study group characteristics

The demographic data of the study population, divided into the COVID-19 with NDM infection, COVID-19 with other infection, and COVID-19 only groups, are shown in Table 1. The p-values represent the significance levels of the differences between the groups. There were no significant differences in age, body height, body weight, or body mass index between the groups.

No statistically significant differences were found in the groups' scores (APACHE II, SAPS II, SOFA—1, 3, 5, and 7 days), morphological test results (WBC, LYM, NEUT, TC, NRL), or biochemical test results (CRP, PCT) repeated at 1, 3, 5, and 7 days (Table 2).

No statistically significant differences were found in the groups regarding comorbidities (AH, IHD, DM) (Table 1).

Median length of hospitalization in the overall group (N = 160) and in the COVID-19 with other infection and COVID-19 only groups was 9 days; median length of hospitalization in the COVID-19 with NDM infection group was 12.5 days. (Table 3).

TABLE 2 Comparison of selected morphological, biochemical, and score tests between the group of: COVID-19 and NDM infection, COVID-19 and other infection and COVID-19 only.

Variables	Day	Total N = 160	COVID-19 and NDM infection	COVID-19 and other infection	COVID-19 only	p-value
		Me (Q1; Q3)	Me (Q1; Q3)	Me (Q1; Q3)	Me (Q1; Q3)	
APACHE II	1	19.0 (15.0; 25.0)	21.5 (19.0; 27.0)	18.0 (15.0; 22.0)	19.0 (15.0; 27.5)	0.11
SAPS II	1	50.5 (37.5; 66.0)	49.5 (40.0; 70.0)	49.0 (36.0; 59.0)	51.5 (37.0; 68.0)	0.41
SOFA	1	8.0 (5.0; 11.0)	9.5 (7.0; 11.0)	7.5 (5.0; 11.0)	8.0 (5.0; 11.0)	0.19
	3	10.0 (7.0; 12.0)	11.0 (9.0; 12.0)	9.0 (7.0; 12.0)	10.0 (7.0; 13.0)	0.39
	5	10.0 (6.0; 13.0)	11.0 (8.0; 13.0)	10.0 (7.0; 12.0)	11.0 (5.0; 14.0)	0.47
	7	10.0 (7.0; 14.0)	12.0 (8.0; 14.0)	10.0 (7.0; 13.0)	10.0 (4.0; 13.0)	0.33
WBC (x10^3/μl)	1	14.0 (9.4; 19.4)	14.2 (10.2; 19.3)	14.0 (9.3; 19.8)	13.4 (8.9; 19.6)	0.98
	3	12.9 (9.4; 18.1)	11.7 (9.5; 15.9)	13.0 (9.0; 20.9)	13.5 (10.6; 19.2)	0.61
	5	13.9 (9.5; 19.8)	13.1 (9.0; 20.1)	13.7 (9.5; 17.8)	14.8 (10.1; 19.9)	0.79
	7	13.3 (10.5; 18.1)	12.7 (10.7; 17.0)	14.4 (10.3; 18.6)	12.6 (10.5; 16.2)	0.63

(Continued)

10.3389/fcimb.2024.1297312

TABLE 2 Continued

Variables	Day	Total N = 160	COVID-19 and NDM infection	COVID-19 and other infection	COVID-19 only	p-value
		Me (Q1; Q3)	Me (Q1; Q3)	Me (Q1; Q3)	Me (Q1; Q3)	
LYM (%)	1	5.6 (3.3; 8.2)	5.5 (4.3; 7.9)	4.7 (2.7; 7.5)	6.0 (3.7; 8.8)	0.18
	3	5.9 (3.4; 8.5)	5.2 (3.4; 8.0)	5.0 (2.9; 8.0)	6.3 (3.9; 8.8)	0.60
	5	5.8 (4.1; 9.2)	5.8 (4.5; 8.3)	5.5 (2.7; 9.2)	6.4 (4.2; 10.4)	0.38
	7	6.2 (3.5; 10.1)	5.5 (4.0; 8.0)	5.4 (2.8; 9.2)	8.0 (4.8; 12.1)	0.11
NEUT (%)	1	88.0 (82.7; 91.2)	87.9 (85.0; 91.8)	88.8 (83.0; 91.0)	87.3 (82.2; 91.2)	0.44
	3	86.8 (82.0; 90.8)	89.1 (84.5; 92.0)	87.7 (80.0; 90.6)	86.2 (82.0; 90.0)	0.17
	5	85.0 (78.2; 89.6)	85.8 (80.8; 90.2)	85.5 (78.4; 90.7)	83.9 (77.0; 87.3)	0.22
	7	83.8 (77.7; 88.8)	85.8 (79.3; 89.8)	84.1 (78.1; 90.4)	81.0 (77.0; 85.0)	0.08
NLR	1	15.6 (10.4; 26.9)	16.2 (10.4; 21.5)	19.0 (11.2; 34.6)	14.0 (9.8; 24.4)	0.18
	3	14.9 (9.9; 27.3)	17.3 (11.0; 27.0)	17.3 (10.4; 31.9)	13.7 (9.6; 23.7)	0.56
	5	14.8 (9.1; 22.0)	15.3 (10.0; 19.2)	15.6 (9.3; 35.0)	12.8 (7.4; 20.4)	0.34
	7	13.9 (8.3; 24.2)	16.0 (10.3; 23.0)	15.9 (8.7; 33.3)	9.7 (6.3; 17.8)	0.10
PLT (x10^3/μl)	1	235.5 (160.5; 328.0)	216.5 (165.0; 291.0)	246.0 (165.0; 341.0)	237.5 (143.0; 317.0)	0.55
	3	241.0 (148.0; 317.0)	265.0 (177.5; 320.0)	234.0 (168.0; 323.0)	240.0 (141.0; 302.0)	0.50
	5	243.5 (159.0; 329.0)	270.5 (163.0; 348.5)	226.5 (149.0; 277.0)	250.0 (156.5; 334.0)	0.46
	7	229.5 (162.5; 313.5)	293.0 (186.0; 334.0)	206.0 (142.0; 296.0)	266.5 (176.0; 354.0)	0.05
CRP (mg/L)	1	154.5 (68.7; 230.0)	160.0 (104.0; 231.0)	124.8 (61.9; 220.0)	164.0 (66.9; 230.0)	0.46
	3	120.0 (71.7; 196.0)	139.1 (84.5; 205.0)	111.4 (55.0; 191.0)	126.5 (88.5; 209.0)	0.22
	5	137.8 (60.7; 224.0)	165.0 (102.5; 273.5)	123.4 (67.0; 213.0)	146.9 (44.9; 216.9)	0.18
	7	150.0 (70.0; 225.0)	172.5 (81.0; 297.0)	137.0 (69.0; 224.0)	140.5 (70.0; 222.0)	0.60
PCT (ng/mL)	1	0.7 (0.2; 2.1)	1.0 (0.3; 3.4)	0.7 (0.2; 1.4)	0.7 (0.2; 2.3)	0.21
	3	0.6 (0.2; 2.5)	0.8 (0.2; 3.5)	0.5 (0.1; 2.3)	0.7 (0.2; 2.3)	0.47
	5	0.5 (0.2; 1.7)	0.9 (0.2; 2.8)	0.5 (0.1; 1.5)	0.5 (0.2; 1.2)	0.35
	7	0.8 (0.2; 2.2)	1.4 (0.4; 3.9)	0.6 (0.2; 1.7)	0.5 (0.1; 1.8)	0.16

SOFA, the Sequential Organ Failure Assessment score; APACHE II, the Acute Physiology and Chronic Health Evaluation II score; SAPS II; WBC, White Blood Cells; LYM, Lymphocytes; NEUT, Neutrophils; NLR, neutrophil to lymphocyte ratio; PLT, platelets; CRP, C-reactive protein; PCT, procalcitonin.

3.2 Mortality

The overall survival rates of the COVID-19 with NDM infection, COVID-19 with other infection, and COVID-19 only groups are compared in Figure 2. Bacterial infection, including with *Klebsiella pneumoniae* NDM type, did not elevate mortality rates. In the group of patients who survive the acute phase of COVID-19 the prolonged survival time was demonstrated. The median overall survival time was 13 days in the COVID-19 with NDM infection group, 14 days in the COVID-19 with other infection group, and 7 days in the COVID-19 only group (Figure 2).

3.3 Independent predictors of outcomes

Both univariate and multivariate Cox proportional hazard regression models were used in the present study to examine the factors linked to mortality. The factors found to be significant in both the univariate and multivariate analyses were regarded as being associated with mortality (as indicated in Table 4). The results of the multivariate analysis revealed that age (HR = 1.017; $p = 0.046$), APACHE II score (HR = 1.042; $p \leq 0.001$), and CRP (HR = 1.002; $p = 0.008$) were predictors of mortality in all the groups. Comparing the COVID-19 with NDM infection and

TABLE 3 Length of hospitalization.

Length of stay (days)	Me	Q1	Q3
Total n=160	9	5	14
COVID-19 and NDM infection	12,5	7	19,75
COVID-19 and other infection + COVID-19 only	9	5	13

n, number of participants; Me, median; Q1, lower quartile; Q3, upper quartile.

COVID-19 only groups, the adjusted model estimated a statistically significant hazard ratio of 0.28 (p = 0.002), indicating that the patients in the COVID-19 with NDM infection group had a lower 28-day mortality rate (Table 4).

3.4 Susceptibility testing of NDM-producing *Klebsiella pneumoniae*

Susceptibility was tested against antibiotics routinely used in the treatment of infections. New therapeutic options, like cefiderocol, were not available in Polish hospitals during the study period. All the identified strains showed resistance to carbapenems. Seventy-five percent of the strains were sensitive to colistin, 84% were sensitive to amikacin, 94% were sensitive to trimethoprim/sulfamethoxazole, and 72% were sensitive to fosfomycin. Only 3% of the tested strains were sensitive to gentamycin. All examined isolates exhibited varied drug resistance. The phenotypic resistance patterns of the analyzed strains are presented in Figure 3.

4 Discussion

A 2022 report by the European Center for Disease Prevention and Control (2022) highlighted an escalating antimicrobial resistance, especially carbapenem-resistant strain of *Klebsiella pneumoniae*, from 2016 to 2020. The data revealed a concerning trend, and the situation in Poland, as confirmed by the National Reference Center for Antimicrobial Susceptibility Testing of Microorganisms, reflected this issue. The number of confirmed cases of carbapenemase-producing *Enterobacterales* strains surged from 2,064 in 2019 to 4,172 in 2021. Specifically, cases of NDM strains from infections increased from 1,527 in 2019 to 3,036 in 2021 (Hryniewicz et al., 2022).

Preventing the spread of multidrug-resistant strains, especially in ICUs, is a great challenge. In addition, during the COVID-19 pandemic, isolating patients with COVID-19 and ensuring an appropriate sanitary regime were significant challenges due to the need to use PPE. There was a rapid spread of infections caused by Gram-negative rods of the Enterobacterales family in this group of patients. Under natural conditions, these microorganisms colonize the digestive tract, being a component of the physiological flora (Pratt et al., 2001; Pelfrene et al., 2021).

In immunocompromised patients with accompanying lymphopenia (e.g., caused by SARS-CoV-2 infection), Gram-negative bacteria can cause respiratory infections, urinary tract infections, soft-tissue infections, and postoperative wound infections, including sepsis and septic shock. Carbapenem-resistant bacilli are currently the biggest clinical problem. The mechanisms responsible for the development of resistance are a decrease in the permeability of the outer sheaths with the simultaneous increased production of extended-spectrum beta-lactamases (ESBLs) or AmpC cephalosporins, as well as carbapenemase-type β-lactamases (Nordmann et al., 2011, 2012; European Centre for Disease Prevention and Control (ECDC), 2017).

During the COVID-19 pandemic, from September 2020 to the end of September 2022, the 4th Military Clinical Hospital in Wroclaw served as a center for the treatment of patients with COVID-19. It ensured the availability of 160 internist beds with an infectious profile, including 16 ICU beds. It provided security for patients with SARS-CoV-2 infection requiring surgery in the fields

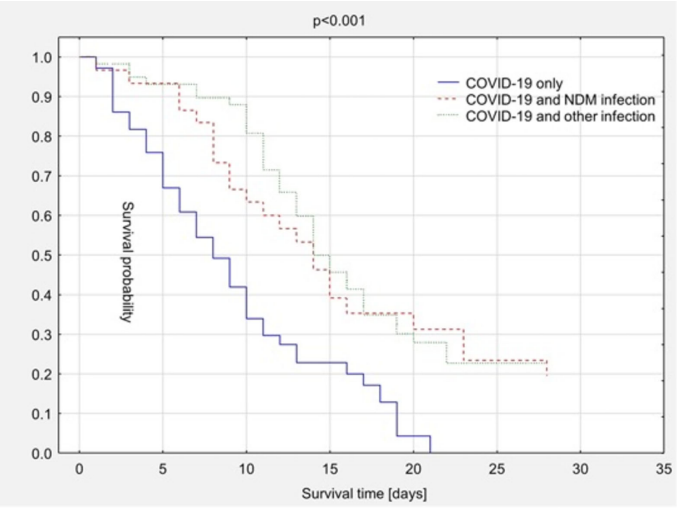


FIGURE 2
Kaplan-Meier survival curves – comparison depending on additional bacterial infection.

TABLE 4 Univariate and multivariate Cox regression analysis of risk factors influence on 28-days mortality.

Variables		Univariate model			
		HR	95% CI		p-value
Age		1.024	1.009	1.039	0.002
APACHE II		1.039	1.019	1.059	0.000
SAPS II		1.022	1.013	1.031	0.000
SOFA		1.111	1.060	1.164	0.000
WBC (x10^3/µl)		1.024	1.001	1.046	0.039
LYM (%)		0.999	0.968	1.030	0.937
NEUT (%)		1.002	0.978	1.026	0.885
NLR		0.998	0.993	1.004	0.514
PLT (x10^3/µl)		0.998	0.996	1.000	0.036
CRP (mg/L)		1.002	1.001	1.004	0.006
PCT (ng/mL)		1.008	1.003	1.013	0.001
Sex (ref. M)		1.102	0.750	1.619	0.621
NDM (ref. non-NDM)		0.751	0.473	1.191	0.223
Bacterial infection group (ref. COVID-19 only)	COVID-19 and NDM infection	0.421	0.252	0.702	0.122
	COVID-19 and other infection	0.370	0.241	0.569	0.006
		Multivariate model 1			
Age		1.018	1.002	1.034	0.032
APACHE II		1.037	1.016	1.059	0.001
CRP (mg/L)		1.002	1.001	1.004	0.005
NDM (ref. non-NDM)		0.526	0.324	0.853	0.009
		Multivariate model 2			
Age		1.017	1.000	1.034	0.046
APACHE II		1.042	1.020	1.063	0.000
CRP (mg/L)		1.002	1.001	1.004	0.008
Bacterial infection group (ref. COVID-19 only)	COVID-19 and NDM infection	0.280	0.161	0.486	0.002
	COVID-19 and other infection	0.365	0.234	0.569	0.075

HR, hazard ratio; CI, confidence interval; SOFA, the Sequential Organ Failure Assessment score; APACHE II, the Acute Physiology and Chronic Health Evaluation II score; SAPS II; WBC, White Blood Cells; LYM, Lymphocytes; NEUT, Neutrophils; PLT, platelets; CRP, C-reactive protein; PCT, procalcitonin.

of neurosurgery, urology, general surgery, laryngology, maxillofacial surgery, vascular surgery, oncology surgery, orthopedics, traumatology, and cardiac surgery. Overall, 1026 patients were hospitalized in the ICU in the analyzed period of which 160 were being treated for COVID-19. Despite the isolation of COVID-19 patients with confirmed infection and the elimination of additional risk factors for infection (e.g., restrictions on the movement of personnel, modification of airlocks, special supervision of hand decontamination, and double protection by protective clothing), an increase in the number of super-infections was observed. In the group of 160 ICU patients treated for COVID-19, *Klebsiella pneumoniae* ESBL or NDM strains were observed. The *Klebsiella* species are among the top 10 pathogens causing nosocomial bacterial infections (Mahmud et al., 2022). No *Klebsiella pneumoniae* infection carbapenemase- (KPC-), VIM-, or IMP-type cases was reported in the aforementioned period.

In our study the percentage of infections caused by CRE in patients on ICU with COVID-19 during the pandemic was 18.75%. According to available publications, the prevalence of CPE in patients with COVID-19 ranges between 0.35% and 54 (Lai et al., 2020; Mędrzycka-Dąbrowska et al., 2021; Yahya, 2022). According to Tiri et al. (2020), the number of cases during the COVID-19 pandemic increased from 6.7% to 50% of the patients treated in the ICU.

In the present study, three groups of ICU patients were extracted: those with NDM bacterial infection, those with other bacterial infection, and those with "pure" COVID-19. The estimated 28-day mortality rate in the whole group of ICU patients was 71.25%. This high mortality rate resulted from the severe course of COVID-19 in most patients in the first days after the onset of the disease. In particular, elderly patients with COVID-19 (COVID-19 only group) who were admitted in a serious condition to the ICU from intermittent wards, where steroids, antibiotics, non-invasive ventilation, prone position, and invasive ventilation had previously been used, which significantly limited their chances of survival. The patients who survived the first phase of the disease developed a bacterial infection. In the COVID-19 only group, there were 72 patients with a median survival time of 7 days. The patients in the COVID-19 and NDM infection and COVID-19 and other infection groups had an average survival time of 13 or 14 days. Fast identification of the pathogen and application of targeted treatment on ICU were shown to be major factors limiting mortality in the case of bacterial infection in the course of COVID-19.

The prolonged survival observed in the bacterial infection groups is hypothesized to be influenced by extended hospitalization durations, thereby establishing an association with a heightened risk of developing multidrug-resistant infections. This suggests a potential relationship between prolonged hospital stays, increased susceptibility to infections, and the subsequent development of multidrug-resistant infections. Patients who survived the acute phase of COVID-19 demonstrated an increased susceptibility to subsequent infections, potentially attributed to prolonged hospitalizations and, consequently, extended survival periods. This observation suggests a correlation between prolonged hospital stays, increased vulnerability to infections, and extended overall survival in this patient cohort.

The results of the multivariate analysis in the present study revealed that age (HR = 1.017; p = 0.046), APACHE II score (HR = 1.042; p ≤ 0.001), and CRP (HR = 1.002; p = 0.008) were predictors of mortality in all the patient groups.

In previous research, COVID-19-associated bacterial infections have resulted in increased mortality, particularly in the group with *Klebsiella pneumoniae*-type NDM infection (Said et al., 2022). In our study, no increase in mortality was demonstrated in the group of patients with additional infections (COVID-19 with NDM

	PHENOTYPE						
	1	2	3	4	5	6	7
MEM	R	R	R	R	R	R	R
IMP	R	R	R	R	R	R	R
AK	S	R	S	R	S	R	R
GE	S	R	R	R	R	R	R
SXT	S	R	S	S	S	R	S
COL	S	S	R	S	S	R	R
FOSF	S	R	S	R	R	R	S

FIGURE 3
Phenotypes of resistance. IMP, imipenem; MEM, meropenem; AK, amikacin; GE, gentamycin; SXT, trimethoprim/sulfamethoxazole; COL, colistin; FOSF, fosfomycin.

infection and COVID-19 with other infection groups). This finding differs from the findings presented in existing publications.

Based on the available data, the variant of the virus causing the infection also influenced mortality in the course of COVID-19. The lowest survival rate was observed during infection with the Delta variant (Lavrentieva et al., 2023). The spread of the Delta variant, which was more transmissible than the previously dominant Alfa variant, in the autumn of 2021 caused the fourth wave of the COVID-19 pandemic in Europe, including Poland. In the early autumn of 2021, the Delta variant of the SARS-CoV-2 virus dominated in Poland. According to the GISDAD EpiCoV database data, as of October 20, 2021, it accounted for over 97% of the SARS-CoV-2 sequences reported from Poland within seven days. According to European Center for Disease Prevention and Control data, in the 42nd week of 2021, the Delta variant accounted for 99.9% of SARS-CoV-2 infections in Poland. In addition, in the fourth wave of the pandemic, a high mortality rate of 62.9% was observed.

4.1 Study limitations

The present study had several limitations. First, it was a single-center study thus the results may not be representative of broader populations or diverse settings. Second, due to the observational nature of the study, several unmeasured confounders could have affected the outcomes. We focused on bacterial infection and ICU mortality. More data on the specific causes of death in COVID-19 could help interpret mortality rates better. The variant responsible for the infection was also not studied, which could have affected mortality.

5 Conclusions

In patients treated for SARS-CoV-2 infection acquiring a bacterial infection due to prolonged hospitalization associated with the treatment of COVID-19 did not elevate mortality rates. The data suggests that in severe COVID-19 patients who survived beyond the first week of hospitalization, bacterial infections, particularly Klebsiella pneumoniae NDM, do not significantly impact mortality. Multivariate analysis revealed that age, APACHE II score, and CRP were predictors of mortality in all the patient groups.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Bioethics Committee at The Military Medical Chamber in Warsaw, Jelinka 48 str., 01-646 Warsaw, Poland (approval no.: KB–3/21, approval date: 21.05.2021). 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

JJ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Visualization, Writing – original draft. NS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft. AW: Data curation, Formal analysis, Investigation, Visualization, Writing – review & editing. LŁ: Formal analysis, Software, Writing – review & editing. MC: Formal analysis, Writing – review

& editing. PL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft.

Funding

This study was not supported by the external fundings. The APC was funded by the subvention of the Faculty of Medicine at the Wrocław University of Science and Technology in Wrocław, Poland.

Acknowledgments

There were no other contributors to the article than the authors as well as there was no writing assistance regarding our paper.

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

Beović, B., Doušak, M., Ferreira-Coimbra, J., Nadrah, K., Rubulotta, F., Belliato, M., et al. (2020). Antibiotic use in patients with COVID-19: a "snapshot" Infectious Diseases International Research Initiative (ID-IRI) survey. *J. Antimicrob. Chemother.* 75, 3386–3390. doi: 10.1093/jac/dkaa326

de Wit, E., van Doremalen, N., Falzarano, D., and Munster, V. J. (2016). SARS and MERS: recent insights into emerging coronaviruses. *Nat. Rev. Microbiol.* 14, 523–534. doi: 10.1038/nrmicro.2016.81

El Mekes, A., Zahlane, K., Ait Said, L., Tadlaoui Ouafi, A., and Barakate, M. (2020). The clinical and epidemiological risk factors of infections due to multi-drug resistant bacteria in an adult intensive care unit of University Hospital Center in Marrakesh-Morocco. *J. Infect. Public Health* 13, 637–643. doi: 10.1016/j.jiph.2019.08.012

European Centre for Disease Prevention and Control (ECDC) (2017) *Antimicrobial resistance surveillance in Europe 2016*. Available at: https://www.ecdc.europa.eu/en/publications-data/antimicrobial-resistance-surveillance-europe-2016 (Accessed August 22, 2023).

European Centre for Disease Prevention and Control and World Health Organization (2022) *Antimicrobial resistance surveillance in Europe: 2022: 2020 data* (LU: Publications Office) (Accessed August 6, 2022).

Fernández-García, O. A., González-Lara, M. F., Villanueva-Reza, M., de-León-Cividanes, N., Xancal-Salvador, L. F., Esteban-Kenel, V., et al. (2022). Outbreak of NDM-1-producing Escherichia coli in a Coronavirus Disease 2019 intensive care unit in a Mexican tertiary care center. *Microbiol. Spectr.* 10, e0201521. doi: 10.1128/spectrum.02015-21

Gupta, N., Limbago, B. M., Patel, J. B., and Kallen, A. J. (2011). Carbapenem-resistant Enterobacteriaceae: epidemiology and prevention. *Clin. Infect. Dis.* 53, 60–67. doi: 10.1093/cid/cir202

Hekmatnia, Y., Rahmani, F., Feili, Z., and Ebrahimzadeh, F. (2022). A review of the effect of COVID-19 on immune responses of the body. *J. Family Med. Prim. Care* 11, 1624–1632. doi: 10.4103/jfmpc.jfmpc_677_21

W. Hryniewicz, A. Kuch, M. Wanke-Ryt and A. Żukowska (Eds.) (2022). *Pałeczki Enterobacterales wytwarzające karbapenemazy (CPE). Epidemiologia, diagnostyka, leczenie i profilaktyka zakażeń* (Warszawa: Ministerstwo Zdrowia).

Lai, C.-C., Wang, C.-Y., and Hsueh, P.-R. (2020). Co-infections among patients with COVID-19: The need for combination therapy with non-anti-SARS-CoV-2 agents? *J. Microbiol. Immunol. Infect.* 53, 505–512. doi: 10.1016/j.jmii.2020.05.013

Langford, B. J., So, M., Raybardhan, S., Leung, V., Westwood, D., MacFadden, D. R., et al. (2020). Bacterial co-infection and secondary infection in patients with COVID-19: a living rapid review and meta-analysis. *Clin. Microbiol. Infect.* 26, 1622–1629. doi: 10.1016/j.cmi.2020.07.016

Lavrentieva, A., Kaimakamis, E., Voutsas, V., and Bitzani, M. (2023). An observational study on factors associated with ICU mortality in Covid-19 patients and critical review of the literature. *Sci. Rep.* 13, 7804. doi: 10.1038/s41598-023-34613-x

Mędrzycka-Dąbrowska, W., Lange, S., Zorena, K., Dąbrowski, S., Ozga, D., and Tomaszek, L. (2021). Carbapenem-resistant Klebsiella pneumoniae infections in ICU COVID-19 patients—A scoping review. *J. Clin. Med.* 10, 2067. doi: 10.3390/jcm10102067

Mahmud, Z. H., Uddin, S. Z., Moniruzzaman, M., Ali, S., Hossain, M., Islam, M. T., et al. (2022). Healthcare facilities as potential reservoirs of antimicrobial resistant Klebsiella pneumoniae: an emerging concern to public health in Bangladesh. *Pharm. (Basel)* 15, 1116. doi: 10.3390/ph15091116

Monnet, D. L., and Harbarth, S. (2020). Will coronavirus disease (COVID-19) have an impact on antimicrobial resistance? *Euro. Surveill.* 25, 2001886. doi: 10.2807/1560-7917.ES.2020.25.45.2001886

Monteiro, J., Abboud, C. S., Inoue, F. M., Tufik, S., and Kiffer, C. R. V. (2023). NDM-producing Enterobacterales prevalence associated to COVID-19 in a tertiary hospital. *Braz. J. Infect. Dis.* 27, 102735. doi: 10.1016/j.bjid.2022.102735

Munoz-Price, L. S., Poirel, L., Bonomo, R. A., Schwaber, M. J., Daikos, G. L., Cormican, M., et al. (2013). Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases. *Lancet Infect. Dis.* 13, 785–796. doi: 10.1016/S1473-3099(13)70190-7

Nordmann, P., Dortet, L., and Poirel, L. (2012). Carbapenem resistance in Enterobacteriaceae: here is the storm! *Trends Mol. Med.* 18, 263–272. doi: 10.1016/j.molmed.2012.03.003

Nordmann, P., Naas, T., and Poirel, L. (2011). Global spread of carbapenemase-producing enterobacteriaceae. *Emerg. Infect. Dis.* 17, 1791–1798. doi: 10.3201/eid1710.110655

Nori, P., Szymczak, W., Puius, Y., Sharma, A., Cowman, K., Gialanella, P., et al. (2020). Emerging co-pathogens: New Delhi metallo-beta-lactamase producing enterobacterales infections in New York City COVID-19 patients. *Int. J. Antimicrob. Agents* 56, 106179. doi: 10.1016/j.ijantimicag.2020.106179

Pelfrene, E., Botgros, R., and Cavaleri, M. (2021). Antimicrobial multidrug resistance in the era of COVID-19: a forgotten plight? *Antimicrob. Resist. Infect. Control* 10, 21. doi: 10.1186/s13756-021-00893-z

Phannajit, J., Takkavatakarn, K., Katavetin, P., Asawavichienjinda, T., Tungsanga, K., Praditpornsilpa, K., et al. (2021). Factors associated with the incidence and mortality of coronavirus disease 2019 (COVID-19) after 126-million cases: A meta-analysis. *J. Epidemiol. Glob. Health* 11, 289–295. doi: 10.2991/jegh.k.210527.001

Porretta, A. D., Baggiani, A., Arzilli, G., Casigliani, V., Mariotti, T., Mariottini, F., et al. (2020). Increased risk of acquisition of New Delhi metallo-beta-lactamase-producing carbapenem-resistant enterobacterales (NDM-CRE) among a cohort of COVID-19 patients in a teaching hospital in Tuscany, Italy. *Pathogens* 9, 635. doi: 10.3390/pathogens9080635

Pratt, R. J., Pellowe, C., Loveday, H. P., Robinson, N., Smith, G. W., Barrett, S., et al. (2001). The epic project: developing national evidence-based guidelines for preventing healthcare associated infections. Phase I: Guidelines for preventing hospital-acquired infections. Department of Health (England). *J. Hosp. Infect.* 47 Suppl, S3–82. doi: 10.1053/jhin.2000.0886

Queenan, A. M., and Bush, K. (2007). Carbapenemases: the versatile beta-lactamases. *Clin. Microbiol. Rev.* 20, 440–458, table of contents. doi: 10.1128/CMR.00001-07

Rawson, T. M., Moore, L. S. P., Zhu, N., Ranganathan, N., Skolimowska, K., Gilchrist, M., et al. (2020). Bacterial and fungal coinfection in individuals with coronavirus: A rapid review to support COVID-19 antimicrobial prescribing. *Clin. Infect. Dis.* 71, 2459–2468. doi: 10.1093/cid/ciaa530

Sacha, P., Giżycka, D., Wieczorek, P., Kłosowska, W. M., Krawczyk, M., Czaban, S., et al. (2012). Genetic similarity and antimicrobial susceptibility of *Klebsiella pneumoniae* - producing carbapenemase (KPC-2) isolated in different clinical specimens received from University Hospitals in Northeastern Poland. *Afr. J. Microbiol. Res.* 6, 6888–6892. doi: 10.5897/AJMR12.266

Said, K. B., Alsolami, A., Moussa, S., Alfouzan, F., Bashir, A. I., Rashidi, M., et al. (2022). COVID-19 clinical profiles and fatality rates in hospitalized patients reveal case

aggravation and selective co-infection by limited gram-negative bacteria. *Int. J. Environ. Res. Public Health* 19, 5270. doi: 10.3390/ijerph19095270

Schoenfeld, D. A. (1983). Sample-size formula for the proportional-hazards regression model. *Biometrics* 39, 499–503. doi: 10.2307/2531021

Sun, K., Chen, J., and Viboud, C. (2020). Early epidemiological analysis of the coronavirus disease 2019 outbreak based on crowdsourced data: a population-level observational study. *Lancet Digit. Health* 2, e201–e208. doi: 10.1016/S2589-7500(20)30026-1

The European Committee on Antimicrobial Susceptibility Testing (2021) *Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0.* Available at: https://www.eucast.org/eucast_news/news_singleview?tx_ttnews%5Btt_news%5D=404&cHash=74a1f440876cb3eefb60e6ad39c622e7.

The European Committee on Antimicrobial Susceptibility Testing (2022) *Breakpoint tables for interpretation of MICs and zone diameters. Version 12.0.* Available at: https://www.eucast.org/eucast_news/news_singleview?tx_ttnews%5Btt_news%5D=464&cHash=ea8540c0fbdaa71b3bbcb3bf765239de.

Tiri, B., Sensi, E., Marsiliani, V., Cantarini, M., Priante, G., Vernelli, C., et al. (2020). Antimicrobial stewardship program, COVID-19, and infection control: spread of carbapenem-resistant Klebsiella pneumoniae colonization in ICU COVID-19 patients. What did not work? *J. Clin. Med.* 9, 2744. doi: 10.3390/jcm9092744

von Elm, E., Altman, D. G., Egger, M., Pocock, S. J., Gøtzsche, P. C., Vandenbroucke, J. P., et al. (2007). Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *BMJ* 335, 806–808. doi: 10.1136/bmj.39335.541782.AD

Wan, S., Xiang, Y., Fang, W., Zheng, Y., Li, B., Hu, Y., et al. (2020). Clinical features and treatment of COVID-19 patients in northeast Chongqing. *J. Med. Virol.* 92, 797–806. doi: 10.1002/jmv.25783

Wang, N., Zhan, M., Wang, T., Liu, J., Li, C., Li, B., et al. (2023). Long term characteristics of clinical distribution and resistance trends of carbapenem-resistant and extended-spectrum β-lactamase Klebsiella pneumoniae infections: 2014–2022. *Infect. Drug Resist.* 16, 1279–1295. doi: 10.2147/IDR.S401807

Yahya, R. O. (2022). Problems associated with co-infection by multidrug-resistant Klebsiella pneumoniae in COVID-19 patients: A review. *Healthc. (Basel)* 10, 2412. doi: 10.3390/healthcare10122412

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K., et al. (2009). Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. *Antimicrob. Agents Chemother.* 53, 5046–5054. doi: 10.1128/AAC.00774-09

Zhong, H., Wang, Y., Shi, Z., Zhang, L., Ren, H., He, W., et al. (2021). Characterization of respiratory microbial dysbiosis in hospitalized COVID-19 patients. *Cell Discovery* 7, 23. doi: 10.1038/s41421-021-00257-2

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Mini Review
PUBLISHED 24 April 2024
DOI 10.3389/fcimb.2024.1339285

OPEN ACCESS

EDITED BY
Sandra Aguiar,
University of Lisbon, Portugal

REVIEWED BY
Sandip Patil,
Shenzhen Children's Hospital, China
Magdalena Zalewska,
University of Warsaw, Poland

*CORRESPONDENCE
Carolina Barrientos-Salcedo
cabarrientos@uv.mx

RECEIVED 15 November 2023
ACCEPTED 25 March 2024
PUBLISHED 24 April 2024

CITATION
Robles Ramirez O, Osuna G, Plisson F and Barrientos-Salcedo C (2024) Antimicrobial peptides in livestock: a review with a one health approach.
Front. Cell. Infect. Microbiol. 14:1339285.
doi: 10.3389/fcimb.2024.1339285

Antimicrobial peptides in livestock: a review with a one health approach

Oscar Robles Ramirez[1], Gabriel Osuna[2], Fabien Plisson[2] and Carolina Barrientos-Salcedo[3]*

[1]Doctorate in Agricultural Sciences, Facultad de Medicina Veterinaria y Zootecnia (FMVZ) Universidad Veracruzana, Veracruz, Mexico, [2]Irapuato Unit, Department of Biotechnology and Biochemistry, Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV-IPN), Irapuato, Mexico, [3]Medicinal Chemistry and Chemogenomics Laboratory, Facultad de Bioanálisis, Universidad Veracruzana, Veracruz, Mexico

Antimicrobial peptides (AMPs), often referred to as nature's antibiotics, are ubiquitous in living organisms, spanning from bacteria to humans. Their potency, versatility, and unique mechanisms of action have garnered significant research attention. Unlike conventional antibiotics, peptides are biodegradable, adding to their appeal as potential candidates to address bacterial resistance in livestock farming—a challenge that has been under scrutiny for decades. This issue is complex and multifactorial, influenced by a variety of components. The World Health Organization (WHO) has proposed a comprehensive approach known as One Health, emphasizing the interconnectedness of human-animal-environment relationships in tackling such challenges. This review explores the application of AMPs in livestock farming and how they can mitigate the impact of this practice within the One Health framework.

KEYWORDS

antimicrobial peptides, veterinary medicine, mastitis, One Health, bacterial resistance

Introduction

Conventional antibiotics have been extensively used for metaphylaxis and as growth promoters in various forms of livestock farming. The correlation between administering antibiotics at sub-therapeutic doses and enhanced animal weight gain is the driving force behind this practice (Dodds, 2017). These sub-therapeutic doses create a favorable environment for beneficial bacteria while impacting intestinal mucosa and motility, *i.e.*, reducing undesirable pathogens and nutrient wastage (Bacanlı and Başaran, 2019) and improving overall animal health (Palma et al., 2020). However, the use and abuse of antibiotics exert selective pressure on microorganisms, contributing to the surges of antimicrobial resistance (AMR) within intensive animal food production systems. Metaphylaxis, in particular, extends this selective pressure to entire animal groups when an infection is identified in one individual (Economou and Gousia,

2015). In both cases, sensitive microorganisms in infected animals or asymptomatic carriers among healthy animals develop resistance (Figure 1) (Mellor et al., 2019).

The rise of AMR has direct and indirect consequences on our public health and global economy. The direct consequences of AMR include impacts on food security and animal health, with infected animals facing slaughter or mutilation in the absence of effective treatment. Indirect consequences include the associated costs of treating and quarantining infected animals and the public health risks of drug-resistant pathogens with zoonotic potential. The World Health Organization (WHO) has proposed a unifying strategy known as One Health to prevent emerging zoonoses such as H1N1 flu and Hendra virus (Becker et al., 2022). The organization suggests shifting the current paradigm in our global food systems by recognizing the interdependence between the health of humans, domestic and wild animals, and the wider environment. This approach relies on the collective participation of communal, federal, and national entities to ensure the surveillance, regulation, and policy over antibiotic use (Figure 1) (Schmidt et al., 2017; Algammal et al., 2020; Bolte et al., 2020; Maity and Ambatipudi, 2021; Molineri et al., 2021; Velazquez-Meza et al., 2022). By working together, government and industries can identify emerging threats and develop more effective therapies.

Most organisms produce antimicrobial peptides (AMPs) as defense mechanisms in response to pathogenic infections. AMPs have gradually emerged as promising alternatives within the One Health approach in addressing the global challenge of AMR (Magana et al., 2020). First, the peptides exhibit broad-spectrum microbicidal activities against various pathogens, including bacteria, fungi, parasites, and viruses (Mookherjee et al., 2020). This makes them invaluable in both human and veterinary medicine. Second, AMPs do not induce bacterial resistance mechanisms due to their non- specific mechanisms of action on the bacterial membranes (Anaya-López et al., 2013; Assoni et al., 2020). This property is crucial in the fight against AMR, aligning with the One Health approach by offering a sustainable solution across human, animal, and environmental health. Finally, AMPs occur in Nature and are often biodegradable, posing fewer ecological risks than conventional antibiotics, which can persist and promote resistance development in microbial communities. By reducing environmental contamination, AMPs support the ecological aspect of the One Health approach. In summary, AMPs represent sustainable, broad-spectrum, and environmental-friendly alternatives to conventional antibiotics, directly supporting the goals of the One Health approach. Here, we describe the promising uses of antimicrobial peptides in veterinary medicine.

Environment and antibiotic bioaccumulation

Antibiotics, much like plastic and fossil fuels, are among the significant advancements of the 20th century that were developed and utilized for their immediate value without a comprehensive understanding of their long-term environmental and biological impacts. Consequently, the presence of antibiotics in various natural environments, including lakes, rivers, and fields fertilized with biosolids, is well-documented in current scientific literature (Storteboom et al., 2010; Booth et al., 2020). The persistence of antibiotics in the environment (Figure 1) is a concern because a substantial proportion of antibiotics is not entirely metabolized and is excreted while retaining their activity (Sukul et al., 2009). These residual antibiotics persist in natural settings, interacting with various bacteria. This includes organisms such as Escherichia coli, which can originate from wastewater discharges, and free-living bacteria like *Vibrio cholerae* (Jang et al., 2017; De, 2021). In these interactions, residual antibiotics can trigger various biological mechanisms that promote bacterial resistance. Among these mechanisms are selective

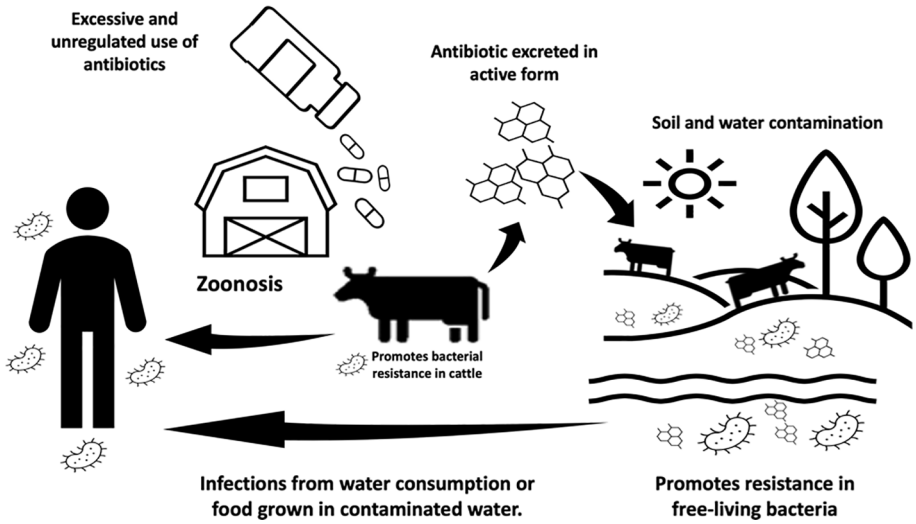


FIGURE 1
Schematic pathway and ramifications of antibiotic contamination in animals and the environment.

pressure and the exchange of mobile genetic elements. Thus, the environment plays a crucial role in disseminating antimicrobial resistance (AMR) (Williams et al., 2016).

Zoonoses and the spread of antimicrobial resistance

Zoonoses are transmissible diseases from animals to humans, such as the well-documented examples of anthrax caused by *Bacillus anthracis*, bovine tuberculosis by *Mycobacterium tuberculosis*, brucellosis by *Brucella abortus*, and hemorrhagic colitis by *Escherichia coli* (Rahman et al., 2020). Beyond bacteria, zoonotic pathogens also include viruses (*e.g.*, Hendra virus, influenza virus A) (Leifels et al., 2022) and parasites (e.g., *Trypanosoma cruzi* and *Toxoplasma gondii*) (Weiss, 2008). These pathogens can reach humans through direct contact with food, water, or the environment (Figure 1). They represent a global health threat due to our close relationships with animals in agriculture, as companions, and in the natural environment. Zoonoses disrupt our current food production systems, leading to the spread of foodborne outbreaks (Abebe et al., 2020; Preena et al., 2020). Zoonoses have also become the sources of AMR with pathogens like extended-spectrum beta- lactamase (ESBL)-producing *E. coli* and methicillin-resistant *Staphylococcus aureus*), becoming notably resistant to our antibiotics. Comprehensive reviews have documented the connections between zoonotic diseases and the spread of antimicrobial resistance (Srivastava and Purohit, 2020; Olaru et al., 2023). The indirect transmission of zoonoses involves vector insects or pets acting as a bridge between production areas and households (Bolte et al., 2020). The need to discover antibacterial molecules that are environmentally non-persistent has become a top priority. Antimicrobial peptides (AMPs) emerge as potential alternatives to contemporary antibiotics. To understand the progress in this domain, we listed peptides tested in livestock production (Table 1), considering associated bacteria for different livestock types and emphasizing achieved outcomes.

Swine livestock

In swine, one of the most important bacteria in veterinary clinics is *Haemophilus parasuis*, which causes Glässer's disease. Teixeira and colleagues, in 2013, isolated a peptide from the culture supernatant of *Bacillus subtilis* subsp. *spizezinii*. Characterization of the peptide revealed antimicrobial activity against *H. parasuis* (Teixeira et al., 2013). On the other hand, using peptides as dietary additives for pigs has proven beneficial, as demonstrated by Tang and colleagues in 2011. They analyzed the effect of dietary supplementation with 100 mg/kg of lactoferricin peptide in a model of gastrointestinal infections by *E. coli* in 21-day-old pigs.

The study yielded positive results for the animals, with one of the main effects being the alteration of the gastrointestinal microbiome. This led to beneficial consequences such as improved nutrient retention and intestinal morphology, reduced incidences of gastrointestinal diseases like diarrhea, and promoted animal growth. This treatment reduced the concentration of *E. coli* and increased the presence of commensal bacteria such as *Lactobacillus* and *Bifidobacterium*. Additionally, it counteracted the effects of E. coli on intestinal architecture by promoting the height of intestinal villi in the jejunum and ileum compared to the group without the peptide. Furthermore, lower concentrations of proinflammatory cytokines such as TNF-alpha, IL-1 beta, and IL-6 were found, along with higher concentrations of growth hormone in the treated pigs (Tang et al., 2011). Another group of peptides evaluated for swine pathogens were surfactins from *B. subtilis* and *Bacillus licheniformis* against intestinal pathogens *Brachyspira hyodysenteriae* and *Clostridium perfringens*, which cause swine dysentery and necrotic enteritis. In this group of peptides, both the dose-response relationship and treatment effectiveness were compared between them. The results showed that *B. subtilis* surfactin is more effective against *B. hydrodysenteriae*, while *B. licheniformis* surfactin is more effective against *C. perfringens*. This suggests that peptide isoforms between species may have selective effectiveness against specific pathogens (Horng et al., 2019).

Goat livestock

Among the peptides studied in goats, researchers have explored recombinant porcine defensin PBD-mI and the peptide isolated from flies, LUC-n. The supplementation of these peptides resulted in a notable alteration of the rumen microbiome, as demonstrated by the analysis of 16S bacterial genes and 18S rRNA genes of ciliated protozoa. Post-treatment analysis revealed an increase in beneficial genera, such as *Fibrobacter*, *Anaerovibrio*, *Succiniclasticum*, and *Ophrysocolex*, while pathogenic genera, like *Selenomonas*, *Succinivibrio*, *Treponema*, *Polyplastron*, *Entodinium*, and *Isotricha* decreased. Additionally, there were changes in enzymatic activity, including xylanase, pectinase, and lipase (Liu et al., 2017). These peptides underwent further evaluation using a different experimental design. Goats were divided into three groups: a control group, one supplemented with 2 grams per day of a combination of AMPs, and another with 3 grams per day. Ren and colleagues conducted this study in 2019 with a distinct experimental setup but arrived at results similar to the previous study: changes in digestion translated into an increase in the mass of treated animals compared to the control group. It is noteworthy that the group administered with 2 grams per day of the peptide combination showed greater mass gain than those given 3 grams, leading to the conclusion that the administered peptide quantity does not linearly affect the increase in body mass of animals, at least in goats. Therefore, an appropriate dosage is more relevant than a high amount administered (Ren et al., 2019). Bacteriocins from *Bacillus thuringiensis* were also evaluated in clinical isolates of goat mastitis. Various pathogenic species, such as *Enterococcus durans*, *Brevibacillus* spp., *Enterobacter* sp., *Escherichia vulneris*, *Pantoea* spp., *Pseudomonas brenneri*, and encapsulated yeast *Cryptococcus neoformans*, as well as several *Staphylococcus* species, were identified. The microbiocidal activity was observed in 67% of these isolated bacteriocins. However, species like *Staphylococcus epidermidis*, *Enterobacter* sp., *E. vulneris*, and *C. neoformans* proved resistant to all bacteriocins (Gutiérrez-Chávez et al., 2016). Finally, a

10.3389/fcimb.2024.1339285

TABLE 1 List of AMPs used as antibiotics, supplements, and food preservatives in animal products.

	Source	Peptide	Target	Application	Reference
Swine	*B. subtilis*	NRWCFAGDD	*H. parasuis*	Antibiotic	(Teixeira et al., 2013)
	Milk	Lactoferricin	*E. coli*	Suplement	(Tang et al., 2011)
	B. subtilis	Surfactins	*B. hydrodysenteriae*	Antibiotic	(Horng et al., 2019)
	B. licheniformis	Surfactins	*C. perfringens*	Antibiotic	(Horng et al., 2019)
Goat	Pigs/flies	PBD–mI/LUC–n	*Selenomonas*, *Succinivibrio*, *Treponema*, *Polyplastron*, *Entodinium*, *Isotricha*	Suplement	(Liu et al., 2017)
	B. thuringiensis	Bacteriocins	*E. durans*, *B. spp.*, *P. spp.*, *P. brenneri.*	Antibiotic	(Gutiérrez-Chávez et al., 2016)
	Silk moth	Cecropin B	*S. aureus*	Antibiotic	(Luo et al., 2013)
Bovine	*P. nigrella*	Plectasin	*S. aureus*	Antibiotic	(Li et al., 2017)
	Bbpi	Bbpi90–99,148-161	*S. aureus*, *E. coli*, *P. aeruginosa*	Antibiotic	(Chockalingam et al., 2007)
	Lactoferrin	Lactoferricin	*E. coli*, *S. aureus*, *S. zopfii*, *yeasts*	Antibiotic	(Bruni et al., 2016)
	S. aureus *S. Epidermidis*	A53/a70	*S. aureus* y *S. agalactiae*	Antibiotic	(Varella Coelho et al., 2007)
	B. Thuringiensis	Morricin 269 Kurstacin 287	*S. aureus*	Antibiotic	(Barboza-Corona et al., 2009)
	Streptococcus equinus	Bovicin HC5	*S. aureus*, *S. agalactiae*, *S. bovis*, *S. uberis*	Food preservative	(Godoy-Santos et al., 2019)
	L. lactis	Nisin	*Staphylococcus spp*	Antibiotic	(Castelani et al., 2019)
	Cows	Tap	*S. aureus*, *E. coli*	Antibiotic	(Sharma et al., 2017)
Poultry	*B. subtilis*	Sublancina	*C. perfringens*	Antibiotic	(Wang et al., 2015)
	Epinephelus lanceolatus	Piscidina	*S. aureus*, *E. coli*, *P. aeruginosa*	Suplement	(Tai et al., 2020)

novel perspective on mastitis treatment in goats involves the generation of peptides directly in milk. This is achieved by transfecting animals with plasmid vectors containing the peptide sequence. For instances, cecropin B, an AMP from the giant silk moth, was transfected into goat mammary glands, resulting in inhibitory effects against *S. aureus* (Luo et al., 2013).

Bovine livestock

Cattle suffer from various zoonotic diseases, including tuberculosis (e.g., *Mycobacterium* bovina) and mastitis (e.g., *Corynebacterium diphtheriae*, *Staphylococcus aureus*), leading severe loss in animal production. Several peptides have been particularly evaluated to treat *S. aureus*-induced bovine mastitis and ulcers on teats, as the pathogen can reside intracellularly within mammary gland epithelial cells (Alva-Murillo et al., 2017). For example, the peptides NZ2114 and MP1102, derived from plectasin, an amphipathic peptide isolated from the fungus *Pseudoplectania nigrella*, were evaluated in murine models of *S. aureus*-induced mastitis and sterile milk cultures. Of note, milk components (among other pathophysiological factors) might significantly affect peptides (Schmelcher et al., 2015). As a result, the peptides were also tested on bovine mammary epithelial cells infected with this bacterium. Both peptides exhibited activity against *S. aureus* in sterile milk cultures, indicating that their effectiveness is not compromised in milk. They demonstrated intracellular activity against *S. aureus* without any cytotoxic effects at concentrations

of up to 100 µg/mL. These peptides were also effective in experimental mastitis treatment (Li et al., 2017). Bacteriocins are another group of antimicrobial peptides that were evaluated against *S. aureus*-induced bovine mastitis. As such example, in 2009, Barbosa and colleagues determined that S. aureus AMR isolates obtained from milk of cows diagnosed with mastitis were sensitive to five bacteriocins from *B. thuringiensis*, with morricin 269 and kurstacin 287 exhibiting the greatest activity (Barboza-Corona et al., 2009). Likewise, bovicin HC5, a bacteriocin obtained from *Streptococcus equinus* HC5 (found in the horse gastrointestinal tract), possesses valuable characteristics for food preservation. It is a thermo-stable peptide with a mechanism of action described as lipid II-dependent, soluble at neutral pH, and effective even in acidic pH. This peptide inhibited the growth of mastitis-causing bacteria such as *S. aureus*, *S. agalactiae*, *Streptococcus bovis*, and *Streptococcus uberis* at concentrations ranging from 40 to 2560 arbitrary units (u.a.)/mL (Godoy-Santos et al., 2019). Finally, in 2021, Sharma and colleagues proposed the directed expression of the tracheal antimicrobial peptide (Tap) to treat *S. aureus*-associated mastitis in mice. They observed significant antibacterial effects in both *in vitro* and *in vivo* experiments by introducing a vector with the TAP peptide into mice infected with S. aureus associated with bovines (Sharma et al., 2021). Similarly, in 2017, the expression of a peptide derived from lactoferricin was carried out using the PiggyBac plasmid in bovine mammary epithelial cells, resulting in successful protection against *S. aureus* and *E. coli* (Sharma et al., 2017). The aforementioned peptides are all derived from external sources. Some peptides originally from cattle or their milk have also been studied for their antimicrobial properties. In 2007, Chockalingam and co-workers synthesized the hybrid peptide named bBPI90–99,148-161, where bBPI stands for bovine bactericidal permeability-increasing protein. This hybrid peptide had an average inhibitory concentration of 16 µg/mL against *E. coli* and 128 µg/mL for *Pseudomonas aeruginosa*, but was ineffective against *Serratia marcescens*. In addition, the antimicrobial activity of the peptide decreased after being suspended in milk, while it remained stable when tested in serum. These results indicated the importance of assessing different fluids in which the peptides may be administered (Chockalingam et al., 2007). Cow milk also holds a rich source of proteins and potential AMPs. Examples include beta-lactoglobulin, alpha-lactalbumin, and lactoferrin (Mohanty et al., 2016). The latter is a glycoprotein with iron- binding properties that plays a significant role in the bovine immune system. This 692-residue protein has demonstrated antimicrobial, anti-inflammatory, and immunomodulatory effects (Woodman et al., 2018). Lactoferricin is a small peptide derived from lactoferrin, which has been effective in treating subclinical mastitis caused by *E. coli and Staphylococci* in cattle. It also exhibited *in vitro* activity against an alga called *Prototheca zopfii*, responsible for protothecal mastitis, and yeast strains causing fungal mastitis (Bruni et al., 2016).

Poultry industry

Clostridium perfringens is one of the most significant pathogens in the poultry industry, being the primary etiological agent of necrotic enteritis, a disease that causes substantial economic losses for producers (Ben Lagha et al., 2017). Factors predisposing to infection and various strategies to control the effects of this pathogen have been extensively studied (Allaart et al., 2013). In 2015, Wang and colleagues reported sublancin, a bacteriocin produced by *B. subtilis*, for its potential antimicrobial effect against *C. perfringens* in chickens. Their results revealed that the peptide displayed similar antimicrobial activity to the commercial antibiotic lincomycin. Unlike the group administered with sublancin, the lincomycin-treated group also experienced a reduction in *Lactobacillus* colonies, a commensal bacterium in the chicken digestive system. Sublancin might not affect other bacterial species in the avian intestinal microbiomes, but there is evidence of potentially greater specificity between species compared to conventional antibiotic treatments (Wang et al., 2015). Alternatively, peptides have also been employed as dietary supplements to promote growth in poultry. One such example is piscidin, isolated from the fish *Epinephelus lanceolatus*, which was utilized as a dietary additive and compared with control groups. Its antimicrobial activity against strains of *S. aureus*, *E.coli*, *P. aeruginosa*, and various strains of *Riemerella antipestifer* was evaluated, with the first three and some *R. antipestifer* strains proving sensitive to the peptide (Tai et al., 2020). Piscidin also exhibited immunomodulatory properties, increasing the secretion of interferon-gamma, immunoglobulins G, and interleukin-10 compared to the control group. Finally, the peptide induced significant changes in bacterial communities, increasing the families *Enterococcaceae* and *Lactobacilliaceae* while decreasing *Enterobacteriaceae* and *Staphylococceaceae*.

Antimicrobial peptides in livestock-relevant clinical strains

Antimicrobial peptides have been used against important clinical strains of the same genus and species. For example, bacteriocins isolated from *S. aureus* and *S. epidermidis* against other strains of *S. aureus* and *Streptococcus agalactiae* isolated from bovine mastitis. In 2007, Varella Coelho and co-workers identified bacteriocin A53 had a moderate effect against some clinical strains. However, when used in combination with A70, a bacteriocin with a similar effect alone, the synergistic effect resulted in increasing the inhibition percentages in *S. agalactiae* (i.e., from 67.6% to 91.9%) and in *S. aureus* (i.e., from 74.4% to 91.5%) (Varella Coelho et al., 2007). These results suggest that antimicrobial peptides from pathogenic bacteria might be helpful against similar pathogens. Another bacteriocin named nisin from *Lactococcus lactis* has been noted for having high antimicrobial activity against Gram-positive bacteria. In 2019, Castelani and co-workers evaluated its antimicrobial potential against *Staphylococcus* spp. isolated from cases of bovine mastitis. The peptide exhibited antibacterial effects against antibiotic-resistant strains. In combination with dioctadecyldimethylammonium bromide, a quaternary amine with broad antimicrobial activity, it enhanced the susceptibility of isolates to the bacteriocin, reducing the minimum bactericidal concentration from 50 to 3 µg/mL

(Castelani et al., 2019). These two examples showed that synergistic effects should be considered in future studies.

Conclusions

The significance of developing therapies in veterinary medicine aligns with the "One Health" concept, acknowledging the interdependence of health across ecosystems, including livestock, pets, wildlife, and plants. Embracing this holistic approach, the urgency to invest in novel peptide-based antimicrobials becomes apparent. These peptides exhibit dual mechanisms of action: direct effects on microorganisms and stimulation of the host's immune response, enhancing their effectiveness against infections. A particularly challenging scenario is observed in bovine mastitis, where the need for peptides capable of remaining stable in the presence of milk components is crucial. Regulatory challenges and concerns about resistance to AMPs complicate their applications in livestock. Despite these challenges, the slower pace of bacterial adaptation to these peptides compared to conventional antibiotics places AMPs as novel therapeutic agents against infections, extending beyond the realms of veterinary clinics and mastitis. AMPs offer a potential solution to the scarcity of effective antibiotics against multidrug-resistant bacteria, emphasizing the need for responsible antibiotic use across all sectors. Efforts to manage antibiotic use, including exploring strategies like genetically engineered microbes for environmental clean-up, are imperative for global health and food security.

Author contributions

OR: Investigation, Writing – original draft. GO: Writing – review & editing. FP: Validation, Writing – review & editing. CB-S: Writing – review & editing, Conceptualization, Funding acquisition, Supervision.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Dirección General de Investigaciones (DGI-UV), Universidad Veracruzana. 2023 Call for Support for Publication Payments.

Acknowledgments

We thank the Dirección General de Cómputo y de Tecnologías de Información y Comunicación (DGTIC) of the Universidad Nacional Autónoma de México (UNAM) for allocation of computer time on the Miztli supercomputer and DGAPA-UNAM grant PAPIIT- IN210023. And to Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo (CYTED) (219RT0573). Red Temática en Salud. Desarrollo de Péptidos Antivirales y Antimicrobianos para Cepas Multi-resistentes.

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

Abebe, E., Gugsa, G., and Ahmed, M. (2020). Review on major food-borne zoonotic bacterial pathogens. *J. Trop. Med* 2020, e4674235. doi: 10.1155/2020/4674235

Algammal, A. M., Hetta, H. F., Elkelish, A., Alkhalifah, D. H. H., Hozzein, W. N., Batiha, G. E.-S., et al. (2020). Methicillin-resistant *staphylococcus aureus* (MRSA): One health perspective approach to the bacterium epidemiology, virulence factors, antibiotic- resistance, and zoonotic impact. *Infect. Drug Resist* 13, 3255–3265. doi: 10.2147/IDR.S272733

Allaart, J. G., van Asten, A. J. A. M., and Gröne, A. (2013). Predisposing factors and prevention of Clostridium perfringens-associated enteritis. *Comp. Immunol. Microbiol. Infect. Dis* 36, 449–464. doi: 10.1016/j.cimid.2013.05.001

Alva-Murillo, N., Ochoa-Zarzosa, A., and López-Meza, J. E. (2017). Sodium Octanoate Modulates the Innate Immune Response of Bovine Mammary Epithelial Cells through the TLR2/P38/JNK/ERK1/2 Pathway: Implications during *Staphylococcus aureus* Internalization. *Front. Cell Infect. Microbiol* 7. doi: 10.3389/fcimb.2017.00078

Anaya-López, J. L., López-Meza, J. E., and Ochoa-Zarzosa, A. (2013). Bacterial resistance to cationic antimicrobial peptides. *Crit. Rev. Microbiol* 39, 180–195. doi: 10.3109/1040841X.2012.699025

Assoni, L., Milani, B., Carvalho, M. R., Nepomuceno, L. N., Waz, N. T., Guerra, M. E. S., et al. (2020). Resistance mechanisms to antimicrobial peptides in gram- positive bacteria. *Front. Microbiol* 11. doi: 10.3389/fmicb.2020.593215

Bacanlı, M., and Başaran, N. (2019). Importance of antibiotic residues in animal food. *Food Chem. Toxicol* 125, 462–466. doi: 10.1016/j.fct.2019.01.033

Barboza-Corona, J. E., de la Fuente-Salcido, N., Alva-Murillo, N., Ochoa-Zarzosa, A., and López-Meza, J. E. (2009). Activity of bacteriocins synthesized by Bacillus thuringiensis against *Staphylococcus aureus* isolates associated to bovine mastitis. *Vet. Microbiol* 138, 179–183. doi: 10.1016/j.vetmic.2009.03.018

Becker, D. J., Eby, P., Madden, W., Peel, A. J., and Plowright, R. K. (2022). Ecological conditions predict the intensity of Hendra virus excretion over space and time from bat reservoir hosts. *Ecol. Letter* 26, 23–36. doi: 10.1111/ele.14007

Ben Lagha, A., Haas, B., Gottschalk, M., and Grenier, D. (2017). Antimicrobial potential of bacteriocins in poultry and swine production. *Vet. Res* 48, 22. doi: 10.1186/s13567-017-0425-6

Bolte, J., Zhang, Y., Wente, N., Mahmmod, Y. S., Svennesen, L., and Krömker, V. (2020). Comparison of phenotypic and genotypic antimicrobial resistance patterns associated with *Staphylococcus aureus* mastitis in German and Danish dairy cows. *J. Dairy Sci* 103, 3554–3564. doi: 10.3168/jds.2019-17765

Booth, A., Aga, D. S., and Wester, A. L. (2020). Retrospective analysis of the global antibiotic residues that exceed the predicted no effect concentration for antimicrobial resistance in various environmental matrices. *Environ. Int* 141, 105796. doi: 10.1016/j.envint.2020.105796

Bruni, N., Capucchio, M. T., Biasibetti, E., Pessione, E., Cirrincione, S., Giraudo, L., et al. (2016). Antimicrobial activity of lactoferrin-related peptides and applications in human and veterinary medicine. *Molecules* 21, 752. doi: 10.3390/molecules21060752

Castelani, L., Arcaro, J. R. P., Braga, J. E. P., Bosso, A. S., Moura, Q., Esposito, F., et al. (2019). Short communication: Activity of nisin, lipid bilayer fragments and cationic nisin-lipid nanoparticles against multidrug-resistant Staphylococcus spp. *isolated bovine mastitis. J. Dairy Sci* 102, 678–683. doi: 10.3168/jds.2018-15171

Chockalingam, A., Zarlenga, D. S., and Bannerman, D. D. (2007). Antimicrobial activity of bovine bactericidal permeability–increasing protein–derived peptides against gram-negative bacteria isolated from the milk of cows with clinical mastitis. *Am. J. Vet. Res* 68, 1151–1159. doi: 10.2460/ajvr.68.11.1151

De, R. (2021). Mobile genetic elements of vibrio cholerae and the evolution of its antimicrobial resistance. *Front. Trop. Dis* 2. doi: 10.3389/fitd.2021.691604

Dodds, D. R. (2017). Antibiotic resistance: A current epilogue. *Biochem. Pharmacol* 134, 139–146. doi: 10.1016/j.bcp.2016.12.005

Economou, V., and Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial- resistant bacteria. *Infect. Drug Resist* 8, 49–61. doi: 10.2147/IDR.S55778

Godoy-Santos, F., MS, P., Barbosa, A. A. T., Brito, M. A. V. P., and Mantovani, H. C. (2019). Efficacy of a ruminal bacteriocin against pure and mixed cultures of bovine mastitis pathogens. *Indian J. Microbiol* 59, 304–312. doi: 10.1007/s12088-019-00799-w

Gutiérrez-Chávez, A. J., Martínez-Ortega, E. A., Valencia-Posadas, M., León-Galván, M. F., de la Fuente-Salcido, N. M., Bideshi, D. K., et al. (2016). Potential use of Bacillus thuringiensis bacteriocins to control antibiotic-resistant bacteria associated with mastitis in dairy goats. *Folia Microbiol* 61, 11–19. doi: 10.1007/s12223-015-0404-0

Horng, Y.-B., Yu, Y.-H., Dybus, A., Hsiao, F. S.-H., and Cheng, Y.-H. (2019). Antibacterial activity of Bacillus species-derived surfactin on Brachyspira hyodysenteriae and Clostridium perfringens. *AMB Express* 9, 188. doi: 10.1186/s13568-019-0914-2

Jang, J., Hur H, -G., Sadowsky, M. J., Byappanahalli, M. N., Yan, T., and Ishii, S. (2017). Environmental Escherichia coli: ecology and public health implications—a review. *J. Appl. Microbiol* 123, 570–581. doi: 10.1111/jam.2017.123.issue-3

Leifels, M., Khalilur Rahman, O., Sam, I.-C., Cheng, D., Chua, F. J. D., Nainani, D., et al. (2022). The one health perspective to improve environmental surveillance of zoonotic viruses: lessons from COVID-19 and outlook beyond. *ISME Commun* 2, 107. doi: 10.1038/s43705-022-00191-8

Li, L., Wang, L., Gao, Y., Wang, J., and Zhao, X. (2017). Effective Antimicrobial Activity of Plectasin-Derived Antimicrobial Peptides against Staphylococcus aureus Infection in Mammary Glands. *Front. Microbiol* 8. doi: 10.3389/fmicb.2017.02386

Liu, Q., Yao, S., Chen, Y., Gao, S., Yang, Y., Deng, J., et al. (2017). Use of antimicrobial peptides as a feed additive for juvenile goats. *Sci. Rep* 7, 12254. doi: 10.1038/s41598-017-12394-4

Luo, C., Yin, D., Gao, X., Li, Q., and Zhang, L. (2013). Goat mammary gland expression of cecropin B to inhibit bacterial pathogens causing mastitis. *Anim. Biotechnol* 24, 66–78. doi: 10.1080/10495398.2012.745417

Magana, M., Pushpanathan, M., Santos, A. L., Leanse, L., Fernandez, M., Ioannidis, A., et al. (2020). The value of antimicrobial peptides in the age of resistance. *Lancet Infect. Dis* 20, e216–e230. doi: 10.1016/S1473-3099(20)30327-3

Maity, S., and Ambatipudi, K. (2021). Response to Comments on "Mammary microbial dysbiosis leads to the zoonosis of bovine mastitis: a One-Health perspective" by Maity and Ambatipudi. *FEMS Microbiol. Ecol* 97(1), fiaa241. doi: 10.1093/femsec/fiab079

Mellor, K. C., Petrovska, L., Thomson, N. R., Harris, K., Reid, S. W. J., and Mather, A. E. (2019). Antimicrobial resistance diversity suggestive of distinct *salmonella typhimurium* sources or selective pressures in food-production animals. *Front. Microbiol* 10. doi: 10.3389/fmicb.2019.00708

Mohanty, D., Jena, R., Choudhury, P. K., Pattnaik, R., Mohapatra, S., and Saini, M. R. (2016). Milk derived antimicrobial bioactive peptides: A review. *Int. J. Food Prop* 19, 837–846. doi: 10.1080/10942912.2015.1048356

Molineri, A. I., Camussone, C., Zbrun, M. V., Suárez Archilla, G., Cristiani, M., Neder, V., et al. (2021). Antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis: Systematic review and meta-analysis. *Prev. Vet. Med* 188, 105261. doi: 10.1016/j.prevetmed.2021.105261

Mookherjee, N., Anderson, M. A., Haagsman, H. P., and Davidson, D. J. (2020). Antimicrobial host defence peptides: functions and clinical potential. *Nat. Rev. Drug Discovery* 19, 311–332. doi: 10.1038/s41573-019-0058-8

Olaru, I. D., Walther, B., and Schaumburg, F. (2023). Zoonotic sources and the spread of antimicrobial resistance from the perspective of low and middle-income countries. *Infect. Dis. Poverty* 12, 59. doi: 10.1186/s40249-023-01113-z

Palma, E., Tilocca, B., and Roncada, P. (2020). Antimicrobial resistance in veterinary medicine: An overview. *Int. J. Mol. Sci* 21, 1914. doi: 10.3390/ijms21061914

Preena, P. G., Swaminathan, T. R., Kumar, V. J. R., and Singh, I. S. B. (2020). Antimicrobial resistance in aquaculture: a crisis for concern. *Biologia* 75, 1497–1517. doi: 10.2478/s11756-020-00456-4

Rahman, M. T., Sobur, M. A., Islam, M. S., Ievy, S., Hossain, M. J., El Zowalaty, M. E., et al. (2020). Zoonotic diseases: Etiology, impact, and control. *Microorganisms* 8, 1405. doi: 10.3390/microorganisms8091405

Ren, Z., Yao, R., Liu, Q., Deng, Y., Shen, L., Deng, H., et al. (2019). Effects of antibacterial peptides on rumen fermentation function and rumen microorganisms in goats. *PLoS One* 14, e0221815. doi: 10.1371/journal.pone.0221815

Schmelcher, M., Powell, A. M., Camp, M. J., Pohl, C. S., and Donovan, D. M. (2015). Synergistic streptococcal phage λSA2 and B30 endolysins kill streptococci in cow milk and in a mouse model of mastitis. *Appl. Microbiol. Biotechnol* 99, 8475–8486. doi: 10.1007/s00253-015-6579-0

Schmidt, K., Mwaigwisya, S., Crossman, L. C., Doumith, M., Munroe, D., Pires, C., et al. (2017). Identification of bacterial pathogens and antimicrobial resistance directly from clinical urines by nanopore-based metagenomic sequencing. *J. Antimicrob. Chemother* 72, 104–114. doi: 10.1093/jac/dkw397

Sharma, A., Gaur, A., Kumar, V., Sharma, N., Patil, S. A., Verma, R. K., et al. (2021). Antimicrobial activity of synthetic antimicrobial peptides loaded in poly-Ɛ-caprolactone nanoparticles against mycobacteria and their functional synergy with rifampicin. *Int. J. Pharm* 608, 121097. doi: 10.1016/j.ijpharm.2021.121097

Sharma, N., Huynh, D. L., Kim, S. W., Ghosh, M., Sodhi, S. S., Singh, A. K., et al. (2017). A PiggyBac mediated approach for lactoferricin gene transfer in bovine mammary epithelial stem cells for management of bovine mastitis. *Oncotarget* 8, 104272–104285. doi: 10.18632/oncotarget.v8i61

Srivastava, S., and Purohit, H. J. (2020). Zoonosis: an emerging link to antibiotic resistance under "One health approach. " *Indian J. Microbiol* 60, 139–152. doi: 10.1007/s12088-020-00860-z

Storteboom, H., Arabi, M., Davis, J. G., Crimi, B., and Pruden, A. (2010). Identification of antibiotic- resistance-gene molecular signatures suitable as tracers of pristine river, urban, and agricultural sources. *Environ. Sci. Technol* 44, 1947–1953. doi: 10.1021/es902893f

Sukul, P., Lamshöft, M., Kusari, S., Zühlke, S., and Spiteller, M. (2009). Metabolism and excretion kinetics of 14C-labeled and non-labeled difloxacin in pigs after oral administration, and antimicrobial activity of manure containing difloxacin and its metabolites. *Environ. Res* 109, 225–231. doi: 10.1016/j.envres.2008.12.007

Tai, H.-M., Huang, H.-N., Tsai, T.-Y., You, M.-F., Wu, H.-Y., Rajanbabu, V., et al. (2020). Dietary supplementation of recombinant antimicrobial peptide Epinephelus lanceolatus piscidin improves growth performance and immune response in Gallus gallus domesticus. *PLoS One* 15, e0230021. doi: 10.1371/journal.pone.0230021

Tang, Z. R., ZH, S., XS, T., ZM, F., Zhou, D., DF, X., et al. (2011). Dietary bovine lactoferricin improved gut microflora, benefited intestinal mucosal morphology and inhibited circular cytokines delivery in 21-day weaned piglets. *J. Appl. Anim. Res* 39, 153–157. doi: 10.1080/09712119.2011.565570

Teixeira, M. L., Rosa, A. D., and Brandelli, A. (2013). Characterization of an antimicrobial peptide produced by *Bacillus subtilis* subsp. *spizezinii showing inhibitory activity towards Haemophilus parasuis. Microbiol* 159, 980–988. doi: 10.1099/mic.0.062828-0

Varella Coelho, M. L., Do Santos Nascimento, J., Fagundes, P. C., Madureira, D. J., de Oliveira, S. S., Vasconcelos de Paiva Brito, M. A., et al. (2007). Activity of staphylococcal bacteriocins against *Staphylococcus aureus* and *Streptococcus agalactiae* involved in bovine mastitis. *Res. Microbiol* 158, 625–630. doi: 10.1016/j.resmic.2007.07.002

Velazquez-Meza, M. E., Galarde-López, M., Carrillo-Quiróz, B., and Alpuche-Aranda, C. M. (2022). Antimicrobial resistance: One Health approach. *Vet. World* 15, 743–749. doi: 10.14202/vetworld.2022.743-749

Wang, S., Zeng, X. F., Wang, Q. W., Zhu, J. L., Peng, Q., Hou, C. L., et al. (2015). The antimicrobial peptide sublancin ameliorates necrotic enteritis induced by *Clostridium perfringens* in broilers. *J. Anim. Sci* 93, 4750–4760. doi: 10.2527/jas.2015-9284

Weiss, L. M. (2008). Zoonotic parasitic diseases: Emerging issues and problems. *Int. J. Parasitol* 38, 1209–1210. doi: 10.1016/j.ijpara.2008.05.005

Williams, M. R., Stedtfeld, R. D., Guo, X., and Hashsham, S. A. (2016). Antimicrobial resistance in the environment. *Water Environ. Res* 88, 1951–1967. doi: 10.2175/106143016X14696400495974

Woodman, T., Strunk, T., Patole, S., Hartmann, B., Simmer, K., and Currie, A. (2018). Effects of lactoferrin on neonatal pathogens and Bifidobacterium breve in human breast milk. *PLoS One* 13, e0201819. doi: 10.1371/journal.pone.0201819

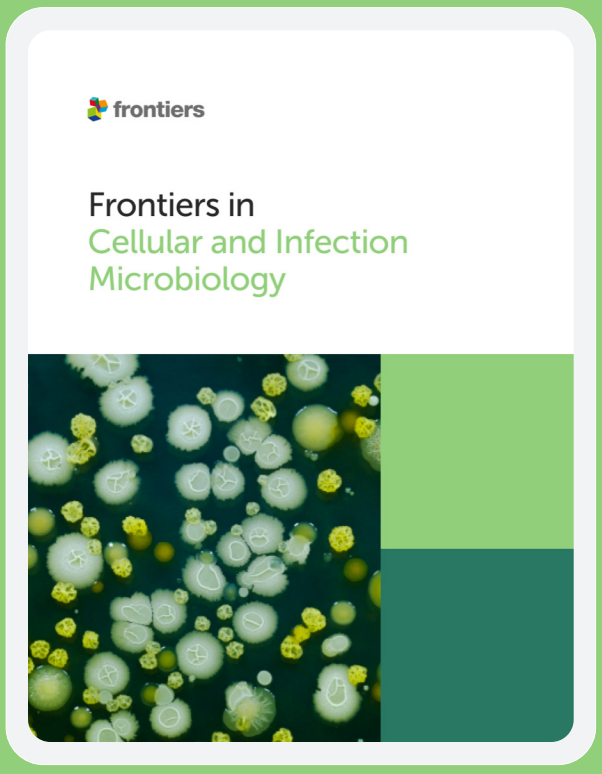
Frontiers in
Cellular and Infection Microbiology

Investigates how microorganisms interact with their hosts

Explores bacteria, fungi, parasites, viruses, endosymbionts, prions and all microbial pathogens as well as the microbiota and its effect on health and disease in various hosts.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact