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Antimicrobial resistance in pediatric infectious diseases: Antimicrobial resistance, resistance mechanisms and antimicrobial use

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Antimicrobial resistance in pediatric infectious diseases: Antimicrobial resistance, resistance mechanisms and antimicrobial use

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Editorial: Antimicrobial resistance in pediatric infectious diseases: antimicrobial resistance, resistance mechanisms and antimicrobial use

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Editorial on the Research Topic
Antimicrobial resistance in pediatric infectious diseases: antimicrobial resistance, resistance mechanisms and antimicrobial use

Antimicrobial Resistance (AMR) occurs when bacteria, viruses, fungi and parasites no longer respond to antimicrobial agents. As a result of drug resistance, antibiotics (usually used for bacteria) and other antimicrobial agents become ineffective and infections become difficult or impossible to treat, increasing the risk of disease spread, severe illness and death. AMR is a global concerning problem, especially in pediatrics. AMR is a serious threat to public health worldwide. Between 2020-2030, patients with antimicrobial resistant infections in Western Pacific region will spend 172 million extra days in hospital, and an estimated 5.2 million deaths will be caused by drug-resistant bacterial infections in the region (WHO, 2023). Children are major consumers of antimicrobial agents and have high rates of AMR. Children's underdeveloped immune systems make them more susceptible to infectious diseases such as pneumonia and meningitis and are treated with antibiotics. Insufficient understanding of the resistance mechanisms of common pediatric pathogens and lack of pediatric-specific data have both contributed to the overuse and misuse of antibiotics, making antibiotic resistance in pediatric infections a growing threat to public health. Macrolide- and clindamycin-resistant *Streptococcus pneumoniae* and *Bordetella pertussis* are serious problems for children in some countries, such as China. The detection rate of carbapenem-resistant *Enterobacteriaceae* was also higher in children than in adults. Because children are in a special period of growth and development, their pharmacokinetic (PK) and pharmacodynamic (PD) characteristics vary widely, making it difficult to determine age-dependent doses. The lack of pediatric-specific data is also an important

cause of the irrational use of antibiotics in children, leading to treatment failure and antibiotic resistance.

This research topic focuses on the antimicrobial-resistant pathogens associated with pediatric patients including *Enterobacteriaceae*, β-hemolytic *Streptococcus* species (*Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae subsp. equisimilis*, etc), *Streptococcus pneumoniae*, *Staphylococcus*, *Bordetella pertussis*, *Mycoplasma pneumoniae* and *Mycobacterium tuberculosis*. A better understanding of the resistance phenotype, transfer, and mechanism of antibiotic-resistant bacteria can fill gaps and expand our knowledge of resistant bacterial epidemiology while uncovering interesting patterns of distribution of strain types. Strategies in the battle against antibiotic-resistant bacteria should be compiled. In addition, a understanding of the current situation of most commonly prescribed pediatric medications can lead to create new guidelines to improve antibiotic stewardship. Antibiotic prescribing patterns for children including hospitalized children, outpatients, or children admitted to the emergency department and General Practice, the multi-center survey will be better. In addition, the search for natural antibacterial compounds and chemical synthesis of novel antibacterial products will help to influence antibiotic resistant bacteria. Finally, the study of COVID-19 associated antibiotic resistant bacterial pathogens circulating in Intensive Care Units can reduce the severity of morbidity and mortality caused by COVID-19.

We seek papers on, but not limited to, the following topics:—Antibiotic resistances and multidrug resistances in pediatric pathogens, including resistance phenotypes and genotypes, antibiotic resistance genetic determinants, antibiotic resistance gene transfer.—Antibiotic resistance epidemiology in children.—PK and PD studies of antibiotics in children.—The rationale for use of antibiotics in children.—Novel antibacterial substances for pediatric use. The result of this call is a relatively comprehensive Research Topic of 17 articles regarding such aspects.

Antibiotic resistances and multidrug resistances

Li et al. give a comprehensive review of the severe problem of macrolide resistance to common pathogens in China. Various common pathogens have shown high resistance rates and high resistance level to macrolides in Chinese children. Yu et al. give a review of penicillin binding protein and *S. pyogenes* (group A *Streptococcus*, GAS) with reduced β-lactam susceptibility. They summarize the current published data on GAS penicillin binding proteins and β-lactam susceptibility, to explore the relationship between them, and to be alert to the emergence of GAS with reduced susceptibility to β-lactams. Cao et al. present a case of spinal muscular atrophy with extensively drug-resistant *Acinetobacter baumannii* pneumonia treated with nebulization combined with intravenous polymyxin B. They present their experience in the diagnosis and treatment of this case and review it in the context of the literature. Wang et al. provide an experimental study about a novel mechanisms of macrolide resistance revealed by *in vitro* selection and genome analysis in *Mycoplasma pneumoniae*. This study presented the first *in vitro* data of induced midecamycin resistance in *M. pneumoniae* and the potential advantage of using midecamycin as an alternative first treatment choice for *M. pneumoniae* infections in patients. Zhuang et al. review the trends and challenges of multi-drug resistance in childhood tuberculosis. This review provides an overview of the current epidemiology of childhood tuberculosis (TB) and drug-resistant tuberculosis (DR-TB), including prevalence, incidence, and mortality. They highlight the urgent need for improved diagnosis and treatment of DR-TB in children.

Antibiotic resistance epidemiology

Guo et al. provide a dynamic change of serotype distribution and antimicrobial resistance of pneumococcal isolates since PCV13 administration and COVID-19 control in Urumqi, China. Su et al. describe the 10-year trends in multicenter investigation in China about the antibiotic susceptibility and clonal distribution of *Staphylococcus aureus* from pediatric skin and soft tissue infections. Wu et al. present an antimicrobial resistance profile of methicillin-resistant *S. aureus* isolates in children reported from the Infectious Disease Surveillance of Pediatrics (ISPED) surveillance of bacterial resistance, 2016–2021. Wang et al. present the characterization of resistance genes and plasmids from sick children caused by *Salmonella enterica* resistance to azithromycin in Shenzhen, China. Kawata et al. describe the fecal carriage rate of extended-spectrum β-lactamase–producing or carbapenem-resistant *Enterobacterales* among Japanese infants in the community at the 4-month health examination in a rural city. Xiao et al. present the antibiotic susceptibility of *Escherichia coli* isolated from neonates admitted to neonatal intensive care units across China from 2015 to 2020. Jiang et al. discuss the clinical significance of macrolide resistance in pediatric *Mycoplasma pneumoniae* infection during COVID-19 pandemic. Weidmann et al. report the assessing respiratory viral exclusion and affinity interactions through coinfection incidence in a pediatric population during the 2022 resurgence of influenza and RSV.

PK and PD studies of antibiotics

Peng et al. demonstrate that the vancomycin dosages of 40-60 mg/kg/d are effective and have no vancomycin-related nephrotoxicity adverse effects in children with Gram positive bacterial sepsis. Vancomycin trough concentrations >15 mg/L are not an essential target for these Gram-positive bacterial sepsis patients.

The rational use of antibiotics

Antibiotics are a double-edged sword, and when used, we must avoid not only overuse, but also underuse. In recent years, countries around the world have introduced antibiotic action plans to control antibiotic resistance in bacteria and strictly control the clinical use of

antibiotics, which has led to the underuse of clinical antibiotics (Hsia et al., 2019; Zhang et al., 2019; WHO, 2023). Yu et al. give a case report about rational use of antibiotics, in which 3 cases of septic arthritis in children caused by *S. pyogenes* were reported. Once *S. pyogenes* infection is confirmed, β-lactam antibiotics provide effective treatment, avoiding use of broad-spectrum antibiotics.

Underuse is common, as shown by: 1. not using it in time when it should be used; 2. insufficient dosage; 3. insufficient duration. These lead to an increase in bacterial infections and even epidemics. This phenomenon can be confirmed by the outbreak of GAS epidemic in the UK in 2022 (The Lancet Microbe, 2023; Venkatesan, 2023). Based on the great controversy in the implementation of β-lactam antibiotic skin tests, especially the controversial cephalosporin skin tests in pediatrics, the mechanism and reasons of anaphylaxis to β-lactam antibiotics, the significance of β-lactam antibiotic skin tests, the current state of β-lactam antibiotic skin tests at home and abroad, and the problems of domestic and international skin tests were analyzed to determine a unified standard of β-lactam antibiotic skin tests in pediatrics to prevent and decrease adverse drug reactions, avoid waste of drugs, and a large amount of manpower and material resource consumption. Gao et al. review the state and consideration for skin test of β-lactam antibiotics in pediatrics.

Novel antibacterial substances

Multi-drug resistant TB (MDR-TB) is often undiagnosed in children due to lack of awareness or under-diagnosis, and the target for children's DR-TB treatment has only been met in 15% of goals. However, due to age and weight differences, adults and children require different dosages. New medications such as bedaquiline and delamanid have been approved for treating DR-TB. Zhu et al. give a comprehensive review of the advances of new drugs bedaquiline and delamanid in the treatment of multi-drug resistant tuberculosis in children. Their review highlights the use of bedaquiline and delamanid as potential treatments for children with MDR-TB. They summarize their development history, efficacy, safety and potential adverse effects. Further research is necessary to determine the optimal use of these drugs for treating MDR-TB in children.

Concluding remarks

The problem of antimicrobial resistance in children cannot be ignored. We sincerely thank all contributors and reviewers for their support in putting this timely Research Topic together and hope that the readers will find useful answers to their questions.

Author contributions

DY: Writing – original draft, Writing – review & editing. YZ: Funding acquisition, Writing – review & editing. AS: Writing – review & editing. FW: Writing – review & editing. AD: Writing – review & editing. MK: Writing – review & editing. YY: Funding acquisition, Resources, Writing – review & editing.

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Conflict of interest

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

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Case Report: Septic arthritis in children caused by *Streptococcus pyogenes*–rational use of antibiotics

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To investigate the clinical characteristics and treatment of septic arthritis caused by *Streptococcus pyogenes*(*S. pyogenes*) in children, we retrospectively analyzed the clinical data, laboratory results, treatments and outcomes of three pediatric cases of septic arthritis caused by *S. pyogenes* occurring from 2016–2018. The three cases of septic arthritis included 1 boy and 2 girls, aged from 2–7 years. Two patients experienced fever, and in all three cases, the affected joints showed redness, swelling, an increased local skin temperature, tenderness and restricted limb movement. At the first visit, all three cases showed a significantly increased white blood cell count [(27.68–32.02)×10^9/mL] and a significantly increased erythrocyte sedimentation rate (113–134 mm/h). The C-reactive protein level was significantly increased in two cases (67 mg/L, 147.7 mg/L) and normal in one case. The procalcitonin level was normal in 1 case, elevated in 1 case, and undetected in 1 case. *S. pyogenes* isolated from cases 1 and 2 were *emm*1/ST28 and from case 3 was *emm*12/ST36. All patients were treated by abscess incision and drainage, and *S. pyogenes* was cultured in the abscess puncture fluid. All patients were treated with intravenous antibiotics after admission, and all patients were cured and discharged. The patients were followed up for 2 months, and their condition was improved and stable. No sequelae such as heart and kidney damage were detected. In conclusion, for children with septic arthritis, early diagnosis and timely treatment with incision and drainage followed by culture of the abscess puncture fluid are important. Once *S. pyogenes* infection is confirmed, β-lactam antibiotics provide effective treatment, avoiding use of broad-spectrum antibiotics.

KEYWORDS

***Streptococcus pyogenes*, child, septic arthritis, antibiotics, resistance**

Introduction

Group A *Streptococcus* (GAS, also known as *Streptococcus pyogenes*), is an important pathogen that most commonly colonizes the upper respiratory tract and skin epithelium (Rouchon et al., 2017). Its pathogenicity corresponds to a broad spectrum of conditions, including pharyngitis, impetigo, scarlet fever, nephritis, rheumatic fever, rheumatic heart disease, and many other diseases (Chaudhary et al., 2018) and can also lead to severe invasive infections such as sepsis, necrotizing fasciitis, and toxic shock syndrome, which are associated with high morbidity, mortality, and disability rates (McMillan et al., 2007). The pathogenic bacteria most commonly found to be responsible for septic arthritis are *Staphylococcus aureus* (Jin et al., 2015), *Haemophilus influenzae* (Zhang et al., 2019), and *Streptococcus agalactiae* (Dhekane et al., 2020), and although less common, septic arthritis caused by *S. pyogenes* infection has been reported. In the present case series, we retrospectively analyzed the clinical data of children treated for septic arthritis due to *S. pyogenes* infection in our hospital to provide a basis for the diagnosis and treatment of septic arthritis cases due to *S. pyogenes* infection.

Data and methods

General data

Three cases with a clinical diagnosis of septic arthritis for which pus culture in the microbiology laboratory identified *Streptococcus pyogenes* between May 2016 and January 2018 were selected as the study subjects. The diagnosis of septic arthritis was made with reference to the literature criteria (Xu et al., 2016), which include: (1) signs and symptoms of infection, such as fever, local pain, swelling, and limitation of activity; (2) imaging examinations (such as ultrasound, computed tomography [CT] or magnetic resonance imaging [MRI]) suggesting local soft tissue swelling and joint cavity effusion; (3) X-ray examination suggesting joint dislocation or subluxation or local bone destruction; (4) significant elevation of inflammatory indexes such as white blood cell (WBC) count, C-reactive protein (CRP) level, and erythrocyte sedimentation rate (ESR); and (5) extraction of pus by puncture of the joint cavity. The diagnosis of septic arthritis is made only when three or more of the above criteria are met.

Methods

This retrospective study analyzed the clinical characteristics, laboratory results and pathological findings for three pediatric cases of septic arthritis, and the patients were followed up by telephone for more than 2 months. For bacterial culture detection, pus specimens were inoculated in 5% defibrinated sheep blood culture dishes. According to the morphological characteristics of the colony in the blood plate and its β- hemolytic ring, strains were further identified as GAS with Lancefield group specific antisera. Genomic DNA was obtained from freshly grown *S. pyogenes* using a Chelex-based DNA extraction kit for genetic analysis, according to the instructions of the DNA extraction kit (Beijing Sangong Bioengineering Co.). The *emm* sequence types were determined using a previously reported protocol (https://www2.cdc.gov/vaccines/biotech/strepblast.asp). Amplicons were sequenced by Guangzhou BGI Genomics Co. Ltd., and *emm* type was determined based on the sequence identity (>95%) of the first 180 bp of the *emm* gene between the tested sequence and the reference *emm* gene.

Results

Clinical presentation

The three pediatric cases included in this retrospective analysis included two male patients and one female patient, between the ages of 2 and 7 years. All three children presented with inflammatory manifestations such as localized skin erythema, localized pain, increased local skin temperature, visible pus on puncture and identification of *S. pyogenes* by bacterial culture, confirming the diagnosis of septic arthritis. Details are shown in Table 1.

Laboratory findings

At the first visit, the peripheral WBC count was significantly elevated in all three cases [$(27.68–32.02)\times10^9$/mL], as was the ESR for all three cases (113–134 mm/h). The CRP level was significantly elevated in two cases (67 mg/L and 147.7 mg/L) and normal in one case. The procalcitonin level was normal in one case, mildly elevated in one case, and not measured in 1 case. Joint X-ray and ultrasound showed local soft tissue swelling and fluid accumulation in the joint cavity in all three cases. For all three cases, treatment included incision and drainage, and pathological examination indicated septic inflammation. Subsequent microbiological culture of the puncture fluid indicated the growth of *S. pyogenes*, and for one patient (case 3), blood culture also identified infection by *S. pyogenes* strains isolated from case 1 and case 2 were *emm*1/ST28 genotype, and case 3 was *emm*12/ST36 genotype. The details are shown in Table 1.

Treatment and follow-up

All three children also received treatment with intravenous antibiotics. Patient 1 was treated with vancomycin intravenously upon initial presentation, and the antibiotic was changed to amoxicillin sodium + sulbactam delivered intravenously after the results of joint fluid culture were obtained. Patient 2 was treated with Cefuroxime for 1 day, desmethylvancomycin for 4 days, vancomycin intravenously for 33 days and then both vancomycin and amoxicillin sodium + sulbactam intravenously for 10 days. Patient 3 was treated with amoxicillin sodium + sulbactam intravenously for 24 days. All three cases showed improvement and were followed up by telephone. No sequelae such as nephritis, rheumatic fever or rheumatic heart disease were reported. Details are shown in Table 1.

TABLE 1 Clinical data of three pediatric cases of septic arthritis caused by *Streptococcus pyogenes*.

Case	Diagnosis	Chief complaint	WBC ($\times 10^9$/mL)	CRP (mg/L)	ESR (mm/h)	PCT (ng/mL)	Pathology	Culture results	Major antibiotic treatment
Case 1 Girl, 2y7m	Left suppurative ankle arthritis, sepsis, left fibula distal osteitis	Left foot and ankle swelling and pain for 3 days, accompanied by fever for >1 day	31.81	67	116	3.53	(Left ankle capsule) Fibroadipose tissue, collagen fiber hyperplasia, visible infiltration of lymphocytes, monocytes and some neutrophils	Septic culture: *S. pyogenes*	Vancomycin 4 days, amoxicillin+ sulbactam 22 days
Case 2 Boy, 7y2m	Left purulent hip arthritis, sepsis	Left lower limb pain and limp for 12 h	32.02	Normal	134	Not measured	Not submitted for inspection	Septic culture: *S. pyogenes*	Cefuroxime for 1 day, desmethylvancomycin for 4 days vancomycin for 33 days, and amoxicillin+ sulbactam for 10 days
Case 3 Girl, 7y11m	Right purulent hip arthritis, right femur bone, femoral neck osteomyelitis	Right hip pain, limited activity with fever for 2 days	27.68	147.7	113	0.06	(Right hip necrosis tissue) No coated epithelium on the tissue surface, interstitial collagen fibers showed glasslike changes, visible more neutrophil infiltration, vasodilation and congestion, in line with acute suppurative inflammation changes	Septic culture: *S. pyogenes* Blood culture: *S. pyogenes* (penicillin, ceefotaxime, linazolamide, levooxygen, chloramphenicol, ceftriaxone, vancomycin sensitive; clindamycin, erythromycin, tetracycline resistant)	Amoxicillin+ sulbactam for 24 days

WBC, white blood cell; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PCT, procalcitonin.

Discussion

Septic arthritis is a rapidly progressive, highly destructive and life-threatening joint disease. A study in the UK found that the incidence of septic arthritis increased from 5.5/100,000 in 1998 to 7.8/100,000 in 2013 (Rutherford et al., 2016). According to the literature, the most predominant pathogen causing sepsis in joints is *Staphylococcus*, followed by *Streptococcus pneumoniae* and *Klebsiella*, but also *Salmonella spp*, *Haemophilus influenzae* type b, and group B streptococci (Wang et al., 2003; Frazee et al., 2009; Rutherford et al., 2016; Xu et al., 2016; Dernoncourt et al., 2019; van den Boom et al., 2021; Yagupsky, 2021). *S. pyogenes* can also cause septic arthritis and is easily overlooked. A case of septic arthritis caused by *S. pyogenes* infection was first reported in 1984, and in that case, a serious infection caused by *S. pyogenes* occurred in a nursing home patient and resulted in sepsis, necrotizing fasciitis, septic arthritis, and cellulitis (Ruben et al., 1984). Septic arthritis after toxic shock syndrome caused by *S. pyogenes* was reported in 1995 (González-Ruiz and Ridgway, 1995). *S. pyogenes* infection causing multifocal septic arthritis has also been reported (Marti and Anton, 2007). In contrast, a 10-year case summary in Hong Kong found that in 31 cases of septic arthritis in children, the predominant causative organism was *S. aureus* (42%), followed by *S. pyogenes* (23%) (Kuong et al., 2012). In another study in France, 1 of 26 cases of diffuse infection in infants younger than 3 months of age was septic arthritis due to *S. pyogenes* infection (Germont et al., 2020). Another study in France isolated bacteria from 377 children with suspected joint infections, of which *S. pyogenes* accounted for 15% (Brehin et al., 2020). Methicillin-resistant *S. aureus* infection and septic arthritis combined with osteomyelitis have been reported in the literature as risk factors for the development of sequelae (Wang et al., 2003). A study in Spain reported that the invasive diseases caused by *S. pyogenes* in the Spanish pediatric population include septic arthritis/osteomyelitis, and the common types of *S. pyogenes* are *emm*1/ST28, *emm*12/ST36-ST242, (Sanchez-Encinales et al., 2019). In the present pediatric case series, cases 1 and 2 were *emm*1/ST28 and case 3 was *emm*12/ST36, and these results were basically consistent with those of previous studies. Overall, our case serious combined with the literature reports show that *S. pyogenes* cannot be overlooked as a potential cause of septic arthritis.

Previous research has shown that *S. pyogenes* can invade the joint microenvironment, and step in the development of septic arthritis (Le Hello et al., 2009). Volzke et al (Volzke et al., 2020) inoculated mice intravenously with *S. pyogenes* and observed septic arthritis 3~20 days after infection with increased levels of interleukin (IL)-1β and IL-6 in the joints along with an increased amount of nuclear factor (NF)-κB receptor activator ligand (RANKL), which is a key cytokine for osteoclast formation. *S. pyogenes* infection can increase RANKL expression by increasing the production of activators to induce infectious arthritis.

Septic arthritis occurs in lower limb joints in 90% of cases, with the hip being the most commonly involved, followed by the knee

(Wang et al., 2003; Xu et al., 2016). Sternoclavicular joint involvement has also been reported (Dhekane et al., 2020; Kwon et al., 2020). Joint pain (81%) is the most common presentation, followed by fever, swelling and limitation of movement, and 89% of patients show an increased ESR (≥20 mm/h) (Wang et al., 2003). The age at onset of septic arthritis due to *S. pyogenes* varies, with reports of onset in neonates and small infants less than 3 months of age (Umadevi et al., 2013; Germont et al., 2020). The age of onset in our pediatric case series ranged from 2–7 years. A study in Hong Kong reported that only 52% of 31 children with septic arthritis had a temperature below 38.5°C on admission; i.e., nearly half of their patients had a temperature above 38.5°C on admission. Additionally, 71% of their patients had a WBC count below 12×10^9/L, and the rate of positive blood culture was not high (negative in 74% of cases) (Kuong et al., 2012). In the present case series, all three children presented with a significantly elevated WBC count (30×10^9/L or more) and a significantly increased ESR. The CRP level was also significantly increased in two cases, while the procalcitonin level was mildly increased in only one case. Previous studies have suggested that procalcitonin is more sensitive than ESR and CRP level for the diagnosis of septic arthritis, and that the combination of these three indicators is beneficial for improving diagnostic sensitivity and specificity (Wang et al., 2014; Wei et al., 2015). In contrast, Chen (Chen et al., 2013) found that the CRP level is more sensitive than procalcitonin for the identification of local bacterial infection. The results in our pediatric case series are more consistent with the findings of Chen et al (Chen et al., 2013).

The standard treatment for septic arthritis in children is arthrocentesis combined with antibacterial drug therapy (Kwon et al., 2020). Studies have shown that adequate use of antimicrobial drugs and a single arthrocentesis is sufficient to treat septic arthritis in most pediatric cases, regardless of the infecting agent or site of infection, as long as the clinical response is good (Peltola et al., 2009). Additional research had indicated that early antibiotic treatment, incision and drainage, and combined non-pharmacological treatments such as drainage and early physiotherapy should be given (Couderc et al., 2020). More recent studies have suggested that targeted synovial cell therapy may be a promising treatment for septic arthritis (Volzke et al., 2020). Penicillin has been widely used worldwide for many years, and so far, no penicillin-resistant strains of *S. pyogenes* have been identified. The reasons for this are not yet known. In recent years, increased minimum inhibitory concentration (MIC) values of penicillin have been reported, and penicillin-nonsusceptible *S. pyogenes* strains have emerged. In 2006, Capoor et al. (Capoor et al., 2006) reported *S. pyogenes* with elevated MIC values to penicillin (0.19~0.25 μg/mL), and *S. pyogenes* with elevated MIC values to penicillin (0.25~0.75 μg/mL) were also found in Mexico (Ogawa et al., 2011). Additionally, several resistance surveillance networks in China have reported *S. pyogenes* "resistance" to β-lactam antimicrobials, but we confirmed that these strains are not truly resistant, and whether they carry a mutated gene for penicillin-binding protein 2X (pbp2x) needs to be confirmed by further studies (Vannice et al., 2020; Musser et al., 2020; Yu et al., 2020). In the present case series, *S. pyogenes* was cultured from the joint cavity pus of all three children, and they were considered sensitive to β-lactam antibiotics based on the outcomes in these cases. No antibiotic susceptibility testing was performed. However, in case 3, *S. pyogenes* was cultured from the blood, and antibiotic susceptibility testing suggested sensitive to β-lactam antibiotics such as penicillin and cephalosporin. For this case, amoxicillin sodium + sulbactam was selected and provided satisfactory treatment. Therefore, in clinical practice, once pus culture identifies *S. pyogenes*, the choice of β-lactam antibiotics is sufficient, and prompt step-down treatment should be given, as it is not advisable to continue advanced antibiotic therapy. In case 1 of our series, after the pus culture result was clear, vancomycin was promptly changed to amoxicillin sodium + sulbactam with good effect, while in case 2, vancomycin combined with β-lactams treatment was considered to be related to the doctor's insufficient knowledge of *S. pyogenes*. In case 3, intravenous infusion of amoxicillin sodium + sulbactam was administered from the time of admission with good effect.

Conclusion

In conclusion, antimicrobial agents commonly used to treat *S. pyogenes* infections are highly active against clinical strains.*S. pyogenes* is an important pathogen causing septic arthritis, and WBC count, ESR, and CRP level are sensitive indicators for the diagnosis of septic arthritis. If *S. pyogenes* infection is confirmed upon culture of pus drained from an infection joint, β-lactam antibacterial therapy with antibiotics such as penicillin or cephalosporin can be selected for treatment.

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 human participants were reviewed and approved by Shenzhen Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

WW and YY contributed to conception, design, and administrative support. DY, QL, WG, DG,YC, and YZ provided study materials and patients. DY contributed to the collection and assembly of data, data analysis, interpretation and the manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

The reviewer MK is currently organizing a Research Topic with the authors DY, YZ, and YY.

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Antimicrobial resistance profile of methicillin-resistant *Staphylococcus aureus* isolates in children reported from the ISPED surveillance of bacterial resistance, 2016–2021

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Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious threat to public health worldwide. In December 2015, the Infectious Disease Surveillance of Pediatrics (ISPED) program was organized to monitor bacterial epidemiology and resistance trends in children.

Methods: This retrospective study was conducted from January 2016–December 2021 on patients at eleven ISPED-group hospitals.

Results: From 2016–2021, a total of 13024 MRSA isolates were obtained from children. The most common age group for patients with MRSA infection was less than 3 years old, and newborns were an important group affected by MRSA infection. MRSA was most commonly isolated from the lower respiratory, an abscess, a secretion, or blood in neonates and from the lower respiratory, an abscess, or the upper respiratory in non-neonates. All isolates were susceptible to vancomycin and linezolid and resistant to penicillin; additionally, 76.88%, 54.97%, 22.30%, 5.67%, 5.14%, 3.63%, and 1.42% were resistant to erythromycin, clindamycin, tetracycline, levofloxacin, sulfamethoxazole-trimethoprim (TMP-

SMX), gentamicin, and rifampin, respectively. Between 2016 and 2021, a significant increase was seen in the levofloxacin- and TMP-SMX-resistance rates (from 5.45% to 7.14% and from 4.67% to 6.50%, respectively) among MRSA isolates, along with a significant decrease in the rates of resistance to erythromycin (from 82.61% to 68.08%), clindamycin (from 60.95% to 46.82%), tetracycline (from 25.37% to 17.13%), gentamicin (from 4.53% to 2.82%), and rifampin (from 1.89% to 0.41%).

Discussion: The antibiotic-resistance rates varied among MRSA isolated from different sources. Because of the high antibiotic resistance rate to clindamycin, this antibiotic is not recommended for empirical treatment of MRSA infections, especially in osteomyelitis.

KEYWORDS

methicillin-resistant *Staphylococcus aureus*, antimicrobial resistance, children, infectious disease surveillance of pediatrics (ISPED), neonates

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), which was first identified in 1961, poses a serious threat to public health worldwide owing to its significant resistance to antibiotics. Among community-associated *S. aureus* infections in children, 63.6% were found to be due to MRSA (Wang et al., 2017). Previous studies in children found that MRSA was responsible for 45.8%–75.6% of *S. aureus* pneumonia cases (Doudoulakakis et al., 2017; Song et al., 2017), 44% of *S. aureus* bacteraemia cases (Kumarachandran et al., 2017), and 32.8% of cases with an *S. aureus* central nervous system infection (Vallejo et al., 2017). Recently, in a study of bloodstream infections in China, the percentage of cases due to MRSA in non-intensive care unit (ICU) patients increased significantly from 8.4% in 1998–2002 to 68.3% in 2013–2017 (Tian et al., 2019).

MRSA can cause invasive, life-threatening systemic infections, e.g., in severe sepsis and necrotizing pneumonia, which are particularly problematic in children (Liu et al., 2011; Lim et al., 2014). The high antibiotic resistance of MRSA is very concerning because it can lead to treatment failure in clinical practice. Currently, MRSA infections remain prevalent and account for significant morbidity and mortality worldwide. MRSA infections in children have been associated with a longer duration of bacteraemia, longer length of hospital stay, higher likelihood of complications, and greater mortality rate compared with methicillin-sensitive *S. aureus* (MSSA) infections (Burke et al., 2009; Hamdy et al., 2019).

Given the heavy burden associated with MRSA infections, there is an urgent need to understand the distribution and antimicrobial susceptibilities of MRSA. Here, to investigate the profiles of MRSA infection and MRSA drug resistance in children, we compared the distribution and antimicrobial susceptibilities of MRSA isolates in cases from eleven hospitals within the Infectious Diseases Surveillance of Pediatrics (ISPED) group of China over a six-year period (2016–2021).

Patients and methods

Surveillance population

This retrospective study was conducted across eleven hospitals within the ISPED group of China from January 2016 to December 2021. We reviewed the medical records of patients who were younger than 18 years and had any clinical culture that yielded an isolate of MRSA. We collected the following data from the patient medical records: sex, age, infection site, and antibiotic resistance profile. Our analysis included a total of 13024 MRSA clinical isolates obtained from the following eleven hospitals: Children's Hospital of Chongqing Medical University, Children's Hospital of Fudan University, Children's Hospital of Zhejiang University School of Medicine, Qilu Children's Hospital of Shandong University, Shenzhen Children's Hospital, Shanghai Children's Medical Center of Shanghai Jiaotong University School of Medicine, Xi'an Children's Hospital, Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University, Children's Hospital of Shanghai Jiaotong University School of Medicine, Bethune First Hospital of Jilin University, and Kaifeng Children's Hospital.

Antimicrobial susceptibility testing (AST)

The antibiotic susceptibility testing was performed in each participating site. Antimicrobial susceptibility testing (AST) was performed using the Kirby-Bauer method or automated systems. AST was conducted for penicillin, oxacillin, erythromycin, clindamycin, levofloxacin, sulfamethoxazole-trimethoprim (TMP-SMX), gentamicin, rifampin, and minocycline. MRSA were identified based on their resistance to oxacillin. The standard strains ATCC 25922, ATCC 29213, and ATCC 29212 were used as quality-control strains for the antimicrobial susceptibility tests. The

AST breakpoint criteria of the Clinical and Laboratory Standards Institute (CLSI) were adopted (Clinical and Laboratory Standards Institute, 2019).

Statistical analysis

Statistical significance was calculated by applying the χ^2 test, or the Fisher's exact test in the case of small sample sizes, using the SPSS (Version 20) statistics program. Statistical significance in this study was defined as a *P*-value of < 0.05.

Results

Distribution of MRSA

From 2016 to 2021, the yearly total number of *S. aureus* isolates obtained ranged from 4231 to 8561. During these years, the respective proportions of *S. aureus* isolates that were MRSA isolates were 31.50%, 36.80%, 34.10%, 34.41%, 35.00%, and 32.31%. A total of 13024 MRSA isolates, of which 6.10% (794) were collected from outpatients and 93.90% (12230) were collected from inpatients, were obtained from children in this study. The distribution of MRSA in inpatients was distinct from that in outpatients; MRSA isolates were detected much more commonly from inpatients than from outpatients (34.85% vs. 25.56%, χ^2 = 109.564, *P* = 0.000).

Among the included 13024 cases, 7763 (59.61%) of the patients were male. The median patient age was 5 months (range: 1 day to 17 years), and 2902 (22.73%) of the patients were newborn infants (aged ≤28 days). MRSA cases were detected much more commonly during the months of January, November, and December (Figure 1).

MRSA was most commonly isolated from the lower respiratory (58.28%), followed by from an abscess (16.78%), a secretion (7.61%), blood (4.64%), the upper respiratory (4.45%), urine (0.98%), a bone or joint (0.27%), the central nervous system (0.24%), and other sources. Notably, compared with those in non-neonates, the constituent proportions of MRSA isolates obtained from blood, secretions, and urine were higher, while those obtained from respiratory sites and bone or joint sites were lower, in neonates (Table 1).

Bacterial identification and AST

The antimicrobial resistance profiles of the 13024 MRSA isolates from this study are provided in Table 2 and Table 3. All study isolates were susceptible to vancomycin and linezolid and were resistant to penicillin; additionally, 76.88% of the study isolates were resistant to erythromycin, 54.97% to clindamycin, 22.3% to tetracycline, 5.67% to levofloxacin, 5.14% to TMP-SMX, 3.63% to gentamicin, and 1.42% to rifampin. The rifampin-resistance rate of MRSA isolates derived from inpatients was significantly higher than those of MRSA isolates derived from outpatients (1.51% vs. 0.13%, χ2 = 10.119, P = 0.001), while tetracycline-resistance rate of inpatients-derived MRSA isolates was lower than that of outpatients-derived strains (22.00% vs. 28.57%, χ2 = 11.111, P = 0.001). Changes in the antibiotic resistance rates among the MRSA isolates from 2016 to 2021 were observed, with an increase in the rates of resistance to levofloxacin (from 5.45% to 7.14%) and TMP-SMX (from 4.67% to 6.50%) and a decrease in the rates of resistance to erythromycin (from 82.61% to 68.08%), clindamycin (from 60.95% to 46.82%), tetracycline (from 25.37% to 17.13%), gentamicin (from 4.53% to 2.82%), and rifampin (from 1.89% to 0.41%) (Table 3, Figure 2).

The activity of the tested antimicrobials against the 13024 MRSA isolates obtained from different infection sites is summarized in Table 4. The erythromycin-resistance rates of blood-derived (79.62%) and abscess-derived (79.29%) MRSA isolates were significantly higher than that of urine-derived isolates (70.87%; χ^2 = 4.645 and 5.089, *P* = 0.031 and 0.024, respectively). The clindamycin-resistance rates of bone and joint-derived (72.73%) and urine-derived (63.49%) MRSA isolates were significantly higher than that of lower respiratory-derived isolates (54.61%; χ^2 = 4.351 and 3.942, *P* = 0.037 and 0.047, respectively). The tetracycline-resistance rate of bone and joint-derived MRSA isolates was significantly higher than those of MRSA isolates derived from other sources (50.00% vs. 20.42%–31.82%, χ^2 = 63.809, *P* = 0.000). The levofloxacin-resistance rate of blood-derived MRSA isolates (8.07%) was significantly higher than those of abscess-derived (4.48%) and secretion-derived (5.15%) isolates (χ^2 = 11.548 and 5.098, *P* = 0.001 and 0.024, respectively). The TMP-SMX-resistance rates of secretion-derived (6.15%) and blood-derived (6.12%) MRSA isolates were significantly higher than that of upper respiratory-derived isolates (3.50%; χ^2 = 5.144 and 4.352, *P* = 0.023 and 0.037, respectively).

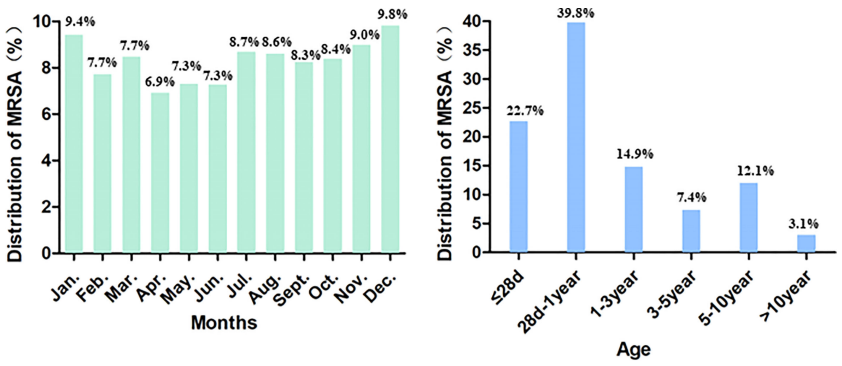

FIGURE 1
Demographics of patients with MRSA infections.

TABLE 1 Distributions of MRSA in neonates and nonneonates in 2016 to 2021.

Specimen type	Neonates % (n=2960)	Nonneonates % (n=10064)	χ^2	P-Value
Lower respiratory	48.55%	61.73%	160.180	0.000
Upper respiratory	1.97%	5.27%	56.281	0.000
Blood	6.19%	4.16%	20.888	0.000
Abscess	18.00%	16.53%	3.406	0.065
Secretion*	13.56%	5.96%	174.783	0.000
Urine	1.49%	0.84%	9.493	0.002
bone and joint	0.07%	0.30%	4.047	0.032
Cerebrospinal fluid	0.10%	0.28%	2.301	0.089
Other	10.07%	4.92%	–	–

*Secretion: Umbilical secretion, wound.

Discussion

We observed the antimicrobial resistance trends of MRSA isolates obtained from children participating in ISPED over the six-year period from 2016 to 2021. Despite the reduction in the proportion of MRSA among *S. aureus* infections that was noted over the recent 10-year span (Kramer et al., 2019), the incidence of MRSA has remained high in some areas (Takemura and Mochizuki, 2018), and MRSA remains a predominant cause of infection. The proportions of MRSA among *S. aureus* infections in adults, according to the China Antimicrobial Surveillance Network (CHINET) report, decreased from 69.94% in 2005 to 31.00% in 2020 (Hu et al., 2016; Hu et al., 2017; Hu et al., 2018; Hu et al., 2020a; Hu et al., 2020b; Hu et al., 2021). In contrast, the proportions of MRSA among paediatric *S. aureus* infections found in our study remained in the range of 31.50%–36.80% over the past six years. Here, MRSA cases were detected much more commonly in the months of January, November, and December, during which the weather in China becomes colder. The seasonal nature of MRSA infections varied based on specimen source, with wound infections more prevalent in warmer months, and respiratory infections more prevalent during colder months (Delorme et al., 2017; Marcelin et al., 2017). The most common age group of patients with MRSA infection is <5 years old (Perovic et al., 2015; Ilczyszyn et al., 2016). In the present study, the median patient age was only 5 months old. The most common age stage of patients participating in our study was 28 d–1 year (39.8%), and 77.4% of study participants were aged less than 3 years old, which is younger compared with the participants studied in other reports (Milstone et al., 2010). In addition, 22.73% of the patients in our study were newborn infants, and newborns are also an important group affected by MRSA infections.

MRSA is a particularly threatening pathogen in children because it causes infections of multiple organ systems. Previous research has found that the most common sites of MRSA infection are the respiratory tract (36.3%–48.0%), followed by the blood (15%–35.7%), wound or intravenous sites (25.2%–44%), and the urinary tract (2.8%–28%), while the least common source of MRSA isolates is puncture fluid (containing hydrothorax, ascites, pericardial fluid,

TABLE 2 Antimicrobial resistance rates of MRSA strains in inpatients and outpatients.

Antibiotic	Inpatients (n=12230)	Outpatients (n=794)	Total (n=13024)	χ2	P-Value
Penicillin	100.00	100.00	100.00	–	–
Oxacillin	100.00	100.00	100.00	–	–
Erythromycin	76.84	77.41	76.88	0.134	0.714
Clindamycin	54.97	54.87	54.97	0.003	0.954
Tetracycline	22.00	28.57	22.30	11.111	0.001
Levofloxacin	5.78	4.09	5.67	3.775	0.052
TMP-SMX	5.06	6.46	5.14	3.014	0.083
Gentamicin	3.63	3.65	3.63	0.001	0.977
Rifampin	1.51	0.13	1.42	10.119	0.001
Vancomycin	0.00	0.00	0.00	–	–
Linezolid	0.00	0.00	0.00	–	–

TABLE 3 Antimicrobial resistance rates of MRSA strains in 2016 to 2021.

Antibiotic	2016 % (n=2100)	2017 % (n=2258)	2018 % (n=2241)	2019 % (n=2946)	2020 % (n=1481)	2021 % (n=2000)	χ^2	P-Value
Penicillin	100.00	100.00	100.00	100.00	100.00	100.00	–	–
Oxacillin	100.00	100.00	100.00	100.00	100.00	100.00	–	–
Erythromycin	82.61	79.03	78.98	77.01	74.44	68.08	135.964	0.000
Clindamycin	60.95	61.28	55.69	52.37	61.10	46.82	137.725	0.000
Tetracycline	25.37	23.39	24.33	22.11	20.94	17.13	30.224	0.000
Levofloxacin	5.45	4.98	4.66	5.71	6.74	7.14	16.114	0.007
TMP-SMX	4.67	4.51	4.87	4.96	5.97	6.50	12.242	0.032
Gentamicin	4.53	5.22	3.48	2.81	2.68	2.82	30.908	0000
Rifampin	1.89	1.94	1.21	1.73	1.29	0.41	21.895	0.000
Vancomycin	0.00	0.00	0.00	0.00	0.00	0.00	–	–
Linezolid	0.00	0.00	0.00	0.00	0.00	0.00	–	–

cerebrospinal fluid, or articular cavity fluid; 21.32%) (Rahimi and Shokoohizadeh, 2016; Huang et al., 2019; Samadi et al., 2019). MRSA is an important cause of bloodstream infections (BSI), and in 2009–2017, the MRSA detection rate was 40%–50% among BSI-associated *S. aureus* (Zhang et al., 2022). Additionally, *S. aureus* was reported to be the main pathogen (67.5%) in paediatric osteomyelitis, and the proportion of MRSA among the cases due to *S. aureus* was 44% (Chen et al., 2021). In the present work, the most common clinical sources of MRSA infection were the respiratory tract (62.73%), followed by an abscess (16.78%), a secretion (7.61%), and blood (4.64%), and the constituent proportions isolated from blood, secretions, and urine were higher in neonates compared with those in non-neonates. An increasing incidence of MRSA infections was observed among neonates (Dolapo et al., 2014). Because immunologically immature children are more susceptible to MRSA infection, neonates are more likely to develop MRSA BSIs compared with non-neonates. In neonates with MRSA infection, the proportion of umbilical infection is high, and BSI may be associated with umbilical cord infection. Therefore, it is important to monitor the MRSA epidemiology and resistance trends in neonates.

MRSA antibiotic resistance has been reported in many countries. The significant antibiotic resistance of MRSA is a particular concern because it can lead to treatment failure in clinical practice. Several studies of MRSA antimicrobial susceptibility have revealed their high rates of resistance to erythromycin, clindamycin, levofloxacin, and ciprofloxacin (Decousser et al., 2018; Liang et al., 2019). Similarly, among MRSA isolates from paediatric patients, the highest percentage of isolates was resistant to erythromycin (62%), followed by those resistant to clindamycin (14%–57%), TMP-SMX (3%–24%), gentamicin (24%), rifampicin (12%), or minocycline (10%), while all isolates were susceptible to both vancomycin and linezolid (Milstone et al., 2010; Miguel et al., 2019). In the CHINET surveillance report of MRSA resistance trends between 2005 and 2020, decreases were observed in the percentages of isolates resistant to clindamycin (from 90.1% to 58.6%), erythromycin (from 92.7% to 78.9%), levofloxacin (from 83.3% to 32.6%), gentamicin (from 77.3% to 20.7%), rifampin (from 34.9% to 8.2%), and TMP-SMX (from 36.3% to 6.4%) (Hu et al., 2016; Hu et al., 2019; Hu et al., 2020; Hu et al., 2021). In the present study, our MRSA isolates had high rates of resistance to erythromycin and clindamycin but low rates of resistance to levofloxacin, TMP-SMX, gentamicin, and rifampin, and all these isolates were susceptible to vancomycin and linezolid. Changes in the antibiotic-resistance profile of MRSA isolates from 2016 to 2021 were noted, with an increase in the levofloxacin- and TMP-SMX-resistance rates (from 5.45% to 7.14% and from 4.67% to 6.50%, respectively), and a decrease in the erythromycin-, tetracycline-, gentamicin-, and rifampin-resistance rates. The decreased rates of resistance to these antibiotics may be related to their decreased use in recent years. In our monitoring, the clindamycin-resistance rate remained at 50%–60% from 2016 to 2020, and although this rate decreased to 47% in 2021, it remained close to 50%.

MRSA isolates obtained from different clinical sources may exhibit different levels of antimicrobial susceptibility. A study in Chicago suggested that MRSA isolates obtained from the blood are more likely to be drug resistant compared with MRSA skin and soft tissue isolates (Acree et al., 2017). Liang et al. reported that the rates of gentamicin and ciprofloxacin resistance were significantly higher in MRSA isolates derived from the respiratory tract than in those

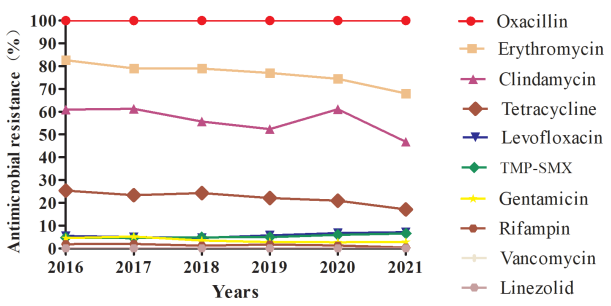

FIGURE 2
Profile of resistance to ten main antimicrobials (%) of MRSA isolates collected from 2016 to 2021.

TABLE 4 Antibiotic resistance rates of MRSA from different site of infection.

Antibiotic	Upper respiratory % (n=579)	Lower respiratory % (n=7590)	Blood % (n=604)	Abscess % (n=2185)	Secretion % (n=991)	Urine % (n=128)	bone and joint %(n=35)	Cerebrospinal fluid %(n=31)	χ^2	P-Value
Penicillin	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	–	–
Oxacillin	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	–	–
Erythromycin	78.37	76.71	79.62	79.29	76.19	70.87	78.79	80.65	12.231	0.093
Clindamycin	58.16	54.61	57.67	58.70	59.77	63.49	72.73	54.84	25.464	0.001
Tetracycline	22.43	20.42	28.61	28.13	23.21	25.00	50.00	31.82	63.789	0.000
Levofloxacin	5.72	6.07	8.07	4.48	5.15	7.94	0.00	3.23	17.064	0.017
TMP-SMX	3.50	4.75	6.12	5.87	6.15	7.09	8.57	3.45	12.961	0.073
Gentamicin	4.63	3.79	3.70	3.06	3.11	3.94	2.86	0.00	6.154	0.522
Rifampin	0.87	1.60	1.58	1.06	2.12	0.79	0.00	0.00	8.667	0.277
Vancomycin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	–
Linezolid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	–

MRSA, methicillin-resistant Staphylococcus aureus; TMP-SMX, sulfamethoxazole-trimethoprim.

isolated from the skin and soft tissue or the blood (Liang et al., 2019). Resistance to antibiotics was prevalent among MRSA isolates cultured from patients with ocular infections at US centres, with 72.7% of isolates resistant to fluoroquinolones and 92.9% resistant to azithromycin (Asbell et al., 2020). We found that the erythromycin-resistance rates of blood- and abscess-derived MRSA isolates were higher than that of MRSA isolates obtained from other clinical sources. Additionally, the rates of clindamycin and tetracycline resistance among bone and joint-derived MRSA isolates were significantly higher than those obtained from other clinical sources.

An understanding of the distribution and antimicrobial susceptibilities of MRSA strains will be crucial for guiding antibiotic treatment in MRSA cases. Presently, vancomycin, linezolid, and tigecycline are the most active agents against MRSA. Based on our monitoring results, erythromycin is not recommended for treating respiratory infections, bacteraemia, or abscesses in children. Intravenous vancomycin is recommended for the treatment of children with invasive MRSA infections. It has been reported that MRSA strains outside of China have retained high susceptibility to clindamycin (Aglua et al., 2018). Greenberg evaluated the effectiveness of clindamycin in infants. They found that 76% of the infants who had MRSA bacteraemia cleared the infection after clindamycin treatment (Greenberg et al., 2020). Clindamycin should be considered for inclusion in the initial antibiotic regimen for treating osteomyelitis and septic arthritis because patients whose initial antibiotic regimens included vancomycin had a longer hospitalization compared with those initiated on a treatment regimen of clindamycin without vancomycin (Weiss et al., 2020). However, we found a particularly high rate of clindamycin resistance among bone and joint-derived MRSA isolates (72.73%), and clindamycin is not suitable to be recommended for the empirical treatment of osteomyelitis. Because of the low number of bone and joint-derived isolates in our study, it needs for more studies. The use of antibiotic treatment is a particular concern owing to the limitation that some antibiotic classes are not suitable for use among neonates, e.g., TMP-SMX, rifampin, gentamicin. Vancomycin and linezolid are recommended for use in treating MRSA infections in neonates, and clindamycin may be considered for the treatment of patients with susceptible isolates who have non-invasive MRSA infections. Although the levofloxacin-resistance rate was low compared to other antibiotics, levofloxacin is not recommended for use in children due to serious side effect.

Conclusions

The most common age group of patients with MRSA infection is less than 3 years old, and newborns are an important group affected by MRSA infections. MRSA isolates had high rates of resistance to erythromycin and clindamycin but low rates of resistance to levofloxacin, TMP-SMX, gentamicin, and rifampin. No isolate was found to be resistant to vancomycin or linezolid. Changes in the antibiotic resistance rates among the MRSA isolates obtained from 2016 to 2021 were observed, with an increase in the levofloxacin- and TMP-SMX-resistance rates and a decrease in the erythromycin-, clindamycin-, tetracycline-, gentamicin-, and rifampin-resistance rates. The clindamycin-resistance rate of bone and joint-derived MRSA isolates was higher than that of isolates derived from other clinical sources. In areas where clindamycin-resistant MRSA strains are a concern, empirical vancomycin therapy is suggested for the treatment of paediatric osteomyelitis, and clindamycin is not recommended for the treatment of osteomyelitis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The study protocol was approved by the Ethics Committee of the Children's Hospital of Fudan University (No. (2020)321). The need for Informed Consent was waived by the Ethics Committee of the Children's Hospital of Fudan University due to the retrospective nature of the study

Author contributions

All authors have contributed to the manuscript. XW designed and wrote the draft. XW, CW, LH, HX, CJ, YinC, JD, AL, HD, HC, YipC, JY, TZ, QC, JH and YH collected and analyzed the data, and helped with the data interpretation. HY reviewed the manuscript for its intellectual content and revised the entire work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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The dynamic change of serotype distribution and antimicrobial resistance of pneumococcal isolates since PCV13 administration and COVID-19 control in Urumqi, China

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Objective: This study aims to analyze the serotype distribution and drug resistance of *Streptococcus pneumoniae* isolated from children aged 8 days to 7 years in Urumqi, China, between 2014 to 2021, during which PCV13 was introduced in the private sector's immunization program and COVID-19 control was administrated in the last 2 years.

Methods: Serotypes of *S. pneumoniae* isolates were determined by Quellung reaction, and their susceptibility against 14 antimicrobials were tested. According to the start year of PCV13 administration (2017) and COVID-19 control (2020), the study period was divided into three stages: 2014–2015, 2018–2019, and 2020–2021.

Results: A total of 317 isolates were involved in this study. The most common serotypes were type 19F (34.4%), followed by 19A (15.8%), 23F (11.7%), 6B (11.4%), and 6A(5.0%). The coverage rate of both PCV13 and PCV15 was 83.0%. The coverage of PCV20 was a little higher at 85.2%. The resistance rate against penicillin was 28.6% according to the breakpoints of oral penicillin, which would reach up to 91.8% based on the breakpoints of parenteral penicillin for meningitis. The resistance rates to erythromycin, clindamycin, tetracycline, and sulfamethoxazole-trimethoprim were 95.9%, 90.2%, 88.9%, and 78.8%, respectively. The PCV13 isolate was more resistant to penicillin than the non-PCV13 ones. There was not any significant change found in the serotype distribution since the PCV13 introduction and the COVID-19 control. The resistance rate against oral penicillin slightly elevated to 34.5% in 2018–2019 from 30.7% in 2014–2015 and then decreased significantly to 18.1% in 2020–2021 (χ^2 = 7.716, P < 0.05), while the resistance rate to ceftriaxone (non-meningitis) continuously declined from 16.0% in 2014–2015 to 1.4% in 2018–2019 and 0% in 2020–2021 (Fisher = 24.463, P < 0.01).

Conclusion: The common serotypes of *S. pneumoniae* isolated from children in Urumqi were types 19F, 19A, 23F, 6B, and 6A, which we found to have no marked change since the PCV13 introduction and the COVID-19 control However, the resistance rate to oral penicillin and ceftriaxone significantly declined in the COVID-19 control stage.

KEYWORDS

***Streptococcus pneumoniae*, serotype, drug resistance, children, COVID-19**

1 Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is the main pathogen causing pneumonia, meningitis, bacteremia, and other serious diseases in children. It is also a common cause of acute otitis media and sinusitis. It is an important cause of morbidity and death of infants in China. The number of pneumococcal disease cases in children under 5 years old in China accounts for 12% of the total number of cases in the world, thus ranking as the second country in the world with the highest occurrence rate (Chinese Preventive Medicine Association and Vaccine and Immunology Branch of the Chinese Preventive Medicine Association, 2020). Universal immunization with pneumococcal conjugate vaccines (PCVs) in children can effectively prevent invasive and some non-invasive pneumococcal infections (Moore et al., 2015). Therefore, the World Health Organization recommends that countries should include PCVs in children's immunization program (World Health Organization, 2019). A systematic study on the epidemiology of pneumococci in children in mainland of China from 2000 to 2018 showed that the 13-valent PCV (PCV13, including 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A, and 23F serotypes) coverage was 90.4%, which suggested the potential effect of this vaccination (Men et al., 2020). Another systematic review including data from 2006 to 2016 found no significant difference in the coverage of PCV13 in invasive isolates compared to that in non-invasive strains (Lyu et al., 2017). However, the previous domestic investigations on the serotypes of *S. pneumoniae* isolates in children were mainly performed in developed areas and cities, which were mainly settled by the Han people. Similar investigations in underdeveloped areas or on ethnic minorities, especially of the population in the northwest, were rare. PCV13 was available for private choice in China in November 2016. It has not been widely administrated, especially in undeveloped areas, because of its high price. Since the beginning of 2020, COVID-19 controls were globally preformed, which have been implemented until now in China. The change in the epidemiology of *S. penumonaie* following the epidemic of COVID-19 has attracted the attention of researchers. Sempere et al. reported that the use of antimicrobials to prevent co-infections in patients with COVID-19 might have affected the increased proportion of pneumococcal-resistant strains (Sempere et al., 2022).

In the present study, the serotypes and drug resistance of *S. pneumoniae* isolated from children in Urumqi Children's Hospital from 2014 to 2021 were analyzed, which would add new knowledge on the epidemiology of *S. pneumoniae* in China and on the effect of PCV administration and COVID-19 control.

2 Methods

2.1 Bacterial isolates and study period

S. pneumoniae isolates identified and frozen at -80°C during daily work at the Medical Laboratory Department of the Children's Hospital in Xinjiang Urumqi from 2014 to 2021 were recovered and re-identified by optochin test, bile solubility test, and capsule swelling test (Satzke et al., 2013). The isolates should be cultured from various children. According to the start year of PCV13 administration (2017) and COVID-19 control (2020), the study period was divided into three stages: 2014–2015, 2018–2019, and 2020–2021.

This retrospective study did not record the information that can confirm the identity of the children or their guardians. Informed consent was not required, and this was approved by the hospital ethics committee (2017-142).

2.2 Serotyping

Antisera (Statens Serum Institute, Copenhagen, Denmark) were used to determine the serotype of the isolates by capsular swelling test, that is, Quellung test (Satzke et al., 2013). The constituent ratios of each serotype were calculated, and the coverage rate of *S. pneumoniae* vaccines was estimated by the sum of the serotype constituent ratio of vaccine serotypes.

2.3 Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) of penicillin, amoxicillin, cefotaxime, and ceftriaxone was detected with E-test stripes, and the susceptibility of meropenem, erythromycin, sulfamethoxazole-trimethoprim (SMZ-TMP), tetracycline, chloramphenicol, linezolid, vancomycin, levofloxacin, and moxifloxacin was detected by VITEC 2 Compact System (bioMerieux Inc., NC, USA). The susceptibility of clindamycin was evaluated by disk diffusion test (Oxoid Ltd., Basingstoke, UK). The quality control strain for this test was *S. pneumoniae* ATCC49619. The susceptibility was judged according to the breakpoints recommended by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI), 2019).

2.4 Statistical analysis

A database including the age, nationality, and sex of the patients and the serotype and drug resistance of the corresponding isolate was constructed in Microsoft Excel 2003. The susceptibility rate, intermediary rate, and drug resistance rate of different antimicrobials were calculated. SPSS25.0 (IBM SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The significance of each group was compared by χ^2 test and Fisher's exact test. P <0.05 was considered to be statistically significant.

3 Results

3.1 Characteristics of strains and specimens and demography

A total of 317 strains were successfully recovered and were tested in the present study, which included 75 strains isolated in 2014 to 2015, 148 strains in 2018 to 2019, and 94 strains in 2020 to 2021. Among all strains, 298 were isolated from sputum (94.0%). A few strains were cultured from eye secretions (3.5%), ear secretions (1.3%), blood (0.9%), and bronchoalveolar lavage fluid (0.3%).

The age composition of host children ranged from 8 days to 7 years old, which included 188 cases of <2 years old (59.3%), 113 cases of 2–< 5 years old (35.6%), and 16 cases of 5–7 years old (5.0%). There were 194 male children (61.9%) and 123 female children (38.8%). Among the 317 cases, there were 202 cases from Han people (63.7%) and 115 cases from ethnic minority people (36.3%), including 92 cases from Uygur, 17 cases from Kazak, four cases from Mongolian, and two cases from Hui.

3.2 Serotype distribution and pneumococcal vaccine coverage

A total of 30 serotypes were identified in the present 317 strains. The serotype distribution and the cumulative coverage of PCV13 and 23-valent capsular polysaccharide vaccine (PPV23, covering serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14,15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) are shown in Figure 1. The most common serotype was 19F (109 strains, 34.4%), followed by 19A (50, 15.8%), 23F (37, 11.7%), 6B (35, 11.4%), 6A (16, 5.0%), 15A (11, 3.5%), and 34 (10, 3.2%). The coverage rate of PCV13 reached 83.0% (263/317). The additional types 22F and 33F in PCV15 were not identified in the study. The coverage of PCV20 increased slightly to 85.2% (270/317), in which the further additional types 8, 10A, and 12F based on PCV15 were not found either. The coverage rate of PPV23 was 80.8% (256/317).

A recent and newly reported PCV, V116 (containing types 3, 6A, 7F, 8,9N, 10A, 11A, 12F, 15A, 16F, 17F, 19A, 20, 22F, 23A, 23B, 24F, 31, 33F, 35B, and de-O-acetylated 15B) (Platt et al., 2022), covered 30.0% (95/317) of the present isolates, 22.7% (72/317) of which were the same serotypes with PCV13 (types 3, 6A, 7F, and 19A), and the other 7.3% (23/317) were unique types to V116 (types 11A, 15A, 17F, 20, 23A, 23B, and de-O-acetylated 15B).

The comparison of serotype composition and PCV13 coverage by age groups and different ethnic peoples is shown in Figure 2. The type

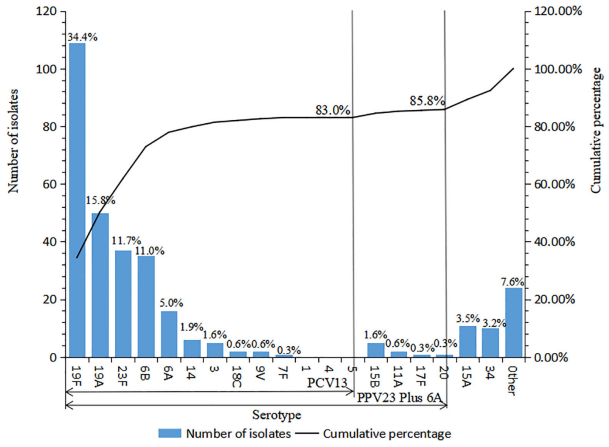

FIGURE 1
Serotype distribution of 317 *S. pneumoniae* strains isolated in Urumqi Children's Hospital during 2014-2021" .The note of Figure 1 is "Other serotypes included 4 strains of type 6C, 3 strains of each type 23A, 7C, 16F, 2 strains of type 42, and 1 strain of each type 29, 13, 37, 23B, 21, 15C, 10C, 28A, and 15F".

distributions of the isolates cultured from children <2 years old, 2–5 years old, and ≥5 years old were similar, and the PCV13 coverage rates were also comparable with each other (80.3% *vs.* 87.6% *vs.* 81.2%, χ^2 = 2.690, P > 0.05). No significant difference in serotype distribution was found between Han children and ethnic minority children as well as in terms of the PCV13 coverage rates (82.7% *vs.* 83.5%, χ^2 = 0.034, P > 0.05).

The serotype distribution of the strains and the coverage of PCV13 in different study stages are shown in Table 1. The coverage rate of PCV13 in 2018–2019 and 2020–2021, respectively, was lower than the corresponding data in 2014–2015, although these differences were not statistically significant. For common serotypes, the proportion of serotype 19A decreased significantly since the PCV13 administration, and that of type 23F was found obviously fluctuated in the three stages. The rates of non-PCV13 types increased from 8.0% in 2014–2015 to 20.3% in 2018–2019 and to 19.1% in 2020–2021. However, this increase did not reach statistical significance (χ^2 = 5.725, P > 0.05).

3.3 Antimicrobial susceptibility

The susceptibility of the 317 strains of *S. pneumoniae* to 14 antimicrobials is shown in Table 2. All strains were susceptible to vancomycin and moxifloxacin; almost all were also susceptible to levofloxacin and linezolid (99.7% and 99.3%, respectively). The susceptibility rate to erythromycin, clindamycin, SMZ-TMP, and tetracycline was very low and less than 12%. The susceptibility to penicillin, ceftriaxone, and cefotaxime appeared to have been divided based on the breakpoints—for example, when the breakpoints of parenteral penicillin (non-meningitis) were adopted (susceptible ≤2 mg/L, intermediate = 4 mg/L, and resistant ≥8 mg/L), the susceptibility rate could reach 97.5%. However, the susceptibility rates based on the parenteral penicillin (meningitis) breakpoints (susceptible ≤0.06 mg/L and drug resistant ≥0.12 mg/L) or the oral penicillin points (susceptible ≤0.06 mg/L, intermediate 0.12–1 mg/L, and drug resistant ≥2 mg/L) were only 8.2% and 7.9%.

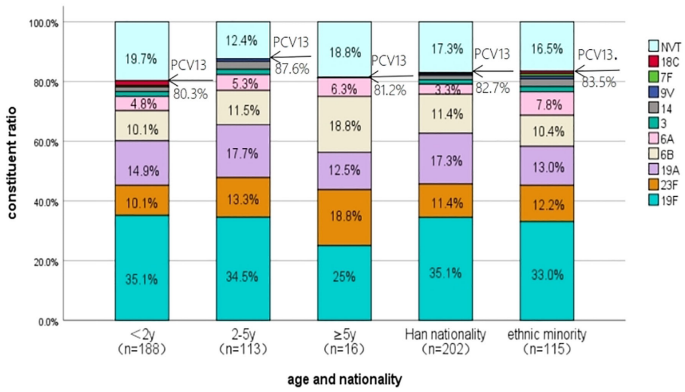

FIGURE 2
The serotype distribution of *S. pneumoniae* isolated from children in different age groups and different ethnic peoples in Urumqi Children's Hospital during 2014-2021". The note of Figure 2 is " NVT represents the serotypes not covered by the PCV13.

The comparison of the susceptibility of different serotypes to penicillin and ceftriaxone is shown in Tables 3, 4. The isolates covered by PCV13 showed higher I% and/or R% or MIC90 or maximum MICs to penicillin and ceftriaxone than the non-PCV13 types. According to the MICs and intermediate and resistance rates on oral penicillin breakpoints, the common serotypes covered by PCV13, including types 19F, 19A, 6B, 23F, and 6A, showed higher resistance than those of uncommon PCV13 types and non-PCV13 types. Overall, a similar trend could be found in the resistance to ceftriaxone according to the MICs and meningitis breakpoints.

The intermediate, resistance, and non-susceptibility rates of the present strains to oral penicillin and ceftriaxone (non-meningitis), meropenem, and tetracycline are found to be significantly different between various stages, as shown in detail in Table 5. No other statistically significant difference is achieved when drug susceptibility is compared between different years.

4 Discussion

The present results revealed that the common serotypes of *S. pneumoniae* isolated from children in Urumqi were types 19F (34.4%), 19A (15.8%), 23F (11.7%), 6B (11.4%), and 6A (5.0%), which showed some difference against previous data in China. The previous systematic review showed that the common serotypes of *S. pneumoniae* in Chinese children from 2006 to 2016 were types 19F, 19A, 23F, 14, and 6B (Lyu et al., 2017). In this study, serotype 14 is rarely found. It may be associated with the fact that almost all the present strains were isolated from non-invasive respiratory tract samples. The systematic review has reported that serotype 14 could be determined more frequently in the invasive strains (5.6%–35.9%) than in the non-invasive ones (0%–12.7%) (Lyu et al., 2017).

The coverage rate of PCV13 in this investigation was 83.0%, which was about the middle level as the previous surveys in Chinese children for non-invasive strains (59.0%–95.2%) and for invasive strains (76.2%–98.8%) (Lyu et al., 2017). The high coverage suggested the potential preventive effect of this vaccination for these children in this region. To date, PCV13 is only available in the private sector and inoculated infrequently. One investigation showed that less than 17% of eligible Chinese children could be immunized with PCVs (Wang et al., 2021). This study revealed that the coverage rates of PCV13 decreased from 92.0% in 2014–2015 to 79.7% in 2018–2019 and 80.9% in 2020–2021, which was hard to pinpoint with certainty as an effect of vaccination. Except for serotype 19A, most of the PCV13

TABLE 1 Distribution of serotypes of *S. pneumoniae* strains isolated from different year periods.

Serotype	2014–2015 (*n* = 75)	2018–2019 (*n* = 148)	2020–2021 (*n* = 94)	χ^2	*P*
PCV13	69 (92.0%)	118 (79.7%)	76 (80.9%)	5.725	0.057
19F	25 (33.3%)	53 (35.8%)	31 (33.0%)	0.253	0.881
19A	17 (22.7%)	25 (16.9%)	8 (8.5%)	6.554	0.038
6B	8 (10.7%)	14 (9.5%)	13 (13.8%)	1.132	0.568
23F	13 (17.3%)	10 (6.8)	14 (14.9%)	6.746	0.034
6A	4 (5.3%)	9 (6.1%)	3 (3.2%)	1.018	0.601
Others[a]	2 (2.7%)[b]	7 (4.7%)[c]	7 (7.4%)[d]	2.047	0.359
Non-PCV13	6 (8.0%)	30 (20.3%)	18 (19.1%)	5.725	0.057

[a]Other serotypes in PCV13 except 19F, 19A, 6B, 23F, and 6A.
[b]Others include one serotype 9V strain and one serotype 14 strain in 2014–2015.
[c]Others include four strains of serotype 3, two strains of serotype 14, and one strain of serotype 18C in 2018–2019.
[d]Others include three strains of serotype 14 and one strain of serotype 3, 9V, 18C, and 7F in 2020-2021.

TABLE 2 Antimicrobial susceptibility test results of 317 isolates of *S. pneumoniae* isolated in Urumqi, 2014–2021.

Antimicrobials	Number	Susceptibility			MIC (mg/L)		
		S (%)	I (%)	R (%)	MIC50	MIC90	MIC range
Penicillin	317				1	2	≤0.06–≥16
Oral	317	7.9	63.5	28.6			
Parenteral, non-meningitis	317	97.5	1.3	1.3			
Parenteral, meningitis	317	8.2	–	91.8			
Ceftriaxone	317				1	1	≤0.06–≥4
Non-meningitis	317	91.8	3.8	4.4			
Meningitis	317	40.1	51.7	8.2			
Cefotaxime	317				1	2	≤0.06–≥4
Non-meningitis	317	87.4	6.6	6			
Meningitis	317	29	58.4	12.6			
Erythromycin	315	3.2	0.9	95.9	≥ 1	≥ 1	≤0.25–>8
Clindamycin	317	8.8	1.0	90.2	–	–	–
Sulfamethoxazole-trimethoprim	292	11.3	9.9	78.8	8	≥ 16	≤0.25–≥16
Tetracycline	316	9.8	1.3	88.9	≥ 16	≥ 16	≤1–>16
Chloramphenicol	292	92.1	–	7.9	≤ 2	4	≤0.06–≥32
Levofloxacin	317	99.7	0.3	0	≤ 0.5	1	≤0.06–4
Vancomycin	317	100	–	–	≤ 1	≤ 1	≤0.5–8
Moxifloxacin	317	100%	0	0	≤ 0.25	0.25	≤0.25–1
Amoxicillin	317	89.6	7.3	3.1	1	3	≤0.06–≥8
Meropenem	312	79.5	15.4	5.1	0.25	0.5	≤0.06–2
Linezolid	273	99.3	–	–	≤ 2	≤ 2	≤1–≥16

S, susceptible; I, intermediate; R, resistant; -, no such interpretation standards.

types were not found to be decreased since the PCV13 administration. It was noted that the proportion of non-PCV13 types increased without statistical significance. After routine immunization with PCVs abroad, the proportion of invasive pneumococcal disease in children caused by some non-vaccine types (NVTs) was found to have increased in many surveys, and serotypes 33F, 22F, 12F, 15B, 15C, 23A, 24F, 23B, 10A, and 38 were the common NVTs (Balsells et al., 2017). The epidemic of non-PCV13 serotypes could counteract the benefits of universal immunization and require additional formula on the PCV13. However, the coverages of PCV15 or PCV20 did not reach up to higher levels significantly in this analysis. In the present study, the non-PCV13 serotypes were very dispersed and included 20 types. Among the above-mentioned common NVTs after PCV13 immunization, only types 15B, 15C, 23A, and 23B were found, which respectively accounted for only 1.6%, 0.3%, 0.9%, and 0.3% in all isolates. These findings make it very important to monitor the serotype distribution in the future.

The previous study revealed obvious differences of serotype distribution between the southern regions and the northern regions of China (Lyu et al., 2017). However, it is not clear whether these differences were associated with the ethnic composition. The present results showed that there was no difference in serotype distribution between children from different ethnic peoples. This finding suggested that the difference of serotype distribution of *S. pneumoniae* between different regions was not related to the composition of ethnic peoples. This means that it is more important to involve subjects from many sites for the investigations than to recruit subjects from different ethnic peoples.

The limited administration of PCV13 and the inconsistent epidemiological changes of PCV13 serotypes 19A and 23F in the three temporal stages complicated the interpretation for the changes of serotype distribution in this study. Considering that the serotypes were proved to be associated with other bacterial characteristics, including virulence and antimicrobial susceptibility (Zhou et al., 2022), these changes of types might be related to multiple factors, including the disease and the severity of inpatients and the increasing improvement and implementation of the antibiotic management system in recent years, especially during COVID-19 control. After the epidemic of COVID-19, various parts of China have introduced measures to control the individual purchasing of antibiotics, such as not to buy antimicrobials at drugstores or the buyer must have a nucleic acid test report. At the same time, more rules were made to control antibiotic prescription in hospitals. These measures may lead to a reduction of the spreading of drug-resistant isolates. According to the China Antimicrobial Surveillance Network, the proportion of penicillin-insensitive and penicillin-resistant strains of non-meningococcal *S. pneumoniae* in Chinese children before COVID-

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TABLE 3 Susceptibility of *S. pneumoniae* to penicillin according to serotype.

Serotype	Number	MIC50(mg/L)	MIC90 (mg/L)	MIC range (mg/L)	Oral penicillin		Parenteral penicillin		
							Meningitis	Non-meningitis	
					I (%)	R (%)	R (%)	I (%)	R (%)
PCV13	263	1	2	≤0.06–≥16	63.1	33.5	96.2	1.5	1.5
19F	109	1	2	0.12–≥16	60.6	39.4	100.0	3.7	1.8
19A	50	1	≥2	≤0.06–8	48.0	50.0	98.0	0	2.0
6B	35	1	2	≤0.06–2	80.0	17.1	97.1	0	0
23F	37	1	2	≤0.06–2	73.0	24.3	97.3	0	0
6A	16	0.75	2	0.12–2	87.5	12.5	100.0	0	0
3	5	≤0.06	≤0.5	≤0.06–≤0.5	40.0	0	20.0	0	0
14	6	1	8	1–8	50.0	50.0	100.0	0	16.7
Other[a]	5	≤0.06	0.12	≤0.06–0.12	40.0	0	40.0	0	0
Non-PCV13	54	0.25	1	≤0.06–2	64.8	5.6	70.4	0	0
15A	11	1	1	≤0.06–2	72.7	9.1	81.8	0	0
15B	5	0.5	1	≤0.06–1	80.0	0	80.0	0	0
34	10	0.25	0.25	≤0.06–0.5	80.0	0	80.0	0	0
Total	317	1	2	≤0.06–16	63.5%	28.6	91.8	1.3	1.3

I, intermediate; R, resistant.
[a]Other includes two isolates for type 9V, two isolates for type 18C, and one isolate for type 7F which is covered by PCV13.

19 control was higher than that after COVID-19 control, and the detection rate of MRSA and MRSE in 2020–2021 was also lower than that before COVID-19 control (China antimicrobial surveillance network(CHINET), 2021). In this study, the drug resistance to ceftriaxone decreased significantly with stages, which may reflect the strict management of broad-spectrum antimicrobials and reduce the advantage of the spread of highly drug-resistant strains. However, the above-mentioned hypothesis may not be the complete

TABLE 4 Comparison of the susceptibility of *S. pneumoniae* to ceftriaxone between different serotypes.

Serotype	Number	MIC50 (mg/L)	MIC90 (mg/L)	MIC range (mg/L)	Meningitis		Non-meningitis	
					I (%)	R (%)	I (%)	R (%)
PCV13	263	1	1	≤0.06–≥4	56.7	9.9	4.6	5.3
19F	109	1	≥ 4	0.12–≥4	58.7	18.3	5.5	12.8
19A	50	1	1	≤0.06–2	64.0	10.0	10.0	0
6B	35	1	1	≤0.06–1	62.9	0	0	0
23F	37	1	1	≤0.06–2	48.6	2.7	2.7	0
6A	16	0.5	1	≤0.06–1	43.8	0	0	0
3	5	≤ 0.06	0.5	≤0.06–0.5	0	0	0	0
14	6	1	1	1–1	100.0	0	0	0
Other[a]	5	≤ 0.06	0.12	≤0.06–0.12	0	0	0	0
Non-PCV13	54	0.25	1	≤0.06–1	27.8	0	0	0
15A	11	1	1	≤0.06–1	63.6	0	0	0
15B	5	0.5	1	≤0.06–1	40.0	0	0	0
34	10	0.12	0.25	≤0.06–0.25	0	0	0	0
Total	317	1	1	≤0.06–≥4	51.7	8.2	3.8	4.4

I, intermediate; R, resistant.
[a]Other includes two isolates for type 9V, two isolates for type 18C, and one isolate for type 7F which is covered by PCV13.

TABLE 5 Comparison of the susceptibility of *S. pneumoniae* to penicillin (oral) and ceftriaxone (non-meningitis) between different years.

Antimicrobials	2014–2015 (*n* = 75)	2018–2019 (*n* = 148)	2020–2021 (*n* = 94)	χ^2/Fisher	*P*
Penicillin (oral)[a]					
I (%)	62.7	56.8	74.5	7.795	0.020
R (%)	30.7	34.5	18.1	7.716	0.021
NS (%)	93.3	91.2	92.6	0.343	0.843
Ceftriaxone (non-meningitis)[a]					
I (%)	6.7	4.1	1.1	3.651	0.156
R (%)	16.0	1.4	0	24.463	<0.01
NS (%)	22.7	5.4	1.1	24.924	<0.01
Meropenem[a]					
I (%)	61.3	0	2.1	142.546	<0.01
R (%)	14.7	2.1	2.1	18.464	<0.01
NS (%)	76.0	2.1	4.3	186.598	<0.01
Tetracycline					
I (%)	0	2.0	1.1	1.240	0.814
R (%)	97.3	85.0	88.3	7.681	0.021
NS (%)	97.3	87.1	89.4	6.011	0.052

I, intermediate; R, resistant.

interpretation for the present results. Other COVID-19 control measures, such as wearing a mask and keeping social distance, could also play a role to limit the spread of pathogens, especially in the hospital. Since COVID-19 occurred, the nosocomial infection control measures had been strengthened as never before. These measures could influence the spread of drug-resistant pathogens.

The penicillin susceptibility of *S. pneumoniae* was analyzed to get different distributions according to breakpoints for administration route and type of disease, which could be 91.8% based on the parenteral administration and meningitis breakpoints. The same was true for susceptibility to other cephalosporins. The beta-lactam antimicrobials were often recommended as the initial empirical treatment for children with *S. pneumoniae* meningitis (van de Beek et al., 2016; China National Clinical Research Center for Respiratory Diseases et al., 2020). However, the present results suggested that these drugs should not have been the initial choice if *S. pneumoniae* meningitis was suspected in Urumuqi. For the patient infected with low susceptibility or resistance to cephalosporins, vancomycin combined with cefotaxime or ceftriaxone should be prescribed. If the child is allergic to beta-lactam antimicrobials, vancomycin combined with rifampicin can be used for empirical treatment (van de Beek et al., 2016; China National Clinical Research Center for Respiratory Diseases et al., 2020). Similar to a previous report (Lyu et al., 2017), this study also found that the isolates covered by PCV13 were more non-susceptible to antimicrobials than the NVT isolates. This suggests that PCV13 immunization would not only prevent pneumococcal infections but also control the spread of drug resistance of *S. pneumoniae* (Ben-Shimol et al., 2020).

There are several limitations in the present study. First, the isolates frozen in 2016 to 2017 could be recovered for some technological reasons. The natural fluctuation of serotype distribution and drug resistance could be shown to some extent if the data from 2016 to 2017 was available. However, *S. pneumoniae* infections and PCV immunization are mainly in children under 5 years of age. This uneven distribution is consistent with the actual situation and enhanced the importance of this pathogen to younger children. Next, some important information, such as the subjects' antibiotic use and vaccination experience, have not been collected, which could have contributed to an in-depth analysis of the current results. Finally, almost of the isolates were cultured from non-invasive specimens, sputum in particular (298/317), and only three strains were from invasive specimens. In the latest PCV position paper, the World Health Organization affirms that continuous or regular non-invasive isolate surveillance is helpful to understand the epidemiological characteristics of *S. pneumoniae* (World Health Organization, 2019).

In summary, the common serotypes of *S. pneumoniae* isolated from the children in Urumqi were types 19F, 19A, 23F, 6B, and 6A, in which there was no marked change found since the PCV13 introduction and the COVID-19 control. The serotype distribution of isolates from ethnic minority children was consistent with those from Han children in the same area. The coverage of PCV13 decreased since the vaccine was introduced into immunization, although this change was not statistically significant and some paradoxical phenomenon existed in specific PCV13 serotypes: 19A and 23F. The resistance rate to oral penicillin and ceftriaxone significantly declined in the COVID-19 control stage. Continuous survey will provide more perfect data for understanding the serotype distribution and drug resistance of *S. pneumoniae* in this region.

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 human participants were reviewed and approved by Beijing Children's Hospital ethics committee. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

X-HS, J-LT, JY, DW, JC, RA, and W-LZ collected the isolates in their local sites. K-HY and W-LZ applied the funds to support this work. M-YG, X-HS, K-HY, and W-LZ designed the study and interpreted the results. M-YG, WG, and LY confirmed the isolates and determined the serotypes of the isolates as well as the susceptibility test with E-test stripes. X-HS, J-LT, JY, DW, JC, and RA re-cultured the present isolates and determined the susceptibility with Compact system and KB discs. M-YG, X-HS, WG, and LY collected the data and performed statistics. M-YG, W-LZ, and K-HY draw the pictures and made the tables. M-YG and X-HS wrote the first draft of the manuscript. K-HY and W-LZ revised the manuscript according to all authors' comments before submission. K-HY and W-LZ are responsible for the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Characterization of resistance genes and plasmids from sick children caused by Salmonella enterica resistance to azithromycin in Shenzhen, China

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Introduction: *Samonella* is 1 of 4 key global causes of diarrhoeal diseases, sometimes it can be serious, especially for yong children. Due to the extensive resistance of *salmonella* serotypes to conventional first-line drugs, macrolides (such as azithromycin) have been designated as the most important antibiotics for the treatment of *salmonella*. Antimicrobial resistance is a major public health problem in the world, and the mechanism of azithromycin resistance is rarely studied.

Methods: This study determined the azithromycin resistance and plasmids of *Salmonella* enterica isolates from children attending the Shenzhen Children's Hospital. The susceptibility of ampicillin (AMP), ciprofloxacin (CIP), ceftriaxone (CRO), sulfamethoxazole (SMZ), chloramphenicol (CL), and azithromycin (AZM) were detected and the genes and plasmids from azithromycin-resistant *Salmonella* were detected by Illumina hi-seq and Nanopore MinIone whole genome sequencing (WGS) using a map-based method, and the genomic background of these factors was evaluated using various bioinformatics tools.

Results: In total, 15 strains of nontyphoid *Salmonella* strains that were isolated (including *S.* typhimurium, *S.*London, *S.* Goldcoast, and *S.*Stanley) demonstrated resistance to azithromycin (minimum inhibitory concentration,MIC from 32 to >256 μg/mL), and the resistance rate was 3.08% (15/487). The sensitivity test to other antibiotics demonstrated 100% resistance to AMP, and the resistance to SMZ and CL was 86.7% and 80.0%, respectively. Through WGS analysis, all isolates were positive for a plasmid-encoded *mphA* gene. Plasmid incompatibility typing identified five *IncFIB(K)*, five *IncHI2/HI2A/Q1*, two *IncC*, one *IncHI2/HI2A/N*, one *IncR*, one *IncFII* and one *IncHI2/HI2A* plasmids. Sequence analyses of plasmids revealed extensive homology to various plasmids or transposons in regions involved in plasmid replication/maintenance functions and/or in antibiotic resistance gene clusters.

Conclusion: *mphA* is the main gene involved in azithromycin, a macrolide, and resistance to *Salmonella*. It is usually located on plasmids and easily spreads, hence posing a great threat to the current treatment of *Salmonella* infection. The plasmid sequence similarities suggest that the plasmids acquired resistance genes from a variety of enterica bacteria and underscore the importance of a further understanding of horizontal gene transfer among enterica bacteria.

KEYWORDS

azithromycin, *mphA* gene, plasmid, children, *Salmonella* enterica

1 Introduction

Salmonella is a gram negative rods belonging to the *Enterobacteriaceae* family and are divided into serotypes according to the structures of H and O antigens on their surface. The two species of *Salmonella* are *S*.bongori and *S*.enterica, *S*.enterica including more than 2,600 serotypes have been shown to be main sources of infections in human. These serogroups include *S*.Typhi,*S*.Paratyphi, *S*.Typhimurium, *S*.Enteritidis, *S*.Choleraesuis, and so on, which can be grouped into typhoidal and nontyphoidal *Salmonella* (NTS) serovars (Gilchrist et al., 2015).

Samonella is 1 of 4 key global causes of diarrhoeal diseases, most of salmonellosis is mild, diarrhea, fever and stomach cramps are the main symptoms, people should not take antibiotics and recover in 4 to 7 days (World Health Organization, 2018). Sometimes it can be serious, especially for yong children, it was reported by WHO that 550 million people falling ill and 220 million chidlren under the age of 5-year-old each year (World Health Organization, 2018). Current recommendations are that antibiotics be reserved for patients with severe disease or patients who are at a high risk for invasive disease (Guarino et al., 2014). Children with suspected or confirmed invasive infections, including infants younger than three months of age with immune deficiency, chronic basic diseases, and severe enteritis need antibiotics (Nair et al., 2021). However, with the wide application of antibiotics, the drug resistance rate is increasing yearly, which poses a severe challenge to treating *Salmonella* infection. The rational selection and use of antibiotics have become important issues worldwide.

Ampicillin, chloramphenicol and cotrimoxazole were the first-line antibiotics for the treatment of salmonellosis, resistance to first-line antibiotics used to treat infections caused by *Salmonella* is increasing. The emergence and spread of multi-drug resistance (MDR) pose a major threat to the effective treatment and control of Salmonellosis, macrolides(such as azithromycin) and carbapenems have been designated as the most important antibiotics for the treatment of *Salmonella* disease (Carey et al., 2021).

Azithromycin is the only remaining oral drug for the treatment of extensively drug resistant (XDR) *Salmonella* infection (Plumb et al., 2019). Particularly noteworthy is the emerging resistance to azithromycin, which will cause people to worry about incurable infection. It is necessary to monitor and diagnose azithromycin resistance to guide rational use and prevent the prevalence and expansion of drug resistance.It was reported that the azithromycin resistance rate of NTS isolates from Taiwan (3.1%) is much higher than that of NTS isolates from European countries and the United States (Chiou et al., 2023).Antimicrobial resistance is a major public health problem in the world, and the mechanism of azithromycin resistance is rarely studied.

This study aimed to determine the azithromycin resistance genes and plasmids of *Salmonella* enterica isolates from children attending the Shenzhen Children's Hospital by susceptibility testing and whole genome sequencing (WGS), and provide information of monitoring and periodic review of sensitivity data to ensure the adequacy of treatment guidelines.

2 Materials and methods

2.1 Ethics approval and consent to participate

The data were approved by the ethics committee of Shenzhen Children's Hospital under document number SEY0132407.

2.2 Bacterial collection

Salmonella enterica strains were isolated from clinical blood and fecal culture samples collected from the Shenzhen Children's Hospital between January 2014 and December 2021. The data, including information of children and isolations were collected from Clinical Microbiology Laboratory, Department of clinical Laboratory, We excluded data on contaminated bacteria and duplicate strains detected from the same patient.

2.3 Bacterial culture and identification

A BACTEC^{TM} FX-200 automatic blood culture instrument (BD Diagnostic Systems, Sparks, MD, USA) and BacT/Alert 3D blood culture system (BTA3D; bioMerieux, Marcy lEtoile, France) were

used for blood culture, 1~10 ml of blood specimens were subjected to blood culture bottles and incubated at 37°C in automated system. Bottles that positive alerts were detected would be removed and samples would be cultured on Columbia blood agar plates and chocolate agar plates. Other samples were cultured and all isolates were identified according to methods of Manual of Clinical Microbiology [11th edition] (James et al., 2015). The bacteria were identified to the genus level using a VITEK 2 COMPACT automatic microbial identification drug sensitivity instrument (Biomerieux, France) and a mass spectrometry system (MALDI-TOF MS, Merier, France), and the serotypes were divided using the Danish Statens Serum Institut diagnostic serum according to structures of somatic O and flagellar H antigens(the Kauffman-White classification).

2.4 Drug sensitivity test

The susceptibility of five antimicrobial agents (Oxoid, UK), including ampicillin (AMP), ceftriaxone (CRO), chloramphenicol (CL), trimethoprim-sulfamethoxazole (SXT), ciprofloxacin (CIP), and azithromycin (AZM), was determined using the disk diffusion method to screen azithromycin resistant strains. The MIC value of azithromycin resistant stains were calculated using the E-test method (Biomerieux,France). The results, evaluated according to the judgment results of the breaking point standards recommended by the Clinical and Laboratory Standards Institute (CLSI) M100 2021 (Clinical and Laboratory Standards Institute[CLSI], 2021), were divided into sensitivity, mediation, and drug resistance. Since there is no definite azithromycin CLSI break point for any *Salmonella* serotype except *S.*Typhi, the azithromycin resistance standard of *S.* Typhi was used, i.e., inhibition zone ≤ 12 mm and MIC ≥ 32 mg/mL was determined as drug resistance.

2.5 Whole-genome sequencing

The *S.* enterica isolates for azithromycin resistance were further subjected to whole-genome sequencing. Genomic DNA was extracted from overnight cultures using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. DNA quality was assessed using Nanodrop spectrophotometry (Thermo Fisher), and quantity was assessed using the Qubit 4.0 system (Thermo Fisher). The DNA libraries were constructed with 150-bp paired-end whole-genome sequencing using the Illumina HiSeq 2500 system (Huada, Shenzhen, China) (Ma et al., 2020). The obtained paired-end Illumina reads were assembled *de novo* using SPAdes v3.6.2 (default parameters except -careful and -k 21,33,55,77,99,127). In addition, to obtain long read sequences, selected strains were further sequenced using Oxford Nanopore MinION flowcell R9.4 (Li et al., 2018). *De novo* hybrid assembly was performed using a combined Illumina HiSeq and Nanopore sequencing approach (Nextomics). Genome assembly was performed with Unicycler version 0.4.1 using a combination of short and long reads, followed by error correction with Pilon version1.12 (Wick et al., 2017) (Walker et al., 2014).

2.6 Data analysis

An in silico multilocus sequence typing (MLST) scheme was used to subtype the isolates using mlst software (version 2.19.0) (Larsen et al., 2012). The chromosome and plasmid sequences were annotated using the prokaryotic gene prediction tool Prokka (Seemann, 2014). The plasmid incompatibility type was searched using the online tool PlasmidFinder (https://cge.cbs.dtu.dk//services/PlasmidFinder/) (Carattoli et al., 2014). Antibiotic resistance genes were identified using both the Comprehensive Antibiotic Resistance Database (CARD) database (Alcock et al., 2020). Comparative plasmid illustration was implemented by BRIG (http://brig.sourceforge.net) (Alikhan et al., 2011). BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for comparative analysis through coverage and identities (Camacho et al., 2009).

Genomic sequences and the associated metadata of 10561 *Salmonella* strains stored in the NCBI GenBank database were obtained. Bacterial core genome single nucleotide polymorphism (cgSNP) analysis between 15 azithromycin-resistant clinical isolates and 10561 complete or draft genomic sequences of *Salmonella* enterica strains was performed to construct a maximum likelihood phylogenetic tree using Parsnp software (Kaas et al., 2014).This analysis was performed using the default parameters, and *S.* enterica subsp. enterica serovar Typhimurium str. LT2 (RefSeq ID: NC_003197.2) as the reference genome. Phylogenetic trees were visualized and annotated by the Interactive Tree of Life (iTOL) V5 web server (Letunic and Bork, 2021).

2.7 Statistical analysis

We adopted WHONET 5.6 software for data analysis, and the comparison of rates adopts χ (2) inspections.

3 Results

3.1 Clinical informations

A total of 15 *Salmonella* strains were detected in 13 children. If the time interval between the detection of *Salmonella* in a child exceeded three days, it was collected as a new strain and tested accordingly. The clinical, demographic, and laboratory characteristics of the 13 patients with azithromycin-resistant Salmonella infections are displayed in Table 1.

3.2 Serotypes of azithromycin-resistant *S.* enterica

After routine drug sensitivity test screening of 487 retained *S.* enterica strains, 15 azithromycin-resistant *S.* enteric strains were detected. The serotype distribution was identified using traditional *Salmonella* serum, and the results of the data analysis after WGS and assembly are displayed in Figure 1. It was demonstrated four

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TABLE 1 General information and clinical features of pediatric patients with azithromycin-resistant *Salmonella enteric*.

Characteristics	Number (n=13)
Age, in median months	14 [3,116]
Sex	
Male	9
Female	4
Underlying diseases	1
Clinical symptoms	
Fever 37.4-39°C	3
39.1-40 °C	9
>40.1 °C	1
Abdominal pain and diarrhea	13
anemia	3
Laboratory findings	
Total leucocyte count, $\times10^9$ cells/L	11.15 [3.67, 33.10]
Lymphocyte count, $\times10^9$ cells/L	3.37 [1.86, 6.56]
Eosinophil count, $\times10^9$ cells/L	0.11 [0.00, 0.74]
C-reactive protein mg/L	23.47 [2.34, 92.50]
Aspartate aminotransferase IU/L	27.0 [12.0, 38.0]
Course of disease, days	5 [3,14]
Clinical Outcomes	
Cure	13

serotypes in the 15 *Salmonella* strains, including *S*.Stanley, *S*.London, *S*.Goldcoast or Brikama and *S*.I 4,[5],12:i:-.

3.3 Antimicrobial susceptibility profiles

Testing of the susceptibility of 15 *Salmonella* isolates (Table 2) to 6 antibiotics. Among these isolates, 100% (16/16) were resistant to AZM, while all isolates were also resistant to AMP. The resistance rates against SMZ, CL, CRO, and CIP were 87.3%, 80.0%, 46.7%, and 20.0%, respectively. For AZM, 26.67% of the isolates showed the highest MICs of 256 μg/mL, 20.0% showed MICs of 64 μg/mL, and 53.33% showed MICs of 32 μg/mL.

3.4 Phylogenetic analyses

In silico MLST analysis indicated that 15 isolates represented four sequence types, which were assigned to ST 29 (1/16), ST 34 (6/15), ST 155 (5/15), and ST 2529 (3/16) (Figure 1). We performed phylogenetic analysis of 15 *S. enterica* isolates and generated a phylogenetic tree with 68 strains and 3,067 SNP loci (Figure 2). The

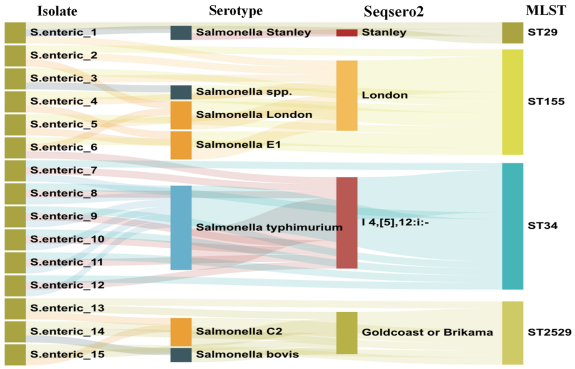

FIGURE 1
Serotype and MLST type distribution of 15 azithromycin resistant Salmonella strains.

phylogenetic tree showed that the 15 self-tested strains clustered and formed four independent branches. The predominant ST type of the clade containing five samples is ST34; however, sample 1 is the rare ST type ST 29. The ST types of clades containing four and three samples were ST 155 and ST 2529, respectively. According to the results of phylogenetic analysis, the closest relative of ST34 isolates was identified in 2007 from a fecal sample in Australia, and the closest relative of ST34 isolates was identified in 2010 from a poultry small intenstine in Nigeria.

3.5 Genotypic characterization of antimicrobial resistance

We performed antimicrobial resistance gene analysis on 15 strains, which were extracted from whole genome sequencing (WGS) analysis. A total of 91 ARGs were found in the 15 isolates (Figures 3, 4), of which 39 ARGs were shared by the 15 isolates (Figure 3). The results shown in Figure 3 indicate that the 15 self-tested azithromycin-resistant strains all carried the azithromycin resistance gene *mphA* (Figure 3B), and *the S.* enterica_1 isolate carried two *mphA* genes. *S.* enterica_7 also carried the other azithromycin resistance gene *ErmB*. In addition, some CTX-M-type extended-spectrum beta-lactamase (ESBL) genes *(blaCTX-M-14, blaCTX-M-55*) and aminoglycoside resistance genes (*APH(3')-Ia, APH(3'')-Ib*, and *APH(6)-Id*) were identified among these isolates. Additional *AMR* genes (*emr* family genes, *dfrA* family genes and *sul* family) were also identified among these isolates. Overall, phenotypic resistance was highly correlated with the presence of known resistance determinants.

3.6 Genetic characterization of *mphA*-carrying plasmid

Among the 15 isolates (S1-S15) with complete genome sequences, *mphA* was positive in all isolates and was located on several different plasmids。 The upstream and downstream parts of the *mphA* resistance gene fragments shared the same backbone

TABLE 2 Distribution of drug sensitivity and MIC value of azithromycin resistant *S.* enterica.

	Disk Diffusion Test						E-TEST
Isolate	AMP	CRO	SXT	CIP	CL	AZM	AZM (MIC, μg/mL)
S.enteric 1	R	S	R	I	R	R	256
S.enteric 2	R	S	R	I	R	R	32
S.enteric 3	R	R	R	I	R	R	32
S.enteric 4	R	S	R	I	R	R	32
S.enteric 5	R	S	R	R	R	R	32
S.enteric 6	R	S	R	I	R	R	32
S.enteric 7	R	R	S	S	S	R	256
S.enteric 8	R	R	R	R	R	R	32
S.enteric 9	R	R	R	R	R	R	32
S.enteric 10	R	R	S	I	R	R	32
S.enteric 11	R	R	R	I	R	R	256
S.enteric 12	R	R	R	I	R	R	256
S.enteric 13	R	S	R	I	S	R	64
S.enteric 14	R	S	R	I	R	R	64
S.enteric 15	R	S	R	I	S	R	64
Resistant (%)	100	46.7	86.7	20	80	100	
Intermediate (%)	0	0	0	73.3	0	0	
Sensitivity (%)	0	53.3	13.3	2.2	20	0	

The bold values of 'R" means "Resistance", "I" means " Intermediate" and "S" means " Sensitive".

FIGURE 2
The phylopene relationship between 15 *Salmonella* enterica isolates and a total of 68 *Salmonella* enterica strains currently deposited in the NCBI GenBank database. The distance of SNPSs is represented by the branch length.

sequence containing genes for 2 mobile element protein genes, transcriptional regulator (*TetR* family), transcriptional regulator *NanR*, and the genetic structure of the *mphA* gene (Figure 5). Blast alignment showed that stable sequences containing *mphA* appeared in multiple plasmid structures, and the sequence identity was 100%.

We used Blastn to compare the plasmid sequence in this study with that on the NCBI website, to find the closest homologous plasmid, the results are shown in the Table 3. Because the plasmid is not species-specific, lead to cannot form a complete phylogenetic tree containing all the plasmids in this study. Among the 15 isolates (S1-S15), we detected 8 diffrenet plasmids, and found cloest homologous plasmids to these eight plasmids(>82% coverage, >99.9% nucleotide sequence identity). 4 plasmids are homologous with plasmids from *Salmonella* spp., 2 plasmids are homologous with plasmids from *Escherichia coli*, 1 plasmid is homologous with plasmid from *Klebsiella pneumoniae*, and 1 plasmid is homologous with plasmid from *Shigella flexneri*. The plasmid carried is closely related to the phylogenetic aggregation of the strains. Strains with close branches carry the same plasmid, such as, strain S2-S6, strain S8-S9, strain S11-S12, strain S13-S15 (Figures 6–9).

The plasmids integrating multiple functional mobile elements and azithromycin resistance genes, which can be transferred

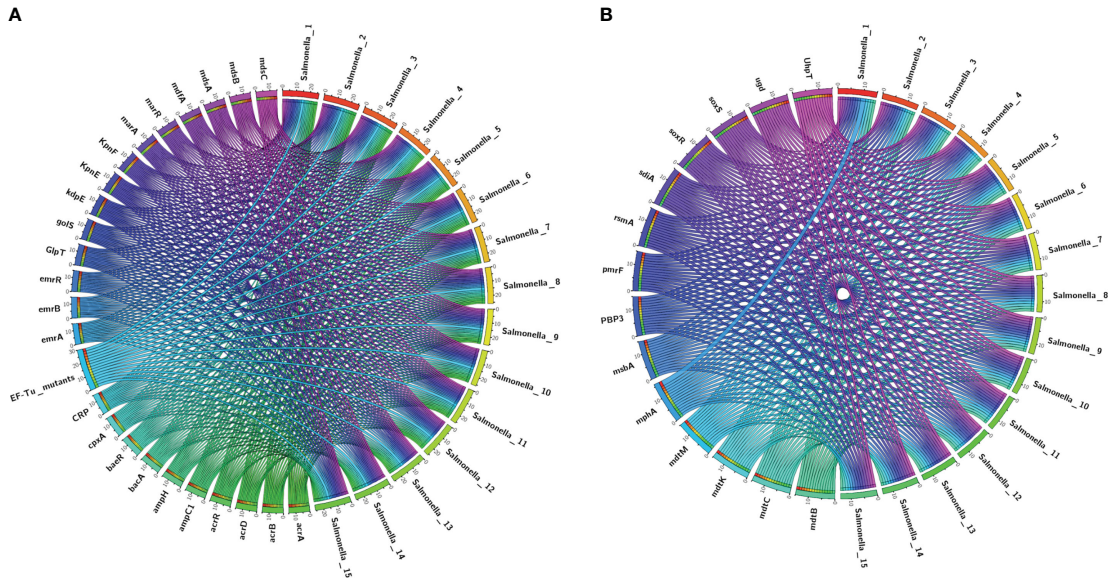

FIGURE 3
Detection of ARGs over the 15 plates. **(A, B)** Chord diagram illustrating the correlations between ARGs and the ARG-carrying pathogenic species. Thickness of the lines represents the number of samples observing such correlation. A total of 39 ARGs were shared by the 15 isolates.

without antibiotic selection pressure. Our plasmids sequence analysis indicates that the *mphA*-bearing *IncX1* plasmids were hypothetically mobilizable and could move into the chromosome *via* insertion sequences such as *IS21/IS26/ISVsa5/IS15*. The typical *IS26-mphA-tap* transposition unit was embedded in the *IncX1* plasmid and other MDR plasmids, such as *IncHI2* (Figures 6, 7, 9). This highlights the pivotal role of *IS26* and tap in the transmission of *mphA* among plasmids and chromosomes. Detailed analysis of *mphA*-bearing contigs in the 15 *mphA*-positive isolates showed that the core structure *IS-mphA-tap* (n = 15) and seven additional core structures were prevalent among these isolates (Figures 6–9). However, the complete structures around *IS-mphA-tap* were identified because of short and long fragmented assembled contigs based on Illumina short-read data and Nanopore long-read data.

4 Discussion

Azithromycin was discovered in 1980 by the Yugoslav pharmaceutical company Pliva and was on the List of Essential Medicines of World Health Organization's List (Wang et al., 2020).

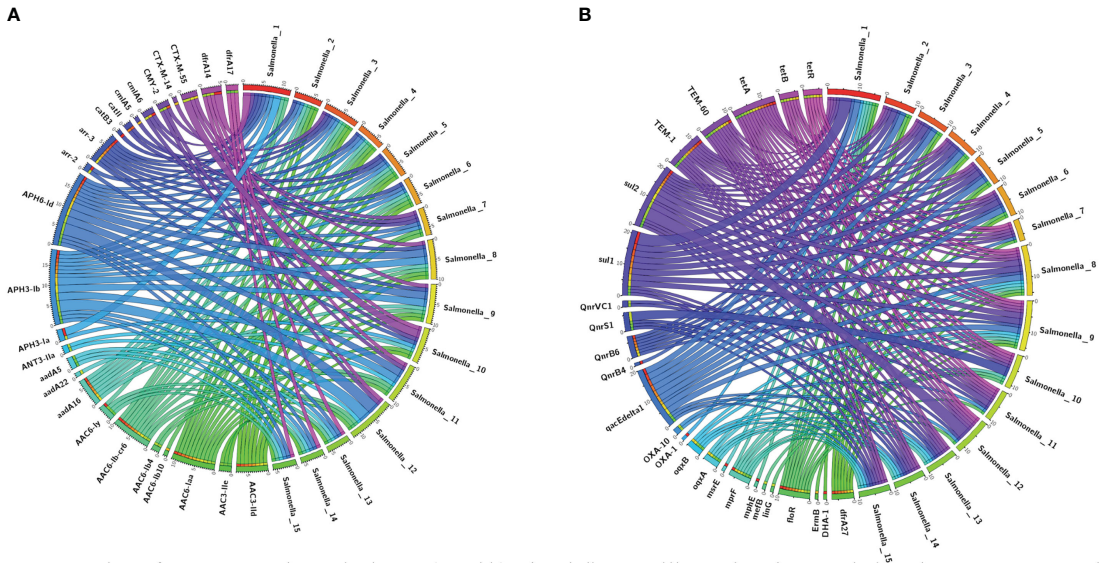

FIGURE 4
The upstream and downstream parts of the mphA resitance gene fragments shared the same backbone sequence containing genes.

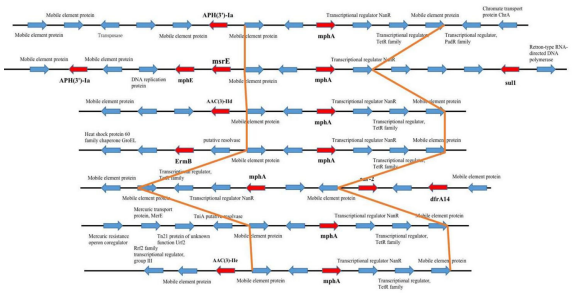

FIGURE 5
mph(A) were located on two different plasmids in S. enteric-7. **(A)** The large plasmid. **(B)** The small plasmid.

Similar to other macrolides, azithromycin mainly targets the P site of the ribosomal 50S subunit, one of the most conserved biomolecules in bacteria. At present, research on the mechanism of azithromycin resistance is increasing. Various reports on azithromycin-resistant *Salmonella* associated with *mphA* found that macrolide-2'-phosphotransferase encoded by *mphA* can mediate the increased resistance of NTS to azithromycin (Centers for Disease Control and Prevention(CDC)). In 2016, Nair Satheesh used WGS and drug resistance phenotype identification to determine the potential mechanism of azithromycin resistance of *S.enteritidis* isolates in the UK. Among 685 strains, 15 were resistant to azithromycin, and 12 encoded *mphA* (Nair et al., 2016). *mphA*-mediated azithromycin resistance has been reported in China.Among the 32 strains of azithromycin-resistant *Salmonella typhimurium* collected in Shanghai by Wang J et al (Wang et al, 2017).15 strains had MIC ≥ 128 μg/mL, from which *mphA* was detected.Hong et al. in Taiwan found that *mphA* existed in azithromycin-resistant *Salmonella* Typhi by identifying the drug resistance genes of serotypes of five strains from humans, pigs, and chickens (Hong et al., 2018). The emergence of *mphA* and its horizontal transmission ability therefore threaten the use of azithromycin for *Salmonella* infection. However, the mechanism of *mphA* level transfer is still unclear and needs to be further clarified using molecular epidemiology and comparative genomics.

This study demonstrated that *mphA* was detected in 15 azithromycin-resistant *Salmonella* strains, with no other azithromycin-related drug resistance gene detected, suggesting that the drug resistance gene is prevalent in Shenzhen Children's Hospital. Among the 15 azithromycin-resistant *Salmonella* strains detected in 13 children, only one child had the basic disease (after neuroblastoma surgery) and was treated with special grade antibiotics (meropenem). One case was complicated with adenovirus infection with severe

TABLE 3 NCBI retrieval of homologous plasmids.

Strian	Plasmid	Homologous plasmid	Host bacteria	Query Cover(%)	Per. ident(%)
*S. enterica*1	pS1_1	pSG17-135-HI2	*S. enterica* subsp. enterica serovar Agona strain SG17-135	82%	99.99
*S. enterica*1	pS1-2	pKP19-3138-5	*Klebsiella pneumoniae* strain KP19-3138	94%	99.97
*S. enterica*2	pS2	pYUHAP1	*S. enterica* subsp. enterica serovar London strain HA3-IN1	100%	99.97
*S. enterica*3	pS3	pYUHAP1	*S. enterica* subsp. enterica serovar London strain HA3-IN1	100%	99.97
*S. enterica*4	pS4	pYUHAP1	*S. enterica* subsp. enterica serovar London strain HA3-IN1	100%	100
*S. enterica*5	pS5	pYUHAP1	*S. enterica* subsp. enterica serovar London strain HA3-IN1	100%	99.95
*S. enterica*6	pS6	pYUHAP1	*S. enterica* subsp. enterica serovar London strain HA3-IN1	100%	99.97
*S. enterica*7	pS7	unnamed1	*Shigella flexneri* strain STLEFF_34	100%	99.99
*S. enterica*8	pS8	pEC22-CTX-M-15	*Escherichia coli* strain EC20	97%	99.99
*S. enterica*9	pS9	pEC22-CTX-M-15	*Escherichia coli* strain EC20	97%	99.99
*S. enterica*10	pS10	pNDM-M121	*Escherichia coli* strain ECNB21-M121	89%	99.9
*S. enterica*11	pS11	pSa1753	Salmonella sp. strain Sa1735	100%	99.99
*S. enterica*12	pS12	pSa1753	Salmonella sp. strain Sa1735	100%	99.99
*S. enterica*13	pS13	unnamed1	*S. enterica* subsp. enterica serovar Indiana strain 222	90%	99.98
*S. enterica*14	pS14	unnamed1	*S. enterica* subsp. enterica serovar Indiana strain 222	92%	99.97
*S. enterica*15	pS15	unnamed1	*S. enterica* subsp. enterica serovar Indiana strain 222	92%	99.98

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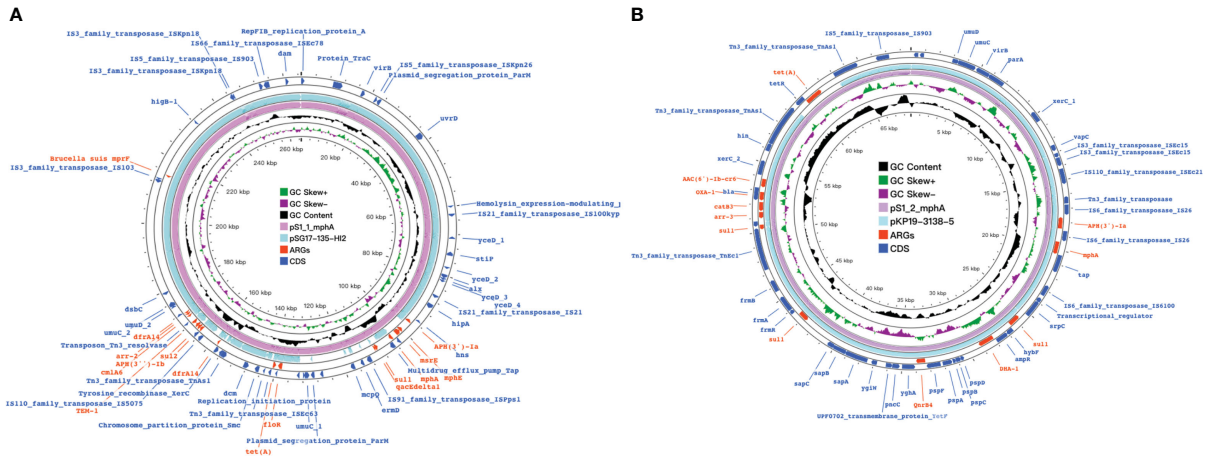

FIGURE 6
mph(A) were located on two different plasmids in S. enteric-2 (also include S.enteric-3, 4, 5, 6) and S. enteric-7. **(A)** The plasmid in S.enteric-2 (also include S.enteric-3, 4, 5, 6) **(B)** The plasmid in S.enteric-7.

diarrhea symptoms. The antibiotics used were all third-generation cephalosporins. The treatment effect was good, and the patient was cured and discharged.

In this study, the correlation of the MIC with a resistance gene showed that the MIC ranged between 32 and 256 μg/mL among the study isolates and that the *mphA* gene was found in all *S.* enterica isolates. There are also reports of E. coli isolates with an MIC of ≥256 μg/mL carrying the *mphA* gene, followed by *ermB* and *mphB* in isolates with an MIC of >1024 and 128 μg/mL, respectively (Phuc Nguyen et al., 2009).

Generally, azithromycin-resistance genes such as *mphA* and *ermB* were reported to be carried in plasmids (Darton et al., 2018).Remarkably, in this study, the plasmid carrying the *mphA* gene was found to be carried in 8 different plasmids, which might be possible due to the presence of various insertion sequences and other mobile elements in *S.* enterica. Apart from this unique finding, genome analysis revealed the presence of multiple resistance genes that were expected. The genome also contained various mobile genetic elements that are reported to play a significant role in AMR dissemination in *S.* enterica (Yu et al., 2012).

An earlier study by Cho S showed that *E.* coli acts as a reservoir for macrolide-resistance genes from which resistant *S.* enterica might have emerged through horizontal gene transfer (Cho et al., 2019). This phenomenon has been previously demonstrated with E. coli donating *mphA* to *S.* sonnei (Phuc Nguyen et al., 2009). In this study, we looked for the occurrence of a similar event among the studied isolates. We compared the plasmid profile carried by *S.* enterica and *E. coli* carrying the macrolide resistance gene to identify the backbone similarity. Although the analysis revealed several genes in common, the *S.* enterica plasmid harbored an additional tra operon compared to *E.coli*, which might have been

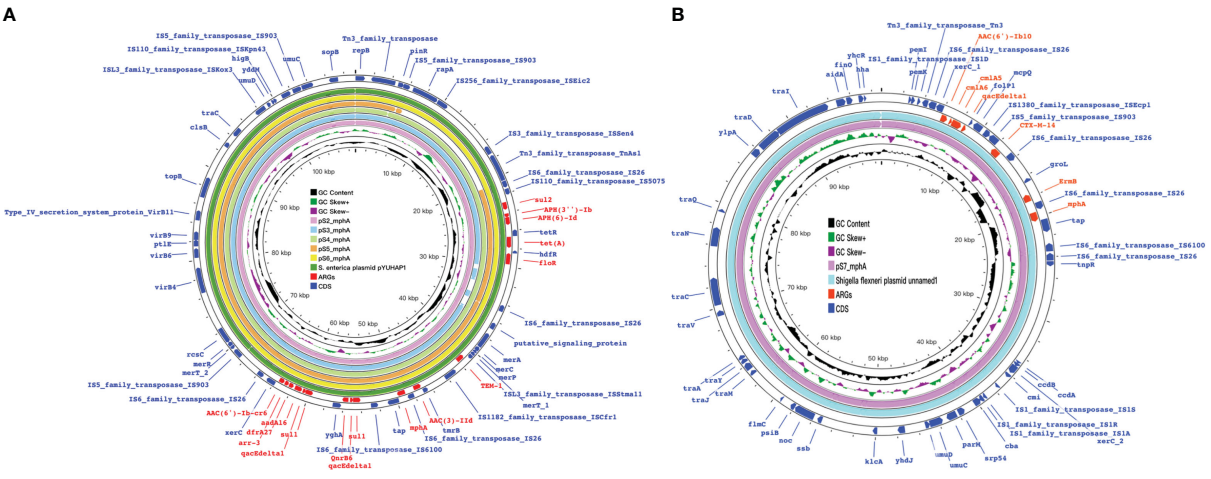

FIGURE 7
mph(A) were located on two different plasmids in S. enteric-8(also include S.enteric-9) and S.enteric-10. **(A)** The plasmid in S. enteric-8 (also include S. enteric-9) **(B)** The plasmid in S.enteric-10.

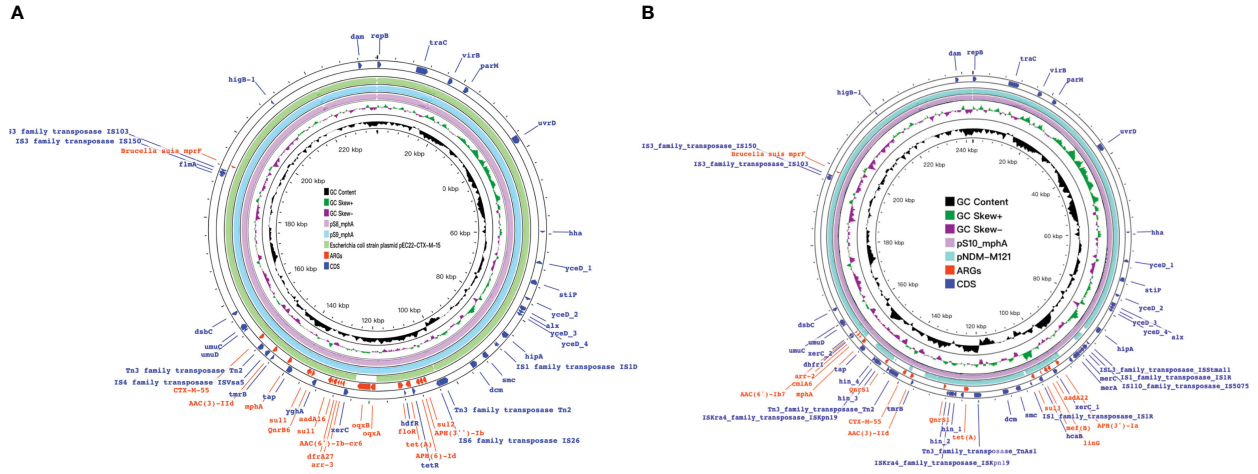

FIGURE 8
Detection of ARGs over the 15 isplates. **(A, B)** Chord diagram illustrating the correlations between ARGs and the ARG-carying pathogenic species. Thickness of the lines represents the number of samples observing such correlation. A total of 52 ARGs were not shared by the 15 isolates.

acquired due to evolution over time. The *tra* genes have always been the only genomic factors that make the plasmid conjugative. Interestingly, Benz et al. showed that plasmid transfer is mainly based on functional *tra* (transfer) genes rather than plasmid types (Benz et al., 2021). Furthermore, earlier studies have shown that plasmids carrying required functional *tra* genes can spread even without antibiotic selection pressure. These results highlight the potential risk of plasmids with resistance genes carrying functional *tra* genes being transferred by natural conjugation.

The widespread emergence of MDR *S.* enterica with changing AMR patterns has also been reported. Generally, in *S.* enterica, acquired resistance is more common. β-Lactam resistance is mainly due to the presence of OXA-type β-lactamases, followed by *TEM* and *CTX-M*. Trimethoprim/sulfamethoxazole resistance is encoded by the *dhfr1A* and *sul* genes. Quinolone resistance involves the accumulation of mutations in QRDR and plasmid-mediated quinolone resistance (PMQR) genes. Furthermore, resistance to tetracycline, chloramphenicol and streptomycin has been shown to be due to the presence of *tetA/B*, *catB3* and *aadA* genes or both (Bustamante and Iredell, 2017; Wang et al, 2017; Wang et al., 2018). These results show the ability of species to acquire AMR determinants.

In conclusion, as the extensive resistance of *salmonella* serotypes to conventional first-line drugs, azithromycin have been designated as the most important antibiotics for the treatment of *salmonella*, the novel finding of an integrated plasmid in this study indicates the potential risk of *S.* enterica isolates becoming resistant to azithromycin in the future. Our study highlights the significance of the hybrid assembly approach in complete genome analysis. These findings suggest that it is imperative to monitor *S.* enterica susceptibility and to study the resistance mechanism of *S.* enterica against azithromycin, considering azithromycin is the only remaining oral drug for the treatment of XDR *Salmonella* infection.

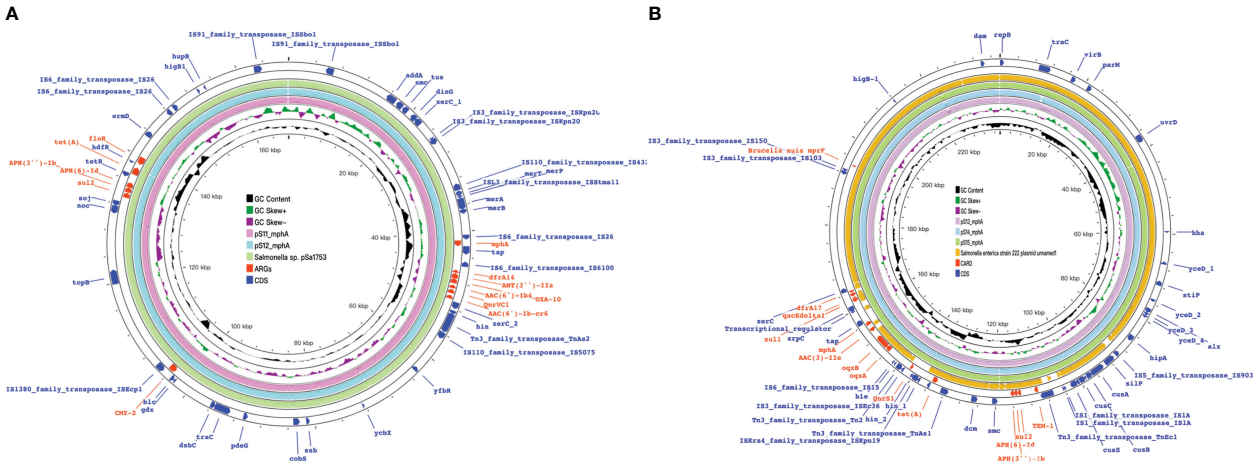

FIGURE 9
mph(A) were located on two different plasmids in S. enteric-11(also include S. enteric-12) and S.enteric-13(also include S.enteric-14, 15) **(A)** The plasmid in S.enteric-11(also include S.enteric-12) **(B)** S.enteric-13(also include S.enteric-14, 15).

Data availability statement

All clinical isolates sequence data used in the present study has been deposited in the NCBI database under project ID PRJNA879416.

Author contributions

QW and JD designed the experiments. HW, HC and BH performed the experiments. HW, HC, XH and YC analyzed the data. HW and HC wrote the manuscript. QW, JD, LY and LZ critically commented and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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A review of penicillin binding protein and group A *Streptococcus* with reduced-β-lactam susceptibility

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With the widespread use of antibiotics, antimicrobial resistance (AMR) has become a global problem that endangers public health. Despite the global high prevalence of group A *Streptococcus* (GAS) infections and the global widespread use of β-lactams, β-lactams remain the first-line treatment option for GAS infection. β-hemolytic streptococci maintain a persistent susceptibility to β-lactams, which is an extremely special phenomenon in the genus *Streptococci*, while the exact current mechanism is not known. In recent years, several studies have found that the gene encoding penicillin binding protein 2X (*pbp2x*) is associated with GAS with reduced-β-lactam susceptibility. The purpose of this review is to summarize the current published data on GAS penicillin binding proteins and β-lactam susceptibility, to explore the relationship between them, and to be alert to the emergence of GAS with reduced susceptibility to β-lactams.

KEYWORDS

group A *Streptococcus* (GAS), *Streptococcus pyogenes*, antibiotic resistance, Penicillin binding protein, Pbp2x, reduced-penicillin susceptibility, β-lactam

Introduction

Group A *Streptococcus* (GAS), also known as *Streptococcus pyogenes* (*S. pyogenes*), is a very important human pathogen. With the widespread use of antibiotics, the incidence of GAS infection has decreased considerably, but it remains an important human pathogen, ranking in the top 10 causes in terms of morbidity and mortality of infectious diseases (Bessen et al., 2019), responsible for more than 700 million infection,1.8 million severe infections and 517,000 deaths worldwide each year (Carapetis et al., 2005; Ralph and Carapetis, 2013). Importantly, so far, there is no licensed vaccine to prevent GAS infections (Steer et al., 2016). GAS can cause a wide range of clinical conditions, from mild pharyngitis to life-threatening invasive infections (Brockmann et al., 2018; Lamagni et al., 2018; Liu et al., 2018).

According to the World Health Organization (World Health Organization, 2014), antimicrobial resistance (AMR) is a global challenge that poses a serious threat to public health and the world economy. In 2014, the WHO Global Antimicrobial Resistance Surveillance System (GLASS) released the first global report on AMR surveillance (World Health Organization, 2014). β-lactams are the of first-line antibiotics of choice for the treatment of most GAS infections. An anomaly in the biology of *S. pyogenes* is the persistent high susceptibility to β-lactams. To date, no naturally occurring penicillin-resistant strain in *S. pyogenes* has been identified (Horn et al., 1998; Suzuki et al., 2015; Chochua et al., 2017; Yu et al., 2020; Yu et al., 2021b). This is unusual because resistance to β-lactams has emerged independently several times in many other important Gram-positive human bacterial pathogens (Zapun et al., 2008). Resistance to β-lactams in *Streptococcus pneumoniae*, an important example of a Streptococcal pathogen, has been described globally. While rare, *Streptococcus agalactiae* (group B *Streptococcus* [GBS]) and *Streptococcus dysgalactiae* subspecies *equisimilis* (SDSE) reported *pbp2x* point mutations resulting in reduced susceptibility to β-lactams (Dahesh et al., 2008; Fuursted et al., 2016; Metcalf et al., 2017). In recent years, reduced-penicillin-susceptibility of GAS has been reported (Kimura et al., 2008; Metcalf et al., 2017). As previously observed in other streptococci, the emergence of mutations in certain PBP genes is considered a first step towards potential full penicillin resistance, and warrants continued surveillance (Jamin et al., 1993; Kimura et al., 2008; Hayes et al., 2020b).

Interpretation criteria for antimicrobial susceptibility testing results of GAS to β-lactams

The Clinical and Laboratory Standards Institute (CLSI) and the European Commission on Antimicrobial Susceptibility Testing (EUCAST) guidelines are widely recognized and have long recommended penicillin for the treatment of GAS infections. There are no "intermediate" or "resistant" breakpoints to penicillin according to CLSI or EUCAST guideline. According to CLSI criteria, whose criteria have remained unchanged for many years, a minimum inhibition zone ≥24mm or a minimum inhibitory concentration (MIC)≤0.12 μg/mL for β-hemolytic streptococci indicates susceptibility to penicillin, and also to other β-lactams (amoxicillin, ampicillin and cefaclor).However, there have been some reports of GAS isolates being described as "non-susceptible" or "resistant" to β-lactams (Amábile-Cuevas et al., 2001; Capoor et al., 2006; Ogawa et al., 2011; Berwal et al., 2018). After reviewing these papers, we found that the terms "intermediate" or "resistant" used in these reports were not used accurately for interpretation of results (Yu et al., 2020; Yu et al., 2021b). The authors of these papers should have described the isolates as "non-susceptible" but instead referred to them as "intermediate" or "resistant", while CLSI and EUCAST do not define breakpoints for these terms for GAS. Therefore, highlighting the current lack of understanding of GAS susceptibility breakpoints and interpretation for β-lactam antibiotics by many researchers.

Penicillin and other β-lactams

β-lactam antibiotics, including penicillin, are a class of antibiotic molecules that disrupt bacterial cell walls during cell proliferation. They are fungal, natural or synthetic antimicrobial agents (Fleming, 1929; Sheehan and Henery-Logan, 1959). There are five classes of penicillin antibiotics including natural penicillins, aminopenicillins, penicillins resistant to penicillinase, extended-spectrum penicillins and aminopenicillin/β-lactamase inhibitor combinations (Miller, 2002). Other antibiotics also have typical β-lactam ring structures, including cephalosporins, carbapenems and monoamides, which together with penicillin are known as β-lactam antibiotics.

After the introduction of penicillin in the early 1940s, *Staphylococci* and *enterococci* (Miller et al., 2014) developed resistance within just a few years (Kirby, 1944; Lakhundi and Zhang, 2018). From the mid-1960s to the 1970s, intermediate strains of *Streptococcus pneumoniae* were sporadically reported (Hansman et al., 1974; Jacobs et al., 1979; Klugman, 1990). While still rare, from the mid-1990s, reports of reduced susceptibility to GBS has been documented (Kimura et al., 2008; Gaudreau et al., 2010). GBS strains with reduced susceptibility to β-lactams are been described in Japan and the United States (Dahesh et al., 2008; Seki et al., 2015; Kobayashi et al., 2021). SDSE is the species most closely related to GAS (Oppegaard et al., 2017). During 2010 to 2012, four incidents of penicillin-resistant (PR) SDSE isolated from blood cultures of three patients were detected in Denmark (Fuursted et al., 2016).

However, there are exceptions. A key exception to Fleming 's warning about the relationship between antimicrobial use and the development of resistance is the persistent susceptibility of GAS to β-lactam antibiotics (Horn et al., 1998). Eighty years after the introduction of penicillin, GAS strains still maintain consistent susceptibility to various β-lactams, and even the MICs of GAS to β-lactams remain low and stable (Yu et al., 2021a). The reasons for the persistent susceptibility of GAS to β-lactams are unclear, but may include differences in the rate and mechanism of horizontal gene transfer between GAS and other *Streptococci*.

Mechanism of resistance

There are three main mechanisms of resistance to β-lactams, including destruction of the antibiotic by β-lactamases, reduced affinity for PBP binding, or reduced access to PBPs (Ambler, 1980). Resistance of Gram-positive organisms to β-lactams is mainly due to target modifications, in which PBPs undergo structural changes (Fisher and Mobashery, 2016). Reduced susceptibility to penicillin in GAS has been demonstrated due to amino acid substitutions within PBPs that affect the ability to bind penicillins (Jamin et al., 1993; Kimura et al., 2008; Hayes et al., 2020b).

Mutations within PBPs

Identification and subsequent genetic analysis of antimicrobial resistant strains revealed that resistant strains have chimeric high molecular mass penicillin-binding proteins (HMM PBPs) compared to susceptible strains (Zighelboim and Tomasz, 1980; Dowson et al., 1989). In resistant Streptococci, the evolution from penicillin susceptible to reduced susceptibility and then to non-susceptible occurs through the progressive accumulation of amino acid substitutions in HMM PBPs rather than through single-event horizontal gene transfer of β-lactamase or low β-lactam affinity HMM PBPs (Zighelboim and Tomasz, 1980; Barcus et al., 1995; Kimura et al., 2008; Zapun et al., 2008; Fuursted et al., 2016). Thus, the identification of the genetic polymorphism in pathogenic streptococci leading to reduced antibiotic susceptibility has been observed from phenotype to genotype. This phenotype-to-genotype workflow has dominated the molecular basis of antibiotic resistance research for decades and is responsible for the discovery of novel mechanisms. GAS with reduced susceptibility to β-lactams have acquired mutations in genes encoding PBPs, including PBP1a, PBP2a, PBP2b and PBP2x. Some mutations have been identified, but the most common mutation in the GAS results in the substitution of amino acid in PBP2x transpeptidase for T553K (Vannice et al., 2020; Chochua et al., 2022) (Table 1).

Prevalence of GAS with reduced-β-lactam susceptibility

All reports of GAS with reduced-β-lactam susceptibility worldwide to date are detailed in Table 1. Detailed characterization of GAS with reduced β-lactams susceptibility were first reported in 2001 (Amábile-Cuevas et al., 2001). Since then, it has also been reported in India (Capoor et al., 2006; Berwal et al., 2018), Japan (Ogawa et al., 2011; Ikeda et al., 2021), in Iceland (Southon et al., 2020) and in the United States (Musser et al., 2020; Vannice et al., 2020; Chochua et al., 2022).

In 2020, Hayes et al. investigated the relative frequency of PBP sequence variations in 9,667 *S. pyogenes* isolates worldwide (Hayes et al., 2020a). The majority of these genomic sequences were derived from UK and US datasets that focused on invasive diseases (Ben Zakour et al., 2015; Davies et al., 2015; Athey et al., 2016; Chalker et al., 2017; Chochua et al., 2017; Kapatai et al., 2017; Turner et al., 2017; Bergin et al., 2018; Coelho et al., 2019; Davies et al., 2019; Dickinson et al., 2019; Lynskey et al., 2019). They found that mutations in *S. pyogenes* PBPs occured rarely in this global database, with less than 3 amino acid changes differing in more than 99% of the world population. Only 4 of 9 667 strains contained mutations near the active sites of PBP2x or PBP1a transpeptidase. No reported PBP2x T553K mutation was found. Their findings

TABLE 1 Reports of reduced susceptibility to β-lactams amongst group A *Streptococcus* isolates and the associated amino acid substitutions identified.

Country (study period)	Sample source	Number OR Rates	β-lactam antibiotics	Antibiotic resistance test	MIC	Breakpoint standards	Mutations identified					References
							PBP2x	*PBP2a*	*PBP2b*	*PBP1a*	*PBP1b*	
Mexico 2001	Pharyngotonsillitis	10(5%)	Penicillin G	E-test	0.25–0.75 μg/ml	NCCLS	—	—	—	—	—	(Amábile-Cuevas et al., 2001)
India 2002-2003	Acute pharyngotonsillitis	7(20.6%)	Penicillin	E-test	0.19-0.25μg/ml	CLSI	—	—	—	—	—	(Capoor et al., 2006)
Japan 2006-2008	Pharyngitis	2/93	Penicillin G	Broth microdilution	>2.0μg/ml	CLSI 2007	—	—	—	—	—	(Ogawa et al., 2011)
India 2016-2017	Upper respiratory tract infections	4(8%), 2 (4.2%), 2(5.3%)	Ampicillin cefotaxime ceftriaxone	MALDI-TOF Mass Spectrometry (VITEK MS, bioMerieux)	MIC, 0.12-8μg/ml	CLSI	—	—	—	—	—	(Berwal et al., 2018)
Australia 2020	—	4/9667	NC	NC	NC	NC	STMK to SAMK STMK to STIK	—	—	—	—	(Hayes et al., 2020a)
USA 2017-2018	Blood and wound isolates	2/282	ampicillin & cefotaxime	Broth Microdilution & E-test	Ampicillin 8-fold higher, cefotaxime 3-fold higher	CLSI	T553K substitution	—	—	—	—	(Vannice et al., 2020)
USA 2020	Mouse Model of Necrotizing Myositis	—	—	—	—	—	Pro601Leu amino acid replacement in PBP2X	—	—	—	—	(Olsen et al., 2020)

(Continued)

TABLE 1 Continued

Country (study period)	Sample source	Number OR Rates	β-lactam antibiotics	Antibiotic resistance test	MIC	Breakpoint standards	Mutations identified					References
							PBP2x	*PBP2a*	*PBP2b*	*PBP1a*	*PBP1b*	
Iceland 1995-2006	—	*emm*12 strains, 332/1575	A 2-fold increased penicillin G and ampicillin MIC	Disk diffusion method	—	CLSI, EUCAST	Met593Thr Ile502Val Pro676Ser Lys708Glu	—	—	—	—	(Southon et al., 2020)
Japan 2015-2016	Skin, Pus, Tonsil	3	—	Agar dilution method	—	CLSI	M593T, A397V	T459A	—	—	—	(Ikeda et al., 2021)
2022	—		—	—	—	—	Identified 464 *pbp2x* alleles	564 *pbp2a* alleles	—	389 *pbp1a* alleles	427 *pbp1b* alleles	(Beres et al., 2022)
USA 2015-2021	—	55/13727	None isolates exhibited nonsusceptibility to b-lactams	—	—	—	*emm*43.4/ PBP2x-T553K variant, two isolates ampicillin MIC 0.25 mg/ml, 129/340 (37.9%) of isolates with elevated β-lactam MICs	—	—	—	—	(Chochua et al., 2022)
USA 2022	An isogenic mutant strain was generated and virulence assessed in a mouse model of necrotizing myositis	—	—	—	—	—	Strains with the chimeric SDSE-like PBP2X had reduced susceptibility *in vitro* to nine β-lactam antibiotics	—	—	—	—	(Olsen et al., 2022)

NCCLS (National Committee for Clinical Laboratory Standards) is the predecessor of Clinical and Laboratory Standards Institute (CLSI) ; WGS, Whole-genome sequencing; MALDI-TOF, Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry; —, No data OR No clear.

imply that while heavy antibiotic pressure may select for mutations in the PBPs, there is currently no evidence that such mutations become fixed in the *S. pyogenes* population or that mutations in the PBPs are being sequentially acquired. However, because low levels of resistance to subclinical lactams could theoretically confer a biological advantage to GAS, vigilance in monitoring population GAS for PBP mutations is encouraged (Musser et al., 2020).

In 2022, Beres et al. (Beres et al., 2022) analyzed 26,465 *S. pyogenes* genome sequences. Population genomic data identified amino acid changes in PBP1a, 1b, 2a, and 2x. The evolutionary signature of these proteins under positive selection is a potential candidate for reduced susceptibility to β-lactams. In 2022, Olsen et al. (Olsen et al., 2022) again noted that whole genome sequencing identified a GAS strain containing a chimeric PBP2x derived from an SDSE recombinant fragment. The results suggest that mutations such as PBP2x chimeras may lead to reduced susceptibility to β-lactams and increased fitness and virulence.

Hanage et al. (Hanage and Shelburne, 2020) noted that studies have shown that mutations of PBP are associated with reduced susceptibility of *S. pneumoniae* to β-lactam antibiotics (Li et al., 2016), *Streptococcus agalactiae* (Dahesh et al., 2008a), some SDSE (Fuursted et al., 2016) and GAS (Vannice et al., 2020).In another study (Musser et al., 2020), two related *Streptococcus pyogenes* strains with reduced susceptibility to ampicillin, amoxicillin, and cefotaxime, antibiotics commonly used to treat *S. pyogenes* infections, were reported. The two strains had the same nonsynonymous (amino acid-substituting) mutation in the *pbp2x* gene, encoding penicillin-binding protein 2X (PBP2X). They identified 137 strains that together had 37 nonsynonymous mutations in 36 codons of *pbp2x*. The authors propose that GAS with reduced susceptibility to β-lactams associated with mutations in the *pbp2x* gene are geographically widespread. Does this study suggest that we are finally at the beginning of the era of widespread susceptibility of GAS to β-lactams? They believed that this answer is no (Hanage and Shelburne, 2020).

Summary

Currently, penicillin, the first-line treatment of GAS infection, is generally considered effective for GAS. However, reports of reduced susceptibility to β-lactams are becoming more common. For now, clinicians can continue to be confident that β-lactams remain the agents of choice for the treatment of GAS infections. The fluctuating nature of the emergence of GAS strains, including those with reduced

susceptibility to various antimicrobials, means that ongoing surveillance of the GAS population is both in the public health interest and helps clinicians understand the changing nature of medically important bacteria.

Author contributions

YZ and YY proposed the topic of this review. DY and DG conducted a literature search and wrote this review. YZ and YY revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors indicated that this study was conducted without any commercial or financial relationships that could be interpreted as potential conflicts of interest.

Publisher's note

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Vancomycin efficiency and safety of a dosage of 40–60 mg/kg/d and corresponding trough concentrations in children with Gram-positive bacterial sepsis

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Background: Optimal vancomycin trough concentrations and dosages remain controversial in sepsis children. We aim to investigate vancomycin treatment outcomes with a dosage of 40-60 mg/kg/d and corresponding trough concentrations in children with Gram-positive bacterial sepsis from a clinical perspective.

Methods: Children diagnosed with Gram-positive bacterial sepsis and received intravenous vancomycin therapy between January 2017 and June 2020 were enrolled retrospectively. Patients were categorized as success and failure groups according to treatment outcomes. Laboratory, microbiological, and clinical data were collected. The risk factors for treatment failure were analyzed by logistic regression.

Results: In total, 186 children were included, of whom 167 (89.8%) were enrolled in the success group and 19 (10.2%) in the failure group. The initial and mean vancomycin daily doses in failure group were significantly higher than those in success group [56.9 (IQR =42.1-60.0) *vs*. 40.5 (IQR =40.0-57.1), P=0.016; 57.0 (IQR =45.8-60.0) *vs*. 50.0 (IQR =40.0-57.6) mg/kg/d, P=0.012, respectively] and median vancomycin trough concentrations were similar between two groups [6.9 (4.0-12.1) *vs*.7.3 (4.5-10.6) mg/L, P=0.568)]. Moreover, there was no significant differences in treatment success rate between vancomycin trough concentrations ≤15 mg/L and >15 mg/L (91.2% *vs*. 75.0%, P=0.064). No vancomycin-related nephrotoxicity adverse effects occurred among all enrolled patients. Multivariate analysis revealed that a PRISM III score ≥10 (OR =15.011; 95% CI: 3.937-57.230; P<0.001) was the only independent clinical factor associated with increased incidence of treatment failure.

Conclusions: Vancomycin dosages of 40-60 mg/kg/d are effective and have no vancomycin-related nephrotoxicity adverse effects in children with Gram-positive bacterial sepsis. Vancomycin trough concentrations >15 mg/L are not an essential target for these Gram-positive bacterial sepsis patients. PRISM III scores ≥10 may serve as an independent risk factor for vancomycin treatment failure in these patients.

KEYWORDS

vancomycin, Gram-positive bacterial, children, tough concentrations, dosages

Introduction

Sepsis, a life-threatening infection due to a dysregulated host response to infections, is considered as one of the leading causes of morbidity, mortality and accounts for a heavy socioeconomic burden in children globally (Weiss et al., 2020). An estimate 48 children per 100 000 population suffer sepsis, 22 children per 100 000 population suffer severe sepsis, and 2202 neonates per 100 000 livebirths develop neonatal sepsis (Fleischmann-Struzek et al., 2018). Mortality ranged from 1% to 5% for sepsis and 9% to 20% for severe sepsis in children (Fleischmann-Struzek et al., 2018). A 20-year antimicrobial surveillance has shown that Gram-positive bacteria are the predominant cause of sepsis (Diekema et al., 2019). Undoubtedly, early identification and appropriate treatment are critically important for children with sepsis (Weiss et al., 2020). Rigorous evaluation of optimal antimicrobial and other therapeutic strategies are correlated with the clinical outcomes (Randolph et al., 2019).

Vancomycin, as a major glycopeptide antibiotic be used to treat severe infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis*, as well as penicillin-resistant *Corynebacterium jeikeium*, *Streptococcus pneumoniae*, and *Clostridium difficile*, is currently recommended for children with severe Gram-positive bacterial infections (Liu et al., 2011; Rubinstein and Keynan, 2014). Undisputedly, appropriate vancomycin dosages and trough concentrations are crucial for children with Gram-positive bacterial sepsis. The Infectious Diseases Society of America (IDSA) guidelines recommend children with serious or invasive infectious disease should be treated with vancomycin at a dosage of 15 mg/kg/d every 6 h (Liu et al., 2011). Vancomycin exhibits time-dependent bactericidal effect, meaning that its antibacterial activity dependent on the time that the concentration of the drug in the body is above the minimum inhibitory concentration (MIC). Thus area under the curve (AUC) over 24 hours to MIC ≥400 is the best index to evaluate clinical efficacy and ensure safety for children being treated with vancomycin (Rybak et al., 2020). However, measuring AUC/MIC by traditional method is not practical in clinical setting as it needing to obtain multiple serum vancomycin concentrations. Therefore, to attain the recommended target AUC/MIC ≥400, the IDSA guidelines recommended that targeting vancomycin trough concentration of 10-20 mg/L in pediatrics: 10-15 mg/L for uncomplicated infections and 15-20 mg/L for serious infections (Liu et al., 2011). However, the IDSA guidelines do acknowledge that limited data is available to evaluate the efficacy and safety of the aforementioned vancomycin dosages and trough concentrations for children (Liu et al., 2011).

Several studies have investigated the efficacy and safety of vancomycin trough concentrations of 10-20 mg/L (Frymoyer et al., 2013; Liang et al., 2018; Tkachuk et al., 2018; Peng et al., 2021). From a pharmacokinetic perspective, the trough concentration of 6-10 mg/L is likely sufficient to achieve AUC/MIC ≥400 in children (Frymoyer et al., 2013; Tkachuk et al., 2018). Another prospective multicenter study revealed that increasing vancomycin trough concentrations to 15-20 mg/L couldn't benefit Chinese patients with complicated infections in children (Liang et al., 2018). Moreover, a population-based pharmacokinetic modeling study demonstrated that vancomycin trough concentrations ≥15 mg/L were independently associated with a above 2.5-fold increased risk of nephrotoxicity in children (Le et al., 2015). However, two previous studies reported that maintaining trough concentrations of 15-20 mg/L was not correlated with increased risk of nephrotoxicity in children (Cies and Shankar, 2013; Matson et al., 2015). About vancomycin dosages, a revised vancomycin consensus guideline recommended that the initial vancomycin dosage should be 60-80 mg/kg/day to achieve AUC/MIC ≥ 400 for children with normal renal function and suspected serious MRSA infections (Rybak et al., 2020). While Liang et al. (Liang et al., 2018) found an average vancomycin dosage of 37.7 mg/kg/d could treat 96% Gram-positive bacterial infectious children successfully.

As the optimal vancomycin trough concentrations and dosages remain controversial in children and most of the abovementioned studies discussed from a pharmacokinetic perspective (Frymoyer et al., 2013; Le et al., 2015; Tkachuk et al., 2018; Rybak et al., 2020), this retrospective cohort study aimed to explore the efficiency and safety of current vancomycin dosages and corresponding trough

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; IDSA, Infectious Diseases Society of America; MIC, minimum inhibitory concentration; AUC, area under the curve; CRP, C-reactive protein; PCT, procalcitonin; PRISM, Pediatric Risk of Mortality; TDM, therapeutic drug monitoring; IQRs, interquartile ranges; SD, standard deviation; ORs, odds ratios; CIs, confidence intervals; ROC, receiver operating characteristic; MSSA, Methicillin susceptible *Staphylococcus aureus*.

concentrations in children with Gram-positive bacterial sepsis from a clinical perspective.

Methods

Study site and study population

We performed a retrospective cohort study in the Children's Hospital of Chongqing Medical University. The hospital is a 2480-bed tertiary teaching hospital in Chongqing, China and ranks among the top three domestic children's hospitals (rank list: http://top100.imicams.ac.cn/home). We retrospectively enrolled 186 children hospitalized between January 2017 and June 2020. The inclusion criteria were all of the following: (i) aged 1m-18 years, (ii) diagnosed with sepsis or septic shock according to the 2020 Pediatric Surviving Sepsis Campaign guidelines (Weiss et al., 2020), (iii) at least one Gram-positive pathogen was obtained from sterile sites with accompanying clinical signs, (iv) received intravenous vancomycin administered as intermittent infusion for at least 48 hours, (v) serum trough concentrations were collected at steady state conditions. If multiple trough concentrations were available, only the first trough was included in this study. The exclusion criteria included any of the following: (i) children with incomplete clinical information, (ii) renal impairment at the initial administration of vancomycin, (iii) considered to have Gram-positive bacteria colonization or pollution, (iv) the vancomycin dosing regimen was not 40-60 mg/kg/d. (v) received linezolid or teicoplanin therapy ≥24 hours concurrently when undergoing vancomycin treatment. This study was approved by the institutional review board of the Children's Hospital of Chongqing Medical University (Approved ID: 2022-463). A waiver of informed individual consent was granted given the retrospective design of this study.

Data collection

Trained staff used a standardized data collection form to extract patients' information from the electronic medical records. Patients' demographic characteristics (age, weight, gender), source of infection, comorbid illnesses, culture pathogens, the MIC values, baseline serum creatinine, liver and kidney function before and after vancomycin treatment, glucocorticoids and gamma globulin administration, laboratory data at the beginning of vancomycin treatment [C-reactive protein (CRP), procalcitonin (PCT), hemoglobin, neutrophil, albumin], the Pediatric Risk of Mortality (PRISM) III scores, concomitant antibiotics, antibiotics used before vancomycin therapy and vancomycin-related details (daily dose, duration and trough concentrations) were collected.

Vancomycin administration and therapeutic drug monitoring

All enrolled children received a starting vancomycin (Vianex S.A., Athens, Greece) dosage of 40-60 mg/kg/day intravenously (maximum daily dose <2000 mg) as recommended by drug instructions and IDSA guidelines. Clinicians adjusted vancomycin dosage according to the therapeutic drug monitoring (TDM). IDSA recommends that the TDM target for the vancomycin trough concentration is 10-15 mg/L for common infections and 15-20 mg/L for children with complicated infections (Liu et al., 2011). However, clinicians could continue at the same vancomycin dose and track the concentration weekly if patients' clinical and microbiological outcomes improved when trough concentrations were lower than the recommended range. Serum trough concentrations were obtained at steady state conditions [defined as any concentrations had been obtained within 1 hour after at least the third scheduled vancomycin administration (Heble et al., 2013)]. If multiple trough concentrations were available, only the first trough was included in this study. The chemiluminescence immunoassay (Abbott Laboratories, Chicago, IL, USA), which has an analytical range of 0.0–100.0 mg/L with a between-run coefficient of variation of <15% throughout the analytical range, was used to analyze vancomycin trough concentrations. A central laboratory was responsible for testing all serum samples within 24 hours, and intra- and inter-batch quality control was measured following the China National Accreditation Service for Conformity Assessment standard.

Definition

The outcome of treatment success included clinical cure and microbiological cure. Clinical cure was defined as the improvement of patient's presenting signs, symptoms and laboratory data (Ma et al., 2020). Microbiological cure was defined as the clearance of the original microbiological from the infection site during or up to 14 days after the administration of vancomycin therapy (Ma et al., 2020). Treatment failure included any of the following: (i) culture the original microorganism from the infection site (Liang et al., 2018), (ii) a lack of improvement of clinical symptoms, signs, and laboratory data requiring a change of a new antibiotic with a similar spectrum due to lack of response (Liang et al., 2018; Ma et al., 2020), (iii) readmission within 14 days of discharge for infection recurrence (Goldstein et al., 2013), (iv) all-cause mortality (Tong et al., 2020). When the children had renal impairment and occurred acute kidney injury (AKI) during vancomycin treatment, vancomycin-related nephrotoxicity was diagnosed. According to Kidney Disease Improving Global Guidelines (KDIGO) Clinical Practice Guidelines, AKI was defined as an increase in serum creatinine of ≥26.5 μmol/L within 48 hours or a known or presumed increase in serum creatinine to ≥1.5 times the baseline within 7 days, or a urine volume <0.5 mL/kg/hour for 6 hours (Khwaja, 2012). Baseline serum creatinine was defined as the lowest level obtained 1 week prior to the initial administration of vancomycin (Ma et al., 2020). Vancomycin-related nephrotoxicity was assessed after 48 hours of initiation of vancomycin to avoid confounding associated with increases in serum creatinine due to sepsis rather than exposure to vancomycin (Goldstein et al., 2013). The source of infection was identified by reviewing the clinical records, radiographic studies, surgical findings and laboratory

records of the children (Yang et al., 2018). The severity of illness estimated by the PRISM III scores (Pollack et al., 1996) at the beginning of vancomycin treatment.

Outcomes

The primary outcome was to investigate vancomycin efficiency and safety with current dosages and corresponding trough concentrations in children with Gram-positive bacterial sepsis. Secondary outcomes included the risk factors for treatment failure in this population.

Statistical analysis

Descriptive statistics were performed to evaluate all variables of interest. The median [interquartile ranges (IQRs)] or mean [standard deviation (SD)] was used to describe the quantitative variables, whereas the categorical variables were displayed as counts (n) and percentages (%). In the univariate analysis, the chi-square or Fisher exact test was assessed for categorical variables, the Mann-Whitney *U* analysis was employed for non-normally distribution variables and two-sample t-test was used to analyses the normally distribution variables. Wilcoxon signed-rank test or paired sample t-test was used to examine any significant difference in the pre- and post-vancomycin treatment in kidney and liver function. Univariate and multivariable logistic regression analyses were performed to identify the independent association between potential factors and treatment failure, and odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. We employed a receiver operating characteristic (ROC) curve and the maximum Youden's index to select the appropriate cutoff value of PRISM III scores and we also evaluated the specificity and sensitivity of the prediction model. All statistical testing was performed using IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). P values were from 2-sided analyses, and a P value <0.05 was deemed statistically significant.

Results

Enrollment and basic characteristics of all enrolled patients

During this study, 1098 patients were screened for eligibility. Of these, 372 patients were excluded due to no Gram-positive pathogens cultured, 285 patients for lacking available steady-state trough concentrations, 164 patients for having incomplete clinical information, 53 patients for receiving other vancomycin dosing regimens, 25 patients for having renal impairment at the initial administration of vancomycin, 8 patients for receiving linezolid or teicoplanin therapy ≥24 hours concurrently when undergoing vancomycin treatment and 5 patients for receiving vancomycin therapy <48 hours. The remaining 186 patients were included with 167 (89.8%) in the success group, while the remaining 19 (10.2%) were in the failure group (Figure 1).

The median age of all patients was 2.0 (IQR =0.8-4.6) years and male gender accounted for 53.2% (99/186) of the patients. The initial and mean vancomycin daily dose was 41.7 (IQR =40.0-57.7) mg/kg/day and 50.0 (IQR =41.5-58.2) mg/kg/day, respectively. The median duration of vancomycin therapy was 12.0 (IQR =8.0-17.0) days. Bacteremia (56.5%) was the most common focus of infections. This was followed by respiratory system infections (27.4%) and skin and soft tissue infections (18.8%). The *Streptococcus pneumoniae* (21.0%) accounted for the most common infectious pathogen, followed by Methicillin susceptible *Staphylococcus aureus* (MSSA)

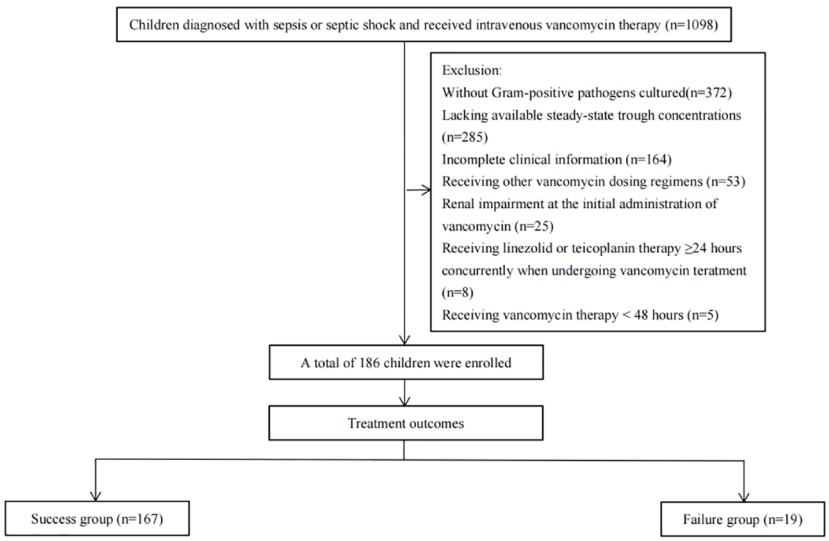

FIGURE 1
Flow chart of patients enrollment.

(17.7%) and MRSA (14.0%). The treatment success rate was 89.8% (167/186). Moreover, no vancomycin-related nephrotoxicity adverse effects were observed in all enrolled patients (Table 1). All patients in the success group achieved microbial clearance, while in the failure group, 26.3% (5/19) patients cultured the original microorganism from the infection site which leaded to requiring antibiotic alteration or death and the remaining 73.7% (14/19) patients did not receive microbiological retesting.

Basic characteristics of success and failure groups

Table 2 presented the basic characteristics of success and failure groups. The success group patients had a significantly longer duration of vancomycin therapy when compared to that in the failure group [12.0 (IQR =9.0-17.0) *vs.* 8.0 (IQR =7.0-14.0) days, P=0.037]. Intriguingly, initial and mean vancomycin daily doses in the failure group were significantly higher than those in the success group [56.9 (IQR =42.1-60.0) *vs.* 40.5 (IQR=40.0-57.1), P=0.016; 57.0 (IQR =45.8-60.0) *vs.* 50.0 (IQR =40.0-57.6) mg/kg/d, P=0.012, respectively]. The albumin level was remarkably lower in treatment failure group patients when compared to those who were treated successfully [(29.9 ± 7.2) *vs.* (33.6 ± 7.0) g/dL, P=0.030]. The success group patients had a significantly lower proportion of septic shock than that in failure group (13.8% *vs.* 42.1%, P=0.005). Moreover, PRISM III scores in success group were significantly lower when compared to those in the failure group [5.0 (IQR =3.0-9.0) *vs.* 12.0 (IQR =5.0-23.0), P<0.001]. The failure group patients had a significantly higher proportion of Gram-negative bacteria co-infections than that in the success group (36.8% *vs.* 15.0%, P=0.017). Also, carbapenems were more commonly used concomitant with vancomycin therapy in failure group when compared to those in success group (68.4% *vs.* 43.1%, P=0.036). Demographics, source of infections, underlying conditions, other treatments, MIC values and antibacterial drugs before vancomycin therapy were similar between two groups (P>0.05). No patients had AKI and liver impairment in two group patients with a vancomycin dosage of 40-60 mg/kg/d and corresponding trough concentrations (Table S1). Tables S2, S3 presented the data before and after concentration adjustment in the two group patients.

Risk factors for treatment failure

Table 2 disclosed that duration of vancomycin therapy, initial and mean vancomycin daily doses, albumin, Gram-negative bacteria co-infections, septic shock, PRISM III scores and carbapenems concomitant with vancomycin therapy might be associated with treatment response. Inter-relationships between PRISM III scores and septic shock may result in confounding of associations between PRISM III scores and treatment failure. Thus, PRISM III scores were finally included in the logistic regression model to avoiding collinearity. The ROC curve (0.77; 95% CI: 0.65-0.90) supported that PRISM III scores could be used to evaluate for treatment failure; PRISM III scores ≥10 could be an appropriate cut point with 73.7% sensitivity and 85.6% specificity in this study (Figure 2). Further multivariable logistic analysis revealed that a PRISM III score ≥10 (OR =15.011; 95% CI: 3.937-57.230; P<0.001) was the only independent clinical risk factor for vancomycin treatment failure in these patients (Table 3).

TABLE 1 Basic characteristics and demographic data of all enrolled patients.

Variables	All patients (n = 186)
Demographics	
Male, n (%)	99 (53.2)
Age (years), median (IQR)	2.0 (0.8-4.6)
Body weight (kg), median (IQR)	11.8 (8.0-16.5)
Vancomycin therapy	
Initial vancomycin daily dose (mg/kg/day), median (IQR)	41.7 (40.0-57.7)
Mean vancomycin daily dose (mg/kg/day), median (IQR)	50.0 (41.5-58.2)
Duration of vancomycin therapy (days), median (IQR)	12.0 (8.0-17.0)
Trough concentration (mg/L), median (IQR)	7.3 (4.5-10.8)
PRISM III scores, median (IQR)	5.0 (3.0-9.0)
Infection sites, n (%)[a]	
Bacteremia	105 (56.5)
Respiratory system	51 (27.4)
Skin and soft tissue	35 (18.8)
Nervous system	25 (13.4)
Bone and joint	22 (11.8)
Other system	11 (5.9)
Microorganisms, n (%)	
Streptococcus pneumoniae	39 (21.0)
MSSA	33 (17.7)
MRSA	26 (14.0)
Staphylococcus epidermidis	23 (12.4)
Staphylococcus hominis	19 (10.2)
Enterococcus faecium	10 (5.4)
Staphylococcus haemolyticus	8 (4.3)
Other Gram-positive bacteria	28 (15.1)
Gram negative bacteria co-infections	32 (17.2)
Fungal co-infection	2 (1.1)
Underlying conditions, n (%)	
Hematologic malignancy	38 (20.4)
Congenital heart disease	16 (8.6)
Nephrotoxicity, n (%)	0 (0)
Treatment success, n (%)	167 (89.8)

[a]The total percentage may >100 due to a patient having multiple infectious sites.
IQR, interquartile range; PRISM, Pediatric Risk of Mortality; MSSA, Methicillin susceptible Staphylococcus aureus; MRSA, Methicillin-resistant Staphylococcus aureus.

TABLE 2 Basic characteristics of success and failure groups.

Variables	Success group (n = 167)	Failure group (n = 19)	P Value
Demographics			
Age (years), median (IQR)	2.0 (0.9-4.6)	1.7 (0.8-11.1)	0.514
Male, n (%)	88 (52.7)	11 (57.9)	0.667
Body weight (kg), median (IQR)	12.0 (8.5-16.5)	9.5 (6.5-31.0)	0.298
Vancomycin therapy			
Trough concentration (mg/L), median (IQR)	7.3 (4.5-10.6)	6.9 (4.0-12.1)	0.568
Duration of vancomycin therapy (days), median (IQR)	12.0 (9.0-17.0)	8.0 (7.0-14.0)	0.037
Initial vancomycin daily dose (mg/kg/day), median (IQR)	40.5 (40.0-57.1)	56.9 (42.1-60.0)	0.016
Mean vancomycin daily dose (mg/kg/day), median (IQR)	50.0 (40.0-57.6)	57.0 (45.8-60.0)	0.012
Laboratory data			
Neutrophil (*10^9/L), median (IQR)	5.8 (1.0-12.9)	10.9 (2.7-18.3)	0.070
CRP (mg/L), median (IQR)	40.0 (17.0-72.0)	69.0 (9.0-83.0)	0.214
PCT (ng/ml), median (IQR)	1.2 (0.3-6.2)	7.8 (0.7-47.0)	0.064
Hemoglobin, (g/dL), (SD)	95.8 (15.8)	93.8 (16.1)	0.619
Albumin (g/dL), (SD)	33.6 (7.0)	29.9 (7.2)	0.030
Infection site[a], n (%)			
Bacteremia	94 (56.3)	11 (57.9)	0.893
Respiratory system	44 (26.3)	7 (36.8)	0.331
Skin and soft tissue	34 (20.4)	1 (5.3)	0.132
Nervous system	20 (12.0)	5 (26.3)	0.145
Bone and joint	19 (11.4)	3 (15.8)	0.476
Other system	11 (6.6)	0 (0)	0.607
Microorganisms, n (%)			
Streptococcus pneumoniae	35 (21.0)	4 (21.1)	1.000
MSSA	29 (17.4)	4 (21.1)	0.751
Staphylococcus epidermidis	22 (13.2)	1 (5.3)	0.476
MRSA	22 (13.2)	4 (21.1)	0.312
Staphylococcus hominis	18 (10.8)	1 (5.3)	0.698
Enterococcus faecium	10 (6.0)	0 (0)	0.602
Staphylococcus haemolyticus	7 (4.2)	1 (5.3)	0.585
Other Gram-positive bacteria	24 (14.4)	4 (21.1)	0.485
Gram negative bacteria co-infections	25 (15.0)	7 (36.8)	0.017
Fungal co-infections	2 (1.2)	0 (0)	1.000
Underlying conditions, n (%)			
Hematologic malignancy	37 (22.2)	1(5.3)	0.130
Congenital heart disease	14 (8.4)	2 (10.5)	0.670
Other treatments, n (%)			
Glucocorticoids	17 (10.2)	1 (5.3)	0.492
Gamma globulin	43 (25.7)	8 (42.1)	0.173

(Continued)

TABLE 2 Continued

Variables	Success group (n = 167)	Failure group (n = 19)	P Value
Severity of the disease			
Septic shock, n (%)	23 (13.8)	8 (42.1)	0.005
PRISM III scores, median (IQR)	5.0 (3.0-9.0)	12.0 (5.0-23.0)	<0.001
MIC ≤ 1, n (%)	154 (92.2)	18 (94.7)	1.000
Antibacterial drugs before vancomycin therapy[b], n (%)			
Penicillins+β-lactamase inhibitors	60 (35.9)	3 (15.8)	0.123
Third generation Cephalosporins	56 (33.5)	10 (52.6)	0.099
Second generation Cephalosporins	25 (15.0)	2 (10.5)	1.000
Carbapenems	33 (19.8)	5 (26.3)	0.549
Fourth generation Cephalosporins	12 (7.2)	0 (0.0)	0.615
First generation Cephalosporins	9 (5.4)	0 (0)	0.601
Macrolides	6 (3.6)	2 (10.5)	0.191
Metronidazole	4 (2.4)	1 (5.3)	0.420
Penicillins	5 (3.0)	2 (10.5)	0.152
Antibacterial agent concomitant with vancomycin therapy[c], n (%)			
Carbapenems	72 (43.1)	13 (68.4)	0.036
Third generation Cephalosporins	39 (23.4)	4 (21.1)	1.000
Penicillins+β-lactamase inhibitors	29 (17.4)	5 (26.3)	0.351
Fourth generation Cephalosporins	5 (3.0)	0 (0)	1.000
Rifamycin	5 (3.0)	0 (0)	1.000

[a]The total percentage may >100 due to a patient having multiple infectious sites.
[b]The total percentage may >100 because a patient received multiple antibacterial drugs before vancomycin therapy.
[c]The total percentage may >100 because a patient received multiple antibacterial drugs concomitant with vancomycin therapy.
IQR, interquartile range; SD, standard deviation; CRP, C-reactive protein; PCT, procalcitonin; PRISM, Pediatric Risk of Mortality; MSSA, Methicillin susceptible Staphylococcus aureus; MRSA, Methicillin-resistant Staphylococcus aureus; MIC, minimum inhibitory concentration.

The treatment success rate between trough concentrations ≤15 mg/L and >15 mg/L

The treatment success rate was similar between vancomycin trough concentrations ≤15 mg/L and >15 mg/L in all enrolled patients (91.2% *vs*. 75.0%, P=0.064). We further divided the patients into MRSA infection patients and non-MRSA infection patients and found that the treatment success rate was also no significant differences between trough concentrations ≤15 mg/L and >15 mg/L (87.5% vs. 50.0%, P=0.289; 91.8% vs. 78.6%, P=0.129, respectively) (Figure 3).

Discussion

We found that nearly 90% patients had been treated successfully and there were no vancomycin-related nephrotoxicity adverse effects among all enrolled Gram-positive bacterial sepsis patients. Intriguingly, we found that the treatment success rate was similar between vancomycin trough concentrations ≤15 mg/L and >15 mg/L groups. Furthermore, we demonstrated that a PRISM III score ≥10 was an independent risk factor for treatment failure with the vancomycin dosage of 40-60 mg/kg/d and corresponding trough concentrations in children with Gram-positive bacterial sepsis.

It is widely accepted that appropriate vancomycin dosage is undoubtedly important for children with Gram-positive bacterial sepsis. Our study presented that about 90% patients could be treated successfully with the vancomycin dosage of 40-60 mg/kg/d and corresponding trough concentrations in children with Gram-positive bacterial sepsis, which was similar to the results of the study by Liang et al. (Liang et al., 2018). However, in contrast to our findings, the previously investigators recommended different vancomycin dosages (Rybak et al., 2020; Van Der Heggen et al., 2021). Van Der Heggen et al. suggested that children with severe infections should be treated with 80 mg/kg/d (Van Der Heggen et al., 2021), while the revised US consensus guideline recommended that the initial vancomycin dosage for children with normal renal function and suspected serious MRSA infections should be 60-80 mg/kg/d (Rybak et al., 2020). The reasons for this inconsistency may be attributable to the follows. Firstly, there are different definitions of standards for adjusting

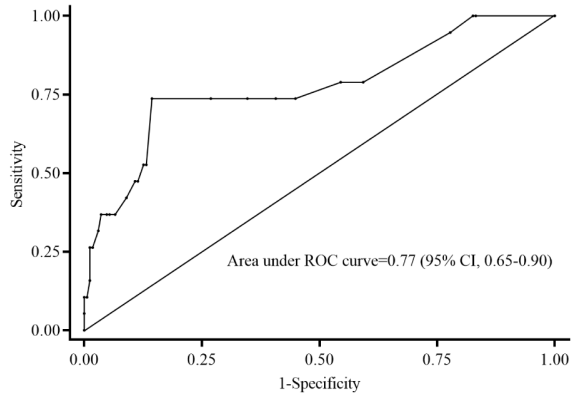

FIGURE 2
Receiver operating characteristic curve of predictive level of PRISM III scores for treatment failure. The PRISM III score of 10 cut point indicates 73.7% sensitivity and 85.6% specificity on treatment failure. ROC, receiver operating characteristic; CI, confidence interval; PRISM: Pediatric Risk of Mortality.

vancomycin doses. We combined vancomycin trough concentrations as well as clinical and microbiological therapeutic effects to comprehensively evaluate vancomycin doses, however Van Der Heggen et al. (Van Der Heggen et al., 2021) just used the vancomycin trough concentration of 10-15 mg/L and the revised US consensus guideline (Rybak et al., 2020) based on an AUC target of 400 mg*h/L from adult data to guide the dose adjustment of vancomycin. Secondly, we assessed different bacterial species and infections with different bacterial species, while the revised US consensus guideline focused on MRSA infections or suspected MRSA infections which might correspond to different vancomycin doses recommendation.

It was interesting to note that the treatment success rate was similar between vancomycin trough concentrations ≤15 mg/L and >15 mg/L groups. This observation supports several studies which revealed that vancomycin trough concentrations had no relationship with clinical outcomes (Hirano et al., 2016; Finch et al., 2017; Liang et al., 2018). Currently, guidelines recommend that TDM for children with poor or augmented renal clearance and serious infections and suggest using vancomycin trough concentrations as a surrogate measure for AUC/MIC (Liu et al., 2011; Rybak et al., 2020). Thus, IDSA recommend targeting vancomycin trough concentration of 10-20 mg/L in both adults and pediatrics; 10-15 mg/L for uncomplicated infections and 15-20 mg/L for serious infections (Liu et al., 2011). Nevertheless, the IDSA guidelines do acknowledge that the efficacy of targeting trough concentrations of 15-20 mg/L in children need additional studies due to this recommendation derives from adult studies (Liu et al., 2011). In adults, the trough concentration of 15-20 mg/L had an AUC range from 405 to 792 (Haeseker et al., 2016), while Tang et al. (Tang et al., 2021) recommended an AUC of 240-480 was an optimal exposure target of vancomycin in children. Moreover, some pharmacodynamic studies reported that the trough concentration of 6-10 mg/L was likely sufficient to achieve AUC/MIC ≥400 in children (Frymoyer et al., 2013; Tkachuk et al., 2018). Another recent study also showed that the median vancomycin trough concentration at steady state that related with the AUC/MIC ≥400 and <800 were 11.18, 9.50, 7.91 and 6.55 mg/L in children receiving vancomycin 40, 60, 80 and 100 mg/kg/day, respectively (Issaranggoon Na Ayuthaya et al., 2020). Moreover, researchers have presented that the trough concentration ≥15 mg/L was correlated with nephrotoxicity in children (Le et al., 2015).

TABLE 3 Logistic analysis of risk factors for treatment failure.

Variables	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P Value	OR (95% CI)	P Value
PRISM III scores ≥10	16.683 (5.504-50.566)	<0.001	15.011 (3.937-57.230)	<0.001
Initial vancomycin daily dose (mg/kg/day)	1.067(1.013-1.123)	0.014	1.017 (0.936-1.106)	0.682
Albumin (g/dL)	0.929 (0.867-0.994)	0.033	1.000 (0.924-1.083)	0.997
Mean vancomycin daily dose (mg/kg/day)	1.053 (1.005-1.102)	0.029	1.052 (0.975-1.135)	0.194
Gram negative bacteria co-infections	3.313 (1.189-9.230)	0.022	1.866 (0.519-6.715)	0.339
Carbapenems concomitant with vancomycin therapy	2.859 (1.036-7.886)	0.042	1.110 (0.296-4.159)	0.877
Duration of vancomycin therapy (days)	0.946 (0.876-1.021)	0.155		

OR, odds ratio; CI, confidence interval; PRISM, Pediatric Risk of Mortality.

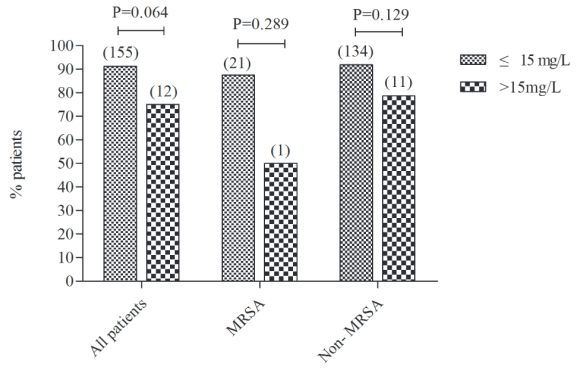

FIGURE 3
The treatment success rate between trough concentrations ≤15 mg/L and >15 mg/L in all enrolled patients, MRSA infection patients and non-MRSA infection patients. The number in brackets above the columns reflects the total number of children. MRSA, Methicillin-resistant *Staphylococcus aureus*.

Therefore, the trough concentration of 15-20 mg/L for children with Gram-positive bacterial sepsis might be reconsidered.

We found that a PRISM III score ≥10 was an independent risk factor for treatment failure with the vancomycin dosage of 40-60 mg/kg/d and corresponding trough concentrations in children with Gram-positive bacterial sepsis. A higher PRISM III score is associated with an increased probability of mortality (Pollack et al., 1996). Yang et al. (Yang et al., 2018) reported that disease severity was the most relevant factor predicting treatment failure in patients with bacteria. Consistent with the observation by Yang et al., findings from our study showed that a PRISM III score ≥10 was a risk factor for treatment failure in children with sepsis. In this study, the failure group patients had a significantly higher proportion of septic shock than that in success group. Severe patients combined with organ dysfunctions and had multi-drug interactions and other therapeutic interventions, which may affect antimicrobial pharmacokinetics (Scaglione and Paraboni, 2008). Hence, caution should be applied when giving a vancomycin dosage of 40-60 mg/kg/day to the sepsis children with PRISM III scores ≥10.

In this study, it was interesting to note that although the failure group patients received a higher vancomycin dosage than that in the success group, they were still treated unsuccessfully. The reasons may be attributable to the follows. One possible explanation might be associated with illness severity. In our research, the failure group patients had significantly higher PRISM III scores than those in the success group. Higher PRISM III scores indicate more severe illness. Thus, some pediatricians might give these severe patients a higher initial vancomycin dosage or adjust to a higher vancomycin dosage, which leaded the initial and mean vancomycin daily doses in failure group were remarkably higher than those in the success group. Another possible explanation might be reaching the target trough concentration of 10-20 mg/L. Truong et al. (Truong et al., 2018) demonstrated that severe sepsis and higher disease severity scores were correlated with higher vancomycin treatment failure rates. Although studies have showed that there was no need to attain trough concentrations of 10-20 mg/L when using AUC-guided vancomycin dosing in children with sepsis (Lanke et al., 2017). Some pediatricians still adjusted the dose of vancomycin based on the target trough concentration of 10-20 mg/L (Sosnin et al., 2019). Therefore, failure group children might be exposed to overuse of vancomycin. Inappropriate use of antibiotics was associated with an elevated risk of antibiotic-related adverse events and healthcare costs (Principi and Esposito, 2019). We have demonstrated PRISM III scores ≥10 might serve as an independent risk factor for vancomycin treatment failure in children with Gram-positive bacterial sepsis. Individualized vancomycin dosages were required in these children. Moreover, the pediatricians should avoid to increase vancomycin dosages blindly in clinical practice when the treatment effect is poor, but should reevaluate the whole treatment strategy. As sepsis comprehensive management not only focuses on antimicrobial treatment, but also pays attention to early identification, source control, appropriate supportive care and hemodynamic optimization (Weiss et al., 2020; Mehta et al., 2022).

We found that failure group patients had a higher proportion of concomitant utilization with carbapenems, but they did not obtain higher treatment success rate. Researches have been reported that vancomycin has many shortcomings, including poor tissue penetration and slow killing time (Tong et al., 2020). Hence, some studies investigated combination therapy using vancomycin with other antibiotics for MRSA infections (Mohammadi-Berenjestanaki et al., 2020; Tong et al., 2020). Mohammadi-Berenjestanaki et al. demonstrated that co-administration of vancomycin and imipenem could effective against MRSA and MSSA infections (Mohammadi-Berenjestanaki et al., 2020). This observation was inconsistent with the results of our study. One possible explanation for this inconsistency could be Mohammadi-Berenjestanaki *et.al*'s study was designed to evaluate the efficacy of combination vancomycin with imipenem only on MRSA and MSSA infections, while our study investigated the efficacy of co-administration of vancomycin and carbapenems on Gram-positive bacterial. Another explanation might be about 74% (14/19) patients in failure group had PRISM III scores ≥10 in our study, which lead to treatment failure. As meropenem therapy was associated with an increased risk of *Clostridioides difficile* infection (Lee et al., 2021). Thus, when there is no evidence of Gram-negative bacterial infections, co-administration of vancomycin and carbapenems should be applied with caution.

There are some limitations of our study. Firstly, the sample size of MRSA-infected children in this study was relatively small, therefore the results of this study should be interpreted with caution when applied to MRSA-infected children. Secondly, we could not estimate the vancomycin nephrotoxicity cutoff value in this population due to no reports of nephrotoxicity adverse effects. Thirdly, although we have tried our best to control the possible bias, it might still exist and potentially skew our results owing to its retrospective design. A prospective randomized multicenter study with larger sample size participants is needed to investigate the vancomycin efficiency and safety with current dosages and corresponding trough concentrations in children with Gram-positive bacterial sepsis.

Conclusions

Vancomycin dosages of 40-60 mg/kg/d are effective and have no vancomycin-related nephrotoxicity adverse effects occur in children with Gram-positive bacterial sepsis. Vancomycin trough concentrations >15 mg/L are not an essential target for these Gram-positive bacterial sepsis patients. PRISM III scores ≥10 may serve as an independent clinical risk factor for vancomycin treatment failure in these patients.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by The Institutional Review Board, Children's Hospital of Chongqing Medical University. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conception and design: LP, ZG, and ZL; Methodology: LP, ZG, and GZ; Collection and assembly of data: GZ, XT, and RG; Data analysis and interpretation: LP, QL, and YL, Writing-original draft: LP; Writing- review and editing: ZL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1117717/full#supplementary-material

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The clinical significance of macrolide resistance in pediatric *Mycoplasma pneumoniae* infection during COVID-19 pandemic

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Background: *Mycoplasma pneumoniae* (MP) is a commonly occurring pathogen causing community-acquired pneumonia (CAP) in children. The global prevalence of macrolide-resistant MP (MRMP) infection, especially in Asian regions, is increasing rapidly. However, the prevalence of MRMP and its clinical significance during the COVID-19 pandemic is not clear.

Methods: This study enrolled children with molecularly confirmed macrolide-susceptible MP (MSMP) and MRMP CAP from Beijing Children's Hospital Baoding Hospital, Capital Medical University between August 2021 and July 2022. The clinical characteristics, laboratory findings, chest imaging presentations, and strain genotypes were compared between patients with MSMP and MRMP CAP.

Results: A total of 520 hospitalized children with MP-CAP were enrolled in the study, with a macrolide resistance rate of 92.7%. Patients with MRMP infection exhibited more severe clinical manifestations (such as dyspnea and pleural effusion) and had a longer hospital stay than the MSMP group. Furthermore, abnormal blood test results (including increased LDH and D-dimer) were more common in the MRMP group ($P<0.05$). Multilocus variable-number tandem-repeat analysis (MLVA) was performed on 304 samples based on four loci (Mpn13-16), and M3562 and M4572 were the major types, accounting for 74.0% and 16.8% of the strains, respectively. The macrolide resistance rate of M3562 strains was up to 95.1%.

Conclusion: The prevalence of MRMP strains in hospitalized CAP patients was extremely high in the Baoding area, and patients infected with MRMP strains exhibited more severe clinical features and increased LDH and D-dimer. M3562 was the predominant resistant clone.

KEYWORDS

Mycoplasma pneumoniae, drug resistance, children, clinical feature, genotyping

Introduction

Mycoplasma pneumoniae (MP) is a prevalent pathogen responsible for respiratory tract infections, comprising 10%-30% of community-acquired pneumonia (CAP) cases in children and young adults (Principi et al., 2001; Michelow et al., 2004). Although MP pneumonia is generally mild and self-limited, there have been increasing reports of severe cases, including myocarditis, urticarial, hepatitis, hemolytic anemia, and others (Narita, 2016).

Macrolides are the recommended first-line antibiotics for treating children with MPP (Principi and Esposito, 2013). However, the rapid global emergence of macrolide-resistant MP (MRMP) has become a significant concern. High rates of MRMP have been observed in Asian countries, particularly in China, Japan and Korea, where rates range from 7.7% to 78.5% (Oishi and Ouchi, 2022). The relationship between MRMP and clinical characteristics remains inconsistent. Some studies in Asia have reported increased disease severity in patients infected with MRMP (Chen et al., 2020), while others have found no significant differences in the clinical course between MRMP and macrolide-susceptible MP (MSMP) infections (Waites et al., 2019). Molecular typing of MP is a useful tool for surveillance and outbreak investigation. The Multilocus Variable-Number Tandem-Repeat Analysis (MLVA) method based on five loci (Mpn1, Mpn13-16) was developed in 2009 (Degrange et al., 2009). However, an amended 4-locus MLVA scheme was later proposed due to concerns over the Mpn1 locus's instability (Sun et al., 2013). Molecular characterization of MP genotypes and macrolide resistance has been widely investigated, with geographic and temporal variations observed.

In late 2019, the emergence of the COVID-19 pandemic drastically affected daily life worldwide. The widespread implementation of non-pharmaceutical interventions (NPIs) has had a significant impact on the transmission of respiratory infections, including MP. However, the prevalence of MRMP and its impact on the clinical manifestations of CAP in children in Baoding during the COVID-19 pandemic have not been well documented. Therefore, this study aimed to investigate the prevalence of macrolide resistance in MP strains isolated from children with CAP in Baoding and to explore the clinical manifestations and genotypes of MRMP.

Materials and methods

Study population

The study recruited children under the age of 18 who were admitted to the Baoding Hospital of Beijing Children's Hospital, Capital Medical University with CAP between August 2021 and July 2022. Diagnosis of CAP was made in accordance with Chinese guidelines and included clinical symptoms and signs consistent with pneumonia, as well as radiographic evidence of consolidation, infiltrate, or pleural effusion (Respiratory Group of Pediatric Branch of Chinese Medical Association, 2015). Patients with immunodeficiency disorders, those receiving immunosuppressive medications, those with a hospital stay of less than one day, and those with incomplete clinical data were excluded. Demographic and clinical information was collected from medical records.

The institutional review board approved the study protocol, and written informed consent was not required since leftover DNA samples from the clinical microbiology laboratory were used.

Laboratory testing

The suspected CAP patients underwent a battery of routine laboratory tests, which included a complete blood count, serum biochemistry, C-reactive protein (CRP), procalcitonin (PCT), creatine kinase-myocardial band isoenzyme (CKMB), and D-dimer. Oropharyngeal swabs were collected for detection of MP and macrolide resistance-associated mutations using a multiplex real-time PCR assay (Mole, Jiangsu, China) as previously described (Feng X et al., 2016). Briefly, the P1 gene was targeted to confirm the presence of MP, with a probe labelled with VIC. Two point mutations (A2063G and A2064G) located in domain V of the 23S rRNA gene were analyzed to identify macrolide resistance, with a probe labelled with FAM. The sample was considered as MRMP if both VIC and FAM fluorescence signals were detected. Any remaining DNA from MP-positive samples was stored at -80°C for future use.

MP genotyping

The genotyping of MP was performed using DNA samples extracted directly from oropharyngeal swabs of MP positive patients, as previously described (Dumke and Jacobs, 2011; Wang et al., 2021). Nested PCR was employed to amplify the four highly discriminatory variable-number tandem-repeat (VNTR) loci (Mpn13, Mpn14, Mpn15, Mpn16). The PCR products were subsequently sequenced by Tianyihuiyuan Biotechnology Co. Ltd. to determine the number of repeats for each locus. The MLVA type was assigned as a string of allele numbers in the order of Mpn13 to Mpn16.

Case definitions

A patient hospitalized with CAP who tested positive for MP *via* PCR was considered to have MP CAP. A MRMP patient was classified as having a positive result for the point mutation test. Severe MPP was defined as a child with MPP exhibiting at least one of the following characteristics according to the Chinese guidelines (Respiratory Group of Pediatric Branch of Chinese Medical Association, 2015): (1) poor general status; (2) increased respiratory rate; (3) cyanosis and dyspnea; (4) infiltration involving multiple lobes or more than two-thirds of the lung; (5) transcutaneous oxygen saturation of 92% or less in room air; (6) extrapulmonary complications.

Statistical analysis

The demographic information and laboratory test results of the study population were presented as percentages for categorical variables and medians (interquartile range [IQR]) for continuous variables. Statistical analysis was performed using SPSS Statistics version 23 for Windows. Categorical variables were compared using chi-square or Fisher's exact test, and non-normally distributed continuous variables were compared using the Mann-Whitney test. Statistical significance was set at a two-tailed P value of less than 0.05.

Result

Study population

Among the 864 children hospitalized with CAP who underwent MP PCR testing, 551 (63.8%) were MP positive. Of these, 520 met the inclusion criteria and were enrolled (Figure 1). The median age of the eligible children was 6.63 years (28 days to 17 years), and 50.4% (262/520) were male. The highest prevalence of MP was observed in the 5-9 age group (64.8%, 337/520), followed by the 2-4 age group (15.0%, 78/520), and the 10-18 age group (14.6%, 76/520) (Table 1). Severe MP infection was present in 21.2% (110/520) of the enrolled patients.

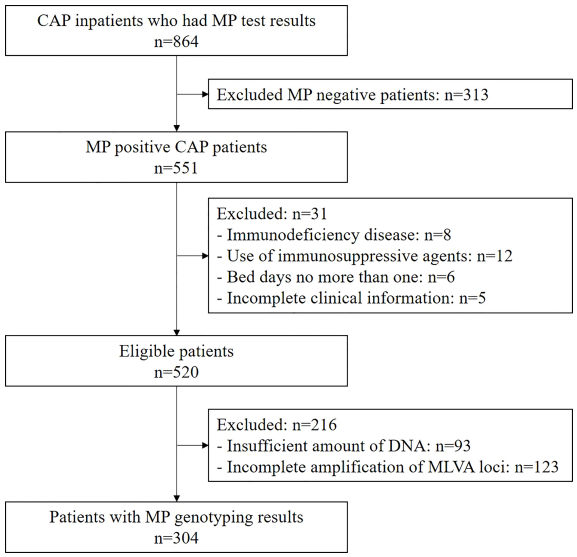

FIGURE 1
Flow chart of patient enrollment.

Macrolide susceptibility testing of MP

Of the 520 MP-positive patients, 482 (92.7%) were found to be macrolide resistant and were classified into the MRMP group. The rate of macrolide resistance did not significantly differ among different age groups, ranging from 90.0% in the 0-1 age group to 93.6% in the 4-6 age group (P=0.826, Table 1). Likewise, there was no significant difference in the rate of macrolide resistance between genders (P=0.699, Table 1).

Relationship between MP drug resistance and clinical characteristics

The clinical manifestations were compared between patients with MRMP and MSMP. As shown in Table 1, MRMP patients had a significantly higher incidence of dyspnea (P=0.040) and were also more likely to experience fever, though the difference was not statistically significant (P=0.059). Gastrointestinal symptoms were only observed in MRMP patients (29/482, 6.0%). However, there was no significant difference in other clinical symptoms, including cough, wheezing, rash, and severe MPP, between the two groups.

Regarding radiological findings, MRMP patients did not show a higher incidence of consolidation or bilateral lung involvement compared to MSMP patients, although the differences were not significant (P=0.395 and 0.180, respectively) (Table 1). Among MP-CAP patients, 126 (24.2%) had pleural effusion, of which 124 patients were in the MRMP group (25.7%, 124/482), indicating that MRMP patients were more likely to develop pleural effusion (P=0.005).

The median duration of fever was 8 (IQR 6-11) days for MRMP patients, which was slightly longer than for MSMP patients (7 days, IQR 5.75-10 days). MRMP patients were significantly associated

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TABLE 1 Relationship between MP drug resistance and clinical characteristics.

Characteristic	Total (n=520) n (%) or median (IQR)	MRMP patients (n=482), n (%) or median (IQR)	MSMP patients (n=38), n (%) or median (IQR)	*P* value
Basic information				
Age				
0-1	29 (5.6)	24 (5.0)	5 (13.1)	Reference
2-4	78 (15.0)	72 (14.9)	6 (15.8)	0.164
5-9	337 (64.8)	312 (64.7)	25 (65.8)	0.076
10-18	76 (14.6)	74 (15.4)	2 (5.3)	0.017 [a]
Gender				
Male	262 (50.4)	244 (50.6)	18 (47.4)	0.699
Female	258 (49.6)	238 (49.4)	20 (52.6)	
Clinical manifestations				
Cough	509 (97.9)	473 (98.1)	36 (94.7)	0.189
Fever	490 (94.2)	457 (94.8)	33 (86.8)	0.059
Wheezing	27 (5.2)	24 (5.0)	3 (7.9)	0.437
Dyspnea	71 (13.7)	70 (14.5)	1 (2.6)	0.040 [a]
Gastrointestinal symptoms	29 (5.6)	29 (6.0)	0 (0.0)	0.257
Skin rash	23 (4.4)	22 (4.6)	1 (2.6)	1
Severe MPP	110 (21.2)	105 (21.8)	5 (13.2)	0.210
Imaging presentations				
Findings				
Infiltrates	386 (74.2)	360 (74.7)	26 (68.4)	0.395
Consolidation	134 (25.8)	122 (25.3)	12 (31.6)	
Involved parts				
Unilateral lung	377 (72.5)	353 (73.2)	24 (63.2)	0.180
Both lungs	143 (27.5)	129 (26.8)	14 (36.8)	
Pleural effusion	126 (24.2)	124 (25.7)	2 (5.3)	0.005 [a]
Treatment features				
Total days with fever [b]	8 (6-11)	8 (6-11)	7 (5.75-10)	0.214
Days of hospitalization [b]	9 (7-12)	9 (7-12)	7 (6-9.25)	0.003 [a]

MRMP, macrolide resistant *M. pneumoniae*; MSMP, macrolide susceptible *M. pneumoniae*; MPP, *M. pneumoniae* pneumonia; IQR, interquartile range; a: P<0.05; b: non-normal distribution.

with a longer duration of hospitalization (9 days vs. 7 days, *P*=0.003). Notably, no deaths occurred among the enrolled children.

Correlation between MP drug resistance and laboratory test indexes

The laboratory findings of children with MRMP and MSMP are presented in Table 2. With the exception of LDH and D-dimer, most indicators, such as WBC, platelet, CKMB, ALT, and PCT, did not differ significantly between the two groups. MRMP children had a higher proportion of increased LDH (*P*=0.003) and D-dimer (*P*=0.029) compared to MSMP children. Moreover, a marginal trend towards significance was observed for increased CRP in the MRMP group (60.4% vs. 44.7%, *P*=0.059).

MP genotyping using MLVA

Upon genotyping, 304 samples were found to be viable for analysis, with the remaining 216 samples either lacking sufficient DNA or incomplete MLVA amplification. Among the genotyped

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TABLE 2 Correlations between MP drug resistance and laboratory indicators.

Laboratory indicators [a]	Total (n=520) n (%) or median (IQR)	MRMP patients (n=482), n (%) or median (IQR)	MSMP patients (n=38), n (%) or median (IQR)	*P* value
WBC [b]				
Normal	423 (81.3)	393 (81.5)	30 (78.9)	Reference
Decreased	18 (3.5)	15 (3.1)	3 (7.9)	0.144
Increased	79 (15.2)	74 (15.3)	5 (13.2)	0.807
Platelet [c]				
Normal	416 (80.0)	387 (80.3)	29 (76.3)	Reference
Decreased	76 (14.6)	68 (14.1)	8 (21.1)	0.28
Increased	28 (5.4)	27 (5.6)	1 (2.6)	0.71
CRP (mg/L) (<8 mg/L)				
Normal	212 (40.8)	191 (39.6)	21 (55.3)	0.059
Increased	308 (59.2)	291 (60.4)	17 (44.7)	
LDH (110-295 U/L)				
Normal	277 (53.2)	248 (51.5)	29 (76.3)	0.003 [e]
Increased	243 (46.8)	234 (48.5)	9 (23.7)	
CKMB (0-24 U/L)				
Normal	451 (86.7)	418 (86.7)	33 (86.8)	0.983
Increased	69 (13.3)	64 (13.3)	5 (13.2)	
ALT [d]				
Normal	451 (86.7)	416 (86.3)	35 (92.1)	Reference
Decreased	7 (1.3)	7 (1.5)	0 (0)	1
Increased	62 (12.0)	59 (12.2)	3 (7.9)	0.605
PCT (<0.25 ng/mL)				
Normal	425 (81.7)	392 (81.3)	33 (86.8)	0.394
Increased	95 (18.3)	90 (18.7)	5 (13.2)	
D-dimer (0-0.256 U/L)				
Normal	227 (43.7)	204 (42.3)	23 (60.5)	0.029 [e]
Increased	293 (56.3)	278 (57.7)	15 (39.5)	

MRMP, macrolide resistant *M. pneumoniae*; MSMP, macrolide susceptible *M. pneumoniae*; WBC, white cell count; CRP, C reactive protein; LDH, lactate dehydrogenase; CKMB, creatine kinase-myocardial band isoenzyme; PCT, procalcitonin.

a: The reference intervals are listed in parentheses after the test indicators.

b: The reference intervals of WBC: 28 days-<6 months: 4.3-14.2 ($\times10^9$/L), 6 months-<1 year: 4.8-14.6 ($\times10^9$/L), 1 year-<2 years: 5.1-14.1 ($\times10^9$/L), 2 years-<6 years: 4.4-11.9 ($\times10^9$/L), 6 years-<13 years: 4.3-11.3 ($\times10^9$/L), 13 years-18 years: 4.1-11.0 ($\times10^9$/L)(China, 2021a).

c: The reference intervals of platelet: 28 days-<6 months: 183-614 ($\times10^9$/L) for boys, 203-653 ($\times10^9$/L) for girls; 6 months-<1 year: 190-579 ($\times10^9$/L) for boys, 172-601 ($\times10^9$/L) for girls; 1 year-<2 years: 190-524 ($\times10^9$/L) for boys, 191-516 ($\times10^9$/L) for girls; 2 years-<6 years: 188-472 ($\times10^9$/L) for boys, 187-475 ($\times10^9$/L) for girls; 6 years-<12 years: 167-453 ($\times10^9$/L) for boys, 177-446 ($\times10^9$/L) for girls; 12 years-18 years: 150-407 ($\times10^9$/L) for boys, 148-399 ($\times10^9$/L) for girls (National Health Commission of the People's Republic of China, 2021a).

d: The reference intervals of ALT: 28 days-<1 year: 8-71 U/L, 1 year-<2 years: 8-42 U/L, 2 years-<13 years: 7-30 U/L, 13 years-18 years: 7-43 U/L for boys, 6-29 U/L for girls (National Health Commission of the People's Republic of China, 2021b).

e: $P<0.05$ (National Health Commission of the People's Republic of China, 2021b).

samples, nine distinct MLVA types were identified, with the majority of cases exhibiting either the M3562 (225/304, 74.0%) or M4572 (51/304, 16.8%) type. Less common types included M3572 (14/304, 4.6%), M3662 (6/304, 2.0%), and several others (M3472, M4562, M4672, M3462, M4462) that accounted for only 2.6% (8/304) of total cases.

Relationship between MLVA types and macrolide resistance

Of note, the macrolide resistance rate was found to be 93.8% (285/304) in patients with MLVA genotyping results, indicating a predominance of MRMP cases among different MLVA types. The

macrolide resistance rates of all MLVA types were high (from 78.6% to 100%). Further analysis revealed a significant association between MLVA type and macrolide resistance, with the M3572 type exhibiting a lower resistance rate (P=0.039). The M3572 is a rare MLVA type and has been reported previously (Xiao et al., 2020). Its emergence in Baoding area remained unclear. Interestingly, all MP strains with rare MLVA types (3472/4562/4672/3462/4462) were found to be resistant to macrolide (Figure 2; Table 3).

Based on the association between MLVA types and P1 lineages reported in previous studies (Sun et al., 2013; Xiao et al., 2020), we determined the P1 subtypes for 296 patients. As presented in Table 3, P1-2 subtype was found to be predominant in this study, accounting for 78.0% of the cases. Notably, the prevalence of macrolide resistance was higher in the P1-2 subtype (219/296, 94.8%) compared to P1-1 (58/65, 89.2%). However, no significant correlation was observed between macrolide resitance and P1 subtype (P=0.147).

Discussion

During the COVID-19 pandemic, despite strict implementation of NPIs, we observed a high rate of macrolide resistance in MP-positive pediatric patients (92.7%). As reported in previous studies, MRMP was associated with dyspnea, pleural effusion, longer hospital stays, and abnormal blood test results, including increased LDH and D-dimer. The MLVA type 3562 was the most prevalent (74.0%) among the genotyped samples, with a very high macrolide resistance rate (95.1%).

Macrolide resistance of MP has been increasing globally, with a particularly severe situation in Asian countries (Oishi and Ouchi, 2022). In China, previous studies have reported that more than 90% of MP infections were caused by macrolide-resistant strains (Xin et al., 2009; Zhou et al., 2015). However, a recent prospective, multicenter, population-based surveillance study among Chinese children during the COVID-19 pandemic (2020.1-2021.6) reported a remarkable decrease in MP infections and a decrease in the rate of MRMP to 7.7% (Chen et al., 2022). In contrast, our study found a high rate of macrolide resistance (92.7%) among children with CAP during the COVID-19 pandemic. Possible reasons for the difference in rates of macrolide resistance could be attributed to several factors. Firstly, the enrolled patients in our study were CAP inpatients, while Chen et al. focused on outpatient children with mild respiratory tract infections. Macrolide resistance rates might be higher among inpatients or patients with pneumonia (Guo et al., 2019; Katsukawa et al., 2019). Secondly, macrolide therapy has been shown to promote the development of macrolide resistance (Suzuki et al., 2017). In the study by Chen et al., only 17.6% of the outpatients had prior macrolide prescriptions, while almost all inpatients in our study received macrolide therapy prior to hospitalization. Thirdly, the primary macrolide resistance of MP was higher in the Baoding area, which was similar to data from adjacent cities (93.9% in Beijing in 2020, 90.0% in Shijiazhuang in 2015-2016) (Wang et al., 2016; Wang et al., 2022). Fourthly, the selective pressure for the development of drug resistance may also result from the excessive or inappropriate use of macrolides. It has been reported that the overall rate of antibiotic use in children with CAP in China was 89.08%, with macrolides being the most commonly used antibiotics as the first choice for the treatment of MP-caused CAP (Wei et al., 2019).

MP infection was most commonly observed in the 5-9 age group, and the prevalence of MRMP infection was higher among older children. This is in line with previous findings that school-aged children, who come into contact with more people and are often in closed spaces, are more susceptible to the rapid spread of pathogens (Guo et al., 2019). We conducted further investigations to examine the relationship between macrolide resistance of MP and clinical characteristics. Our findings revealed a significantly higher incidence of dyspnea among children infected with MRMP (P=0.040), with a similar trend observed for fever (P=0.059). Gastrointestinal symptoms were only observed in MRMP patients (6.0%). Previous studies from Italy and various regions in China

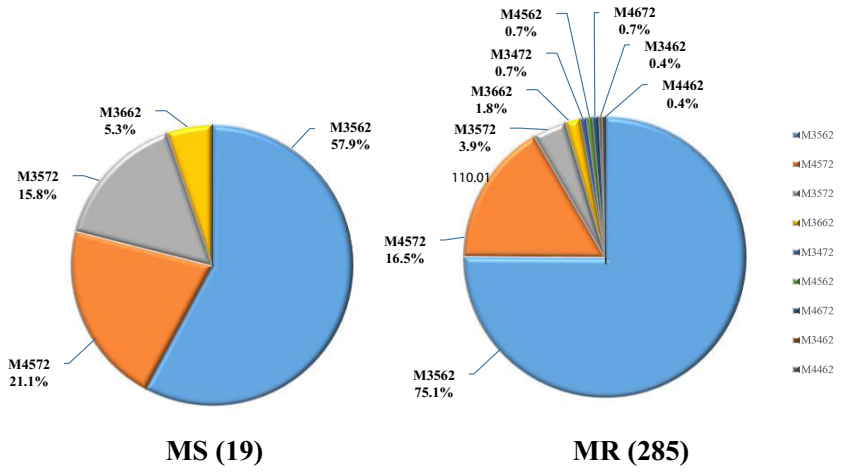

FIGURE 2
Macrolide resistance in MLVA types.

TABLE 3 Relationship between genotypes and MP drug resistance.

Genotype	Total (n=304) n (%)	MRMP patients (n=285), n (%)	MSMP patients (n=19), n (%)	*P* value
MLVA type				
3562	225 (74.0)	214 (95.1)	11 (4.9)	reference
4572	51 (16.8)	47 (92.2)	4 (7.8)	0.490
3572	14 (4.6)	11 (78.6)	3 (21.4)	*0.039
3662	6 (2.0)	5 (83.3)	1 (16.7)	0.277
3472/4562/4672/3462/4462	8 (2.6)	8 (100)	0 (0)	1.00
P1 subtype[#]				
1 (4572, 3572)	65 (22.0)	58 (89.2)	7 (10.8)	reference
2 (3562, 3662)	231 (78.0)	219 (94.8)	12 (5.2)	0.147

MLVA, multilocus variable-number tandem-repeat analysis; MRMP, macrolide resistant *M. pneumoniae*; MSMP, macrolide susceptible *M. pneumoniae*; *: $P<0.05$; #: The P1 subtypes were determined based on the relationship between MLVA types and P1 lineages (Sun et al., 2013; Xiao et al., 2020).

have also reported a trend towards higher rates of dyspnea, fever, and extrapulmonary symptoms in MRMP patients (Cardinale et al., 2013; Ma et al., 2014; Yang et al., 2019). However, they did not observe any significant differences in clinical features among MRMP and MSMP patients. The possible reasons for this finding could be the relatively small sample size in previous studies, with the total number of patients with dyspnea being less than 60, or the huge disparity in the number of MRMP and MSMP patients, with MRMP patients being predominant in most Chinese reports while MSMP patients being more common in Europe (Oishi and Ouchi, 2022).

The presence of pleural effusion in MP pneumonia may indicate the severity and prolonged lesions on chest radiography, and it is one of the most common reasons for transfer to tertiary hospitals in patients with MP pneumonia. In this study, pleural effusion was observed in 24.2% of children with MP-CAP, which was slightly higher than previous reports (16%-20.7%) (Fine et al., 1970; Miyashita et al., 2009; Kim et al., 2021). Further analysis revealed that pleural effusion occurred significantly more frequently in the MRMP group ($P=0.005$). While Kim et al. reported that pleural effusion was not associated with macrolide resistance, all 24 patients with pleural effusion in their study belonged to the MRMP group, which is consistent with our findings.

The length of hospital stay was longer in MRMP patients than in MSMP patients ($P=0.003$). This is in line with a recent systematic review, which found that MRMP infections were associated with prolonged hospitalization compared with MSMP infections ($P<0.001$). Although macrolide-resistant strains were associated with more pleural effusion and longer hospital stays, there was no significant correlation between macrolide resistance and severe MPP ($P=0.210$).

We compared common laboratory indicators between CAP patients with MRMP infection and MSMP infection. In accordance with the new clinical biochemistry and blood cell analysis standards for children implemented in October 2021, reference intervals for each indicator vary by age group. Therefore, we used classification indices instead of absolute numbers. As a result, more MRMP patients were found to have elevated LDH and D-dimer levels ($P<0.05$). Previous studies have suggested that serum LDH is a biomarker for predicting refractory MPP and evaluating the need for corticosteroid therapy during early hospitalization (Inamura et al., 2014; Lu et al., 2015; Huang et al., 2021). Similarly, higher D-dimer levels have been associated with more severe clinical manifestations and longer treatment durations (Huang et al., 2021; Zheng et al., 2021). Our findings suggest that elevated levels of LDH and D-dimer are significantly greater in CAP patients with MRMP infection, indicating the severity of disease caused by MRMP. Additionally, we observed a trend towards significance for increased CRP in the MRMP group ($P=0.059$). However, previous studies have typically used absolute numbers for comparison, and no association has been found between CRP and MRMP (Chen et al., 2020).

In this study, we successfully obtained MLVA genotype data of 304 MP strains in specimens and categorized them into nine types. The predominant type was M3562 (74.0%), followed by M4572 (16.8%). However, the most prevalent type reported in Beijing, Taiwan, and Spain was M4572 (79.3%, 73.2%, and 50.1%, respectively), suggesting geographical differences (Rivaya et al., 2020; Wang et al., 2021; Wu et al., 2021). The macrolide resistance rates of M3562 and M4572 were 95.1% and 92.2%, respectively. Previous studies have suggested that the spread of MP appears to be polyclonal, associated with multiple genotypes (Pereyre et al., 2012; Suzuki et al., 2019). Interestingly, Wang et al. found that the prevalence of M3562 increased from 11.63% to 24.67% during 2016-2019 in northern China, with a drastic increase in the macrolide resistance rate from 60% to 93.48% (Wang et al., 2021). This suggests that expansion of drug-resistant MP strains with specific genotype may play an important role in the high rate of macrolide resistance. It appears that the high prevalence of M3562 in this study is the result of drug-resistant genotype expansion. We derived P1 subtypes based on the relationship between MLVA type and P1 lineage and found that MR was more common in the P1-2 subtype, which is inconsistent with previous reports showing that most MRMP strains harbored the P1-1 subtype (Katsukawa et al.,

2019; Xiao et al., 2020). However, this may be due to the predominant prevalence of M3562, which belongs to the P1-2 subtype and is highly drug-resistant. Continuous monitoring of MP genotypes will be necessary to understand the epidemiological trends.

Several limitations of our study should be noted. Firstly, the study was conducted in a single center, which may limit the generalizability of our findings to other populations. Secondly, MP detection was not performed for all CAP patients during the study period, which may introduce selection bias. Thirdly, mutations associated with macrolide resistance other than 23S rRNA A2063G and A2064G were not included in this study, which may underestimate the prevalence of macrolide resistance. Fourthly, not all MP-positive samples could be genotyped due to insufficient amounts of DNA or incomplete amplification of MLVA loci, which may have led to bias in the results. Fifthly, the low number of MSMP patients included in our study (compared to MRMP patients) may also have introduced bias, even though we have statistically processed the data. Finally, as with all observational studies, we cannot infer causality, and further studies are needed to confirm our findings.

In conclusion, our study highlights the severity of macrolide resistance in Baoding during the COVID-19 pandemic. Our findings suggest that MRMP infection is associated with more severe clinical manifestations and increased levels of LDH and D-dimer. Additionally, our results indicate that the M3562 clone is the prevailing macrolide-resistant strain in Baoding. Based on our findings, substitution therapy using tetracycline and quinolone should be considered for the treatment of MRMP infections. Furthermore, continuous monitoring of drug resistance and genotypes is recommended to guide treatment strategies and inform public health policies in children.

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 human participants were reviewed and approved by Ethics committee of Baoding hospital of Beijing Children's Hospital, Capital Medical University. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

A-DS, JB, W-WJ were responsible for study design. T-TJ, HQ, T-YW, QH, X-QS collected and organized data. T-TJ, T-YW and Y-CW did the genotyping. T-TJ, LS, HT, W-WJ, A-DS analyzed the data. T-TJ, LS, W-WJ wrote the manuscript, JB and A-DS revised the manuscript. JB and A-DS provided research funds. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Antibiotic susceptibility of *Escherichia coli* isolated from neonates admitted to neonatal intensive care units across China from 2015 to 2020

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Background: *Escherichia coli* is one of the most common pathogens causing neonatal infections. Recently, the incidence and drug resistance of *E. coli* have increased, posing a major threat to neonatal health. The aim of this study was to describe and analyze the antibiotic resistance and multilocus sequence typing (MLST) characteristics of *E. coli* derived from infants admitted to neonatal intensive care units (NICUs) across China.

Methods: In this study, 370 strains of *E. coli* from neonates were collected. *E. coli* isolated from these specimens were subjected to antimicrobial susceptibility testing (by broth microdilution method) and MLST.

Results: The overall resistance rate was 82.68%, with the highest rate of methicillin/sulfamethoxazole (55.68%) followed by cefotaxime (46.22%). Multiple resistance rate was 36.74%, 132 strains (35.68%) had extended-spectrum β-lactamase (ESBL) phenotype and 5 strains (1.35%) had insensitivity to the tested carbapenem antibiotics. The resistance of *E. coli* isolated from different pathogenicity and different sites of infections varied, strains derived from sputum were significantly more resistant to β-lactams and tetracyclines. Currently, the prevalence spectrum in NICUs was dominated by ST1193, ST95, ST73, ST69 and ST131 across China. And the multidrug resistance of ST410 was the most severe. ST410 had the highest resistance rate to cefotaxime (86.67%), and its most common multidrug resistance pattern was β-lactams + aminoglycosides + quinolones + tetracyclines + sulfonamides.

Conclusions: Substantial proportions of neonatal *E. coli* isolates were severely resistant to commonly administered antibiotics. MLST results can suggest the prevalent characteristics of antibiotic resistance in *E. coli* with different ST types.

KEYWORDS

neonate, neonatal infection, *Escherichia coli*, antimicrobial resistance, MLST, epidemiology

1 Introduction

Newborns admitted to the Neonatal Intensive Care Units (NICUs), and particularly those born preterm, are at high risk of infection for several reasons, including relative immunocompromise from an immature immune system, prolonged hospitalization, and frequent use of invasive devices and antibiotics (Collins et al., 2018). Infectious diseases are also the main causes of neonatal morbidity and mortality (Zhang et al., 2019; GBD 2019 Diseases and Injuries Collaborators, 2020). Globally, 2.6 million newborns still die each year, with preterm birth and infections the two leading causes. Neonatal sepsis and meningitis were responsible for an estimated 420,000 deaths annually, accounting for 16% of neonatal mortality (Khan et al., 2017).

Escherichia coli is one of the most well-adapted and pathogenically versatile bacterial organisms (Riley, 2020). It is the main pathogen causing neonatal meningitis and sepsis especially in developing countries (Tan, 2020; van der Flier, 2021; Wen et al., 2021; Hallmaier-Wacker et al., 2022), also a common pathogen of ventilator associated pneumonia (VAP) in hospitals(Scamardo et al., 2020). According to the data from the Neonatal Monitoring Network released by NICHD (the Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network) in 2020 (Stoll et al., 2020), the most frequent pathogens of early onset neonatal sepsis were *E. coli* (36.6%) and Group B *streptococcus* (30.2%), and *E. coli* mainly occurred in premature infants (51.9%). Besides, a retrospective cohort study in China found that the complications and mortality of *E. coli* meningitis were higher than those of other pathogens (Collaborative Study Group for Neonatal Bacterial Meningitis, 2018).

At the same time, bacterial antimicrobial resistance (AMR) further increases the difficulty of treatment and the speed of transmission of infection—has emerged as one of the leading public health threats of the 21st century (Antimicrobial Resistance Collaborators, 2022). Recent surveillance data from the 2000s indicate that antibiotic resistance to all major antibiotic classes exists among *E. coli* strains. These include the production of extended-spectrum-beta-lactamases (ESBLs) (including TEM, SHV, CMY, and CTX-M types), production of carbapenemases (including KPC, NDM, VIM, OXA-48 and IMP types), resistance to fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole and recently also plasmid-mediated colistin resistance (Paitan, 2018). A multicenter cohort study in the United States showed that the majority of neonatal *E. coli* isolates were insensitive to commonly used antibiotics, with 66.8% of isolates insensitive to ampicillin and 16.8% insensitive to aminoglycosides (Flannery et al., 2021).

One major challenge to tackling AMR is understanding the true burden of resistance and its epidemiological distribution. Neonatal clinicians must balance concerns of inadequate empirical coverage for suspected infection with the risks of indiscriminate antibiotic use. Currently, there is a paucity of contemporary, large-scale, neonatal-specific antibiotic susceptibility data for *E. coli* in China. Therefore, this study retrospectively analyzed the antimicrobial resistance characteristics of 370 clinical isolates of *E. coli* among infants admitted to NICUs across China, aiming to provide reasonable guidance for the use of antibiotics in neonatal infections.

2 Methods

2.1 Sample origin

Patients hospitalized in the NICUs between November 2015 and October 2020, aged ≤28 days, with *E. coli*-positive cultures from any of the specimens described below were included in this study. Specimens were collected from patients who matched these conditions: blood was collected from sepsis, cerebrospinal fluid was collected from meningitis, sputum was collected from lower respiratory tract infection, gastric fluid was collected from early-onset sepsis, ear secretions, umbilical cord secretions were collected for a routine test from neonates without systemic symptoms. A total of 370 *E. coli* strains isolated from clinical culture-positive specimens across China were ultimately included. The specimens were stored in a refrigerator at -20°C. The study was conducted in accordance with the Declaration of Helsinki, met all ethical requirements, and was approved by the ethics committee of Beijing Children's Hospital.

2.2 Identification of *E. coli* strains and antimicrobial susceptibility testing

MacConkey agar (CM00078) and chromogenic *E. coli* media (EC166) (Beijing Land Bridge Technology Co., Ltd., Beijing, China)

were used to isolate and identify *E. coli* strains. The *E. coli* strains were stored in the refrigerator at -80°C. The antimicrobial susceptibility tests was performed by the broth dilution method according to the instructions of the Sensititre TM Gram Negative GNX2F Plate (Thermo Fisher Scientific, USA) and included 21 antimicrobial agents, namely Imipenem, Ertapenem, Doripenem, Meropenem, Aztreonam, Ceftazidime, Cefotaxime, Cefepime, Ticarcillin/clavulanic acid, Piperacillin/tazobactam, Tobramycin, Gentamicin, Amikacin, Levofloxacin, Ciprofloxacin, Doxycycline, Tigecycline, Minocycline, Trimethoprim/sulfamethoxazole, Colistin, and Polymyxin B. Antimicrobial susceptibility testing results were classified as susceptible (S), intermediate (I), or resistant (R), in accordance with Clinical and Laboratory Standards Institute (CLSI), 2023 standards (Clinical and Laboratory Standards Institute. CLSI M100-ED33: 2023 Performance Standards for Antimicrobial Susceptibility Testing, 33rd Edition (2023). M100-ED33). ESBL phenotype, defined as any isolate with at least 1 nonsusceptibility result to cefotaxime, ceftazidime, or cefepime; and carbapenem-resistant Enterobacteriaceae, defined as any isolate with at least 1 nonsusceptibility result to imipenem, meropenem, doripenem, or ertapenem sodium. Definitions of ESBL and carbapenem-resistant Enterobacteriaceae were based on updated Centers for Disease Control and Prevention (CDC) definition (Flannery et al., 2021; Centers for Disease Control and Prevention, Antibiotic/antimicrobial resistance (AR/ARM): biggest threats and data: 2019. AR threats report. https://www.cdc.gov/DrugResistance/Biggest-Threats.html). *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, USA) was used for routine quality control.

2.3 DNA extraction

DNA was extracted using bacterial genomic DNA extraction kits (Tiangen Biotech Co., Ltd., Beijing, China). Briefly, after the bacteria were collected by centrifugation, the cell wall was removed by lysozyme digestion. DNA was released from the cell after adding the lysate and proteinase K. Then the binding solution was added to adjust the optimal binding conditions. Next, the solution was transferred to the purification column and centrifuged. DNA was bound to the filter membrane, and impurities such as proteins were filtered out into the filtrate. Residual contaminants and enzyme inhibitors were removed after two washing steps, and the DNA was finally eluted with a small amount of buffer.

2.4 Multi-locus sequencing typing

MLST was performed on all isolates. Seven housekeeping genes were targeted as follows: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *rec*A. The genes were amplified using polymerase chain reaction (PCR) and sent to Tiangen Biotech Co., Ltd., Beijing, China for sequencing. Allelic patterns of these genes were used to determine the sequence type. Sequence data were analyzed based on the *E. coli* MLST database (https://pubmlst.org/organisms/escherichia-spp).

2.5 Statistical analysis

Data analysis was performed by SPSS 27.0 (IBM SPSS, Chicago, IL, USA). The χ 2 test was performed for comparing antibiotic and multidrug resistance proportions of *E. coli* strains. Differences with $P < 0.05$ were considered statistically significant.

3 Results

3.1 General characteristics of *E. coli* strains

Among the 370 *E. coli* strains isolated from newborns aged less than 28 days, 104 were from blood, 31 from cerebrospinal fluid, 96 from sputum, 45 from gastric juice, and 94 from other secretions (ear secretions, umbilical cord secretions). The strains were divided into three groups according to the source, the invasive infection group (n=135) deriving from blood and cerebrospinal fluid, the respiratory tract infection (RTI) group (n=96) consisted of strains from sputum, and the others (n=139) being isolated from gastric juice, ear secretions, and umbilical cord secretions.

3.2 Antimicrobial susceptibility testing

In all the *E. coli* isolates, 315 were resistant to at least one antimicrobial drug (the total resistance rate was 82.68%), and the resistance rate of trimethoprim/sulfamethoxazole was the highest (55.68%, 206/370), followed by cefotaxime (46.22%; 171/370), ciprofloxacin (35.41%; 131/370). No *E. coli* strains were found to be resistant to polymyxin B. We defined multidrug resistance in *E. coli* as resistance to at least three distinct antibiotic families and estimated this rate at ~36.74% (136/370) across all isolates. More details about antimicrobial resistance rates were presented in Table 1. And a total of 132 (35.68%) *E. coli* strains had the ESBL phenotype and 5 (1.35%) strains had insensitivity to the carbapenem antibiotics tested (Table 2).

3.3 Resistance of strains from respiratory tract infection, invasive infection and others

According to distinct pathogenic characteristics and various isolation sites, we divided the strains into RTI group, invasive infection group, and others for comparison. *E. coli* isolated from sputum had generally higher resistance to commonly used antibiotics. Almost all those of the β-lactams and tetracyclines were statistically different between the three groups, with aztreonam, ceftazidime, cefotaxime, cefepime, ticarcillin/clavulanic acid, piperacillin/tazobactam, doxycycline, and minocycline were the most significant (P<0.001, Table 3).

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3.4 MLST results

Of the 370 *E. coli* neonatal isolates in this study, 313 strains (84.60%) were assigned to 85 known STs, and the remaining 57 strains were unknown ST. Among known STs, the most common sequence type was ST1193 (16.76%; 62/370), followed by ST95 (8.92%; 33/370), ST73 (6.49%; 24/370), ST69 (6.22%; 23/370), ST131 (5.42%; 20/370) and ST410 (4.05%; 15/370). ST410 had the highest multidrug resistance rate (80.00%; 13/16). The multidrug resistance rates of ST410, ST1193 and ST131 were much higher than those of ST95 and ST73 (Table 4).

3.5 Relationship between antimicrobial resistance and MLST

ST410, ST1193 and ST131 with the top three multidrug resistance rates were all sensitive to amikacin, and were highly sensitive to tigecycline, polymyxin B and colistin. ST410 isolates demonstrated the highest resistance rate to cefotaxime (86.67%; 13/15), and they were found to have serious resistance to β-lactam, quinolones and sulfonamides. The most common multidrug resistance pattern of ST410 was β-lactams + aminoglycosides + quinolones + tetracyclines + sulfonamides (Figure 1A). ST1193 exhibited a 90.32% resistance rate to levofloxacin and ciprofloxacin and the common patterns of resistance were β-lactams + quinolones + sulfonamides, aminoglycosides + quinolones + sulfonamides (Figure 1B). Among 20 ST131 isolates, 14 (70.00%) were resistant to gentamicin, with 12 resistant to ceftazidime (60.00%), and the most frequent multidrug resistance pattern was β-lactams + aminoglycosides + sulfonamides (Figure 1C) (Table 5).

TABLE 2 ESBL phenotype and carbapenem resistance status.

	ESBL phenotype[a]	Carbapenems[b]
No. of strains (%)	132 (35.68)	5 (1.35)

ESBL, extended-spectrum β-lactamase.
[a]Includes testing for cefotaxime, ceftazidime, and/or cefepime.
[b]Includes testing for imipenem, meropenem, doripenem, and/or ertapenem.
The bold values marked representative values with high resistance rates.

4 Discussion

E. coli is a gram-negative bacillus and resident of the normal intestinal microbiota. However, some pathogenic *E. coli* strains are capable of causing human disease, and can be broadly divided into

TABLE 1 Susceptibility of 370 *E. coli* strains to 21 antimicrobial agents (%).

Antimicrobial agent		R (%)	I (%)	S (%)
β-lactams	Imipenem	7 (1.89)	1 (0.27)	362 (97.84)
	Ertapenem	14 (3.78)	3 (0.81)	353 (95.41)
	Doripenem	8 (2.16)	1 (0.27)	361 (97.57)
	Meropenem	9 (2.43)	4 (1.08)	357 (96.49)
	Aztreonam	82 (22.16)	29 (7.84)	259 (70.00)
	Ceftazidime	46 (12.43)	33 (8.92)	291 (78.65)
	Cefotaxime	**171 (46.22)**	2 (0.54)	197 (53.24)
	Cefepime	47 (12.70)	3 (0.81)	320 (86.49)
	Ticarcillin/clavulanic acid	30 (8.11)	92 (24.86)	248 (67.03)
	Piperacillin/tazobactam	16 (4.32)	6 (1.62)	348 (94.05)
Aminoglycosides	Tobramycin	35 (9.46)	68 (18.38)	267 (72.16)
	Gentamicin	110 (29.73)	1 (0.27)	259 (70.00)
	Amikacin	3 (0.81)	1 (0.27)	366 (98.92)
Quinolones	Levofloxacin	126 (34.05)	152 (41.08)	92 (24.86)
	Ciprofloxacin	**131 (35.41)**	16 (4.32)	223 (60.27)
Tetracyclines	Doxycycline	110 (29.73)	95 (25.68)	165 (44.59)
	Tigecycline	1 (0.27)	0 (0.00)	369 (99.73)
	Minocycline	22 (5.95)	38 (10.27)	310 (83.78)
Sulfonamides	Trimethoprim/sulfamethoxazole	**206 (55.68)**	0 (0.00)	164 (44.32)
Polymyxins	Colistin	2 (0.54)	0 (0.00)	368 (99.46)
	Polymyxin B	0 (0.00)	0 (0.00)	370 (100.00)

The bold values marked representative values with high resistance rates.

two groups, extraintestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (InPEC) (Pokharel et al., 2023). In terms of morbidity and mortality, ExPEC has a great impact on neonatal health, and it has become a common causative agent of neonatal infections, especially invasive infections (Ejiofor et al., 2018). Several pandemics of *E. coli* strains, which are highly virulent and antibiotic resistant, have occurred in recent years (Yair and Gophna, 2018). In addition, selection pressures exerted by antibiotic use, overuse, and misuse are driving a gradual increase in antibiotic resistance and leading to the emergence of multidrug resistant bacterial strains, further accelerating the rate of spread of infection and the difficulty of treatment (Wu et al., 2021).

The emergence of multidrug-resistant *E. coli* has been reported in many countries. And the parallel increase in incidence and frequency of multidrug resistance has raised increasing concerns about the treatment of *E. coli* infections (Dunn et al., 2019). *E. coli* tested in this study generally had a high rate of resistance to commonly used antibiotics, with the highest rate of resistance for methotrexate/sulfamethoxazole (55.68%; 206/370), followed by cefotaxime (46.22%; 171/370), and ciprofloxacin (35.41%; 131/370). The Pediatric Surveillance of Infectious Diseases (ISPED) reported bacterial epidemiology and drug resistance in Chinese children in 2020: *E. coli* resistance to methotrexate/sulfamethoxazole was 54.0%, ceftazidime 49.0%, and ciprofloxacin 41.5%, which is broadly consistent with our findings (Fu et al., 2021). Methotrexate/sulfamethoxazole have been

TABLE 4 Multidrug resistance of major E. coli STs.

	ST1193	ST95	ST73	ST69	ST131	ST410
No. of strains (%)	**62 (16.76)**	33 (8.92)	24 (6.49)	23 (6.22)	20 (5.42)	15 (4.05)
No. of multidrug resistant strains (%)	37 (59.68)	8 (24.24)	6 (25.00)	9 (39.13)	13 (65.00)	**12 (80.00)**

Bolded values indicated the subtype with the highest number proportion (ST1193) and the subtype with the highest multi-drug resistance rate (ST410).

TABLE 3 Comparison of resistance between respiratory tract infection (RTI) group, invasive infection group and others.

Antimicrobial agent		RTI group n=96(%)	Invasive infection group n=135 (%)	Others n=139(%)	χ^2	*P*
β-lactams	Imipenem	3(3.1)[a]	4(3.0)[a]	0(0.0)[a]	*	0.083
	Ertapenem	9(9.4)[a]	5(3.7)[a,b]	0(0.0)[b]	13.712	0.001
	Doripenem	3(3.1)[a]	5(3.7)[a]	0(0.0)[a]	*	0.048
	Meropenem	5(5.2)[a]	4(3.0)[a,b]	0(0.0)[b]	*	0.015
	Aztreonam	39(40.6)[a]	23(17.0)[b]	12(8.6)[b]	37.489	**<0.001**
	Ceftazidime	22(22.9)[a]	12(8.9)[b]	8(5.8)[b]	17.900	**<0.001**
	Cefotaxime	70(72.9)[a]	49(36.3)[b]	33(23.7)[b]	58.742	**<0.001**
	Cefepime	28(29.2)[a]	7(5.2)[b]	7(5.0)[b]	40.889	**<0.001**
	Ticarcillin/clavulanic acid	15(15.6)[a]	6(4.4)[b]	5(3.6)[b]	14.744	**<0.001**
	Piperacillin/tazobactam	10(10.4)[a]	4(3.0)[a,b]	1(0.7)[b]	14.378	**<0.001**
Aminoglycosides	Tobramycin	11(11.5)[a]	12(8.9)[a]	11(7.9)[a]	0.878	0.645
	Gentamicin	26(27.1)[a]	32(23.7)[a]	48(34.5)[a]	4.084	0.130
	Amikacin	0(0)[a]	0(0)[a]	3(2.2)[a]	*	0.081
Quinolones	Levofloxacin	38(39.6)[a]	52(38.5)[a]	32(23.0)[b]	10.005	0.007
	Ciprofloxacin	40(41.7)[a]	51(37.8)[a,b]	36(25.9)[b]	7.387	0.025
Tetracyclines	Doxycycline	42(43.8)[a]	23(17.0)[b]	33(23.7)[b]	21.423	**<0.001**
	Tigecycline	0(0)[a]	1(0.7)[a]	0(0.0)[a]	*	0.624
	Minocycline	14(14.6)[a]	7(5.2)[b]	1(0.7)[b]	19.736	**<0.001**
Sulfonamides	Trimethoprim/ sulfamethoxazole	57(59.4)[a]	77(57.0)[a]	72(51.8)[a]	1.480	0.477
Polymyxins	Colistin	1(1.0)[a]	1(0.7)[a]	0(0.0)[a]	*	0.530
	Polymyxin B	0(0)[a]	0(0)[a]	0(0)[a]	*	1.000

* Fisher's Exact Test.
The bold values indicate antibiotics with significant differences among the three groups (p<0.001).
Different small letters represent statistically different between groups.

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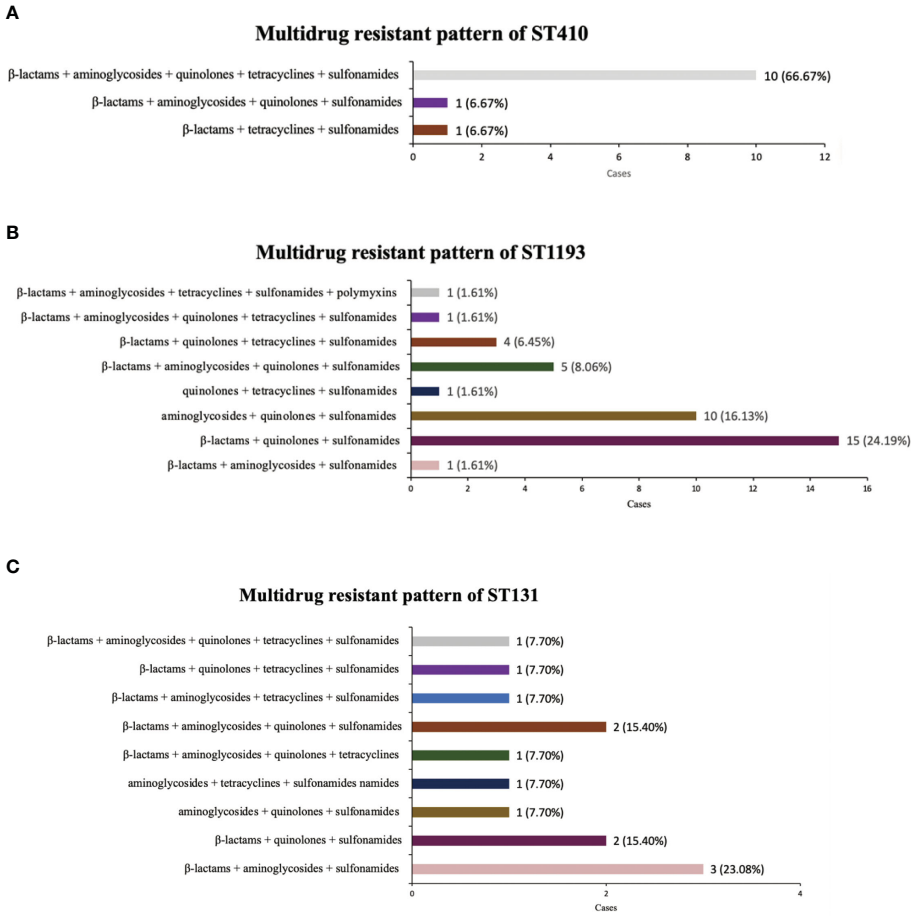

FIGURE 1
Multi-antibiotics resistance patterns of ST410, ST1193, ST131. **(A)** Composition of antibiotics resistance patterns of ST410. **(B)** Composition of antibiotics resistance patterns of ST1193. **(C)** Composition of antibiotics resistance patterns of ST131.

used for decades as effective and inexpensive antimicrobial agents in animals and humans, but extensively resistance has spread widely and rapidly due to the horizontal spread of *sul*1 and *sul*2 genes and expressing dihydropteroate synthases highly resistant to sulfonamide. It is rarely used at present (Sköld, 2001). Notably, the total of 370 neonatal *E. coli* isolates had a multidrug resistance rate of 36.74%, with 35.68% having ESBL phenotype and 5 strains (1.35%) resistant to carbapenem antibiotics. In a cohort study by Flannery et al. of neonatal *E. coli* samples admitted to multicenter NICUs across the United States from 2009 to 2017 (Flannery et al., 2021), cefazolin resistance was 17.1% and ciprofloxacin was 10.2%, with only 5.0% of isolates meeting ESBL phenotype criteria and no resistance to carbapenems was observed. This difference may be due to the different sources of specimens, but it also shows that the form of drug resistance is more severe in China compared to developed countries.

ESBL and CRE have become more prevalent in pediatric patients and are strongly associated with a poorer clinical prognosis, but surveillance data and evidence of medication use for the neonatal population are currently inadequate (Chiotos et al., 2020). Emergence of ESBL-producing Enterobacterales and CRE in neonatal settings is particularly worrisome because such infections may be resistant to most or all conventional antibiotics (Flannery et al., 2022). Horizontal transfer of plasmid-borne ESBL genes causes resistance of *E. coli* to β-lactam antibiotics such as cephalosporin, and cephalosporin treatment failure is a serious problem in infection control worldwide. (Dunn et al., 2019; Ibrahim et al., 2023). A minority of *E. coli* strains were found to be resistant to carbapenem antibiotics such as ertapenem, donipenem, meropenem and imipenem in our study. Carbapenem resistance can result from several mechanisms including, porins coupled with ESBL production, membrane permeability changes *via* mutations in efflux pumps or by hydrolysis of the beta-lactam ring by dedicated carbapenemase enzymes (Paitan, 2018). Treatment options for multidrug-resistant *E. coli* (especially ESBL and CRE) infections in neonates are severely limited. Therefore, there is an urgent need to focus on surveillance, prevention, and management of ESBL and CRE to improve the accuracy of diagnosis of neonatal infections and the rationality of antibiotic use.

We found that *E. coli* with different pathogenicity or isolation sites also differed in drug resistance characteristics. *E. coli* isolated from sputum were generally more resistant to commonly administered antibiotics than those isolated from other sites –even blood and cerebrospinal fluid. Among them, aztreonam, ceftazidime, cefotaxime, cefepime, ticarcillin/clavulanic acid, piperacillin/tazobactam, doxycycline, and minocycline were the most significant ($P < 0.001$). In an adult epidemiological survey conducted in France, pneumonia-specific *E. coli* were also found to be more resistant than

TABLE 5 Susceptibility of major *E. coli* STs to 21 antimicrobial agents (%).

Antimicrobial agent		ST1193 (%)	ST131 (%)	ST410 (%)
β-lactams	Imipenem	1 (1.61)	0 (0.00)	3 (20.00)
	Ertapenem	0 (0.00)	0 (0.00)	9 (60.00)
	Doripenem	1 (1.61)	0 (0.00)	4 (26.67)
	Meropenem	0 (0.00)	0 (0.00)	6 (40.00)
	Aztreonam	4 (6.45)	4 (20.00)	10 (66.67)
	Ceftazidime	5 (8.06)	4 (20.00)	11 (73.33)
	Cefotaxime	28 (45.16)	**12 (60.00)**	**13 (86.67)**
	Cefepime	4 (6.45)	3 (15.00)	10 (66.67)
	Ticarcillin/clavulanic acid	2 (3.23)	1 (5.00)	10 (66.67)
	Piperacillin/tazobactam	0 (0.00)	0 (0.00)	12 (80.00)
Aminoglycosides	Tobramycin	6 (9.68)	3 (15.00)	9 (60.00)
	Gentamicin	21 (33.87)	**14 (70.00)**	4 (26.67)
	Amikacin	0 (0.00)	0 (0.00)	0 (0.00)
Quinolones	levofloxacin	**56 (90.32)**	7 (35.00)	11 (73.33)
	Ciprofloxacin	**56 (90.32)**	9 (45.00)	11 (73.33)
Tetracyclines	Doxycycline	11 (17.74)	7 (35.00)	12 (80.00)
	Tigecycline	1 (1.61)	0 (0.00)	0 (0.00)
	Minocycline	1 (1.61)	0 (0.00)	9 (60.00)
Sulfonamides	Trimethoprim/sulfamethoxazole	47 (75.81)	13 (65.00)	13 (86.67)
Polymyxins	Colistin	1 (1.61)	0 (0.00)	0 (0.00)
	Polymyxin B	0 (0.00)	0 (0.00)	0 (0.00)

The bold values marked representative values with high resistance rates.

commensal isolates to all antimicrobial drugs tested except amikacin and more resistant to cefotaxime and cefoxitin compared to bacteremia isolates (La Combe et al., 2019). *E. coli* is one of the most genetically versatile microorganisms and has the high plasticity of the genome which gives it a tremendous capacity for evolution, resulting in the acquisition of drug resistance genes and virulence factors (Pokharel et al., 2023). Several studies have found that *E. coli* strains containing all virulence genomes had a lower resistance phenotype than that observed in non-virulent *E. coli* strains, and this may be related to the regulation of the bacterial genome (Čurová et al., 2020). As we know, meningitis-associated *E. coli* crosses the blood-brain barrier requiring a combination of virulence factors, such as *OmpA*, *Ibe*, *CNF1* (Yang et al., 2023). We therefore hypothesize that the reason why strains causing invasive infections were instead less resistant than those separated from sputum may be related to the complex regulation between virulence and drug resistance. Moreover, the clinical use of antibiotics has had a selective effect on drug-resistant bacteria (Davies and Davies, 2010). Hospitalized neonates are often in need of respiratory support, which, combined with their immature immune development, makes them highly susceptible to pneumonia. Neonatal pneumonia can be fatal and challenging to diagnose, so empirical antibiotic therapy is often initiated early, further leading to a preferential proliferation of antibiotic-resistant strains in the respiratory tract (Vishnu Bhat and Adhisivam, 2018).

ExPEC strains are comprised of many lineages. MLST is a nucleic acid sequencing-based genotyping method that has been widely used in the study of *E. coli* and the identification of ExPEC-related clonal complexes or lineages. Different ST types have different drug resistance and virulence characteristics (Vanstokstraeten et al., 2022). Manges et al. (2019) using meta-analysis described the type, evolution, distribution and characteristics of ExPEC, which showed that ST131 had the highest proportion, and other major lineages included ST69, ST95, ST10, ST405, ST73, ST410, and ST1193. Among the 370 strains of *E. coli* in the current study, ST1193 (16.76%) was the most prevalent, followed by ST95 (8.92%). In some regions, ST1193 had emerged as a new virulent clone of fluoroquinolone-resistant *E. coli* in several countries (Pitout et al., 2022), and it was also isolated from blood and cerebrospinal fluid specimens of Chinese newborns (Ding et al., 2021). In the bacteremia *E. coli* isolates from newborns in the United States, ST95 and ST131 prevailed; ST1193 emerged recently (Cole et al., 2019), with some similarity to our study, suggesting that there may be an epidemic spectrum in the neonatal population that differs from the adult population.

In this study, we also statistically compared the drug resistance characteristics of isolates from different STs. It is noteworthy that ST410, ST1193 and ST131 had significantly higher multidrug resistance rates than ST95 and ST73, and there was a certain pattern in their resistance profile. ST410 had the most serious multidrug-resistant situation, with very high resistance to β-lactams and quinolones, and its most common multidrug resistance pattern was β-lactams + aminoglycosides + quinolones + tetracyclines + sulfonamides. ST1193 showed the most significant resistance to quinolones (90.32%), in agreement with Johnson et al. (2019), and the common multidrug resistance pattern was β-lactams + quinolones + sulfonamides. ST131 isolate had the highest rate of resistance to gentamicin (70%). Usually, ST131 are reported to produce ESBLs, such as CTX-M-15, and almost all are resistant to fluoroquinolones (Nicolas-Chanoine et al., 2014). The difference may be due to the small number of ST131 *E. coli* isolated in this study, which was not representative enough.

There is an enormous public health burden due to *E. coli* multidrug-resistant high-risk clones such as ST1193, ST131 and ST410. These clones have played pivotal roles in the global spread of AMR. It is notable that ST410, as an emerging multidrug-resistant clone, should raise more serious concerns and be monitored more closely. The results of this study showed the higher level of resistance to fluoroquinolones, cephalosporins, and carbapenems in ST410 compared to other ST types. ST410 belongs to 2 clades namely antimicrobial susceptible ST410-A and ST410-B. Clade B is divided into the following subclades: ST410-B1, ST410-B2 that is associated with fluoroquinolone resistance and *bla*CTX-M-15, while ST410-B3 is linked with fluoroquinolone resistance, *bla*CTX-M-15 and *bla*OXA-181 (Roer et al., 2018). Genomic analysis of ST410 by Chen et al. (2022) revealed that ST410-B2 and ST410-B3, which are resistant to fluoroquinolones, contained identical quinolone resistance determining region (QRDR) mutations, and that the acquisition of these QRDR mutations may be due to a single multiple allele homologous recombination event. And the prevalence of the plasmid-mediated fluoroquinolone resistance determinant *aac(6)-Ib-cr)* was high among ST410 isolates (>90%), especially among the ST410-B3 subclade. Furthermore, the most common ESBLs type in ST410 was CTX-M-15, and the CTX-M-15 genes was mainly carried on the IncF plasmids to move within and between different strains or clones (Pitout et al., 2023). OXA-181 and NDM-5 were the most frequent carbapenemases in ST410 and specifically linked with the ST410-B3 subclade (Roer et al., 2018). The OXA-181 genes were located on near identical broad-host range IncX3 plasmids and NDM-5 genes were located on mosaic narrow-host range IncFII plasmids (i.e. F1:A1:B49) that contained various AMR genes including *bla*CTX-M-15. ST410 high-risk clones acquired MDR determinants (i.e., fluoroquinolone resistance, CTX-M enzyme, carbapenemase) in a stepwise pattern, acting as "hoarders and transmitters" of AMR genes through horizontal and vertical transmission, which together lead to a high level of resistance and risk in ST410. The specimens in our study were obtained from tertiary care hospitals across China, and contained detailed antibiotic susceptibility data for 370 neonatal-specific *E. coli* isolates, which makes the analysis of the prevalence of *E. coli* in Chinese neonates very representative. Nevertheless, this study has some limitations. We only analyzed the antibiotic resistance and susceptibility characteristics of *E. coli* and lacked further classification and analysis of clinical symptoms and diagnostic information. Records on antibiotic dose or frequency of administration and detailed patient-level data, such as maternal antibiotic exposure, gestational age, and mode of delivery, were not available; therefore, we were unable to include these variables in the adjusted analysis.

In conclusion, the results of this study suggest that resistance of *E. coli* clinically isolated from neonates hospitalized in NICUs across China was severe, with a substantial proportion of isolates found to be insensitive to commonly used antibiotics. Particular attention should be paid to the monitoring and management of ESBL-type *E. coli* and strains resistant to carbapenem antibiotics. The resistance phenotype of *E. coli* varied by pathogenicity and by site, with lower respiratory tract infection such as neonatal pneumonia was generally more resistant to antibiotics. Currently, the main prevalent sequence types in the neonatal population in China were ST1193 and ST95, but multidrug resistance was most severe with ST410. Different MLST types existed with different antibiotic resistance patterns, suggesting that the antibiotic resistance characteristics of *E. coli* can be inferred from MLST results.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Beijing Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

RX drafted the manuscript and did the statistical analysis. YL and XL aggregated and analyzed the data. YJD, JL, YFL and WK completed the data curation, investigation, validation. PZ, JW performed experimental operation and searched for literature research. YD, JZ analyzed the data and edited the manuscript. All authors contributed to the article and approved the submitted version. YW conceptualized and designed the study.

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Conflict of interest

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

Publisher's note

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Novel mechanisms of macrolide resistance revealed by *in vitro* selection and genome analysis in *Mycoplasma pneumoniae*

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Mycoplasma pneumoniae is an important pathogen causing upper and lower respiratory tract infections in children and other age groups. Macrolides are the recommended treatments of choice for *M. pneumoniae* infections. However, macrolide resistance in *M. pneumoniae* is increasing worldwide, which complicates the treatment strategies. The mechanisms of macrolide resistance have been extensively studied focusing on the mutations in *23S rRNA* and ribosomal proteins. Since the secondary treatment choice for pediatric patients is very limited, we decided to look for potential new treatment strategies in macrolide drugs and investigate possible new mechanisms of resistance. We performed an *in vitro* selection of mutants resistant to five macrolides (erythromycin, roxithromycin, azithromycin, josamycin, and midecamycin) by inducing the parent *M. pneumoniae* strain M129 with increasing concentrations of the drugs. The evolving cultures in every passage were tested for their antimicrobial susceptibilities to eight drugs and mutations known to be associated with macrolide resistance by PCR and sequencing. The final selected mutants were also analyzed by whole-genome sequencing. Results showed that roxithromycin is the drug that most easily induces resistance (at 0.25 mg/L, with two passages, 23 days), while with midecamycin it is most difficult (at 5.12 mg/L, with seven passages, 87 days). Point mutations C2617A/T, A2063G, or A2064C in domain V of *23S rRNA* were detected in mutants resistant to the 14- and 15-membered macrolides, while A2067G/C was selected for the 16-membered macrolides. Single amino acid changes (G72R, G72V) in ribosomal protein L4 emerged during the induction by midecamycin. Genome sequencing identified sequence variations in *dnaK*, *rpoC*, *glpK*, *MPN449*, and in one of the *hsdS* (*MPN365*) genes in the mutants. Mutants induced by the 14- or 15-membered macrolides were resistant to all macrolides, while those induced by the 16-membered macrolides (midecamycin and josamycin) remained susceptible to the 14- and 15-membered macrolides. In summary, these data demonstrated that midecamycin is less potent in inducing resistance than other macrolides, and the induced resistance is restrained to the 16-membered macrolides, suggesting a potential benefit of using midecamycin as a first treatment choice if the strain is susceptible.

KEYWORDS

***Mycoplasma pneumoniae*, macrolides, midecamycin, resistance mechanisms, mutation**

1 Introduction

Mycoplasma pneumoniae (*M. pneumoniae*) is a small, cell wall-less, and pleomorphic bacterium which belongs to the order Mycoplasmatales, family Mycoplasmataceae, and class Mollicutes (Waites and Talkington, 2004; Waites et al., 2017). It is one of the major mucosal pathogens of the respiratory tract that causes infectious diseases in humans, especially in school-age children and adolescents (Atkinson et al., 2008). Additionally, some researchers have observed that a portion of children typically carry *M. pneumoniae* within the upper respiratory tract asymptomatically (Spuesens et al., 2013; Meyer Sauteur et al., 2016). Under certain conditions, this "atypical" bacterium has also been reported to be responsible for many extrapulmonary manifestations, such as hematologic disorders and central nervous system and dermatological diseases, that can range in severity from mild to life-threatening (Waites et al., 2017).

Because of their lack of a cell wall, *M. pneumoniae* is innately resistant to many classes of antimicrobial agents that act on the cell wall (Waites et al., 2017; Lee et al., 2018). Effective antimicrobials against *M. pneumoniae* include fluoroquinolones (levofloxacin, ciprofloxacin, and moxifloxacin) that inhibit DNA replication and macrolides (erythromycin, azithromycin, and josamycin) and tetracyclines (minocycline and doxycycline) that both inhibit protein synthesis (Waites et al., 2017; Gautier-Bouchardon, 2018). Owing to the potential toxicities of fluoroquinolones and tetracyclines, macrolides are used as the first-line therapy for *M. pneumoniae* infections, particularly in children (Waites et al., 2017). Azithromycin is one of the most commonly used medications throughout the world because of its great tolerance and longer half-life (Waites et al., 2017). However, many studies have reported the increase of macrolide-resistant *M. pneumoniae* (MRMP) strains and related treatment failure worldwide since the early 2000s (Okazaki et al., 2001; Okazaki et al., 2007; Kawai et al., 2012; Kawai et al., 2013; Waites et al., 2017), and over 90% isolates were MRMP in some regions of China and Japan (Liu et al., 2012; Komatsu et al., 2014; Zhou et al., 2015; Wang et al., 2022). Currently, there is no safe and effective secondary treatment option for young children once macrolide resistance develops.

The resistance mechanisms of MRMP have been demonstrated by *in vitro* selection that involves point mutations in the peptidyl transferase loop of *23S rRNA* and point mutations, insertions, or deletions in ribosomal proteins L4 and L22 (Lucier et al., 1995; Pereyre et al., 2004; Ou et al., 2015; Waites et al., 2017). Some of these changes were observed in clinical MRMP isolates (Morozumi et al., 2005; Xin et al., 2009; Wang et al., 2019), while some were not (Pereyre et al., 2004; Ou et al., 2015), probably because these MRMP strains were induced by subminimum inhibitory concentrations of macrolide antibiotics. The investigation of macrolide resistance mechanisms in *M. pneumoniae* has mainly been focused on the single *23S RNA* gene and ribosomal protein genes, and so further analysis on other potential resistance mechanisms is required. To better understand macrolide resistance in *M. pneumoniae* and make better usage of currently available macrolide drugs, we performed an *in vitro* study to select macrolide-resistant mutants in the parent strain by using increasing concentrations of five macrolides and characterized the final mutants by whole-genome sequencing.

2 Materials and methods

2.1 Bacterial strains, growth conditions, and antibiotics

The macrolide-susceptible reference strain *M. pneumoniae* M129 (ATCC 29342) was used as the parent strain to select macrolide-resistant mutants. Before induction, M129 colonies were clone-purified and inoculated at 37°C in mycoplasma broth for 5–7 days until a color change (pink to orange-yellow) occurred. The cultures were then aliquoted and stored at −70°C. As previously reported by Sun et al., mycoplasma broth was prepared using mycoplasma broth base CM403 (Oxoid, Hampshire, United Kingdom), mycoplasma selective supplement G SR59 (Oxoid), 0.002% phenol red, and 0.5% glucose. Mycoplasma agar plates, containing mycoplasma agar base CM401 (Oxoid) and mycoplasma selective supplement G SR59, were also prepared (Sun et al., 2008). Five macrolides and three other drugs were included in the study. Erythromycin and roxithromycin as 14-membered ring macrolides were purchased from the National Institutes for Food and Drug Control (Shanghai, China). Azithromycin (15-membered ring macrolide) and josamycin and midecamycin (16-membered ring macrolides) were purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Tetracycline and moxifloxacin were also from the National Institutes for Food and Drug Control (Shanghai, China). Nemonoxacin was provided by Zhejiang Medicine Co., Ltd. (Xinchang, China).

2.2 Selection of macrolide-resistant *Mycoplasma pneumoniae* mutants and mutation detection

The *in vitro* selection of macrolide-resistant mutants was conducted according to the previously described methods with minor modifications (Reinhardt et al., 2002; Tatay-Dualde et al., 2017). Briefly, the minimum inhibitory concentrations (MICs) of the parent strain to the five macrolides were determined (Waites et al., 2011). The induction inocula contained 1 ml of organisms (approximately 5×10^4 CFU/ml) and each macrolide drug at four concentrations in a two-fold increasing step (0.00064, 0.00128, 0.00256, and 0.00512mg/L) initially (first step). When the medium color changed from red to yellow, approximately 0.1 ml of the culture from the tubes with the most concentrated drugs was transferred into a new culture tube containing the next serial concentrations of the drugs (starting from the highest concentration showing color change from the first step). The remaining cultures were aliquoted and stored at −80°C for further analysis. One aliquot was processed for antimicrobial susceptibility testing and one was used for PCR assays. The selection step was repeated as described above until the inducted strain was confirmed

resistant to the macrolide of induction by antimicrobial sensitivity testing.

The point mutations in the *23S rRNA* gene were detected by nested PCR using a previous method (Sun et al., 2013). The ribosomal protein genes *rplD* (L4) and *rplV* (L22) were amplified using primer pairs described previously (Pereyre et al., 2004). Sanger sequencing was performed on the amplicons, and sequences were compared with those of the reference strain.

2.3 Antimicrobial susceptibility test

The MICs of eight drugs consisting of five macrolides, two quinolones, and tetracycline were determined using the broth microdilution method, according to the recommendation of the CLSI (Waites et al., 2011).

2.4 Whole-genome sequencing and sequence variation detection

Genomic DNA from 50 ml of the end selected culture and the parent strain was extracted using the TIANamp Bacteria DNA Kit (DP302) from Tiangen Biochemical Technology (Beijing) Co., Ltd., China, according to the manufacturer's instructions. Genome sequencing was performed by Shanghai Yuanxu Biotechnology Co., Ltd., China. Briefly, genomic DNA was sheared using KAPA Frag Kit for Enzymatic fragmentation (KK8600, Kapa Biosystems, Wilmington, MA, USA), and sequencing libraries were constructed *via* VAHTS Universal DNA Library Prep Kit for Illumina V4 (ND610-02, Vazyme, Nanjing, China). Pair-end libraries were sequenced on HiSeq X Ten platforms with a read length of 150 bp. Genome reads were deposited to the Sequence Read Archive (SRA, BioProject ID PRJNA954979).The average number of reads per sample was 7,178,870 with more than 200× coverage on average. Sequences were analyzed using CLC Genomics Workbench 23 (Qiagen, Valencia, CA, USA). *Mycoplasma pneumoniae* M129-B7 reference chromosome (GenBank accession number NC_020076) was used to map the reads, and sequence variations, including single nucleotide variants (SNVs), insertions, deletions, and replacements, were detected using the Basic Variant Detection tool. The resulting sequence variations in the mutants were then compared with that of the parent strain and differences were recorded.

3 Results

3.1 Selection of macrolide-resistant mutants

The parent reference strain M129 was susceptible to all drugs tested, with MICs equal to or lower than 0.015 mg/L for the five macrolides and 0.125 mg/L for quinolones and tetracycline (Table 1). Mutants resistant to macrolides were successfully selected by serial passages of the parent strain in the increasing concentrations of erythromycin, roxithromycin, azithromycin, josamycin, and midecamycin. The induction took two to seven passages or 23–87 days for different macrolides. Changes of the MICs and the emergence of mutations in the *23S rRNA* gene and *rplD* were observed during the passages (Table 1). The MICs of the final selected mutants were increased 1,067- to 160,000-fold compared with the parent strain. The selected mutants displayed different MIC characters for the five macrolides, while no changes for quinolones and tetracycline were observed.

3.1.1 Stepwise evolution toward 14-membered ring macrolide resistance

The selection of erythromycin-resistant mutants took 52 days with five passages (Table 1). Resistance and mutations were not detected in the first three passages under the influence of erythromycin concentrations equal to or lower than 0.0256 mg/L (E1–3). At the fourth passage (E4), mutation C2617A in the *23S rRNA* gene emerged, accompanied by slightly increased MICs for erythromycin and roxithromycin (0.125 and 0.5 mg/L). At the fifth passage (E5), A2063G in *23S rRNA* emerged in addition to C2617A, and MICs were increased for all macrolides (>128 mg/L for erythromycin and roxithromycin, 128 mg/L for azithromycin, 64 mg/L for josamycin, and 32 mg/L for midecamycin). The selection of roxithromycin-resistant mutants was the quickest, only taking 23 days and two passages (Table 1). At the second passage (R2), mutation A2064C in the *23S rRNA* gene appeared, and MICs were also increased for all macrolides (>128 mg/L for erythromycin and roxithromycin, 4 mg/L for azithromycin, 64 mg/L for josamycin, and 128 mg/L for midecamycin).

3.1.2 Stepwise evolution toward 15-membered ring macrolide resistance

The selection of azithromycin-resistant mutants took 58 days and four passages (Table 1). Unfortunately, frozen culture aliquots from the second and third passages (A2 and A3) failed to be recovered, and their MICs were not available for them. However, the *23S rRNA* mutations C2617T were detected in passage 2 and C2617A in passage 3. In the fourth passage (A4), A2063G substitution was detected, and increased MICs to all macrolides were observed (>128 mg/L for erythromycin and roxithromycin, 32 mg/L for azithromycin, and 4 mg/L for josamycin and midecamycin).

3.1.3 Stepwise evolution toward 16-membered ring macrolide resistance

The selection of josamycin-resistant mutants was quick, taking 28 days and two passages (Table 1). At the second passage (J2), mutation A2067G in the *23S rRNA* gene emerged, and MICs were increased for the 16-membered macrolides josamycin (16 mg/L) and midecamycin (64mg/L). Interestingly, MICs for the 14- and 15-membered macrolides were still low (≤0.125 mg/L).

The selection of midecamycin-resistant mutants took the longest induction time (87 days), the greatest number of passages (seven), and the highest induction drug concentration (5.12 mg/L). Resistance and mutations were not detected in the first two passages (M1 and M2). From the third to the seventh passages (M3–M7),

TABLE 1 Characteristics of macrolide-resistant *Mycoplasma pneumoniae* mutants collected at each selection step.

Macrolides (total induction days)	Passages	Incubation days	Concentration (mg/L)	Mutations			MICs (mg/L)							
				23S rRNA	*rplD*	*rplV*	ERY	ROX	AZI	JOS	MID	NEM	MOX	TET
–	Parent strain	–	–	–	–	–	0.0025	≤0.00375	0.0002	0.015	0.015	0.125	0.125	0.125
ERY (52 days)	E1	5	0.00064	–	–	–	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.125	0.125	0.125
	E2	4	0.00512	–	–	–	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.125	0.125	0.125
	E3	9	0.0256	–	–	–	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.125	0.125	0.125
	E4	21	0.1024	C2617A	–	–	0.125	0.5	≤0.06	≤0.06	≤0.06	0.125	0.06	0.125
	E5	13	0.64	C2617A, A2063G	–	–	>128	>128	128	64	32	0.125	0.125	0.125
ROX (23 days)	R1	5	0.00512	–	–	–	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.125	0.125	0.125
	R2	18	0.25	A2064C	–	–	>128	>128	4	64	128	0.25	0.25	0.125
AZI (58 days)	A1	11	0.00128	–	–	–	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.125	0.125	0.125
	A2[a]	26	0.00512	C2617T	–	–	–	–	–	–	–	–	–	–
	A3[a]	15	0.0512	C2617A	–	–	–	–	–	–	–	–	–	–
	A4	6	0.512	A2063G	–	–	>128	>128	32	4	4	0.125	0.125	0.25
JOS (28 days)	J1	5	0.005	–	–	–	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.125	0.125	0.125
	J2	23	1.024	A2067G	–	–	≤0.06	0.125	≤0.06	16	64	0.125	0.125	0.125
MDI (87 days)	M1	5	0.00512	–	–	–	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.125	0.125	0.125
	M2	7	0.032	–	–	–	≤0.06	≤0.06	≤0.06	0.125	≤0.06	0.125	0.125	0.125
	M3	17	0.128	–	G214A (G72R)	–	≤0.06	0.125	≤0.06	0.5	0.5	0.125	0.125	0.125
	M4	9	0.128	–	G214A (G72R)	–	≤0.06	0.125	≤0.06	0.5	0.5	0.125	0.125	0.125
	M5	18	0.256	–	G214A (G72R)	–	0.125	0.25	≤0.06	0.5	0.5	0.125	0.125	0.125
	M6	12	0.512	–	G215T (G72V)	–	0.125	0.25	≤0.06	1	0.5	0.125	0.125	0.125
	M7	19	5.12	A2067C	G214A (G72R)	–	0.125	0.125	≤0.06	32	64	0.125	0.125	0.125

ERY, erythromycin; ROX, roxithromycin; AZI, azithromycin; JOS, josamycin; MID, midecamycin; TET, tetracycline; MOX, moxifloxacin; NEM, nemonoxacin.

[a]Failure to resuscitate the strains in the second and third passages of induced resistance by azithromycin. "-" in column "Mutations" means "No mutation detected"; in column (MICs) means "Not available".

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different single point mutations in ribosomal protein L4 gene *rplD* were detected sequentially (nucleotide changes: G214A in M3–M5 and M7, G215T in M6). Slightly increased MICs for midecamycin (0.5 mg/L) and josamycin (0.5 or 1.0 mg/L) were observed in passages M3 to M6. In the last passage (M7), mutation A2067C in the *23S rRNA* gene emerged in addition to the L4 mutation, and the corresponding MICs for josamycin and midecamycin were increased to 32 and 64 mg/L, respectively. Similar to josamycin, the MIC values for the 14- and 15-membered macrolides also remained low (≤0.125 mg/L).

3.2 Genome-wide analysis of selected mutants

The genomes of the parent strain M129 and the five selected mutants were partially assembled, and each genome contained 5–13 contigs. The genome size of the parent strain M129 was 816,516 bp, and the range of the five selected mutants was 813,065 to 815,897 bp, with a GC content of approximately 40% for all genomes (Supplementary Table 1). Whole-genome alignment analysis revealed that they were highly similar with the average nucleotide identity (ANI) ≥99.99% and alignment percentage (AP) ≥97.15%.

There were 7–11 sequence variations per genome in the final selected mutants (Table 2). The variations were distributed in 15 genes and four intergenic regions. Mutations previously detected by PCR in the *23S rRNA* gene and *rplD* were also identified by the genome-wide variant detection. Non-synonymous sequence variations were found in 12 protein-coding genes, namely, *dnaK* (encodes a chaperone protein Hsp70, also named *MPN434* in genome NC_000912), *rpoC* (encodes DNA-directed RNA polymerase beta, *MPN515*), *glpK* (encodes glycerol kinase, *MPN050*), *C985_RS03055* (encodes L-ribulose-5-phosphate 4-epimerase, *MPN498*), *C985_RS02085* (encodes type I restriction-modification system, specificity subunit S, *MPN365*), *C985_RS03730* (encodes YitT family protein, *MPN657*),

TABLE 2 Genetic alterations of genes in mutants.

Mutant	Region	Type	Reference	Allele	Gene name/ Locus Tag	Gene alteration	Amino acid alteration	Function
Parent	62792	SNV	G	T	*glpK*	C710A	T237N	Glycerol kinase (EC 2.7.1.30)
Parent	605306	SNV	C	T	C985_RS03055	C352T	L118F	L-ribulose-5-phosphate 4-epimerase
R2	122095	SNV	A	C	C985_RS00540	A2064G		23S rRNA
R2	167971	SNV	C	T	C985_RS00770	C374T	S125L	Hypothetical protein
R2	436118^436119	Insertion	–	CCGAGCTAAGCG	C985_RS02085	Insertion 454GAGCTAAGCGCC	152ELSA	Type I restriction-modification system, specificity subunit S
R2	497605	SNV	C	A				Intergenic region (C985_RS02375 and C985_RS04390)
R2	547563	SNV	A	G	C985_RS02560	T1172C	I391T	Hypothetical protein
R2	605306	SNV	C	T	C985_RS03055	C352T	L118F	L-ribulose-5-phosphate 4-epimerase
R2	622660.622661	Deletion	TT	–				Intergenic region (between C985_RS02925 and C985_RS02930)
E5	62792	SNV	G	T	*glpK*	C710A	T237N	Glycerol kinase (EC 2.7.1.30)
E5	122094	SNV	A	G	C985_RS00540	A2063G		23S rRNA
E5	122648	SNV	C	A	C985_RS00540	C2617A		23S rRNA
E5	195426	Deletion	A	–				Intergenic region (between C985_RS04175 and C985_RS00880)

(Continued)

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TABLE 2 Continued

Mutant	Region	Type	Reference	Allele	Gene name/ Locus Tag	Gene alteration	Amino acid alteration	Function
E5	528862^528863	Insertion	–	T				Intergenic region (between C985_RS02495 and C985_RS02500)
E5	548224	SNV	G	T	C985_RS02560	G511A	R171S	Hypothetical protein
E5	605306	SNV	C	T	C985_RS03055	C352T	L118F	L-ribulose-5-phosphate 4-epimerase
E5	629581	SNV	C	T	*rpoC*	G2761A	V921M	DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6)
E5	782762	SNV	C	G	C985_RS03730	G380C	R127P	YitT family protein
A4	62792	SNV	G	T	*glpK*	C710A	T237N	Glycerol kinase (EC 2.7.1.30)
A4	122094	SNV	A	G	C985_RS00540	A2063G		23S rRNA
A4	522684	SNV	C	T	*dnaK*	G997A	V333M	Chaperone DnaK
A4	538445	SNV	C	T	C985_RS02535	G3351A	Synonymous	DUF3713 domain-containing protein
A4	547899	SNV	C	A	C985_RS02560	G836T	S279I	Hypothetical protein
A4	605306	SNV	C	T	C985_RS03055	C352T	L118F	L-ribulose-5-phosphate 4-epimerase
A4	782783	SNV	G	A	C985_RS03730	C359T	S120F	YitT family protein
J2	62792	SNV	G	T	*glpK*	C710A	T237N	Glycerol kinase (EC 2.7.1.30)
J2	122098	SNV	A	G	C985_RS00540	A2067G		23S rRNA
J2	497605	SNV	C	A				Intergenic region (C985_RS02375 and C985_RS04390)
J2	497625	SNV	A	C	C985_RS04390	A17C	Stop to S6	Hypothetical protein
J2	497629.497630	MNV	AA	CG	C985_RS04390	A21C, A22G	W7C, R8G	Hypothetical protein
J2	497634	SNV	G	C	C985_RS04390	G26C	R9P	Hypothetical protein
J2	497638	SNV	A	C	C985_RS04390	A30C	Synonymous	Hypothetical protein
J2	527357	SNV	C	G	C985_RS02495	G1311C	Synonymous	DUF3713 domain-containing protein
J2	528862^528863	Insertion	–	T				Intergenic region (between C985_RS02495 and C985_RS02500)
J2	548224	SNV	G	T	C985_RS02560	C511A	R171S	Hypothetical protein
J2	605306	SNV	C	T	C985_RS03055	C352T	L118F	L-ribulose-5-phosphate 4-epimerase
M7	62792	SNV	G	T	*glpK*	C710A	T237N	Glycerol kinase (EC 2.7.1.30)
M7	122098	SNV	A	C	C985_RS00540	A2067C		23S rRNA

(Continued)

TABLE 2 Continued

Mutant	Region	Type	Reference	Allele	Gene name/ Locus Tag	Gene alteration	Amino acid alteration	Function
M7	219011	SNV	G	A	*rplD*	G214A	G72R	LSU ribosomal protein L4p (L1e)
M7	261605	SNV	G	A	C985_RS01220	G25A	V9I	DUF5426 family protein
M7	497605	SNV	C	A				Intergenic region (C985_RS02375 and C985_RS04390)
M7	497625	SNV	A	C	C985_RS04390	A17C	Stop to S6	Hypothetical protein
M7	528862^528863	Insertion	–	T				Intergenic region (between C985_RS02495 and C985_RS02500)
M7	547423	SNV	C	G	C985_RS02560	G1312C	D438H	Hypothetical protein
M7	605306	SNV	C	T	C985_RS03055	C352T	L118F	L-ribulose-5-phosphate 4-epimerase
M7	781991	SNV	G	A	C985_RS03730	C1151T	S384L	YitT family protein

SNV, single nucleotide variation; MNV, multiple nucleotide variation. "-" means "Not available".

C985_RS01220 (encodes DUF5426 family protein, *MPN212*), and four hypothetical protein genes (Table 2). There was a G997A SNV identified in the *dnaK* gene that resulted in amino acid change V333M in the mutant induced by azithromycin (A4). BLAST results showed that V333 is conserved in DnaK protein in mycoplasmas and other bacteria (including *Escherichia coli*) and is located in a helix according to the predicted secondary structure. In the mutant induced by erythromycin (E5), SNV G2761A in the *rpoC* gene which caused amino acid change V921M was also identified. The amino acid residue at this position is not conserved among the *Mollicutes* and other bacteria, and V921 is only present in *M. pneumoniae* and *M. genitalium*. It is interesting to observe that in the parent strain, *glpK* (*MPN050*) and *C985_RS03055* (*MPN498*) existed as a mixture carrying both SNVs [C710A (T237N) in *glpK* and C352T (L118F) in *C985_RS03055*] and wild-type sequences (SNV frequency were 36.74% and 50.51%, respectively). After drug induction, the SNVs were selected to be almost pure in all mutants, except for R2 which was totally selected back to the wild-type sequence. There was a 12-bp tandem repeat sequence insertion in one of the *hsdS* genes *C985_RS02085* (*MPN365*) in the mutant induced by roxithromycin R2. Notably, this insertion was detected in 42.19% of the reads, indicating that a dynamic evolution is still ongoing. It is also interesting to find that C985_RS02560 (*MPN449*), which encodes a hypothetical membrane protein, developed four different SNVs in the five mutants (Table 2).

4 Discussion

We have successfully selected resistant mutants of *M. pneumoniae* to five different macrolides by *in vitro* induction with increasing concentrations of the drugs. Mutations in *23S rRNA* and ribosomal protein L4 emerged during the passages with corresponding changes in MICs. Midecamycin was the least potent drug to induce resistance. The 14- and 15-membered macrolides induced mutations at position 2063 or 2064 in *23S rRNA*, while the 16-membered macrolides selected mutations at position 2067. Genome sequencing revealed that additional SNVs were developed in other protein-coding genes and intergenic regions in the final selected mutants. Induced mutants resistant to 14- and 15-membered macrolides were cross-resistant to 16-membered macrolides, while those resistant to 16-membered macrolides were still susceptible to 14- and 15-membered ones.

This study showed that the potential of the five macrolides to induce resistance in *M. pneumoniae* was different. Roxithromycin, azithromycin, and josamycin selected only one final mutation in *23S rRNA*, while erythromycin and midecamycin finally selected two mutations in *23S rRNA* or the L4 protein. Roxithromycin, josamycin, and azithromycin induced an initial *23S rRNA* substitution quickly at the second passage. In contrast, erythromycin induced the first mutation in the fourth passage. This result is different from the previous selection study where erythromycin selected mutations earlier and josamycin later (Pereyre et al., 2004), probably due to the different induction strategies used in this study. Midecamycin induced an initial mutation in the L4 protein in passage 3. However, the complete induction of resistance took the most passages and induction days (seven passages, 87 days) and the highest final induction concentration (5.12 mg/L), making it the macrolide most difficult to induce resistance. To our knowledge, this is the first *in vitro* selection of midecamycin resistance in mycoplasmas.

Midecamycin is a naturally occurring 16-membered macrolide synthesized by *Streptomyces mycarofaciens* (Cong and Piepersberg,

2007; Arsic et al., 2018). It is active against the strains susceptible or resistant to erythromycin (Schlegel et al., 2001). This antimicrobial and its derivatives are also active against other bacteria and mycoplasma species and have been widely used in the clinical treatment of respiratory tract infections (Ishida et al., 1994; Wang et al., 2020; Lin et al., 2021). The dosage and safety of midecamycin have already been determined in pediatric patients in different countries. Since azithromycin has been the empirical treatment option for *M. pneumoniae* infections in children, increasing resistance has appeared worldwide in the last 20 years (Waites et al., 2017). Finding an alternative treatment for infections caused by *M. pneumoniae* is urgently needed. The results of this study indicated that it is more difficult to select mutants by midecamycin than by other macrolides. Furthermore, even in the case that the strain developed resistance to midecamycin, it is still susceptible to the 14- and 15-membered macrolides. Taken together, these features of midecamycin suggest that it is a promising alternative first choice of antimicrobial agent for the treatment of mycoplasma infections, especially when susceptible strains are present. This treatment strategy may help reduce the occurrence of macrolide resistance and reserve the 14- and 15-membered macrolides as secondary treatment options. Further clinical trials are needed to clarify their efficacies for this purpose.

Mutations A2063G and A2064C in *23S rRNA* in the mutants induced by the 14- and 15-membered macrolides were the same as the naturally occurring mutations in clinical MRMP strains (Lucier et al., 1995; Bébéar et al., 2011; Pereyre et al., 2016). Mutation C2617A in *23S rRNA* was also described in the previous *in vitro* induction study with a subinhibitory concentration of azithromycin (Pereyre et al., 2004; Bebear and Pereyre, 2005). Josamycin induced the mutation A2067G in *23S rRNA*, which is the same as the other *in vitro* studies in *M. pneumoniae* and *M. hominis* (Furneri et al., 2001; Pereyre et al., 2004) and in an *M. genitalium* clinical strain from failed josamycin treatment (Guschin et al., 2015). This study also reported the first finding of the mutations associated with midecamycin resistance in mycoplasmas. Midecamycin induced mutation A2067C in *23S rRNA* and different mutations in L4 protein [G214A (G72R) and G215T (G72V)]. Interestingly, mutants with A2067 alterations in *23S rRNA* (J2 and M7) were resistant to the 16-membered macrolides, while the remaining were susceptible to the 14- and 15-membered macrolides. This observation is similar to the previous reports on mycoplasmas (Furneri et al., 2001; Pereyre et al., 2004) and in *Streptococcus pneumoniae* (Depardieu and Courvalin, 2001). The different MIC profile probably reflects the difference in binding site, drug orientation, and binding kinetics between the 16-membered macrolides and the 14- and 15-membered macrolides (Starosta et al., 2010). Midecamycin is the only macrolide that selected mutations both in *23S rRNA* and in ribosomal protein L4. The L4 mutations were located close to a previously reported mutation A209T (H70R) that occurred in *in vitro* selected isolates as well as in clinical isolates (Pereyre et al., 2004; Cao et al., 2010; Ou et al., 2015). Mutants harboring only L4 mutations showed slightly decreased susceptibility to the 16-membered macrolides with MICs ≤1 mg/L and did not cause resistance to the 14- and 15-membered macrolides.

This study used whole-genome sequencing to investigate the potential novel mechanisms of macrolide resistance on the mutants selected *in vitro* and identified sequence variations in 15 genes and four intergenic regions. DnaK is a molecular chaperone with multiple functions, including stress response, protein folding, and interacting with the host extracellular matrix in *M. pneumoniae* (Hagemann et al., 2017). It is reasonable to speculate that DnaK is involved in the drug-induced stress response. However, the biological function of the V333M mutation in DnaK could not be ruled out in this study because of the co-occurrence of other mutations related to macrolide resistance. The V921M alteration in the RpoC may not be essential since the V921 residue is not conserved. Another interesting finding is the insertion of a 12-bp tandem repeat unit in one of the *hsdS* genes *MPN365* in mutant R2. Although tandem repeat variation in the *hsdS* genes was predicted to be related to the epigenetic regulation of gene function/expression (Xiao et al., 2015), this is the first observation of *hsdS* variation with the corresponding phenotypic change. The exact role of *hsdS* genes in macrolide resistance development warrants further investigation. The increased percentages of the population harboring SNVs in *glpK* (*MPN050*) and *C985_RS03055* (*MPN498*) with the drug selection indicates that these SNVs favor the adaptation of *M. pneumoniae* to the drug pressures. *glpK* is an essential gene for *M. pneumoniae* with a known function in glycerol metabolism (Hames et al., 2009). This study reveals its possible secondary function in drug resistance. It has been reported that mutations in the *glpK* gene contributed to drug tolerance in *Mycobacterium tuberculosis* and *Mycobacterium bovis* (Safi et al., 2019; Dong et al., 2022). MPN449 has predicted transmembrane structures according to CLC Genomics and the computed structure model from AlphaFold DB (AF_Q50363F1) and I-TASSER (Zheng et al., 2021). Its *M. genitalium* homolog MG_314 was annotated as putrescine transporter PotE (Yang et al., 2020). Two of the amino acid changes (S279I and I391T) in the mutants induced by azithromycin (A4) and roxithromycin (R2) were in the predicted membrane coil and strand, and the other two (R171S and D438H) were in the predicted helix and coil in the cytoplasm. It would be interesting to further investigate the biological function of this protein and its role in macrolide resistance. Although the mechanisms of resistance are unknown for the mutations identified in this study, we expect that they would impose potential fitness costs to *M. pneumoniae*, affecting the growth rate of the mutants in conditions without drugs. However, this study was not set up for this measurement to answer this question.

This study has some limitations. First, although resistance is not expected to occur in the parent strain, a passage control was not included in each selection step, and thus, second, background sequence variation could not be ruled out without the passage controls of the final selected mutants when conducting the variant detection. Third, the drug selection was only performed in a single set without biological replicates, which limits the confidence in determining the induction potential of each drug and the chances of catching more mutations. However, these limitations do not affect the conclusions of this study.

In summary, this study presented the first *in vitro* data of induced midecamycin resistance in *M. pneumoniae* and the potential advantage of using midecamycin as an alternative first treatment choice for *M. pneumoniae* infections in patients.

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 below: NCBI, SRA, PRJNA954979.

Author contributions

YL and LX conceived and designed the experiments. NW performed the experiments. NW, XX, and LX analyzed the data. NW wrote the draft of the manuscript. YL and LX reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1186017/full#supplementary-material

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Trends and challenges of multi-drug resistance in childhood tuberculosis

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Drug-resistant tuberculosis (DR-TB) in children is a growing global health concern, This review provides an overview of the current epidemiology of childhood TB and DR-TB, including prevalence, incidence, and mortality. We discuss the challenges in diagnosing TB and DR-TB in children and the limitations of current diagnostic tools. We summarize the challenges associated with treating multi-drug resistance TB in childhood, including limitations of current treatment options, drug adverse effects, prolonged regimens, and managing and monitoring during treatment. We highlight the urgent need for improved diagnosis and treatment of DR-TB in children. The treatment of children with multidrug-resistant tuberculosis will be expanded to include the evaluation of new drugs or new combinations of drugs. Basic research is needed to support the technological development of biomarkers to assess the phase of therapy, as well as the urgent need for improved diagnostic and treatment options.

KEYWORDS

multidrug resistant, childhood tuberculosis, diagnostic, treatment, epidemiology

1 Introduction

Tuberculosis (TB) is a major infectious disease that seriously threatens millions of people worldwide, including children. An estimated 10.6 million new cases and 1.4 million deaths were reported in 2021 alone (World Health Organization, 2022), while cases of TB in children account for 11% of all TB cases (1.2 million out of 10.6 million) and 14% of TB deaths (0.2 million out of 1.413 million).

Drug-resistant TB (DR-TB) is a form of TB that is resistant to the most commonly first-line anti-TB drugs, making it more difficult to treat. The WHO divides DR-TB into five categories: isoniazid-resistant TB, rifampicin-resistant (RR) TB, multi-drug resistant (MDR) TB (TB resistant to isoniazid and rifampicin), pre-extensively drug resistant (preXDR) TB, which is MDR-TB with resistance to a fluoroquinolone, and extensively

drug resistant (XDR) TB, which is TB resistant to rifampicin plus any fluoroquinolone plus at least one other priority A drug (bedaquiline or linezolid). DR-TB is a significant threat to public health, as it is more difficult and expensive to treat than drug-susceptible (DS) TB, and is associated with higher rates of treatment failure, relapse, and mortality.

Children with DR-TB face more unique challenges. Childhood TB was neglected in the first decade of the 21st century - children were not recognized as contributing to the TB epidemic and were therefore not prioritized in the global TB response. It was not until 2012 that the WHO began reporting pediatric (<15 years) TB estimates. After years of neglect (Sandgren et al., 2012), the detection methods are inadequate and the diagnosis of pediatric cases of MDR-TB is a challenge. Of the estimated 1.1 million children who develop TB, only 399, 000 (36.5%) were identified by National Tuberculosis Programs (NTPs) in 2020 (World Health Organization, 2022). Below 5 years old, there are only 27.5% of children are identified (World Health Organization, 2021a; World Health Organization, 2022). The detection rate of multi-drug resistant TB in children is low, due to the difficulty in diagnosing TB. Despite the combination therapy is available for children TB, but not based on clinical trial evidence. The biomarkers to monitor treatment response during TB therapy are urgently needed. The basic research should be a strength to develop diagnosis and treatment against children TB, especially for MDR- children TB. In this review, we summarize the progress of mycobacterial disease diagnosis and the treatment of MDR-TB in children.

2 Epidemiology

Childhood TB is a major public health concern, particularly in low- and middle-income countries, where poverty, malnutrition, and overcrowding contribute to the spread of the disease. The incidence rate of childhood tuberculosis varies widely across different regions of the world, with the highest rates observed in sub-Saharan Africa and Southeast Asia. In 2019, there were 465,000 patients with MDR/RR-TB, with a treatment success rate of 59%. About 180,000 people died from MDR/RR-TB in 2019 (Dean et al., 2022). In 2020, about 132,222 new RR-TB patients were identified, of which about 25% were MDR-TB (World Health Organization, 2022).

According to World Health Organization (WHO), an estimated 1 million children develop TB each year. Children account for 12% of the global TB burden (1.2 million out of 10 million) and 16% of TB deaths (0.20 million out of 1.28 million in 2020). In addition, approximately 25,000-32,000 children develop MDR-TB each year (Jenkins et al., 2014; Dodd et al., 2016; Jenkins and Yuen, 2018), only 3-4% of pediatric MDR-TB are diagnosed and treated, and 21% of children with MDR-TB may die as a result (Jenkins and Yuen, 2018).

TB and MDR-TB in children remain to be major public health challenges worldwide. The burden of MDR-TB in children is highest in settings with high levels of MDR-TB in adults, and MDR-TB is associated with poorer treatment outcomes than DS-TB. MDR-TB also has worse treatment outcomes than DS-TB. Therefore, there is an urgent need for improved strategies to prevent, diagnose, and treat TB and MDR-TB in children.

3 Diagnostic challenges of drug-resistant tuberculosis in children

The serials review has summarized the mechanisms of mycobacterial drug tolerance and resistance (Ramaswamy and Musser, 1998; Singh and Kaur, 2011; Walker et al., 2022). The tolerance and resistance mechanisms include molecular mutations (Walker et al., 2022), epistasis (Borrell et al., 2013; Wong, 2017), DNA epigenetics, and tolerance (Gagneux et al., 2006; McBryde et al., 2017).

The development of new diagnostic tools is needed, that quickly and accurately identify drug resistance in mycobacteria, which will aid for the effective treatment of MDR-TB. Diagnosing TB and DR-TB in children presents several challenges due to the limitations of current diagnostic tools. The current gold standard for diagnosing TB is sputum culture, which can take several weeks to yield results. Children with TB, who harbor small amounts of *Mycobacterium tuberculosis* (MTB), have lower sensitivity to current diagnostic tests. Primary pulmonary TB is more common in children with TB, and the clinical symptoms and signs are often non-specific and other diagnostic tests, such as X-rays and the tuberculin skin test, are easily misdiagnosed and missed, particularly in areas with high rates of tuberculosis and HIV co-infection. Additionally, these tests are unable to differentiate between DR and DS-TB strains, making it challenging to identify patients who require alternative treatments. In addition, an estimated 69% of missed cases occurred among children under 5 years of age, with a low case detection rate relative to other age groups. This is mainly due to the characteristics of children's growth and pathogenesis. Therefore, accurate and accessible point-of-care tests (POCTs) are needed to detect TB in children to reduce TB-related morbidity and mortality in this vulnerable population (Mukherjee et al., 2011; Graham et al., 2015; Reuter et al., 2019; Nicol and Zar, 2020; World Health Organization, 2022).

In recent years, a variety of POCTs for TB have been developed, such as the molecular detection of MTB using loop-mediated isothermal amplification (LAMP) (Mitarai et al., 2011) and the portable polymerase chain reaction (PCR)-based GeneXpert. (Lawn, 2015; Bloom et al., 2017; World Health Organization, 2021a; World Health Organization, 2021b).

According to the 2021 Global Tuberculosis Report, WHO recommended some new diagnostic tests for non-invasive samples like sputum, gastric aspirate, or stool by Xpert MTB/RIF Ultra assay. (WHO consolidated guidelines on tuberculosis Module 5, 2022). The diagnostic of Xpert MTB/RIF Ultra assay was recommended for the initial diagnostic test and the detection of rifampicin resistance (Bloom et al., 2017). According to Kay et al., Xpert Ultra has varying sensitivity to different specimen types. Sputum has the highest sensitivity, followed by gastric aspirate and stool. Nasopharyngeal aspirate has the lowest sensitivity (Kay et al., 2022). A study examines Xpert Ultra on stool to diagnose PTB in children found that it's sensitivity and specificity were 58.6% and

88.1%, respectively, while Xpert on stool had its sensitivity of 37.9% and a specificity of 100.0% (Kabir et al., 2021). However, this test does not always detect the mycobacterial infection accurately, especially in children with severe acute malnutrition (SAM), or those with HIV.

The whole-genome sequencing (WGS) is recommended as a valuable tool for the surveillance of DR-TB by WHO (World Health Organization, 2020). WGS can thoroughly detect all genes related to drug resistance, which can provide valuable information for clinical treatment, particularly for MDR-TB (Votintseva et al., 2017; Doyle et al., 2018). WGS has also been studied for the identification of MDR-TB in children (Zhang et al., 2021). Compared with the conventional phenotypic drug susceptibility test (DST), it provides accurate results for both first-line and second-line anti-TB drugs. Recently, Studies showed the method for sequencing DNA directly from sputum samples provides a promising approach to diagnosis (Votintseva et al., 2017; Doyle et al., 2018). WGS is a promising approach for predicting resistance to isoniazid, rifampin, pyrazinamide, levofloxacin, streptomycin, second-line injectable drugs (SLIDs) and prothionamide. It has satisfactory accuracy, with sensitivity and specificity of over 85.0% (Chen et al., 2019). For the detection of MDR-TB in children, sputum samples are still the limitation of the application, and improving the preparation of test specimens will provide potential solutions for the accurate detection of MDR-TB in children.

In addition, age is also a key consideration in the diagnosis of TB in children (Kay et al., 2022). This may be due to differences in immune status and response to MTB at different ages. When MTB infects the host lung, it causes host inflammation and tissue damage. Recent studies using combined multi-omics such as metabolomics, lipidomics, and cytokine profiling have shown that host metabolism plays a crucial role in driving inflammation against TB (Pitaloka et al., 2022; Yu et al., 2023). These studies have indicated the potential benefits of taking advantage of the host's response for clinical diagnosis.

Metabolomics allows for the quantitative profiling of both the infected host and MTB, enabling the identification of biomarkers for diagnosing active TB, identifying latent TB infection (LTBI), predicting the risk of developing active TB, and monitoring the effectiveness of anti-TB drugs (Szewczyk et al., 2013; Salgado-Bustamante et al., 2018; Magdalena et al., 2022).

Mass spectrometry, gas chromatography-time-of-flight mass spectrometry (GC-TOF MS), ultra-performance liquid chromatography-mass spectrometry (UPLC-MS), or nuclear magnetic resonance spectroscopy are platforms used in metabolomics to characterize metabolites as biomarkers from biological samples such as blood, urine, cerebrospinal fluid (CSF), fecal wastes and breath (Lau et al., 2015; Isa et al., 2018). Biomarkers have been shown to identify DR strains of MTB. A study compared the lipid profiles of drug-sensitive (DS) and DR strains of MTB and found differences in fatty acyls and glycerophospholipids that could potentially be used as biomarkers for identifying drug resistance (Pal et al., 2017). Rego et al. found DS, MDR, and extensively drug-resistant (XDR) strains of TB had different profiles, which could be used to predict their susceptibility or resistance to drugs (Rego et al., 2021). Some other non-sputum diagnostics are also promising for improved pediatric MTB diagnosis. The urine-based lipoarabinomannan (LAM) assay has been endorsed by WHO for TB diagnosis as sensitivity increases significantly in patients with lower CD4 cell counts. The value of urine-based lipoarabinomannan (LAM) antigen tests for diagnosing tuberculosis in children has been shown to vary depending on test used. The sensitivity and specificity of the *Mycobacterium. tuberculosis* enzyme-linked immunosorbent assay (MTB-LAMELISA), Alere Determine TB LAM Ag (Alere LAM) test, and the Fuji LAM diagnostic procedure among pediatric cases below 15 years with TB were 16.0% and 95.61%; 45.90% and 80.42%; and 52.32% and 89.37%, respectively (Seid et al., 2022). Blood transcriptional markers and cell-free DNA in urine are promising additional candidates for non-sputum triage or confirmatory TB tests (Turner et al., 2020; Hiza et al., 2021). These tests have the potential to improve the accuracy and speed of MDR-TB diagnosis, particularly in cases where sputum samples are difficult to obtain or analyze. The sensitivity and specificity of Cepheid's Xpert MTB-HR fingerstick blood test using 3-host mRNA, were 90% and 55.8%, respectively, regardless of HIV status (Sutherland et al., 2022). CD38-based TAM-TB assay is also a sputum-independent, blood sample test that has been shown to have a specificity of 93.4% and a sensitivity of 82.2% (Hiza et al., 2021). As to children, both TAM-TB blood test and MTB-HR fingerstick blood test has been shown to be a reliable and highly specific tool for diagnosing TB in children, which is designed to detect progression from LTBI to active MTB (Hiza et al., 2021; Sutherland et al., 2022). Blood specimens can be used to diagnose children, but biomarkers specific to DR-TB need to be sought.

Other specimen types are also evaluated for TB test suitability. Gastric aspirates can be useful for children and the best way to obtain specimens from children less than 1 year of age. Xpert Ultra pooled sensitivity was 67.3% and 71.5% for children aged 1-4 years (Kay et al., 2022). For stool samples from children less than 1 year of age, Xpert Ultra pooled sensitivity was 65.2% and 43.3% for children 1-4 years. Samples that are more invasive like gastric aspirate show more accuracy with their high sensitivity (70.4%) and specificity (94.1) for children under one year. The WHO recommends that by 2021, the use of artificial intelligence (AI), and computer-aided detection for diagnosis based on X-ray databases in adults, but not in children (Codlin et al., 2021; Qin et al., 2021). As technology develops, AI should also be used to diagnose children. However, there are still limited data available on metabolic alterations that occur in children with TB (Dutta et al., 2020; Comella-Del-Barrio et al., 2021).

4 Treatment challenges for DR-TB in children

The emergence of MDR strains of *M. tuberculosis* presents an added challenge in the battle against TB. The main challenge in treating MDR-TB in children is the lack of effective drugs. Many of the drugs used to treat TB are either ineffective or have toxic side effects in children. This limits the treatment options and can lead to prolonged treatment periods, which can be especially difficult for

children. The treatment of MDR-TB in children follows the same general principles as in adults (Pecora et al., 2021; Bossu et al., 2023). The WHO recommends using injectable-free regimens for children and has classified drugs into three groups: Group A includes levofloxacin/moxifloxacin, bedaquiline, and linezolid; Group B includes clofazimine and cycloserine; and Group C includes delamanid, ethambutol, pyrazinamide, ethionamide, para-aminosalicylic acid, and amikacin (details are listed in Table 1).

The drug regimen used to treat drug-resistant tuberculosis (DR-TB) in children is often complex and lengthy, leading to several challenges. One of the most concerning issues is the side effects associated with the drugs used to treat DR-TB, such as nausea, vomiting, hearing loss, and liver damage. These side effects can be especially challenging for children, who may have a difficulty tolerating the medications they are taking (Seddon et al., 2012; Schaaf, 2019). Additionally, treating DR-TB in children can be prolonged, with regimens lasting up to two years depending on disease severity and drug resistance. This can be difficult for children and their families to adhere to. Children with MDR-TB require specialized care, including close monitoring and support from healthcare providers. This can be difficult to provide in resource-limited settings, where there may be a shortage of trained health workers and limited access to diagnostic tools (Bossu et al., 2023). Furthermore, children with DR-TB may face depression, stigma, social isolation and low self-esteem (Snow et al., 2020).

It is important to develop new therapeutic strategies against MTB (such as monoclonal antibodies, TB immunotherapy, which include cytokines, immune cells and immunomodulatory drugs) or combinations of drug cocktails (combining the use of bedaquiline and/or pretomanid regimens of drugs in the replacement of first-line anti-TB drugs) (Kak et al., 2018; Olin et al., 2018; Rao et al., 2019; Mi et al., 2021; Lu et al., 2022).

In general, most children with TB have less severe disease than adults. Shorter treatment regimens than those used for adults may be effective in treating children with TB, but the evidence is lacking. Shorter treatment regimens may result in lower costs for families and health services, less potential toxicity, less risk of drug interactions in HIV-infected children, and fewer problems with adherence to full treatment. Shorter, safe and effective treatment regimens for children with DS and DR-TB benefit children with TB and their families, and are key interventions to achieve the goals of the End TB Strategy and the child-related targets set at the 2018 United Nations General Assembly High-Level Meeting on TB. New evidence from a recently completed trial on shortening treatment duration for DS-TB in children and adolescents paves the way for new recommendations for shorter treatment courses for this group. The recent WHO consolidated guidelines on TB, Module 5, and the accompanying operational handbook, provide four new recommendations on the treatment of TB disease in children (WHO).

Gaps in the treatment of children with MDR/RR-TB include a lack of safe and effective treatments, and limited access to diagnosis. There is a significant lack of clinical trials of TB drugs in children due to ethical and practical challenges. Conduct clinical trials in children is difficult because of the need for parental consent, the limited number of eligible participants, and the difficulty in measuring outcomes. The regulatory agencies or governments need to develop clear guidelines and requirements for drug development in children, provide incentives to encourage clinical trials for children drugs and address ethical and practical challenges. More research is needed on how best to treat MDR-TB in children, including understanding the long-term effects of different treatments. Antibiotics and vaccination have played important roles in reducing TB, and understanding the function and status of immune cells after MTB infection will provide scientific guidance for immunotherapy of TB. However, the field of pediatric immunology has been limited by the difficulty of collecting samples from children. Systems immunology is a scientific field that combines high-throughput analysis technology, with comprehensive data analysis to study the immune system from a holistic perspective. Single-cell sequencing technology can be used to obtain more comprehensive immunological information from small amounts of blood samples, which could provide scientific guidance for Immunodiagnostics and immunotherapy of TB in children (Olin et al., 2018).

Furthermore, children should be closely monitored to ensure that the treatment is effective and to identify any side effects of the medication. Regular follow-up visits should be scheduled and clinical, radiological and laboratory tests should be used to assess the child's progress and to help and support the family in adhering to the treatment.

5 Discussion

The serious situation of childhood tuberculosis has received more attention. The World Health Organization (WHO) has listed "control of tuberculosis in children" as a priority for infectious disease prevention and control, and proposed the goal of "aiming for zero deaths from tuberculosis in children".

Diagnosing TB in children is challenging. Obtaining samples from children for diagnosis is difficult because they often have fewer bacilli in their sputum. Alternative samples such as blood, urine, cerebrospinal fluid (CSF), feces and breath are being investigated. Corresponding diagnostic tools such as metabolomics and mass spectrometry, especially GC-TOF MS, for clinical testing of MDR/RR-TB are being developed. These methods are not currently recommended for clinical testing due to their limitations, but show promise for future use.

When treating MDR/RR-TB in children, it is important to consider the differences between adults and children when using adult therapies. This includes taking into account age, weight, medical history, drug allergies or sensitivities, and other factors that may affect a child's response to treatment. In addition, different approaches may be needed to treat MDR-TB in younger and older children due to their different levels of maturity and understanding.

The developing interventions to selectively enhance children's immunity is urgently needed and the need for a deeper understanding of children's immunity to identify barriers to early vaccinations and ultimately define new approaches to overcome TB.

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TABLE 1 Treatment Regimen for Children with Multi-Drug and Rifampicin Resistant TB.

Treatment Program		Treatment Drugs		Dosage*	Duration of Treatment
Short-course treatment program	6 months full oral regimen	Bedaquiline, Pretomanid, Linezolid/Moxifloxacin		/	6 months
	9 months full oral regimen	Bedaquiline, Fluoroquinolones, Linezolid, Ethionamide, Pyrazinamide, Ethambutol, Isoniazid, Clofazimine		/	9 months
Long-course treatment program		Group A	Bedaquiline	>12 y and > 30 kg body weight: 400 mg daily x 2 wk followed by 200 mg M/W/F x 24 wk Data on dose in younger children not yet available	Long-term
			Linezolid	Weight-banded dose: 5-9 kg = 15 mg/kg OD 10–23 kg = 12 mg/kg OD >23 kg =10 mg/kg OD	
			Levofloxacin	15–20 mg/kg/d	
			Moxifloxacin	10–15 mg/kg/d	
		Group B	Clofazimine,	2-5 mg/kg/d. Weight-banded dose: 50 mg gel capsule: 5- < 10 kg: 1 caps every 2nd d; 10-20 kg:1 caps/d; > 20 kg: 2 caps/d; 100 mg gel capsule: 5- < 10 kg: 1 caps Mon/Wed/Fri; 10-20 kg: 1 caps every 2nd day; >20 kg 1 caps everyday	
		Cycloserine/ Terizidone	15–20 mg/kg/d		
		Group C	Ethambutol	15–25 mg/kg/d	
		Delamanid	>12 y/>35 kg: 100 mg twice daily 6–12 y/20-34 kg: 50 mg/kg twice daily Data in younger children not yet available		
		Pyrazinamide	30–40 mg/kg/d		
		Amikacin	15–20 mg/kg IMI or IVI daily		
		Ethionamide /Prothionamide	15–20 mg/kg/d		
		Meropenem	20–40 mg/kg 8 hourly (IV)		
		Amoxicillin-clavulanate	80 mg/kg/d in 2–3 divided doses of amoxicillin component – always combine with a carbapenem (not effective on its own)		
		Para-aminosalicylic acid	150–200 mg/kg daily as single or divided dose		

(1) This table is intended to guide the development of individualized MDR-TB long-course regimens (MDR-TB short-course regimens are essentially standardized in composition.) The drugs in Group C are listed in descending order of the strength of evidence for their recommended use. (2) There is insufficient evidence of safety and efficacy for Bedaquiline use beyond 6 months and for use in pediatric patients under 6 years of age, so the use of Bedaquiline in these cases should follow the "over-indication" use protocol. (3) There is insufficient evidence of safety and efficacy for delamanid use beyond 6 months and in pediatric patients under 3 years of age, therefore, the use of delamanid in these cases should follow the "over-indication" use protocol.
* Dosage information adapted from (Schaaf, 2019) (World Health Organization, 2018),

Overall, effective management and follow-up during the treatment of drug-resistant TB in children is crucial to ensure the success of the treatment and to minimize the risk of adverse effects.

Author contributions

KM conceived and designed the article, while KM and XZ contributed to the initial drafting, editing, and supervision of the project. KM and LS secured funding for the study. ZZ and LS led the initial drafting of the manuscript, while XS, HZ, and LL were involved in the critical review and editing of the manuscript. All authors have approved the final version of the article for publication.

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Conflict of interest

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

Publisher's note

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Advances of new drugs bedaquiline and delamanid in the treatment of multi-drug resistant tuberculosis in children

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Tuberculosis (TB) is a major public health problem, with nearly 10 million new cases and millions of deaths each year. Around 10% of these cases are in children, but only a fraction receive proper diagnosis and treatment. The spread of drug-resistant (DR) strain of TB has made it difficult to control, with only 60% of patients responding to treatment. Multi-drug resistant TB (MDR-TB) is often undiagnosed in children due to lack of awareness or under-diagnosis, and the target for children's DR-TB treatment has only been met in 15% of goals. New medications such as bedaquiline and delamanid have been approved for treating DR-TB. However, due to age and weight differences, adults and children require different dosages. The availability of child-friendly formulations is limited by a lack of clinical data in children. This paper reviews the development history of these drugs, their mechanism of action, efficacy, safety potential problems and current use in treating DR-TB in children.

KEYWORDS

tuberculosis (TB), multi-drug resistant TB, children, bedaquiline, delamanid

1 Introduction

Tuberculosis (TB) is a major public health problem that is becoming increasingly concerning in children, with 1.1 million new cases accounting for 11% of all new TB cases worldwide in 2021 (World Health Organization, 2022a). Multi-drug resistant/rifampicin-resistant (MDR/RR) TB is one of the leading causes of death worldwide and has been drawn attention for its severe form in children. The diagnosis and treatment of TB in children are particularly difficult due to their developmental stage, so it is important to understand the opportunities and challenges currently facing TB in children, as well as MDR/RR-TB infection in particular. To reduce the burden of this disease on children's health, improved early diagnosis methods and precise and effective treatments must be developed if we are to achieve our goal of "zero death from tuberculosis in children" (World Health Organization et al., 2013).

Drug-resistant tuberculosis (DR-TB) is a growing problem that makes it more difficult to achieve disease control goals. MDR-TB is a type of TB that is resistant to two important drugs, rifampicin and isoniazid (World Health Organization, 2022b). In 2021 alone, there were 450,000 new patients with MDR-TB (World Health Organization, 2022a). An estimated 25,000 to 32,000 children develop MDR/RR-TB each year (Jenkins et al., 2014; Dodd et al., 2016; Jenkins and Yuen, 2018). MDR/RR-TB treatment cases are 2.5% of the total number of children with MDR/RR-TB starting treatment and only 10.1-12.9% of the estimated number of children with MDR/RR-TB (World Health Organization, 2020). MDR/RR-TB is difficult to treat due to the lack of effective drugs, which are costly and require a long treatment period (Dodd et al., 2016; Guglielmetti et al., 2021).

Treatment of MDR-TB is challenging due to a limited number of effective drugs, associated with high drug burden cost, long treatment duration, and potential for adverse effects (Chan et al., 2013). Studies showed that MDR-TB treatment drugs such as aminoglycosides can cause irreversible ototoxicity, hepatotoxicity, and neurological side effects (Dheda et al., 2017; Liu et al., 2018). After fifty years of stagnation in identifying new targets and drugs to treat TB (Zumla et al., 2013), new drugs, bedaquiline and delamanid, have showed promising efficacy in adults (Skripconoka et al., 2013; Borisov et al., 2017; Guglielmetti et al., 2017a; Mohr et al., 2018). Significant advances have been made in treating MDR-TB in children after years of neglect in developing and using of these new drugs (Sandgren et al., 2012). Shown in Figure 1 is the development history of bedaquiline and delamanid used in the treatment of MDR-TB. The timeline provides an overview of the drug development process, from discovery to regulatory approvals, including preclinical studies, clinical trials for both adults and children. This information highlights the significant efforts put into bringing these drugs to the market, and their potential to revolutionize the treatment of MDR-TB.

In 2022, the World Health Organization (WHO) revised the guidelines for TB treatment in children, recommending the use of new drugs bedaquiline and delamanid to treat MDR-TB (World Health Organization, 2022b). In this review, we will provide an overview of the progress made in treating children with MDR-TB using bedaquiline and delamanid, including their efficacy, safety, and treatment recommendations.

2 Treatment of TB in children using of the new drug bedaquiline and delamanid

Historically, the treatment of TB in children has lagged behind that of adults, largely due to a lack of research specifically tailored to young people. For instance, the duration of TB treatment in children was based on the adult studies, mandating a 6-month combination of daily medications. However, a recent study has recommended that the treatment duration for children with drug-susceptible (DS) TB be shortened from 6 months to 4 months (Turkova et al., 2022). In line with this, the WHO has updated the recommendations for the management of TB, including MDR/RR-

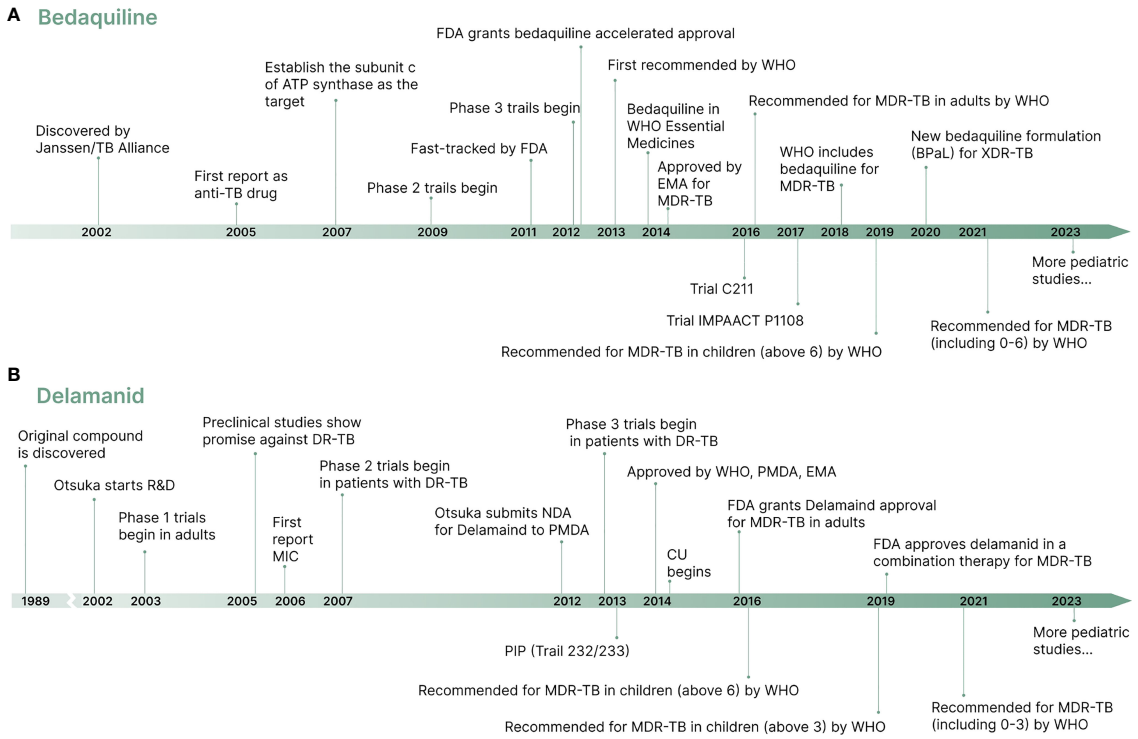

FIGURE 1
A brief timeline of the development of new drugs bedaquiline and delamanid. **(A)** Bedaquiline development timeline. **(B)** Delamanid development timeline.

TB in children. These recommendations include the use of bedaquiline in shorter or longer regimens for treating MDR/RR-TB in children of all ages, and the use of delamanid in longer regimens for treating MDR/RR-TB in children of all ages (World Health Organization, 2022b). The WHO provides guidelines for the dosage recommendations for drugs used in MDR-TB regiments, which are based on body weight and age. Details can be found on the WHO website and are described in full at https://www.ncbi.nlm.nih.gov/books/NBK539514/table/annex2.tab2/. It is important to select the most effective and appropriate treatment plan for each age group. Children are generally expected to respond to TB treatment as well as or better than adults (Ettehad et al., 2012; Donald et al., 2013; Nachman et al., 2015). However, there is a lack of research specifically targeting children, and there are only a limited number of drug regimens available for children. Further research is needed on the effectiveness of different treatment regimens in this group of children.

While the basic principles for designing treatment regimens for children with TB are largely similar to those used for adults (Schaaf and Marais, 2011), there are some differences in the pharmacokinetics and toxicity of drugs when they are administered to children. This makes it challenging to determine which treatments are most effective and appropriate for children, as it is not always possible to directly translate drug dosing recommendations from adults to children.

Further research is needed to determine the efficacy of different children-specific treatment plans to ensure optimal care for children with TB (Poorana Ganga Devi and Swaminathan, 2013). Despite the challenges in the provision of children TB formulations, new drugs like bedaquiline and delamanid have been developed for children with TB. Innovative approaches like these can accelerate the development of more effective treatments tailored to children.

3 Bedaquiline

Bedaquiline, developed by Johnson & Johnson's Janssen Pharmaceuticals, was approved for use in adults with MDR-TB in 2012 (Mahajan, 2013). Its discovery dates back to 2005, when Andries et al. investigated the inhibitory effect of various chemicals on the growth of *M. smegmatis*. They found that bedaquiline, then known as TMC207, had a potent inhibitory effect on several species of mycobacteria, particularly *M. tuberculosis*, due to its ability to inhibit ATP synthase, which is essential for energy production in *M. tuberculosis* (Andries et al., 2005). Blocking the enzyme responsible for ATP synthesis, bedaquiline has demonstrated a reduction in the growth and survival of MDR-TB strains, making it an effective treatment option for TB in children (Andries et al., 2014; Diacon et al., 2014). Bedaquiline is the first new drug approved for the treatment of MDR-TB since rifampin in 1971 (Mahajan, 2013). In the WHO Consolidated Tuberculosis Guidelines (2022), bedaquiline has been conditionally recommended for treating children with MDR-TB aged less than 6 years (World Health Organization, 2022b). Although bedaquiline has many advantages as a promising new adult TB drug, more research is required to understand how young children are affected by bedaquiline and what dose to use for them.

3.1 Mechanisms and pharmacokinetics of bedaquiline

Bedaquiline is a new anti-TB drug that functions by blocking the ion-binding sites of the mycobacterial ATP synthase, which converts ADP to ATP *via* a transmembrane electrochemical ion (H^+ or $Na^{+)}$ gradient, thereby producing energy. Bedaquiline specifically targets the ion-binding sites present in the c-subunit of ATP synthase, which hinders the proton pump and results in a decline in intracellular ATP levels (Andries et al., 2005; Koul et al., 2007). Additionally, bedaquiline also targets the ϵ-subunit of F-ATP synthase by interacting with Trp16 residues (Kundu et al., 2016). By inhibiting mycobacterial ATP synthase, bedaquiline leads to ATP depletion, thereby damaging energy-producing mycobacteria and disrupting pH homeostasis, ultimately inhibiting strain growth. Unlike other quinolones antibiotics that block DNA gyrase, bedaquiline specifically targets the mycobacterial ATP synthase (Andries et al., 2005). Additionally, bedaquiline has a high degree of specificity to mycobacterial ATP synthase compared to other organisms, such as humans or mice. Because bedaquiline has low sensitivity to mitochondrial ATP synthases of humans, it is unlikely to cause toxicity (Haagsma et al., 2009). Studies have shown that bedaquiline exhibits superior *in vitro* antibacterial activity when compared to other antibiotics. This highlights ATP synthase as a promising target for the treatment of TB, as it offers a new mode of action against TB. This differs from traditional antibiotics such as quinolones, which block DNA gyrase (Andries et al., 2005; Diacon et al., 2009; van Heeswijk et al., 2014). Furthermore, bedaquiline does not cross-reaction with other anti-tuberculosis drugs, making it effective against both MDR-TB strains and DS-TB strains (Andries et al., 2005).

Human pharmacokinetic studies have shown that bedaquiline is well absorbed by the body when taken orally with food (Rouan et al., 2012; Diacon et al., 2013; D'Ambrosio et al., 2017). A standard diet containing approximately 22 g of fat has been found to improve bedaquiline bioavailability by a factor of 2 compared to fasting U.S. Food and Drug Administration, (2012). This oral administration method has been used in the treatment of TB. Bedaquiline has a linear relationship between dose and maximum plasma concentration (C_{max}), and peak concentration is reached on average 5-6 hours after treatment, and an effective half-life of 24 hours on average (Andries et al., 2005; Rouan et al., 2012; Diacon et al., 2013; D'Ambrosio et al., 2017). Drug efficacy is related to cumulative weekly doses, suggesting intermittent administration is favorable.

Bedaquiline is mainly metabolized by cytochrome P-450 3A4 (CYP3A4) and is converted to the relatively inactive N-desmethyl metabolite N-monodesmethyl (M2) (Dooley et al., 2012). *In vitro* studies have shown that M2 can cause more disruption of cellular phospholipid deposition than bedaquiline, which may result in adverse effects such as QT interval prolongation and hepatotoxicity (van Heeswijk et al., 2014). However, *in vivo* studies with M2 and bedaquiline have not shown any such side effects, even at C_{max} (Dooley et al., 2012).

Drug resistance is a growing concern in the use of antibiotics, including bedaquiline. The identification of bedaquiline-resistant strains in MDR-TB patients without prior exposure to bedaquiline

(Villellas et al., 2017; Xu et al., 2017) suggests that resistance to this drug can emerge spontaneously. This highlights the importance of careful monitoring of patients receiving bedaquiline and the need for further research to understand the mechanisms of resistance and to develop strategies to prevent or overcome it. The emergence of bedaquiline-resistant strains underscores the need for continued research and development of new treatment options for MDR-TB in both adult and children.

3.2 Bedaquiline recommended for the treatment of MDR-TB

A clinical study was conducted to evaluate the efficacy of bedaquiline, when added to the standard treatment for DR-TB. The study found that the addition of bedaquiline significantly reduced the amount of time for patients' sputum to convert to culture when compared to a placebo. When the patients were followed up for an additional 24 weeks, 50% of the patients in the bedaquiline group achieved culture conversion in 78 days, whereas 129 days in the placebo group (Diacon et al., 2009; Diacon et al., 2012). Similarly, another clinical trial involving patients with DR-TB showed that patients who received bedaquiline had higher rates of sputum conversion compared to those who received a placebo. Specifically, after 24 weeks, 79% of patients in the bedaquiline group achieved sputum conversion compared to 58% in the placebo group (Lakshmanan and Xavier, 2013). In addition, the TMC207-C208 Clinical Trials (NCT00449644) evaluated sputum-culture conversion in patients and found that adding bedaquiline to standard TB treatment for 24 weeks resulted in faster recovery than placebo. After 120 weeks, patients who received bedaquiline showed significantly more improvement compared to those who received a placebo (Diacon et al., 2014). A study of 20 patients with MDR/extensively-drug resistant (XDR)-TB showed that 12 patients (60%) achieved culture conversion within 8 weeks and 20 patients (100%) achieved culture conversion within 6 months when treated with a bedaquiline regimen for at least 6 months (Olaru et al., 2017). A larger group study that looked at treatment outcomes of 428 patients with DR-TB in 15 countries found that 62.4% were cured at the end of their treatment, 13.4% died during treatment, 7.3% dropped out of treatment before completion, and 7.7% did not successfully respond to the prescribed regimen (Borisov et al., 2017). Several studies have supported a significant reduction in the mortality rate of patients who received bedaquiline compared to those who received a placebo (Ahmad et al., 2018; Olayanju et al., 2018; Schnippel et al., 2018; Mbuagbaw et al., 2019). The available evidence suggests that bedaquiline is effective in treating DR-TB. It has been classified by the WHO as the drug of choice for this disease and is recommended for use in all-oral treatment regimens (World Health Organization, 2022b).

3.3 Side effects of bedaquiline

The safety of bedaquiline has been assessed and it is generally considered safe for long-term use with mild side effects such as nausea and headache (Guglielmetti et al., 2017b). However, it can cause QTc prolongation (Darmayani et al., 2022) and caution should be used when combining with other drugs that have similar risks.

In an early evaluation of bedaquiline's adverse effects in 75 patients, Rustomjee et al. found that the administration of 400 mg bedaquiline daily led to very few mild adverse events, including hemoptysis (21.4%), rash (7%), diarrhea (7%), and somnolence (7%) (Rustomjee et al., 2008). Adverse events, mainly related to other drugs in the regimen, occurred in 23 of 75 patients (77%) in the study by Olaru et al. Although QT interval prolongation was observed in 95% of patients, no arrhythmias or deaths were recorded, and none had to discontinue treatment due to bedaquiline-related adverse events (Olaru et al., 2017). A case analysis of 247 MDR-TB patients conducted by Borisov et al. showed that 5.8% discontinued bedaquiline treatment due to adverse events and there was one death, not considered related to bedaquiline use (Borisov et al., 2017). A retrospective analysis by Hewison et al. showed that 84.4% achieved a culture conversion rate, 79.3% experienced some form of the adverse event, and 10 died, two of which may have been related to bedaquiline use (Hewison et al., 2018).

Overall, studies have shown that bedaquiline has a low incidence of adverse events and a high rate of culture conversion, indicating bedaquiline is generally safe and well-tolerable with mild side effects. In the long term, bedaquiline has a potential for adverse effects, including the possibility of death. Consequently, caution should be taken when combining bedaquiline with other drugs that carry similar risks, such as fluoroquinolones or CYP3A4 inhibitors. QTc prolongation may occur. In contrast, levofloxacin, a fluoroquinolone drug, has been found to have fewer drug interactions and cause less QTc prolongation (Harausz et al., 2017; Denti et al., 2018). As such, long-term follow-up of patients is necessary to monitor for any life-threatening adverse effects.

3.4 Safety and effectiveness of bedaquiline in children with drug-resistant TB

The use of bedaquiline in the treatment of DR-TB in children has been limited due to its potential for adverse side effects. While clinical trials are ongoing to assess its safety and efficacy, information on its use in children is scarce because they are often not included in trials. A compassionate use study of bedaquiline to treat MDR-TB in children under 15 indicated that bedaquiline was associated with higher success rates in eliminating TB infection after 24 weeks of treatment. This supports the use of bedaquiline as a treatment option and suggests that bedaquiline is an effective regimen for treating TB in children (Guglielmetti et al., 2015). Studies have also assessed the potential mortality associated with the use of bedaquiline. An analysis of TB cases registered in EDRweb, excluding children under 15, reported that among 1016 MDR-TB patients treated with bedaquiline, there were 128 deaths (12.6%), a lower mortality rate than standard treatment (24.8%) (Schnippel et al., 2018). The study by Koirala et al. found that 74.2% of patients had a successful treatment outcome with the use of bedaquiline, with only 6.5% dying and 2.9% experiencing a failure rate due to its use (Koirala et al., 2021). Another study looked at 27 young patients with MDR-TB and found that although 5 of them

experienced QTc prolongation, it was still safe to continue treatment with bedaquiline alongside drugs that can cause heart problems (Achar et al., 2017). In addition, the safety and efficacy of bedaquiline were evaluated in 15 children, 3 of whom were HIV infected. Of these children, 10 experienced mild QTc prolongation and 2 experienced arthritis. The study also found that the bioavailability of bedaquiline in children was about 57% compared to what it was in adults, which could be partially explained by delaying feeding after administering bedaquiline on days when blood samples were taken for evaluation (Hughes et al., 2022).

Two clinical trials, Janssen C211 and IMPAACT P1108, have been investigating the treatment of pediatric patients with DR-TB since 2016 and 2017 respectively. These trials focus on pediatric MDR-TB patients in different age groups (Moodliar et al., 2021; https://www.impaactnetwork.org/studies/p1108). Despite limited data and small sample sizes, bedaquiline was found to be safe for heart health in both adults and children.

The WHO recommends that children under 6 years of age with TB receive all-oral treatment, with doses adjusted for age and weight (World Health Organization, 2022b). The treatment success rates of children who receive an all-oral regimen are comparable to those who do not receive it. Bedaquiline, having demonstrated efficacy and safety in younger age groups, may be used as an alternative to injectable drugs (Seddon et al., 2018).

Janssen is developing a child-friendly version of bedaquiline, but it may not be available for some time (Garcia-Prats et al., 2018). The practical dose of bedaquiline for children may be supported by the suspension or syrup forms of bedaquiline that are proven to be available (Taneja et al., 2023). Meanwhile, a trial (NCT03032367) sponsored by the IMPAACT Network is comparing the effectiveness of using whole tablets and dissolved bedaquiline for children who cannot swallow whole tablets (Garcia-Prats et al., 2018). Another study of 24 healthy adult volunteers found that bedaquiline tablets dissolved in water worked as well as those swallowed whole (Svensson et al., 2018). According to the WHO, until a child-friendly version is available, the current formulation can still be used to treat MDR-TB in children.

4 Delamanid

Delamanid (OPC-67683), a bicyclic nitroimidazole, is a new TB drug developed by Otsuka that has demonstrated potent TB activity *in vitro* and *in vivo* (Matsumoto et al., 2006; Saliu et al., 2007; Igarashi, 2017). It was originally discovered in 1989, and improvements have been made to eliminate mutagenicity while increasing its anti-TB effects (Ashtekar et al., 1993; Sasaki et al., 2006). Delamanid has the potential to be used as a treatment for TB because it does not show cross-resistance or antagonistic activities with other existing drugs such as isoniazid and rifampicin (Lewis and Sloan, 2015). It has been recommended by the WHO for use in longer regimens for the treatment of MDR-TB in children.

4.1 Mechanisms and pharmacokinetics of delamanid

Delamanid is an antibiotic drug used to treat MDR/RR-TB. Delamanid is a prodrug that works by reducing the nitro group of the F420-dependent nitroreductase, which activates the drug. This process also generates many intermediates that facilitate its action against MDR/RR-TB bacteria, making it more effective in treating mycobacterial infections (Field, 2013; Liu et al., 2018). Delamanid inhibits the production of mycolic acid, which is necessary for *Mycobacterium avium* survival and cell wall formation. By limiting the amount of mycolic acid produced, *M. avium* reproduction is inhibited, and the abnormal cell wall allows the drug to penetrate the cell. This makes delamanid more effective in treating mycobacterial infection because mycolic acids are only found in the cell walls of mycobacteria (Glickman and Jacobs, 2001; Matsumoto et al., 2006; Field et al., 2012; Ryan and Lo, 2014). Delamanid has inhibitory effects on *M. avium*, which may involve the release of free radicals such as nitric oxide (NO). These free radicals are essential in mammalian defense mechanisms against mycobacterial infections, making delamanid more effective in treating such infections (MacMicking et al., 1997; Xavier and Lakshmanan, 2014).

Tanneau et al. established a population pharmacokinetic (PK) model, investigated potential pharmacological interactions with bedaquiline, and determined the concentration-time course of delamanid and DM-6705 in adults with DR-TB. Plasma albumin concentration had no discernible effect on delamanid metabolism. Delamanid PK was not affected by the co-administration of bedaquiline (Tanneau et al., 2022). Using a large body of delamanid clinical data, a PK model for delamanid was developed in individuals with pulmonary MDR-TB. The model had a good fit and sufficient predictive power, taking into account different dosing populations and treatment regimens. Based on this model, the current recommended dose of 100 mg is more acceptable after analyzing the bioavailability of 100 mg, 200 mg, 250 mg, and 300 mg. However, given the number of drugs used to treat MDR-TB, it is critical to adjust the optimized dose in complex situations. The study was conducted in patients aged 18-64 years with MDR-TB, and pediatric data are still lacking (Wang et al., 2020).

A population pharmacokinetic analysis of delamanid and its major metabolite DM-6705 gained insight into the pharmacokinetic profile of these drugs in children with MDR-TB. This analysis utilized data from two clinical trials, involving participants aged 0.67 to 17 years, to provide healthcare providers with a better understanding of how children absorb and metabolize delamanid. The findings of this study can aid healthcare providers in adjusting dosages appropriately to achieve optimal treatment outcomes (Sasaki et al., 2022). Two clinical trials (NCT01856634 and NCT01859923) were conducted to evaluate the pharmacokinetics, safety, efficacy, and appropriate dosing of delamanid in MDR-TB children aged 0-17. The study included a total of 37 children who were grouped into four categories: 12-17, 6-11, 3-5, 0-2. These

children received doses of 100 mg, 50 mg, 25 mg, and 5-10 mg respectively, twice daily. A favorable response was observed in 33 out of 37 children (89.2%) at 24 months. The safety profile of delamanid was found to be similar in children aged 0-17 years and adults who took delamanid (Garcia-Prats et al., 2018). The IMPAACT, 2005 trial is currently underway to evaluate the pharmacokinetics, safety, and tolerability of delamanid in combination with an optimized multidrug background regimen (OBR) for treating of MDR-TB in HIV-infected and uninfected children. The trial is expected to be completed by 2027, and can track its progress on the IMMPACCT Network website (https://test.impaactnetwork.org/studies/impaact2005). Moreover, in a lead-in pediatric PK study, the PHOENIx trial (NCT03568383), is evaluating once-daily delamanid dosing in children. More details about the trial can be found on the ClinicalTrials.gov website (https://clinicaltrials.gov/ct2/show/NCT03568383).

Delamanid has low water solubility, which can impede its absorption into the body. Nevertheless, animal studies have shown that its oral bioavailability ranges from 35 to 60% and it increases with food intake, particularly high-fat foods (Lewis and Sloan, 2015). Furthermore, delamanid has been found to have no interactions with CYP enzymes (Liu et al., 2018) and a low potential to interact with antiretroviral drugs. Consequently, it is feasible to co-administer delamanid with antiretroviral drugs without worrying about drug interactions (Mallikaarjun et al., 2016).

Besides its limited bioavailability, mutations in the *ddn*, *fgd1*, *fbiA*, *fbiB*, *fbiC* genes linked to the F420-dependent bioactivation pathway can result in delamanid resistance (Nguyen et al., 2020; Liu et al., 2022). This implies that some bacteria may develop resistance to delamanid therapy, necessitating the use of alternative drugs. Healthcare providers should monitor for any indications of resistance while prescribing this medication to ensure effective treatment outcomes.

4.2 Delamanid recommended for the treatment of MDR-TB

In 2014, Otsuka launched the first compassionate use (CU) program, which aimed to provide free-of-cost delamanid to patients with limited treatment options. This program also allowed the combination of delamanid and bedaquiline under certain circumstances. Studies have shown that 79% of patients treated with delamanid achieved culture conversion, which is a better outcome than those not treated with the combination of bedaquiline and delamanid (Hafkin et al., 2019).

In a phase II study, Gler et al. investigated the efficacy of delamanid for the treatment of pulmonary MDR-TB in 481 patients. The treatment regimen was supplemented with 100 mg or 200 mg of delamanid or placebo twice daily for 8 weeks. The results showed that the experimental group receiving delamanid had a higher rate of sputum transformation than the control group, indicating the potential as an effective therapy for MDR-TB (Gler et al., 2012). According to Skripconoka et al., a clinical trial study was conducted on 421 patients who were administered delamanid at dosages of 100 and 200 mg twice daily. The results reported that the patients who received delamanid for 6 months experienced a mortality rate of 1%, while those who received the drug for 2 months experienced a higher mortality rate of 8.3% (Skripconoka et al., 2013). A study from South Korea reported that 32 patients with MDR-TB received delamanid for 24 weeks. The study showed that 72.2% of the patients achieved solid media conversion and 50% achieved liquid media conversion after 8 weeks of treatment. After 24 weeks of delamanid treatment, the culture conversion rates significantly improved, with 94.4% and 92.9%, respectively. No serious adverse events or deaths were reported during the treatment period, indicating that delamanid can be safely used for long-term treatment of MDR-TB infection and is effective in achieving the culture conversion rates associated with successful treatment outcomes (Mok et al., 2018). A longer treatment regimen with delamanid may be more effective in reducing mortality associated with MDR-TB infection.

The studies concluded that delamanid is an effective anti-TB drug that can improve the culture conversion rates leading to successful therapeutic outcomes. The studies also indicate that delamanid is safe for long-term use. Delamanid should be considered as a potential treatment option for patients with MDR-TB who are unresponsive to conventional drugs or have limited alternatives.

4.3 Side effects of delamanid

Delamanid can cause QTc prolongation, anorexia, gastritis, malaise, anemia, and psychiatric disorders. In children, it may result in liver damage and low white blood cell counts. Monitoring for these side effects is necessary to ensure the safe use of delamanid (Skripconoka et al., 2013; Mohr et al., 2018). Although delamanid may cause increased QTc prolongation, it may not necessarily be associated with any serious health risks. Vomiting, QTc prolongation, and myalgia were the most commonly reported side effects of delamanid administration. In more severe cases, QTc prolongation could indicate serious health risks (Gler et al., 2012; Skripconoka et al., 2013; Mohr et al., 2018). For example, Hughes et al. conducted a study to determine the adverse effects of the delamanid regimen in 58 RR-TB patients in South Africa. The study found that vomiting, QTc prolongation, and myalgia were the most common adverse reactions reported with delamanid administration. Moreover, one patient exhibited progressively severe QTc prolongation and cardiac symptoms such as chest discomfort, lightheadedness, and palpitations (Hughes et al., 2019), which could indicate more serious health risks from taking the drug. Since delamanid is considered to be an effective drug, it should not be stopped abruptly stopped or discontinued due to QTc prolongation. Monitoring for side effects while taking delamanid is important to ensure its safe use.

One of the major concerns associated with the prolonged utilization of delamanid is its low water solubility. This problem results in suboptimal absorption of delamanid formulations in clinical trials (Tao et al., 2019). Limited bioavailability leads to more frequent dosing, which can increase the overall treatment expenses and affect the efficacy of the drug. Attempts have been

made to optimize the delamanid formulation to increase its bioavailability and reduce dosing frequency. To improve the effectiveness of delamanid, it may be necessary to increase food intake or employ other measures such as liposomal formulations or co-administration with other drugs to ensure its effective delivery and absorption into the body. In a study aimed at optimizing the route of administration, an indigestible nanostructured lipid formulation was found to absorb duration of delamanid, outperforming milk or suspension formulations (Ramirez et al., 2021). This finding could be beneficial in reducing treatment costs for patients in low-income regions by reducing the number of doses required for successful outcomes. Additionally, a recent study has shown the *in vitro* efficacy of delamanid can be improved, and its water solubility increased, through cyclodextrin complexation (Patil et al., 2023).

4.4 Safety and effectiveness of delamanid in children with Drug-Resistant TB

According to the WHO's comprehensive Tuberculosis Guidelines evaluate, delamanid is evaluated as part of a long-term regimen for the treatment of MDR/RR-TB in children. In a study, cultures were found to be negative in 116 (79%) out of 147 MDR-TB patients after 24 weeks of delamanid treatment. This included a negative culture rate of 20 of 25 (80%) in pediatric culture. 8% of participants experienced QTc prolongation, but no other serious adverse events were reported. These results suggest that delamanid can be an effective and safe therapy for the treatment of MDR-TB infection in both adults and children (Ghosh et al., 2021). The pharmacokinetics and safety of delamanid when administered to children with MDR-TB aged 0-17 years were evaluated in two clinical trials (NCT01856634 and NCT01859923) to determine the appropriate dose for this age group. The study results indicated that a good therapeutic response was achieved by 89.2% of patients at 24 months after the first dose. Furthermore, the safety profile of delamanid was found to be similar between adults and patients aged 0-17 years (Garcia-Prats et al., 2022).

According to the study conducted on adult volunteers, it has been shown that the bioavailability of dispersed 50 mg delamanid tablets is equivalent to that of whole tablets. This could be an alternative for patients who are unable to swallow whole tablets, such as children and other patients (Zou et al., 2023).

Despite its potential side effects, delamanid may still be considered a priority drug for certain demographic groups and for patients refractory to standard medications.

5 Combination the new drugs bedaquiline and/or delamanid with other drugs

Recent studies have shown encouraging results in the treatment of multidrug-resistant tuberculosis (MDR-TB) through the combined use of bedaquiline and delamanid. Extensive trials combining these drugs with other promising medications have provided data that can guide the treatment of MDR-TB in children (Table 1).

A systematic review and meta-analysis conducted by Holmgaard et al. included 13 studies with a total of 1031 individuals and reported a combined estimate of a favorable treatment outcome of 73.1%. The review also found that sputum culture conversion rates at 6 months ranged from 61% to 95%. Overall, QTc prolongation was 7.8% (Holmgaard et al., 2023). Culture conversion and Treatment effectiveness of delamanid-containing regimens were evaluated by a comprehensive evaluation of 25 studies (22 observational and 3 experimental studies) including 1276 patients. In observational studies, the group of regimens including delamanid, which included 591 patients, had a treatment success rate of 80.9%; by contrast, the group of regimens combining delamanid and bedaquiline, which included 685 patients, had a success rate of 72.8%. The success rates for delamanid-containing regimens were 72.5% in experimental investigations including 411 patients (Nasiri et al., 2022). Franke et al. conducted a study that analyzed data from 1,109 patients receiving polypharmacy therapy with bedaquiline (63%), delamanid (27%), or both (10%), 939 patients (85%) experienced culture conversion within 6 months. The incidence of culture conversion was lower among HIV patients (Franke et al., 2021). However, the findings suggest that the combination of bedaquiline and delamanid is still an effective regimen for the treatment of MDR-TB, with a low incidence of clinically significant cardiotoxicity. The expanded use of this drug combination could be particularly beneficial for unique cohorts, such as TB patients with AIDS.

Unfortunately, there is a dearth of combination trials available for children, which makes it imperative to collect data on children to provide them with faster and more effective treatment options. In South Africa, an injection-free regimen containing bedaquiline and delamanid was used to treat RR-TB in adolescents (10-19 years). The final outcomes at the end of treatment for 22 participants were 17 (77%) treatment successes, 2 (9%) lost to follow-up, 2 (9%) treatment failures, and 1 (5%) death. These results indicate that injection-free regimens containing bedaquiline and/or delamanid are effective and well-tolerated in adolescents, which is consistent with WHO recommendations for this age group (Mohr-Holland et al., 2020). Another study conducted on children in Mumbai, India showed similar results; an injection-free regimen containing bedaquiline and/or delamanid was found to be effective and well tolerated when administered on an outpatient basis. Therefore, it should be routinely available to these vulnerable groups (Das et al., 2020).

While the potential QTc prolonging effects of bedaquiline and delamanid limit their combined use, there is some preliminary experience with their concomitant use in adults. In the absence of effective alternative treatments, this combination may be considered for use in children under close clinical monitoring (Matteelli et al., 2015; Seddon et al., 2012). Two children with highly DR-TB received bedaquiline and delamanid, resulting in a prolonged QTc interval when assessed using the Bazett formula. However, when assessed using the Fridericia formula, the QTc interval was normal. To ensure patients receive the maximum benefit from treatment, it is crucial to emphasize the use of appropriate monitoring formulas to assess for potential adverse effects, such as prolonged QTc

TABLE 1 The BDQ and/or DLM combination dosing clinical trials for TB treatment on ClinicalTrials.gov.

ClinicalTrials.gov Identifier	Conditions or disease	Age	Enrollment	Intervention/treatment	Phase	Trial Status	Start Date	Completion Date	the source of the clinical trial details information
NCT01215851	Pulmonary TB	18-65	85	PMD, PZA, BDQ, EMB, MXF	II	Completed	Oct 2010	Aug 2011	https://clinicaltrials.gov/ct2/show/NCT01215851
NCT01341184	TB	18-45	33	RFB, RFP, BDQ	I	Completed	Oct 2011	May 2012	https://clinicaltrials.gov/ct2/show/NCT01341184
NCT01691534	Pulmonary TB	18-65	105	PMD, BDQ, PZA, CFZ, EMB	II	Completed	Oct 2012	May 2013	https://clinicaltrials.gov/ct2/show/NCT01691534
NCT02193776	TB	18-75	240	BDQ, PMD, MXF, PZA, INH, EMB, RFP	II	Completed	Oct 2014	Feb 2018	https://clinicaltrials.gov/ct2/show/NCT02193776
NCT02216331	TB	19-55	32	RFT, RFP, BDQ	I	Completed	Mar 2010	May 2010	https://clinicaltrials.gov/ct2/show/NCT02216331
NCT02333799	**Pulmonary TB, MDR-TB, XDR-TB**	**≥14**	**109**	**PMD, BDQ, LNZ**	**III**	**Completed**	**Feb 2015**	**Aug 2020**	https://clinicaltrials.gov/ct2/show/NCT02333799
NCT02409290	**MDR-TB**	**≥15**	**588**	**MXF, CFZ, EMB, PZA, INH, PTH, KAN, LVX, BDQ**	**III**	**Active, not recruiting**	**Apr 2016**	**Estimated Apr 2023**	https://clinicaltrials.gov/ct2/show/NCT02409290
NCT02454205	TB, MDR-TB, XDR-TB	≥18	154	LNZ, BDQ, LVX, PZA, INH, ETA, TRD, MXF, KAN	II/III	Completed	Nov 2015	Aug 2021	https://clinicaltrials.gov/ct2/show/NCT02454205
NCT02583048	TB, HIV Infections	≥18	84	BDQ, DLM, DTG	II	Completed	Oct 2016	Feb 2021	https://clinicaltrials.gov/ct2/show/NCT02583048
NCT02589782	**Pulmonary TB, MDR-TB, XDR-TB**	**≥15**	**552**	**BDQ, PMD, MXF, LNZ, CFZ**	**II/III**	**Active, not recruiting**	**Jan 2017**	**Estimated Dec 2022**	https://clinicaltrials.gov/ct2/show/NCT02589782
NCT02619994	TB, MDR-TB	19-85	238	LNZ, DLM, LVX, PZA	II	Recruiting	Jan 2016	Estimated Jun 2021	https://clinicaltrials.gov/ct2/show/NCT02619994
NCT02754765	**Pulmonary TB, MDR-TB**	**≥15**	**754**	**BDQ, CFZ, MXF, LVX, PZA, LNZ, DLM**	**III**	**Active, not recruiting**	**Dec 2016**	**Estimated Sep 2023**	https://clinicaltrials.gov/ct2/show/NCT02754765
NCT03086486	**Pulmonary TB, MDR-TB, XDR-TB, Pre-XDR-TB**	**≥14**	**180**	**BDQ, PMD, LNZ**	**III**	**Active, not recruiting**	**Nov 2017**	**Estimated Feb 2022**	https://clinicaltrials.gov/ct2/show/NCT03086486
NCT03338621	Pulmonary TB, MDR-TB, DR-TB, DS-TB	≥18	455	BDQ, PMD, MXF, PZA	II/III	Completed	Jul 2018	Jun 2022	https://clinicaltrials.gov/ct2/show/NCT03338621
NCT03474198	Pulmonary TB	18-65	900	RFP, INH, PZA, EMB, LNZ, CFZ, RFT, LVX, BDQ	II/III	Recruiting	Mar 2018	Estimated Mar 2022	https://clinicaltrials.gov/ct2/show/NCT03474198
NCT03678688	Pulmonary TB	18-64	122	BDQ, DLM, OPC-167832	I/II	Completed	Oct 2018	Jun 2022	https://clinicaltrials.gov/ct2/show/NCT03678688

(Continued)

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TABLE 1 Continued

ClinicalTrials.gov Identifier	Conditions or disease	Age	Enrollment	Intervention/treatment	Phase	Trial Status	Start Date	Completion Date	the source of the clinical trial details information
NCT03828201	**TB, MDR-TB**	**≥12**	**220**	**DLM, LVX, BDQ, CFZ, LNZ**	**II**	**Recruiting**	**Jun 2022**	**Estimated Jul 2025**	https://clinicaltrials.gov/ct2/show/NCT03828201
NCT03896685	**Pulmonary TB, MDR-TB**	**≥15**	**324**	**BDQ, DLM, LNZ, CFZ**	**III**	**Recruiting**	**Apr 2020**	**Estimated Nov 2024**	https://clinicaltrials.gov/ct2/show/NCT03896685
NCT03959566	Pulmonary TB	18-65	75	BDQ, DLM, MXF, SZD	II	Completed	May 2021	Sep 2022	https://clinicaltrials.gov/ct2/show/NCT03959566
NCT04062201	**Pulmonary TB, MDR-TB, XDR-TB, Pre-XDR-TB, RR-TB**	**≥6**	**402**	**BDQ, DLM, LNZ, LVX, CFZ, INH, EMB, PZA**	**III**	**Active, not recruiting**	**Aug 2019**	**Estimated Jun 2023**	https://clinicaltrials.gov/ct2/show/NCT04062201
NCT04081077	Pulmonary TB, MDR-TB, XDR-TB	≥18	240	BDQ, PMD, MXF, LNZ, CFZ	II/III	Active, not recruiting	Aug 2019	Estimated Sep 2022	https://clinicaltrials.gov/ct2/show/NCT04081077
NCT04207112	Pulmonary TB, MDR-TB, XDR-TB	≥18	200	BDQ, PMD, MXF, LNZ, CFZ	II/III	Recruiting	Oct 2020	Estimated Jul 2022	https://clinicaltrials.gov/ct2/show/NCT04207112
NCT04545788	RR-TB	18-65	200	LNZ, BDQ, CS	/	Recruiting	Aug 2020	Estimated Dec 2022	https://clinicaltrials.gov/ct2/show/NCT04545788
NCT04550832	Pulmonary TB	18-65	76	DZD, BDQ, DLM, MXF	II	Active, not recruiting	Oct 2021	Estimated Mar 2024	https://clinicaltrials.gov/ct2/show/NCT04550832
NCT04629378	TB	18-65	22	MPM, co-amoxiclav, PZA, BDQ, EMB	II	Completed	Aug 2020	Jun 2021	https://clinicaltrials.gov/ct2/show/NCT04629378
NCT05007821	Pulmonary TB, MDR-TB	≥18	132	LNZ, BDQ, DLM, CFZ	II	Recruiting	Aug 2022	Estimated Sep 2025	https://clinicaltrials.gov/ct2/show/NCT05007821
NCT05040126	Pulmonary TB, MDR-TB, Pre-XDR-TB	18-65	400	LNZ, BDQ, PMD	III	Recruiting	Oct 2021	Estimated Mar 2024	https://clinicaltrials.gov/ct2/show/NCT05040126
NCT05221502	Pulmonary TB	18-65	120	BDQ, DLM, OPC-167832	II	Recruiting	Apr 2022	Estimated Feb 2024	https://clinicaltrials.gov/ct2/show/NCT05221502
NCT05278988	MDR-TB	18-66	60	BDQ, DLM, CFZ, PZA	IV	Recruiting	Apr 2021	Estimated Sep 2024	https://clinicaltrials.gov/ct2/show/NCT05278988
NCT05306223	TB, MDR-TB	18-65	212	BDQ, LVX, LNZ, CS, CFZ, PZA, PTH	IV	Recruiting	May 2022	Estimated Aug 2025	https://clinicaltrials.gov/ct2/show/NCT05306223
NCT05382312	TB	18-65	55	GSK3036656, BDQ, DLM, EMB	II	Recruiting	Jul 2022	Estimated Sep 2023	https://clinicaltrials.gov/ct2/show/NCT05382312
NCT05556746	Pulmonary TB, HIV Infections	≥18	156	BDQ, CFZ, PZA, DLM, RFP, INH, EMB	II	Not yet recruiting	Estimated Mar 2023	Estimated Jun 2026	https://clinicaltrials.gov/ct2/show/NCT05556746

(Continued)

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TABLE 1 Continued

ClinicalTrials.gov Identifier	Conditions or disease	Age	Enrollment	Intervention/treatment	Phase	Trial Status	Start Date	Completion Date	the source of the clinical trial details information
NCT05686356	TB	18-65	352	SZD, NAC, PMD, BDQ, EMB	II/III	Not yet recruiting	Estimated Jan 2023	Estimated Sep 2025	https://clinicaltrials.gov/ct2/show/NCT05686356
NCT05766267	**Pulmonary TB**	**≥12**	**288**	**RFB, DLM, BDQ, MXF, PZA, INH, RFP, EMB**	**II/III**	**Not yet recruiting**	**Estimated Mar 2023**	**Estimated Apr 2026**	https://clinicaltrials.gov/ct2/show/NCT05766267
NCT05807399	Pulmonary TB	18-65	360	SZD, RFP, INH, PZA, MXF, BDQ, DLM	II	Recruiting	Apr 2023	Estimated Feb 2025	https://clinicaltrials.gov/ct2/show/NCT05807399

Data until April 2023. Recent data might not have been updated. Bold black indicates clinical trials that included children. TB, tuberculosis; MDR-TB, multidrug-resistant TB; XDR-TB, extensively drug-resistant tuberculosis TB; Pre-XDR-TB, pre-extensively drug-resistant tuberculosis TB; RR-TB, rifampicin-resistant TB; BDQ, Bedaquiline (TMC-207, R207910); CFZ, Clofazimine (NSC-141046); CS, Cycloserine; DLM, Delamanid (OPC-67683); DTG, Dolutegravir; DZD, Delpazolid; EMB, Ethambutol (Rifafour e-275); ETA, Ethionamide; INH, Isoniazid; KAN, Kanamycin; LNZ, Linezolid; LVX, Levofloxacin; MPM, Meropenem; MXF, Moxifloxacin; NAC, N-acetylcysteine; PMD, Pretomanid (PA-824); PTH, Prothionamide; PZA; RFB, Rifabutin Pyrazinamide; RFP, Rifampicin(Rifampin); RFT, Rifapentine; SZD, Sutezolid; TRD, Terizidone.

intervals. If necessary, the required interventions can be promptly initiated without discontinuing treatment for patients who would otherwise benefit from it (Shah et al., 2020).

6 Discussion

Significant progress has been made in the development of drugs to treat MDR-TB infection in children. Bedaquiline and delamanid have been used successfully used and are recommended by WHO as part of the treatment regimen. However, further studies are required to gather accurate information on the safety and effectiveness of these drugs in pediatric patients. It is important to use anti-TB drugs judiciously until more data are available.

To accelerate the goal of TB elimination, it is critical to continue the search for effective new therapeutic targets and drugs. The development of new drugs should focus on avoiding negative drug-drug interactions, utilizing novel modes of action that reduce cross-resistance, and ensuring compatibility with other drugs that are used in combination. In addition, the use of analogs of existing drugs could provide more effective and safer treatments for TB. It is also important to explore new technologies to discover new targets and new treatments for TB, such as using CRISPR to disrupt chemical genetics platforms in order to discover new drug targets and drug resistance mechanisms (Li et al., 2022). Furthermore, the issue of phage therapy, has become increasingly serious due to concerns about antimicrobial resistance (Strathdee et al., 2023). The development of drugs to treat children with MDR-TB is a challenging task due to the lack of easy ways for children to take the adult formulations correctly, and the limited availability of medicines suitable for children. Urgent action is required to expedite research in creating child-friendly medicines that can be more accessible worldwide, thereby ensuring that children can receive appropriate treatment.

In conclusion, our review highlights the use of bedaquiline and delamanid as potential treatments for children with MDR-TB. We summarize their development history, efficacy, safety and potential adverse effects. Further research is necessary to determine the optimal use of these drugs for treating MDR-TB in children.

Author contributions

KM conceived and designed the article; HZ and XZ wrote the manuscript; ZZ and LL put forward professional opinions; KM and JB revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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The fecal carriage rate of extended-spectrum β-lactamase–producing or carbapenem-resistant *Enterobacterales* among Japanese infants in the community at the 4-month health examination in a rural city

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Background: Extended-spectrum β-lactamase–producing *Enterobacterales* (ESBL-E) is a great public health concern globally not only in hospitals but also in the community. To our knowledge, there have been few studies on the prevalence of ESBL-E and much less about carbapenem-resistant *Enterobacterales* (CRE) among children in the community, and there is no such study in Japan despite such situations. This study aimed to clarify their carriage status among Japanese infants in the community by taking the opportunity of the 4-month health checkup.

Methods: This prospective analysis was conducted from April 2020 to March 2021 in Shimabara City, Nagasaki Prefecture, Japan. The research-related items were mailed to all subjects with official documents for the checkup. The fecal samples were obtained from the diaper by guardians beforehand and were collected with the questionnaire and then screened for ESBL-E and CRE by a clinical laboratory company with selective agars followed by identification and confirmation. Only the positive samples were analyzed about resistant genotypes.

Results: One hundred fifty infants aged 4–5 months, over half of the subjects, participated in this study. The overall ESBL-E carriage rate was 19.3% (n = 29), and no CRE carrier was detected among them. All identified ESBL-E were *E. coli* except for one *K. pneumoniae*. A significantly higher carriage rate was recorded among the infants born at "Hospital A" (25.0%) than the others (11.3%). *Enterobacterales* producing CTX-M-9 ± TEM were broadly distributed among the positive samples (65.5%), whereas the CTX-M-1 group was exclusively detected among those from "Hospital A". Recursive partitioning analysis suggested that delivery facilities might be an important factor for ESBL-E colonization, although the effect could be decreased as they grow. In contrast, no significant effect was observed for other factors such as parent(s) as healthcare worker(s), having a sibling(s), and the mode of delivery.

Conclusion: This study revealed the ESBL-E and CRE carriage status of Japanese infants in the community for the first time, although the setting is somewhat limited. Our findings indicated that environmental factors, especially delivery facilities, influenced ESBL-E colonization among infants aged 4–5 months, implying the need for strengthening countermeasures against antimicrobial resistance at delivery facilities and communities outside the hospitals.

KEYWORDS

extended-spectrum beta-lactamase-producing *Enterobacterales*, carbapenem-resistant *Enterobacterales*, antimicrobial resistance, antimicrobial stewardship, infants, fecal carriage, *Escherichia coli*, obstetrics facilities

1 Introduction

Antimicrobial resistance (AMR) is a serious ongoing problem globally. An estimation from the United Kingdom advocates that AMR in illnesses such as bacterial infections, malaria, tuberculosis, and human immunodeficiency virus will be the first cause of death all around the world in 2050 (O'Neill, 2018). Indeed, patients with urinary tract infections (UTIs) caused by extended-spectrum β-lactamase– producing *Enterobacterales* (ESBL-E) are not rare on the daily clinical scene, even at pediatric clinics in Japan. In addition, we also experienced an outbreak of carbapenemase-producing *Enterobacterales* (CPE) and ESBL-E at the neonatal intensive care units (NICUs) in Nagasaki Prefecture, Japan.

ESBL-E has a higher carriage rate in the community than methicillin-resistant *Staphylococcus aureus*. The global carriage rate of ESBL-E in the community was reported as 16.5% during the period between 1 January 2000 and 13 February 2020. It is increasing continuously from 2.6% (95% CI, 1.6–4.0) in 2003–2005 to 21.1% (95% CI, 15.8%–27.0%) in 2015–2018 and estimated as a 1.5% yearly increase by linear regression analysis (Bezabih et al., 2021). Among six WHO regions (i.e., Southeast Asia, Western Pacific, Africa, Eastern Mediterranean, Americas, and Europe), the Southeast Asian Region had the highest prevalence at 27% (95% CI, 2.9%–51.3%) based on four studies from India, Thailand, and Nepal, whereas the European Region had the lowest at 6.0% (95% CI, 4.6%–7.5%) based on 19 studies.

The ESBL-E carriage rate in Japan was reported to be 6.4% among 218 stool specimens obtained from adult volunteers (Luvsansharav et al., 2011) and 7.5% in 67 stool samples acquired from medical students in 2011 (Niki et al., 2011). There have been subsequent reports of ESBL-E carriage rates among adults in Japan, including 4.8% of 4,314 stool specimens obtained from 2,563 food handlers (Nakane et al., 2016), 12.5% of 257 hospitalized patients and 8.5% of 496 workers at 25 meal supply centers in the same community without significant difference (Nakamura et al., 2016), 12.2% of 263 persons who underwent routine medical checkups (Higa et al., 2019), and 9.7% of 547 healthy individuals (Masui et al., 2022), whereas there have been only a few studies on ESBL-E carriage rates among Japanese children, including 12% of 50 inpatients at a pediatric tertiary care hospital (Minami et al., 2012) and 12.5% of 256 pediatric patients with gastroenteritis among 11 hospitals in Mie Prefecture (Nagai et al., 2017). The worldwide prevalence of ESBL-E carriage in children has been reportedly variable, such as 74% of neonates and 59% of children in Ethiopia's largest tertiary hospital (Desta et al., 2016), 50.4% of hospitalized patients and 11.6% of children in the community in Tanzania (Tellevik et al., 2016), 2.3% of asymptomatic nursery children in Germany (Harries, et al. 2016), and 3.5% of healthy children in the United States (Islam et al., 2018). The ESBL-E carriage rates have shown an increasing trend even among children in the community. The French study showed the rate to be 7.6% of 1886 children aged 6–24 months with a tendency to increase from

2.2% in 2010 to 10.8% in 2016 (Birgy et al., 2016). The study from Sweden reported that 16.8% of 334 children aged 13–45 months were positive in 2016, over a six-fold increase from 2.6% in 2010 (Kaarme et al., 2018).

Meanwhile, relatively few studies have examined the community-acquired carriage of carbapenem-resistant *Enterobacterales* (CRE). In a review article analyzing 15 studies, the rates of the community-associated or community-onset CRE globally were as various as 0.04%–29% in 10 studies across the world, and five studies did not detect CRE (Kelly et al., 2017). These rates should not be interpreted immediately as the community-acquired carriage rates because most of those reports were not based on the research in the community but on the studies conducted at clinics or estimated from the rates of hospitalized patients on admission. In any case, the results suggest that the CRE threat is not limited to the hospital but is spreading to the community as well. For example, the aforementioned report studying hospitalized patients of all ages in Ethiopia revealed five CRE carriers, all of whom were children. Two of them carried *Klebsiella pneumoniae* carbapenemase –producing CPE (Desta et al., 2016).

To our knowledge, there are no data about the ESBL-E carriage rate of children in the community in Japan and about the CRE carriage rate of non-hospitalized individuals whether adults or children. We conducted this study to reveal these carriage rates among infants in the community in a rural area and to determine associated factors in this area by analyzing the questionnaires.

2 Materials and methods

2.1 Study setting

This prospective study was performed between April 2020 and March 2021 in Shimabara City, Nagasaki Prefecture, the westernmost part of Japan (Figure S1). This city has an estimated population of 43,556 people with live births of 280 neonates in January 2020 and 43,228 people with 295 neonates in January 2021, according to information from Nagasaki Prefecture (https://www.pref.nagasaki.jp/shared/uploads/2021/05/1622446419.pdf; https://www.pref.nagasaki.jp/shared/uploads/2022/05/1653363988.pdf).

ESBL-E or CRE carriage was defined with the positive results of both screening cultures and identification of the resistant genotypes using the methods described below.

This study was approved by the Human Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences.

2.2 Study subject

To reveal the actual carriage rate of ESBL-E and CRE among healthy children, we took advantage of the opportunity for a 4-month health checkup, which all children were required to have in Japan. All subjects for the checkup received the supporting documentation, the informed consent documents, questionnaires, and kits for stool sample collection with other documents related to the health checkup through the Shimabara Healthcare Center. All infants with the approval of their parents or guardians were included in this study except those who could not take stool samples or those who were regarded as inappropriate for other reasons.

2.3 Sample processing and data collection

All samples were obtained from the stool on the diaper with two separate swabs including Cary-Blair media for culture named Seed swab γI (Eiken Chemical, Ltd.) at each home mainly by parents following the instruction text or the video created originally and uploaded on YouTube® presented on the supporting documentation beforehand of visiting the health checkup (Figure S2). These stool samples were collected at the Shimabara Healthcare Center on the day of the checkup. If the parents of the participant could not bring the sample but wanted to join the study, then we asked them to take it to the reception for the Pediatric Department at the Shimabara Prefectural Hospital. Every sample was retrieved and screened for ESBL-E and CRE with 48-h aerobic incubation under 35°C in CHROMagar™ ESBL and CHROMagar™ mSuperCARBA (Kanto Chemical Co., Inc.), respectively, at a major Japanese clinical laboratory company. The former yielded colonies of the following colors: mauve red, metallic blue with or without a red halo, and brown with a halo, and the latter yielded those of the following colors: mauve red and metallic blue. Hence, we picked up one colony each from different color groups from these selection agars. Incubation with 5% Trypto-casein Soy Agar was also performed in parallel for each culture to ensure non-contamination and to use for retesting or additional examination. After the bacterial identification and drug susceptibility test using MALDI-TOF MS (matrix-assisted laser desorption/ionization–time-of-flight mass spectrometer) of MALDI Biotyper® microflex® (Bruker) and Dry Plate Eiken (Eiken Chemical, Ltd.) based on the criteria of CLSI M100S-22, respectively, the ESBL production and CRE characteristic were confirmed with the following methods for all screening-positive samples. ESBL-E was identified when Cica Beta Test I was positive and CVA was negative. If Cica Beta Test was inconclusive, then clavulanic acid added double disc synergy test (DDST) test was performed with cefotaxime and cefpodoxime. CRE was identified under the criteria stipulated by the Japanese Infectious Disease Control Law in which the minimum inhibitory concentration (MIC) for meropenem is 2 μg/ml or higher or the MIC for imipenem is 2 μg/ml or higher, and the MIC for cefmetazole is 64 μg/ml or higher.

Only the positive samples were returned and stored in the Microbank™ (Pro Lab Diagnostics, Inc.) at the −70°C deep freezer placed at the Department of Pediatrics, Nagasaki University Hospital.

The following data were obtained from the questionnaires: gender, birth date, sampling date, gestational age, birth weight, mode of delivery, delivery facility, feeding methods within the first week of birth and around 4 months old, hospitalized history (cause of admission, hospital, antibiotic treatment, and length of hospital stay), siblings, ESBL-E or CRE outbreak exposure of siblings, parental occupation (healthcare worker or else), parental history

of staying abroad, and parentally history of hospitalization abroad. The birth date was used for calculating age in days at the time of the health checkup.

2.4 Identification of the resistant genomic pattern

The genotypes of ESBL were detected with TaKaRa PCR Thermal Cycler Dice® Gradient (TaKaRa Bio, Inc.) by using Cica Geneus® ESBL Genotype Detection KIT2 (Kanto Chemical Co., Inc.) following its instruction manual. DNA extraction was demonstrated with the boiling method using Chelex (Bio-Rad Laboratories, Hercules, CA, USA) following the previous report with minor modifications (Motoshima et al., 2010). The targeted genes are *bla* $_{\text{CTX-M (-1, -2, -8, -9, and -25 group)}}$, *bla* $_{\text{CTX-M chimera}}$, *bla* $_{\text{GES (ESBL type)}}$, *bla* $_{\text{TEM}}$, and *bla* $_{\text{SHV}}$. Each abbreviation indicates the following: CTX-M, cefotaximase-Munich; GES, Guiana extended-spectrum β-lactamase; TEM, temoneira; and SHV, sulfhydryl variable.

2.5 Data analysis

All the data obtained from the questionnaires were entered into a Microsoft® Excel® (Microsoft Corporation) worksheet at the venue of the health checkup immediately after the collection, and the parent was asked to fill in if anything remained blank or appeared obscure. JMP® Pro 16.0.0 (SAS Institute, Inc.) was used for every statistical analysis. Fisher's exact test was used to analyze categorical data, and a t-test was used for continuous data. The two-sided p-values <0.05 were considered statistically significant. Hence, the objectives of the presenting study were exploratory ones, the statistical significance cannot be interpreted that it is valid to reject the corresponding null hypothesis.

In analyses of the association between two binomial variables, the p-values were obtained via Fisher's exact test and presented to describe the extent of statistical significance of the association. In the analysis, the candidate of the predictor was a continuous variable; instead of Fisher's exact test, the t-test was used to obtain p-values. To determine associated factors among the background of the study subjects among infants in the community, the odds ratios for ESBL-E and CRE carriages and the confidence intervals were calculated. The 95% confidence intervals of binomial probabilities were obtained using the Wilson score method.

In addition to those, recursive partitioning analysis was conducted to describe the bias of background factors in carriers and non-carriers. The nodes in the tree were generated using the likelihood ratio chi-square. The splitting was stopped with the following criteria: No branching increases the likelihood ratio chi-square in the descendent nodes, the number of individuals allocated into nodes containing positive subjects is less than 10, or it is difficult to interpret the results and make hypotheses.

3 Results

3.1 Number of participants

The research documents and sampling kits were sent to 271 subjects, and 150 infants (55.4%) were eligible to participate in this study except for one infant whose parent had difficulty in informed consent and answering the questionnaire.

3.2 Characteristics of the participants

The characteristics of the participants are summarized in Table 1. The percentages of preterm (3.3%) and low birth weight infants (5.3%) were lower than those previously reported from Japan (5.7% and 9.4%, respectively) (Isayama, 2019; Mine et al., 2021). Some of the characteristics included too few subjects to assess the impact on ESBL-E carriage, such as "antibiotic treatment during hospitalization", "sibling's stay in a relevant ward during the outbreak", and "parental stay abroad".

3.3 Carriage situation of ESBL-E and CRE

Each participant provided two samples for screening ESBL-E and CRE, and all 300 specimens except for those two from the excluded participant were eligible for testing. ESBL-E was detected from a total of 29 samples (19.3%), whereas no CRE-positive samples were found. Among the ESBL-E positive samples, 28 (96.6%) were *Escherichia coli* and one was *Klebsiella pneumoniae*. One *K. oxytoca* was identified through the screening but excluded because any relevant genotype was detected.

Table 2 shows the carriage rates according to the background factors, and Figure 1 exhibits the carriage rates according to the delivery facilities. There was no visible difference in the backgrounds between carriers and non-carriers except for their delivery facilities. "Hospital A", accounting for the largest number of deliveries in the study site, had more than double and significantly higher ESBL-E prevalence than that of the other facilities (22/88 vs. 7/62, 25.0%; 95% CI, 17.1–35.0% vs. 11.3%; 95% CI, 5.6–21.5%; p = 0.039; OR, 2.62; 95% CI, 1.04–6.59).

3.4 Recursive partitioning analysis

Recursive partitioning analysis was performed to describe background factor bias in ESBL-E carriers and non-carriers as presented in Figure 2. All factors obtained from the questionnaire except for the sub-answers (i.e., antibiotic usage and the duration of hospital stay) were included in the explanatory variables. To better reflect the actual situation, "delivery facilities" was arranged for "Hospital A" and others, and "occupation" was changed to whether one of the parents is currently or has been in the medical profession within the past 3 years or not and was included in the explanatory

TABLE 1 Characteristics of the participants.

Characteristic		No.	%
Sex	Male	72	48.0
	Female	78	52.0
Age	Median (IQR), day	146 (134 to 160)	
The period from sampling to collection	Median (IQR), day	−1 (−1 to 0)	
Gestational age	Median (IQR), week	39 (38 to 40)	
Birth weight (BW)	Median (IQR), g	3,101 (2,803.5 to 3,376.0)	
Preterm infants		5	3.3
Low birth weight infants (BW below 2,500 g)		8	5.3
Delivered by cesarean section		35	23.3
Completely bottle-fed, perinatally		2	1.3
Completely bottle-fed, currently		33	22.0
History of hospitalization		18	12.0
	Antibiotic treatment	6	4.0
	Unknown	4	2.7
Sibling(s)	Yes	103	68.7
	The sibling's hospital stay during the outbreak*1	1	1.0
Mother is a healthcare worker	Currently	31	20.7
	In 3 years	8	5.0
	No	111	74.0
Father is a healthcare worker	Currently	17	11.3
	In 3 years	1	0.7
	No	128	85.3
	Not living with father	4	2.7
Mother or father is a healthcare worker	Currently or in 3 years	42	28.0
Parental stay abroad*2	Yes	1	0.7
Parental history of hospitalization abroad	Yes	0	0

*1 The sibling stayed in the perinatal ward during the ESBL-E outbreak in the NICU of a particular facility.
*2 refers more than 2 weeks.

variables. The first branch was the "delivery facilities" ("Hospital A" and "The other facilities") with a significant difference between the nodes as the most major contributors to the tree split (statistical values are the same as described above in 3.3). The descendent nodes created under the node "those from 'Hospital A'" was the "days of age" with a cutoff of 140 days (15/41 vs. 7/47, 36.6% vs. 14.9%, p = 0.026, OR 3.30; 95% CI, 1.21–8.96). The descendent nodes created under the "those from the other facilities" was "birth weight" with a cutoff of 3,332 g (5/15 vs. 2/47, 33.3% vs. 4.3%; p = 0.007; OR, 11.25; 95% CI, 2.14–57.53). The nodes of "days of age" with a cutoff of 153 days among those older than 140 days from "Hospital A" (7/27 vs. 0/20, 25.9% vs. 0.0%; p = 0.015; OR, ∞; 95% CI, 1.68 −∞) were the only descendent node of above nodes with $p < 0.05$.

3.5 Analysis of resistant genes

The genotypes of ESBL were detected for all 30 positive samples except for one of *K. oxytoca* (Table 3). The CTX-M-9 group was the most predominant genotype accounting for 16 *E. coli* isolates, followed by the CTX-M-1 group with the TEM gene (n = 7), CTX-M-9 group with TEM gene (n = 3), CTX-M-1 group (n = 1), and TEM gene (n = 1). The only *K. pneumoniae* isolate harbored SHV with the TEM gene.

In addition, as shown in Table 4 and Figure S3, a total of eight CTX-M-1 group *E. coli* were dominantly detected in the participants born at "Hospital A" with statistical significance compared with those from the other facilities (8/88 vs. 0/62, 9.1% vs. 0.0%; p = 0.021; OR, ∞; 95% CI, 1.58–∞) and of 11 TEM *E. coli* as

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TABLE 2 ESBL-E carriage rates according to the background factors.

Variables		ESBL-E positive n = 29		ESBL-E negative n = 121	
		No.	%	No.	%
Sex	Male	14	48.3	58	47.9
	Female	15	51.7	63	52.1
Age	Median (IQR), days	137 (130.5 to 165.0)		146 (134.5 to 160.0)	
The period from sampling to collection	Median (IQR), days	0 (−1 to 0)		−1 (−1 to 0)	
Gestational age	Median (IQR), weeks	39 (38.5 to 40.0)		39 (38.0 to 40.0)	
Birth weight (BW)	Median (IQR), grams	3,130 (2,821.0 to 3,413.0)		3,078 (2,800.0 to 3,321.0)	
Preterm infants		1	3.5	4	3.3
Low birth weight infants (BW below 2,500 g)		2	6.9	6	5.0
Delivered by cesarean section		6	20.7	29	24.0
Completely bottle-fed, perinatally		0	0.0	2	1.7
Completely bottle-fed, currently		7	24.1	26	21.5
History of hospitalization		3	10.3	15	12.4
	Antibiotic treatment	0	0.0	6	40.0
	Unknown	0	0.0	4	26.7
Sibling(s)	Yes	22	75.9	81	66.9
	The sibling's hospital stay during the outbreak*1	1	1.0	0	0.0
Mother is a healthcare worker	Currently	5	17.2	26	21.5
	In 3 years	2	6.9	6	5.0
	No	22	75.9	89	73.6
Father is a healthcare worker	Currently	3	10.3	14	11.6
	In 3 years	0	0.0	1	0.8
	No	26	89.7	102	84.3
	Not living with father	0	0.0	4	3.3
Mother or father is a healthcare worker	Currently or in 3 years	7	24.1	35	28.9
Parental stay abroad*2	Yes	0	0.0	1	0.8

*1 The sibling stayed in the perinatal ward during the ESBL-E outbreak in the NICU of a particular facility.
*2 refers more than 2 weeks.

well (10/88 vs. 1/62, 11.4% vs. 1.6%; $p = 0.027$; OR, 7.82; 95% CI, 1.24–48.37), whereas CTX-M-9 group isolates, which accounted for 19 (65.5%) of the 29 ESBL positives, were widely distributed regardless of the delivery facilities (12/88 vs. 7/62, 13.6% vs. 11.3%; $p = 0.81$; OR, 1.24; 95% CI, 0.47–3.26).

4 Discussion

To the best of our knowledge, this is the first study about the ESBL-E carriage situation among infants in the community of Japan, as well as about the CRE carriage for non-hospitalized people in Japan although no CRE carrier was detected. In the present study, the ESBL-E carriage rate of infants aged 4 to 5 months in Shimabara city was 19.3%. The infants born at "Hospital A" had a significantly high carriage rate of 25.0% compared with 11.3% in those born at other delivery facilities. The overall prevalence was >1.5-fold higher than those from the previous studies among healthy adults or pediatric samples from hospitals in Japan (Luvsansharav et al., 2011; Niki et al., 2011; Minami et al., 2012; Nakamura et al., 2016; Nakane et al., 2016; Nagai et al., 2017; Higa et al., 2019; Masui et al., 2022); however, it is worthy to note that the ESBL-E prevalence at delivery facilities other than "Hospital A" was comparable to those from the previous studies.

The fecal carriage of ESBL-E can cause UTIs. The annual proportion of ESBL-E among pediatric UTIs in Japan has been reported to be 3.6%–21.1% (Nagata et al., 2015; Yanai et al., 2019; Sakata et al., 2021; Okumiya et al., 2022), except for a study from Shimane Prefecture reporting over 50% prevalence (Horie et al., 2019). These data should not be immediately interpreted as the

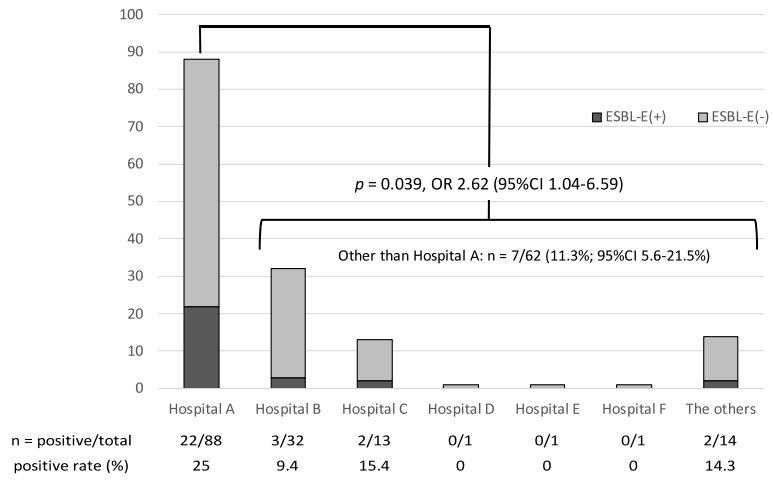

FIGURE 1
The number of ESBL-E–positive and ESBL-E–negative samples by the birth hospital.

ESBL-E carriage rate in the community because the specimens obtained at hospitals tend to show higher carriage rates (Bezabih et al., 2022). However, clinicians should be aware of the ESBL-E prevalence among patients with UTIs at their hospitals for appropriate treatment strategies. For example, in high-prevalence hospitals, intravenous administration of cefmetazole or flomoxef can be selected as the initial therapy for febrile UTI at hospitals with high ESBL-E prevalence in Japan (Matsumura et al., 2015; Fukuchi et al., 2016). Moreover, the escalation to meropenem without waiting for culture results may not be overtreatment, depending on the clinical course of UTI and the prevalence of ESBL-E because 5%–10% of UTIs with gram-negative rods are complicated by bacteremia (Schnadower et al., 2010; Sakata et al., 2021).

Several studies have shown that the younger the child, the higher the carriage rate (Tellevik et al., 2016; Cheng et al., 2022), and the results of our present study suggest that children aged around 4 months may be susceptible to environmental factors, especially the delivery facilities in which they were born. The recursive partitioning analysis indicated that the birth facility significantly contributed to ESBL-E carriage among our study population. Because previous studies have not included "delivery facility" in the survey items, our finding needs to be examined by future studies. Interestingly, further splitting suggested a slightly decreasing trend in the carriage rate over time, and other factors such as the occupation of their parents as medical workers, having siblings, or the mode of delivery did not show statistical significance for the ESBL-E carriage status, although these might be the results of the limitations about our study subjects. We are planning to conduct a similar study on the ESBL-E carriage for the same population when they became age 3 shortly, which will hopefully reveal changes in the carriage status.

In the present study, the CTX-M-9 group was broadly detected among infants born at various delivery facilities, whereas the CTX-M-1 group was exclusively detected among those born at "Hospital A" with statistical significance, strongly suggesting environmental differences among birth facilities and the possibility of CTX-M-9 spreading in the community outside hospitals. In Japan, these two genotypes are common even in non-clinical specimens (Masui et al., 2022; Sekizuka et al., 2022). In particular, the CTX-M-9 group became dominant over the CTX-M-2 group, which was the most prevalent in the early 2000s (Suzuki et al., 2009). These results emphasize the need for action against AMR not only in hospitals but also in the community and birth facilities.

There are several limitations to our study. First, unlike other studies conducted in medical institutions, the samples were collected by the parent, who might technically be less reliable. To address this issue, we have not only provided an instruction manual simply explaining precautions for specimen collection but also directly confirmed that a sufficient amount of stool adhered to the swabs at the time of collection before handing them over to a clinical laboratory company. Contamination from the environment, such as the surface of diapers, is not expected to significantly affect test results, as the number of bacteria in the stool should be overwhelmingly larger.

TABLE 3 ESBL genotypes identified among the screening-positive samples.

ESBL genotypes	Samples (n)	Rate (%)
CTX-M-1 group	1	3.33
CTX-M-1 group, TEM	7	23.33
CTX-M-9 group	16	53.33
CTX-M-9 group, TEM	3	10.00
Not Detected *1	1	3.33
SHV, TEM *2	1	3.33
TEM	1	3.33
Total	30	100.0

*1 refers to one *K. oxytoca* isolate.
*2 refers to one *K. pneumoniae* strain.

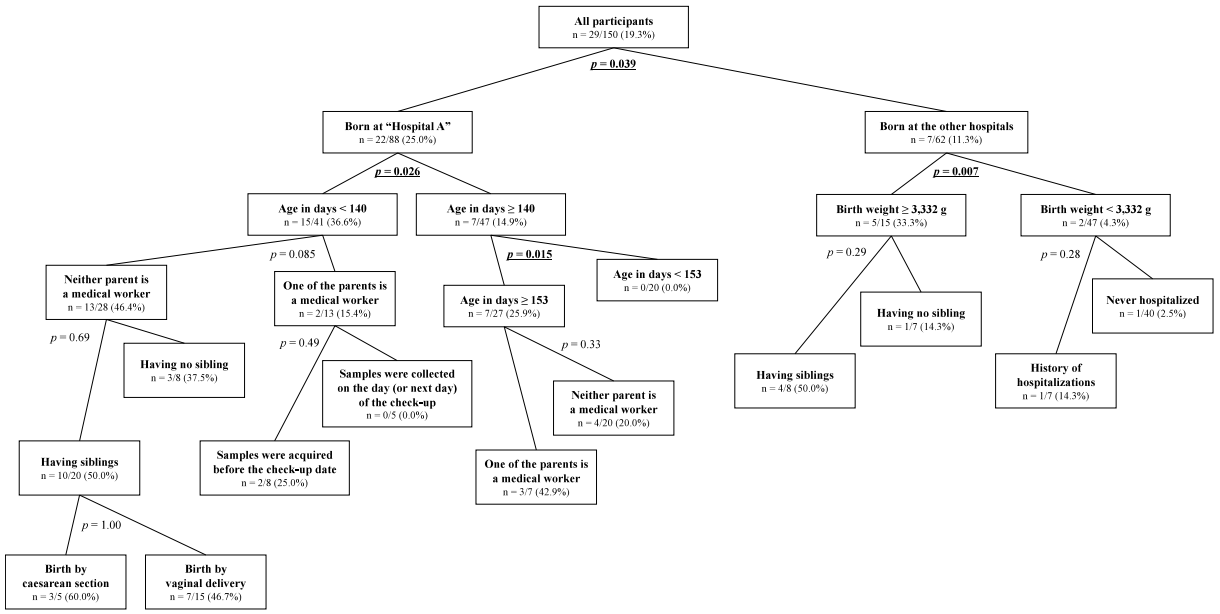

FIGURE 2
The tree of recursive partitioning analysis about ESBL-E carriage among the participants. The number in each node means the prevalence of ESBL-E carriage [i.e., n = positive/total (carriage rate)]. The *p*-values shown beside each branch were presented not for rejection of the null hypothesis but for exploratory purposes, and those that are lower than 0.05 are emphasized with bold and are underlined.

TABLE 4 The differences in detected ESBL genotypes about *E. coli* between birth facilities.

ESBL genotypes	Hospital A n (%*)	The others n (%*)	Statistical values
CTX-M-1 group	8 (9.1)	0 (0.0)	$p = 0.021$, OR ∞ (95% CI 1.58–∞)
CTX-M-9 group	12 (13.6)	7 (11.3)	$p = 0.81$, OR 1.24 (95% CI 0.47–3.26)
TEM	10 (11.4)	1 (1.6)	$p = 0.027$, OR 7.82 (95% CI 1.24–48.37)
Overall ESBL positive *E. coli*	21 (23.9)	7 (11.3)	$p = 0.058$, OR 2.46 (95% CI 0.99–6.07)

* These rates indicate the prevalence of the arbitrary genotype of ESBL-positive *E. coli* among all participants born in each hospital.

Second, parents have not been tested for their carriage status for two reasons: first, performing it would make them hesitate to participate, and, second, submission of plural specimens would increase the risk of sample mix-up.

Third, the results obtained in our study are only from infants aged around 4 to 5 months in a rural city in Japan; therefore, the external validity of the results remains to be assessed. In addition, it is impractical to model the mechanisms of harboring ESBL-E and the network of background factors, thus, we performed the recursive partitioning analysis but not multivariable regression analyses. Future studies in Japan are awaited including the similar survey that we are planning to perform at the age of three as mentioned above.

Finally, because of the influence of the COVID-19 pandemic, the health checkup in May 2020 was canceled and allocated to another month's checkup schedule. Hence, the ages in months of some participants were slightly older than 4 months. However, the data still reflect the prevalence of carriage during early infancy in Shimabara City and showed a slightly decreasing trend in the carriage rate over time as a by-product.

In conclusion, our study revealed the ESBL-E carriage rate as 19.3% and no CRE carriage among infants aged 4 to 5 months in a rural city in Japan. This is the first report on the ESBL-E carriage rates in children in the Japanese community and the CRE carriage rate in any non-hospitalized individuals in Japan. The ESBL-E carriage rate appeared to be associated with delivery facilities: The infants born at "Hospital A" had a higher carriage rate (25.0%) than those born at other facilities (11.3%) and CTX-M-1 was detected only in those from "Hospital A" among the positive samples. Our results suggested that infants aged 4 to 5 months may be more prone to acquire ESBL-E due to environmental factors, especially their delivery facilities. These findings highlighted the importance of AMR countermeasures not only in hospitals but also in the community and maternity facilities.

A part of this study was presented at the 10th Asian Congress of Pediatric Infectious Diseases (Seoul, October 2022).

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 human participants were reviewed and approved by the Human Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

Study design: SK, SM, YN, YM, KY, and HM. Data collection: SK. Data interpretation: SK, SM, KK, and HM. Genotype identification: YK and KK. Statistical analysis: SK and SM. All authors contributed to the initial draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1168451/full#supplementary-material

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Assessing respiratory viral exclusion and affinity interactions through co-infection incidence in a pediatric population during the 2022 resurgence of influenza and RSV

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Introduction: In the Northeast US, respiratory viruses such as influenza and respiratory syncytial virus (RSV), which were largely suppressed by COVID-19-related social distancing, made an unprecedented resurgence during 2022, leading to a substantial rise in viral co-infections. However, the relative rates of co-infection with seasonal respiratory viruses over this period have not been assessed.

Methods: Here we reviewed multiplex respiratory viral PCR data (BioFire FilmArray™ Respiratory Panel v2.1 [RPP]) from patients with respiratory symptoms presenting to our medical center in New York City to assess co-infection rates of respiratory viruses, which were baselined to total rates of infection for each virus. We examined trends in monthly RPP data from adults and children during November 2021 through December 2022 to capture the full seasonal dynamics of respiratory viruses across periods of low and high prevalence.

Results: Of 50,022 RPPs performed for 34,610 patients, 44% were positive for at least one target, and 67% of these were from children. The overwhelming majority of co-infections (93%) were seen among children, for whom 21% of positive RPPs had two or more viruses detected, as compared to just 4% in adults. Relative to children for whom RPPs were ordered, children with co-infections were younger (3.0 vs 4.5 years) and more likely to be seen in the ED or outpatient settings than inpatient and ICU settings. In children, most viral co-infections were found at significantly reduced rates relative to that expected from the incidence of each virus, especially those involving SARS-CoV-2 and influenza. SARS-CoV-2 positive children had an 85%, 65% and 58% reduced rate of co-infection with influenza, RSV, and Rhino/enteroviruses, respectively, after compensating for the incidence of infection with each virus ($p < 0.001$).

Discussion: Our results demonstrate that most respiratory viruses peaked in different months and present in co-infections less than would be expected based on overall rates of infection, suggesting a viral exclusionary effect between most

seasonal respiratory viruses, including SARS-CoV-2, influenza and RSV. We also demonstrate the significant burden of respiratory viral co-infections among children. Further work is necessary to understand what predisposes certain patients for viral co-infection despite this exclusionary effect.

KEYWORDS

COVID-19, SARS-CoV-2, multiplex, co-infection, respiratory, virus, influenza, RSV

1 Introduction

Since the start of COVID-19 pandemic 3 years ago, there have been over 750 million confirmed cases and estimates of 44% of the global population having been infected by the end of 2021. This has transformed global awareness of respiratory viral illness (Organization, G.W.H, 2020; Collaborators, 2022). Amidst a surge of research on SARS-CoV-2, there have also been a wealth of studies assessing the effect of co-infections, or secondary infections, in patients with COVID-19 (Alhumaid et al., 2021; Dao et al., 2021; Kim et al., 2021; Kinoshita et al., 2021; Sreenath et al., 2021; Alhumaid et al., 2022; Hedberg et al., 2022; Krumbein et al., 2023). For the first year of the pandemic, lockdown measures appeared to lower the incidence of most seasonal respiratory viruses (Uhteg et al., 2022), but gradual relaxation of these measures and social distancing norms have led to their re-emergence.

In this setting, assays that can detect multiple respiratory viral and/or bacterial co-infections can play an important role in both treatment decisions and infection control measures. Multiplex molecular assays, such as the FilmArray™ Respiratory Panel v2.1 (RPP), have become increasingly popular due to their ability to rapidly assess for up to twenty pathogen-specific targets simultaneously (Andersson et al., 2014; Hanson and Couturier, 2016). There has been some controversy around the clinical utility of such panels for regular use in the outpatient pediatric setting, amidst concerns for diagnostic stewardship, prior to the COVID-19 pandemic (Esposito et al., 2019; Hanson et al., 2020). More recently, there has been a shift in the role of multiplex polymerase-chain reaction (PCR) assays in rapidly differentiating cases of SARS-CoV-2 infection from other respiratory viruses for public health purposes, and to identify common and treatable viral etiologies such as influenza. However, the role of multiplex respiratory viral testing to assess viral co-infection, particularly the interaction of SARS-CoV-2 with other respiratory viruses, has received relatively little attention.

Multiplex PCR assays also represent a novel opportunity to study the interaction of viruses in real time as they move through human populations. Of all viral co-infections involving SARS-CoV-2, influenza has received the most attention, with a meta-analysis from early in the pandemic demonstrating an overall co-infection rate of 0.7%, but much higher rates in children (3.2%) (Dao et al., 2021). Yet children displayed a very different pattern of respiratory co-infection prevalence with a more recent metanalysis demonstrating the highest prevalence from RSV (1.7%) and rhinovirus (1.0%), with influenza as third most prevalent at only 0.5% of overall SARS-CoV-2 infections (Alhumaid et al., 2022). In contrast, another metanalysis looking at viral co-infections in all age groups found influenza overall ranking third amongst viral co-infections with SARS-CoV-2 (1.2% prevalence), with EBV (1.8%) and HHV6 (1.6%) being more common (Alhumaid et al., 2021). Several factors contribute to the diversity in the rates and types of viral respiratory co-infection seen, including the background rates of infection with each virus, mechanisms of viral exclusion or predisposition for co-infection. The advent of multiplex viral panels allows for relative ease in assessing the background rates of mono vs. co-infection for a given patient population, yet few studies have formally attempted this.

By normalizing for the probability of mono-infection, when comparing the relative incidence of various forms of viral co-infection, we can therefore gain novel insights into viral interactions within human hosts that have thus far only been studied in animal models. Horemheb-Rubio et al. (2022) conducted such an examination of respiratory viral interactions from 2010-2019 in Europe found relatively few synergistic viral interactions, such as between influenza H3N2 and parainfluenza virus 4 or HCoV-NL63 and parainfluenza virus 1, and predominantly viral exclusion between influenza and RSV, rhinovirus and most parainfluenza viruses (Horemheb-Rubio et al., 2022). However, such an analysis has not been conducted on a pediatric population, which have been shown to have higher rates of respiratory viral co-infection (Dao et al., 2021; Chen and Er, 2022; Krumbein et al., 2023), in general, nor been conducted since the beginning of the COVID-19 pandemic and therefore systematically assessed such viral co-infection dynamics in patients with SARS-CoV-2. The resurgence of respiratory viruses seen over the past two Northern Hemisphere winter seasons represents a unique opportunity to study their co-infection rates normalized to background mono-infection.

2 Methods

2.1 Study population

We performed retrospective analysis of a total of 50,022 BioFire FilmArray™ Respiratory Panel v2.1 tests (noted as Respiratory

Pathogen Panel or RPP) (BioFire® Diagnostics, Salt Lake City, UT, USA) were performed over November 1st, 2021 through December 31st, 2022 for 34,610 patients seen at one of several sites at Columbia University Irving Medical Center. Our hospital has instituted policies wherein pediatric patients with upper respiratory symptoms who are seen in the ED and are planned for admission, or inpatients who develop respiratory symptoms, are screened with the BioFire Respiratory Pathogen Panel 2.1 [RPP]. Duplicate results were excluded from the analysis. For patients with multiple positive RPPs, each positive RPP was considered as a separate episode of infection, in order to include new targets detected throughout a patient's hospital course or for different encounters. All RPP tests were performed using nasopharyngeal (NP) swabs on patients suspected of respiratory tract infection. Subsequent analysis was performed solely on the pediatric population, defined as age less than 18 years at time of NP swab collection.

2.2 BioFire FilmArray™ Respiratory Panel v2.1

Nasopharyngeal swab samples were collected in viral transport media and analyzed with the BioFire FilmArray™ Respiratory Panel v2.1 as per the manufacturer's instructions, which includes nucleic acid extraction, non-specific amplification, target-specific amplification, target detection and automatic interpretation of each target as detected, not detected or invalid from melting curve data by BioFire FilmArray™ software. The Panel consists of 21 targets, four of which are specific to bacteria (*Bordetella pertussis*, *Bordetella parapertussis*, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*) and the remaining specific for viruses, including SARS-CoV-2, influenza viruses (A, B, A H3, A H1 2009 variant), RSV, parainfluenza viruses (types 1-4), human rhinovirus/enterovirus, human metapneumovirus (HMpv), and non-SARS coronaviruses (229E, NL63, OC43, and HKU1).

2.3 Data analysis

Data were imported from Cerner (Kansas City, MO) using Discern Analytics 2.0 software. Raw data were analyzed using Microsoft Excel. R Studio (Posit Software, PBC) was used for additional statistical analyses including Chi-squared testing to assess for significance between categorical variables, and Pearson correlation coefficients were used to examine the linear correlation between monthly incidence of viral co-infections. Viral predisposition or exclusion of co-infection with another virus was assessed by comparing the probability of co-infection involving viruses X and Y with the probability of random co-incidence of each viral infection in the same individual as described in (Horemheb-Rubio et al., 2022). While these authors calculated their "co-infection exclusion score" initially for each month of data ((Horemheb-Rubio et al., 2022), supplemental 1.1), and then compiled monthly values into one composite score, here we calculated a similar ratio from our full dataset. If this Viral Co-infection Ratio (VCR) value equals 1, the chance of co-infection is the same as expected from the incidence of each virus, values less than 1 represent a reduced chance of co-infection relative to expected and values greater than one represent an increase relative to expected. We calculated the percent change in probability relative to expected for a particular viral co-infection pair *via* the equation:

$$(-1 + \mathrm{VCR}) * 100$$

3 Results

3.1 Increased rates of RPP positivity and co-infection in pediatric patients

While children (<18yrs) represented a minority of patients tested by RPP (45.3%, Table 1), and a minority of RPPs tested overall were in children (47.0%), they represented a significantly higher proportion of patients with positive RPP (at least one target positive) (69.6%, p<0.001) as well as positive RPPs overall (71.8%, p< 0.001; Table 1). RPP ordering rates were similar in pediatric patients relative to adults (1.5 vs. 1.4 RPPs/patient), indicating that repeat RPP ordering for a single patient was not frequent both amongst children and adults, however pediatric patients had a three-fold higher rate of positive RPPs per patient (15,790/15,675 = 1.01) relative to adults (6,211/18,935 = 0.33) (p< 0.001).

The overall RPP positivity rate was 2.7-fold higher in children relative to adults (67.1% vs. 23.4%, p< 0.001). Further, the percentage of positive RPPs with multiple targets detected (co-infections) in pediatric patients (20.7%, 3,263/15790) was 5.3-fold higher relative to adults (3.9%, 242/6211) (p< 0.001), with significantly higher proportions of two, three and four target-positive RPPs in pediatric patients (Figure 1B). Most positive RPPs contained a single target positive, both for overall and pediatric patients (Table 1; Figure 1A).

3.2 Demographic characteristics of pediatric patients with RPP

The majority of pediatric patients with RPPs ordered were younger than 5 years of age (mean 4.5 years ± 0.06 95% CI), with a trend of lower age for those with positive RPP (3.9 years ± 0.06 95% CI), and particularly those with multiple targets positive (mean 3.0 years ± 0.10 95% CI; Table 2). Pediatric patients with RPPs ordered, as well as positive RPPs, were significantly more likely to be male (p< 0.001 and p< 0.001), for whom there was also a trend towards a higher rate of co-infection (Table 2). The majority of RPPs for pediatric patients were ordered from the ED (65.0%; Table 2), with significantly higher proportions of positive results and co-infection among ED patients (p< 0.001), with corresponding decreases in positive RPPs and co-infections from inpatient units. When stratifying by location, positivity rates and co-infections were highest among outpatient clinics (93.5% and 20.8%, respectively).

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TABLE 1 RPP ordering, positivity and co-infections.

Patients/Testing	Total	Pediatric (%)	Adult (%)
Patients	34,610	15,675 (45.3)	18,935 (54.7)
Patients Positive	18,207	12,681 (69.6)	5,526 (30.4)
Patients Positivity Rate	52.6%	80.9%*	29.2%*
RPP Total	50,022	23,529 (47.0)	26,495 (53.0)
RPP Positive	22,001	15,790 (71.8)	6,211 (28.2)
RPP Positivity Rate	44.0%	67.1%*	23.4%*
Mono-infection	18,496	12,527 (67.7)**	5,969 (32.3)**
Co-infection	3,505	3,263 (93.1)**	242 (6.9)**
Double	3,169	2,941 (92.8)**	228 (7.2)**
Triple	317	303 (95.6)**	14 (4.4)**
Quadruple	17	17 (100)	0 (0)
Quintuple	2	2 (100)	0 (0)

Patients with an RPP ordered, those with positive RPPs, number of RPPs ordered and positive, as well as the number of RPPs positive for a single (mono-infection) or more than one target (Co-infection) are listed for overall and pediatric patient populations. Percent of each category composed of pediatric patients (<18 years) are listed in the rightmost column. Positivity rates are listed as percentages of patients with positive RPPs or positive RPPs out of the total number in each category. *p< 0.001 for pediatric vs. adult, **p< 0.001 for proportion of each category to total RPP for pediatric compared to adult.

3.3 Distinct trends in respiratory viral infections over time in pediatric patients

Assessing monthly trends, a wide range of positivity rates among pediatric patients was observed, following distinct seasonal patterns for most viruses (Figure 2). Rhinovirus/enterovirus demonstrated the highest incidence during all but two months of the study period and peaked in September 2022 (Figure 2A). SARS-CoV-2 demonstrated peaks in January and July-August 2022, whereas seasonal coronaviruses peaked in March-April 2022. Influenza viruses peaked in April-May and December 2022, while metapneumovirus (HMpv) and parainfluenza viruses peaked in June 2022, and RSV peaked in November 2022.

Pairwise Pearson correlations between each of the viral categories depicted in Figure 2 (Table 3) were assessed. Of 28 pairwise correlations, 17 were negative (inverse) and 11 were positive. SARS-CoV-2 showed a strong negative correlation (defined as<-0.5) with Rhino/enterovirus and a moderately negative correlation with seasonal coronaviruses (between -0.3 and -0.5). Influenza showed moderate positive correlations with RSV, adenovirus and seasonal coronaviruses, but a moderate negative correlation with rhinovirus/enterovirus. There was also a strong negative correlation between adenovirus and rhinovirus/enterovirus, as well as strong positive correlations between adenovirus and seasonal coronaviruses, as well as HMpv and parainfluenza viruses (Table 3).

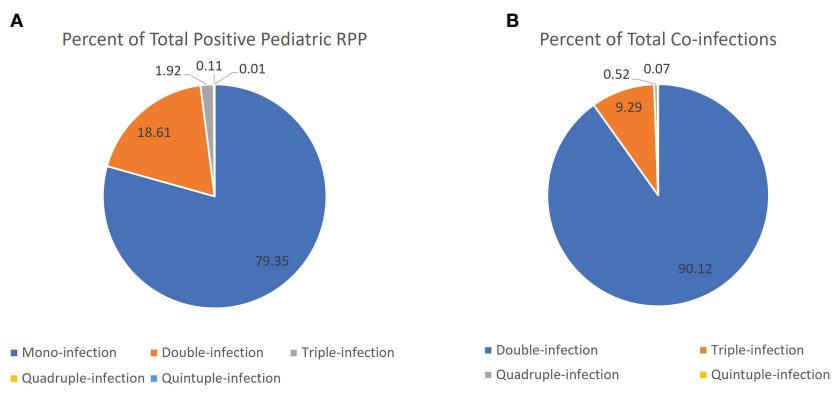

FIGURE 1
Proportion of positive RPPs that were positive for a single vs. multiple viral targets. **(A)** Pie chart comparing percentage of mono-infection (single target positive) vs. co-infection (two or more targets positive) out of total positive RPP in the pediatric patient population. **(B)** Pie chart comparing the percentage of RPPs positive with two or more targets positive out of the total number of RPPs with multiple targets (presumed co-infections) in the pediatric patient population.

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TABLE 2 Positive Respiratory Pathogen Panel demographics and level of care in pediatric patients.

	Demographics			Location of RPP				
	Mean Age	**Male (%)**	**Total Pts**	**ED (%)**	**Inpatient (%)**	**Outpatient (%)**	**ICU (%)**	**RPPs**
RPP Pediatric	4.5	8599 (**54.2**)	15675	15297 (**65.0**)	6858 (**29.1**)	985 (**5.8**)	389 (**2.4**)	23529
RPP Positive Pediatric	3.9	7006 (**55.2**)	12681	11353 (**71.9**)	3516 (**22.3**)	921 (**5.8**)	142 (**1.9**)	15790
Co-infections Pediatric	3.0	1606 (**54.5**)	2948	2390 (**73.3**)	668 (**20.5**)	205 (**6.3**)	27 (**0.8**)	3263
		RPP Positivity Rate (%)		74.2	51.2	93.5	36.5	
		RPP Co-infection Rate (%)		15.6	9.7	20.8	6.9	

Demographics are reported respective to the age, gender and total number of pediatric patients with RPP ordered, positive or multiple targets positive (presumed co-infection). Location of RPP ordering is reported as number of RPPs ordered total, the number of positive RPPs, and the number of RPPs with multiple targets positive at each location. Percentages of the total RPPs in each category are also reported in parentheses for each category.

3.4 SARS-CoV-2 demonstrates viral exclusion and low rates of respiratory viral co-infection

We found a wide variety of co-infection relative to overall infection percentages for individual viral categories, ranging from 59.6% (adenovirus) to 26.2% (SARS-CoV-2, Figure 3). SARS-CoV-2 showed a significantly lower co-infection proportion than every other viral category, except it did not have a significantly lower co-infection rate than influenza, which was next lowest at 27.4%. Adenovirus had a significantly higher co-infection proportion than every other viral category, including seasonal coronaviruses, which had the next highest co-infection proportion at 48.7% ($p< 0.01$).

These findings aligned closely with our comparison of the probability of specific viral co-infections, relative to the probability of co-infection based on the random interaction of each virus involved, based on their overall prevalence in the study

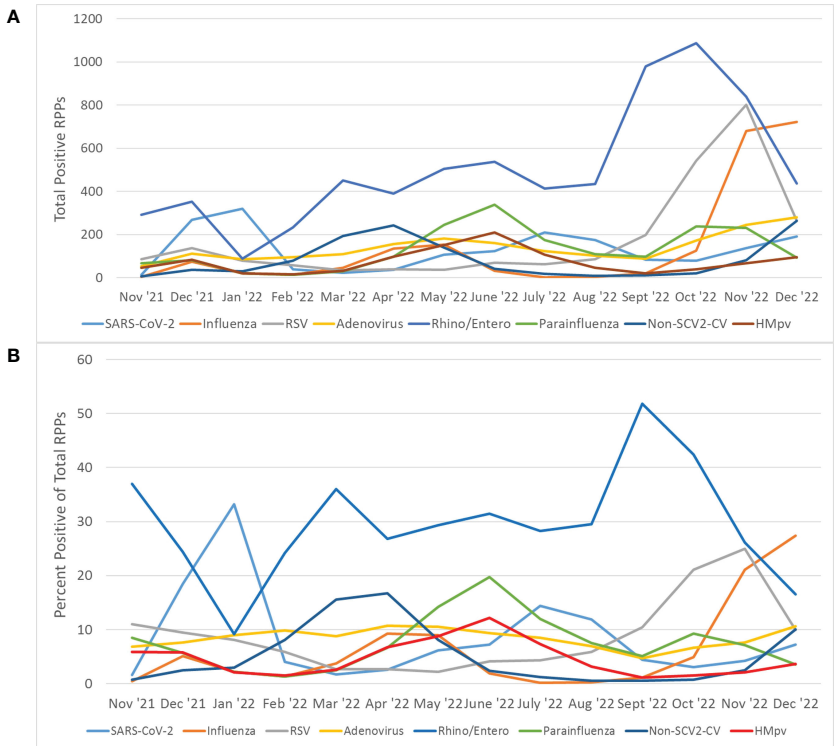

FIGURE 2
Trends overall viral infections in patients with RPP. **(A)** The number of total positive RPPs from each viral category are reported for each month of the study period. **(B)** The percentage of total RPPs ordered each month that were positive for each viral category was plotted for each month of the study period. Rhino/enterovirus was excluded for purposes of visualization. The influenza category refers to RPPs positive for any of the influenza A (non-subtyped), influenza A H3, influenza A H1 2009, and influenza B targets. The parainfluenza category refers to RPPs positive for any of the parainfluenza viruses 1-4. The Non-SCV-2 category refers to RPPs positive for any of the targets specific for HKU1, NL63, OC43 or 229E Coronaviruses.

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TABLE 3 Pearson Correlation in viral incidence trends over study period.

	SARS-CoV-2	Influenza	RSV	Adenovirus	Rhino/Entero	Parainfluenza	Non-SCV2-CV
Influenza	-0.18						
RSV	-0.12	**0.39**					
Adenovirus	0.05	**0.39**	**-0.49**				
Rhino/Enterovirus	-0.64	**-0.36**	0.16	-0.67			
Parainfluenza	-0.15	-0.15	-0.13	0.06	0.24		
Non-SCV-CV	**-0.31**	**0.33**	**-0.46**	0.70	-0.22	**-0.31**	
HMpv	-0.03	-0.11	**-0.49**	**0.41**	-0.08	0.83	0.04

The incidence rates of each viral category, by month, over the study period (as depicted in Figure 1B) were compared by Pearson correlation, with coefficients reported for each pair. Correlations of moderate strength (0.30-0.50) are bolded in green (positive correlation) and red (negative correlation). Strong correlations (>0.50) are highlighted in either green (positive) or red (negative).

population (Figure 4). SARS-CoV-2, which had the lowest co-infection proportion (Figure 3), was found to have a significantly decreased probability of co-infection with every other type of respiratory virus assessed. Amongst co-infections with SARS-CoV-2, the greatest effect was seen with influenza, where there was an 86% lower than expected probability of co-infection, and the least effect with adenovirus, with a 30% reduction from expected (Figure 4). Influenza also demonstrated a significantly lower probability than expected of co-infection with any other virus assessed, from the highest probability with adenovirus, as a 21% reduction from expected, to the lowest with SARS-CoV-2. RSV demonstrated viral exclusion with every other category except adenovirus, with no significant difference from expected, and the largest reduction in probability was seen with HMpv at -79.9%. In general, most viral pairs assessed showed lower-than-expected probability of co-infection, except for adenovirus with seasonal coronaviruses, with a 39.9% increase from expected, while there was no significant difference from the expected co-infection probability for adenovirus co-infection with RSV, rhinovirus/enterovirus, and HMpv (Figure 4). Of note, we did not assess several viral pairs for whom there was no significant Pearson correlation coefficient seen (Table 2)

4 Discussion

This study is the first since the onset of the COVID-19 pandemic to assess respiratory viral co-infections while controlling for viral incidence in pediatric patients. Interestingly, our findings indicate that most common respiratory viruses are found in co-infection much more rarely than would expected based on their overall incidence. This finding is consistent with the assessment of incidence rates of individual RPP viral categories on a monthly basis, which demonstrated distinct peaks in incidence for each viral category, except for parainfluenza and HMpv. SARS-CoV-2, which has never been assessed by this method of estimating viral interactions in human subjects, was found to have a particularly high tendency for viral exclusion in all the viral categories assessed here. Influenza and RSV also demonstrated viral exclusion or no interaction for all viruses tested.

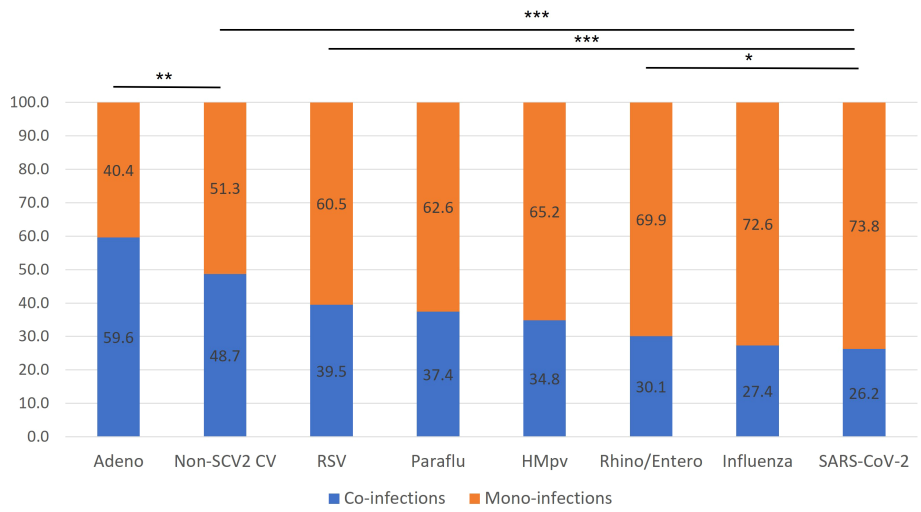

FIGURE 3
Rates of mono-infection vs. co-infection by virus. The percentage of total RPPs positive for each viral category, positive for only a single target (presumed mono-infection) or multiple targets (presumed co-infection) are listed for each viral category. p-values correspond to airs of specific viral categories. *p< 0.05, **p< 0.01, ***p< 0.001.

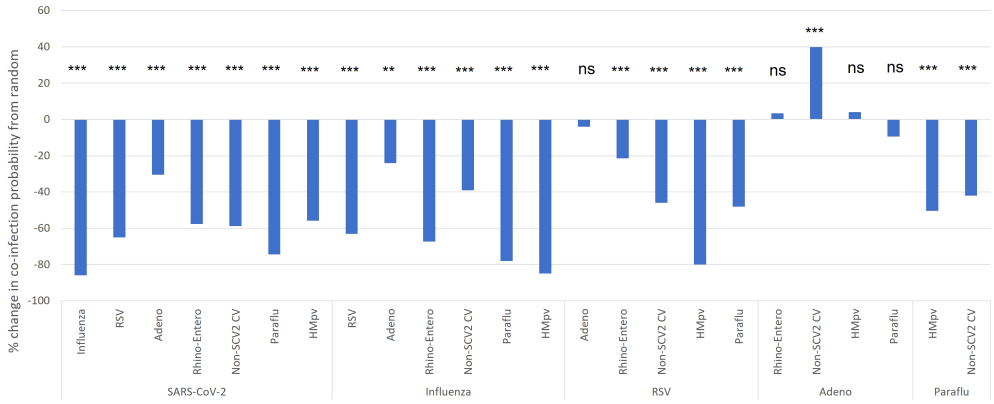

FIGURE 4
Pediatric co-infection probability relative to expected based on overall infection rate. The probability of viral co-infection was calculated based on the overall incidence of each co-infection pair, and compared to the expected probability of each co-infection type based on the incidence of each virus category as would be expected from stochastic interactions (random chance). *p< 0.05, **p< 0.01, ***p< 0.001. ns, not significant.

Influenza and SARS-CoV-2 co-infection in the hospitalized US pediatric population showed similarly low rates during the 2021-2022 influenza season as we saw for our overall pediatric patient population who received RPP, with 5.6% and 5.4% of influenza patients demonstrating SARS-CoV-2 co-infection, respectively (Adams et al., 2022). Despite this low co-infection rate, national data have demonstrated that co-infection carries a far higher risk of mortality, with the mortality rate of co-infected pediatric inpatients nearly three-fold higher (21.9%) than those infected with influenza alone (7.7%) (Adams et al., 2022). While there was no significant difference in rates of anti-viral therapy for patients hospitalized with co-infection vs. influenza alone, only one of the seven (14%) children who died with SARS-CoV-2 and influenza co-infection had received antiviral therapy for influenza, relative to 46% in the influenza-only population (Adams et al., 2022). Early administration of anti-influenza therapy may therefore be an even more significant factor for survival of co-infected patients. Hospitalized pediatric patients with influenza and SARS-CoV-2 co-infections also had significantly lower rates of influenza vaccination (17%) relative to those with influenza alone (42%, $p = 0.02$) (Adams et al., 2022). These national data therefore complement our findings of a high degree of viral exclusion between SARS-CoV-2 and influenza, and suggest that the relatively few patients who develop co-infection may represent a particularly susceptible segment of the pediatric population, with reduced immunity to influenza.

Most studies have found comparably low rates of viral co-infection in adult populations as those we present here. For example, Chen and Er (2022) characterized FilmArray™ RP results from 804 Emergency Department patients in Taiwan, with 27.9% having positive results, 5.3% of patients having co-infection, but only two co-infections involving SARS-CoV-2, with the plurality of co-infections involving Adenovirus and Rhino/Enterovirus (42%) (Chen and Er, 2022). However, these authors did little to examine the rates of specific viral co-infections relative to the frequency each virus was encountered, or to explore correlations between the incidence of specific viral co-infections. A recent metanalysis of viral co-infections in the setting of COVID-19 found a similar rate of co-infection (5.0%) (Krumbein et al., 2023), but the most prevalent co-infecting virus was influenza (1.5%), followed by enterovirus (1.3%), and co-infections were more common in children (9.4%) than adults (3.5%). However, this meta-analysis was conducted on studies published from late 2019 to August 2021, and therefore did not include the most recent two respiratory viral seasons (Krumbein et al., 2023). Similarly, rates of respiratory viral co-infection among COVID-19 patients have been consistently higher in children, with one study reporting up to 15.8% for hospitalized patients, and 33.9% among children less than 5 years of age (Wanga et al., 2021), consistent with the findings in our study.

One limitation of the present study is the inability to distinguish the timeline of multiple infections, with each RPP datapoint representing a snapshot in time. Therefore differentiating superinfection vs. early co-infection was not possible. This makes it more challenging to determine which viruses from co-infected patients increased or decreased the likelihood of infection with another virus, or whether other factors, such as ineffective clearance of a virus, may have been responsible for affinity interactions seen. We suspect that this latter scenario played a role in the overall increased rate of co-infection we saw for adenovirus, and the relatively low exclusion or increased affinity of adenovirus for other viruses. Schjelderup Nilsen et al. (2019) found higher positivity rates of adenovirus in healthy children relative to those with respiratory tract infection, though symptomatic children were significantly more likely to have only adenovirus DNA detected (mono-infection), grow adenovirus in culture and have higher adenovirus viral loads (Schjelderup Nilsen et al., 2019). These authors therefore concluded that qualitative PCR testing for adenovirus DNA alone was not useful in the pediatric population as a diagnostic test. Zadheidar et al. (2022) found similar rates of adenovirus positivity in both symptomatic children and healthy controls, but found different subtypes predominated in each

population (Zadheidar et al., 2022). The increased rates of co-infection involving adenovirus seen here may therefore be partially attributable to asymptomatic colonization.

We also cannot distinguish from our analysis whether reduced probability of viral co-infection was due to, or contributed to, distinct trends in monthly incidence in nearly each viral category assessed over the study period. Due to the limited number of co-infections for some targets, such as individual parainfluenza viruses, coronaviruses, and influenza viruses, their incidence was combined by group, which prevents assessment for specific viruses within each category, such as some of those observed by Horemheb-Rubio et al. (2022). For example, these authors found increases in the probability of interaction between specific HPIV 4 and influenza A(H3N2), as well as HPIV 1 and HCOV-NL63, while we found overall decrease in the interaction probability of these viral categories, but were not able to assess affinity between specific viruses within each category (Horemheb-Rubio et al., 2022). Additionally, viral exclusion/affinity analysis was not performed for individual months in the dataset due to the relatively infrequent co-infections for a given month. However, when we focused on November 2022, a month in which SARS-CoV-2, influenza and RSV were all present at relatively high incidence, we found rates of co-infection of each viral pair comparably low as to results obtained for the full 14 months assessed by this study (Figure S1). Thus, viral co-infection of these viruses is present at rates far lower than would be expected during a month where each is present at high incidence, suggesting that some biological mechanism of viral exclusion or host response also plays a significant role. This may have reduced the sensitivity of our analysis of viral co-infection due to the non-linear relationship between the probability of co-infection and the monthly prevalence of each virus involved

In contrast to the present study, affinity of adenovirus towards co-infection with seasonal coronaviruses was not observed by Horemheb-Rubio et al. Whereas the previous study observed a weak exclusionary interaction between adenovirus and RSV, rhinovirus/enterovirus, and parainfluenza, this interaction was not seen in the present study. However, a weak viral exclusion between adenovirus and both influenza and RSV was consistently observed in both studies (Horemheb-Rubio et al., 2022). The differences between the two studies could have stemmed from a variety of factors in addition to the methodological differences noted above, including differences in the pediatric host population, changes in viral interactions, viral immunity occurring in the setting of SARS-CoV-2, or other viral interactions that cannot be determined here. Further, the reduced incidence of overall viral infection, and particularly viral co-infection, seen for the adult population, did not allow for a robust comparison in the adult population. However, greatly reduced rates of all respiratory viral co-infections, even relative to overall infections in adults, suggests that viral exclusion may be even more common among adults. Though differences in RPP ordering practices make it likely that some proportion of this difference is from artifact due to the different clinical thresholds for which the test was used in these populations.

In summary, the data presented here demonstrate that, despite the expectedly high rates of RPP positivity seen in the pediatric population, viral co-infection occurred significantly less frequently than would be predicted from viral incidence alone. However, viruses included in the panel displayed a range of predilections for co-infection, with Adenovirus and non-SARS-CoV2 Coronaviruses demonstrating the highest frequency of co-infection, while SARS-CoV2 and influenza demonstrated the lowest overall. The distinct peaks in positivity rate for each virus over the course of the study period suggest that low co-infection rates may be in part due to differences in their distribution over time, but biological exclusion of viruses present in the same population likely also play a significant role. Further study is necessary to distinguish to what extent low rates of viral co-infection seen here can be attributed to different temporal trends of viral incidence, biological mechanisms of viral exclusion or virally-induced changes in host immune defense.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Data was extracted from our laboratory information system and has sensitive patient information. Requests to access these datasets should be directed to FW, fw108@cumc.columbia.edu for access to de-identified data from preliminary analysis.

Ethics statement

The studies involving human participants were reviewed and approved by Columbia University Institutional Review Board. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

FW and MW designed the study and submitted for expedited IRB review. FW extracted the data from the LIS and MW performed the data analysis, with feedback from FW. MW composed the manuscript and figures, with initial review by FW. DG and GB revised the manuscript and figures prior to submission. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1208235/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Specific viral co-infection incidence in November, 2022. **(A)** The percentage of RPPs positive for each viral target, or group of targets (influenza), relative to total RPPs ordered for November, 2022. **(B)** The expected incidence (as percentage of total RPPs) of each type of co-infection based on the individual viral incidences listed in **(A)**, relative to the actual incidence of each viral co-infection (as percentage of total RPPs ordered) for November, 2022. **(C)** The percent decrease from the expected incidence of each viral co-infection to the actual incidence reported in **(B)**.

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The state and consideration for skin test of β-lactam antibiotics in pediatrics

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β-lactam antibiotics are the most frequently used drugs and the most common drugs that cause allergic reactions in pediatrics. The occurrence of some allergic reactions can be predicted by skin testing, especially severe adverse reactions such as anaphylactic shock. Thus, penicillin and cephalosporin skin tests are widely used to predict allergic reactions before medication in pediatrics. However, false-positive results from skin tests were more often encountered in pediatrics than in adults. In fact, many children labeled as allergic to β-lactam are not allergic to the antibiotic, leading to the use of alternative antibiotics, which are less effective and more toxic, and the increase of antibiotic resistance. There has been controversy over whether β-lactam antibiotics should be tested for skin allergies before application in children. Based on the great controversy in the implementation of β-lactam antibiotic skin tests, especially the controversial cephalosporin skin tests in pediatrics, the mechanism and reasons of anaphylaxis to β-lactam antibiotics, the significance of β-lactam antibiotic skin tests, the current state of β-lactam antibiotic skin tests at home and abroad, and the problems of domestic and international skin tests were analyzed to determine a unified standard of β-lactam antibiotic skin tests in pediatrics to prevent and decrease adverse drug reactions, avoid waste of drugs, and a large amount of manpower and material resource consumption.

KEYWORDS

β-lactam antibiotics, anaphylaxis, skin test, drug provocation test, pediatrics

1 Introduction

β-lactam antibiotics are the most frequently used drugs and the most common drugs that cause allergic reactions in pediatrics; death could occur in severe cases due to anaphylactic shock. Penicillins and cephalosporins are the two main and most used β-lactam antibiotics, especially in children (Mori et al., 2019). A β-lactam antibiotic skin test is widely used to predict anaphylactic reactions before medication in pediatrics (Azevedo et al., 2019). However, most patients with suspected hypersensitivity reactions to β-lactam antibiotics could tolerate antibiotics. Positive results of skin tests were more often

encountered in pediatrics than in adults (Graham et al., 2018). There has been controversy over whether β-lactam antibiotics should be tested for skin allergy before medication in children and a lack of unified standards and guidelines in the clinical operation of β-lactam antibiotic skin tests.

The primary aim of this review was to determine whether β-lactam antibiotics should be tested for skin allergy before application in children by analyzing the mechanism and reasons for anaphylaxis to β-lactam antibiotics, the significance of β-lactam antibiotic skin tests, the current state of β-lactam antibiotic skin tests at home and abroad, and the problems of domestic and international skin tests.

2 The mechanism of β-lactam antibiotics allergic reactions

2.1 The mechanism of drug hypersensitivity reactions

Drug hypersensitivity reactions (DHRs) are mediated by the immune system after exposure to drugs. Based on immunologic mechanisms, the Gell and Coombs classification divides them into four categories. Type I (immediate hypersensitivity) is mediated by IgE specific for allergens and occurs usually within a few minutes to an hour after administration; typical clinical manifestations include urticaria, angioneurotic edema, bronchospasm, and anaphylactic shock. Type II is characterized by antigen–antibody interactions, of which the vasculitides are classic examples. Type III is mediated by immune complexes, whose typical clinical manifestations include serum disease and drug-associated vasculitis. The clinical manifestations of Type IV hypersensitivity reactions, mediated by T cells, include eosinophilia, systemic symptom syndrome, Stevens–Johnson syndrome, and so on (Rajan, 2003; Dispenza, 2019; Wilkerson, 2022).

β-lactam antibiotic reactions are defined as immediate reactions (IR) or non-immediate reactions (NIR) based on the time interval from the last dose to the onset of symptoms (Romano et al., 2011; Demoly et al., 2014). IR occurs within 1 h after the last dose administration. The clinical manifestations of IR include urticaria and severe anaphylaxis (Demoly et al., 2014; Romano et al., 2020). NIR occurs more than one hour after the last dose administration and up to several hours or days (Romano et al., 2011). The clinical manifestations of NIR include urticaria, angioedema, and maculopapular exanthema.

2.2 The mechanism of penicillin allergy

The chemical structure of penicillin contains a β-lactam ring, a tetrahydrothiazole ring, and an R side chain. *In vivo*, the products of penicillin metabolism bind to self-proteins, resulting in allergic reactions (Lteif and Eiland, 2019). The reactive products of penicillin metabolism, also termed antigenic determinants, are classified into major and minor determinants. Benzyl penicilloyl (95%) is considered the major determinant, and other products (5%) include penicilloate, penicillanyl, and penicillenate, and others are considered minor determinants (Chang et al., 2012). It is the basic principle of skin testing and avoiding administration if a severe anaphylactic IgE reaction is observed.

The major determinant (benzylpenicilloyl polylysine, PPL) is recommended as the ideal skin test reagent. The most significant determinants include benzylpenicillin (penicillin G), benzylpenicilloate, and benzylpenilloate, as well as ampicillin or amoxicillin (Joint Task Force on Practice et al., 2010; Iammatteo et al., 2021), but there are no standardized reagents that contain all major and minor penicillin determinants commercially (Solensky et al., 2019).

2.3 The mechanism of cephalosporins allergy

The chemical structure of cephalosporins contains β-lactam ring, a six-membered dihydrothiazine ring, and R1 and R2 side chains, which differ from penicillins in the six-membered dihydrothiazine ring and R2 side chain. During the degradation of cephalosporins, the β-lactam ring, dihydrothiazine ring, and R2 side chain were disrupted, while the R1 side chain may remain undamaged. Unlike penicillins, for which the antigenic determinants are definite, the antigenic determinants of cephalosporins have not been clear and definite. In addition, cephalosporins' efficiency in forming hapten protein conjugates is inefficient compared to penicillin. Some evidence supports the idea that the degradation of the β-lactam ring destroys the R2 side chain, resulting in unstable conjugates and deficiently identified determinants. The remaining β-lactam moiety and R1 side chain, which can link to host proteins covalently, are central to immune and allergic reactions (Khan et al., 2019).

2.4 Cross-reactivity in β-lactam allergy

The structure of all β-lactam antibiotics includes β-lactam ring, and the structure of penicillin has a thiazolidine ring. Different side chains distinguish different penicillins. Unlike the thiazolidine ring of penicillins, cephalosporins have a dihydrothiazine ring and R1 and R2 side chains, which distinguish different cephalosporins (Romano et al., 2018). During the drug metabolism of cephalosporins, the R1 side chain may remain intact, which can induce cross-reactivity with penicillins. Some evidence supports the idea that cross-reactivity between penicillins and cephalosporins primarily depends on whether their R1 side chain have a similar structure rather than the similarity of the β-lactam ring (Pichichero and Zagursky, 2014).

A meta-analysis of studies (Pichichero and Casey, 2007) indicated that first-generation cephalosporins increased anaphylactic reactions significantly, while there was no increase with second- and third-generation cephalosporins. According to a review of the cross-reactivity of β-lactam antibiotics with anaphylactic reactions (Zagursky and Pichichero, 2018). The prevalence of cross-reactivity between penicillins and

cephalosporins was rare, and the occurrence of cross-reactivity was due to the similar structure of the R1 side chain. Patients with anaphylactic reactions to penicillins could be treated by administration of cefuroxime and ceftriaxone, whose side chains differ from those of penicillins (Romano et al., 2018). In addition, prospective studies demonstrated that cross-reactivity of penicillins and cephalosporins with monobactams and carbapenems was scarce (Gaeta et al., 2015; Mirakian et al., 2015; Romano et al., 2016; Zagursky and Pichichero, 2018), except for ceftazidime, which had the same R1 side chain as aztreonam (Frumin and Gallagher, 2009).

3 The significance of skin test

3.1 Penicillin skin test

Approximately 5% of children report a history of penicillin allergy. However, only a minority of these children were allergic. Due to the fact that penicillin allergy history had a poor prediction of reactivity, skin testing was key to identifying whether patients could be treated with penicillin safely (Picard et al., 2014). The penicillin skin test was the fastest, most sensitive, and most economical method to predict penicillin type I allergic reactions in children (Kulhas Celik et al., 2020). The standard penicillin skin test has a negative predictive value of 97%–99%, and reagents include major determinants, minor determinants, positive controls, and negative controls. Due to the lack of availability of major and minor determinant test reagents, the penicillin skin test is usually performed with diluted penicillin G (Geng et al., 2017).

3.2 Cephalosporin skin test

Unlike penicillins, where the antigenic determinants are stable and definite (Ariza et al., 2015; Khan et al., 2019), anaphylaxis to cephalosporins may occur due to unique antigenic determinants of cephalosporins or antigenic determinants that are shared with other β-lactam antibiotics infrequently, particularly penicillins. Given this reason, parent drugs are recommended as skin test reagents in addition to the classic benzylpenicillin reagents and semisynthetic penicillins (Romano et al., 2021). Although the cephalosporin skin test was less valuable than the penicillin skin test and had not been well validated, it had a good negative predictive value with different R1 side chains of cephalosporins. The ideal concentration for the cephalosporin skin test reagent has not been apparent strictly, and the association of the negative predictive value of the skin test with immediate hypersensitivity is uncertain (Khan et al., 2019). There were few research data available on the predictive values of skin tests for cephalosporins (Hershkovich et al., 2009).

4 The state of skin test in pediatrics

The routine skin test was not required before using β-lactam antibiotics in European and American countries; it was only carried out in China. In China, routine skin tests for cephalosporins had been canceled, but penicillin skin tests were still carried out at present for both adults and children. If penicillin was stopped for more than 72 h, the skin test should be repeated (Joerg et al., 2021; Jiang et al., 2023). In European and American countries, penicillin skin tests were only performed on patients with a history of allergies who needed penicillin (Forrest et al., 2001; Mirakian et al., 2015).

Since few studies have been performed on children, skin testing in the pediatric population has not been standardized. The guidelines, which can diagnose drug allergies in adults, were generally applied to pediatrics (Ibáñez et al., 2018). When the results of the skin test are positive, the patients are hypersensitive to the tested drug, and the administration is suspended (Kulhas Celik et al., 2020). In the past several years, the accuracy of skin tests has been questioned and discussed in some studies (Caubet et al., 2011; Ibáñez et al., 2018; Sousa-Pinto et al., 2021), and these studies highlighted that the diagnostic value of skin tests was not optimal in children. There are many diagnostic shortages in skin tests in children, such as low sensitivity and positive predictive value (PPV), especially for mild skin reactions (Arıkoğlu et al., 2022). A study indicated that skin tests could be false positives in 80% of cases, leading to the unnecessary avoidance of drugs (Ibáñez et al., 2018). A study indicated that higher concentrations of reagent, large injection volumes, and hidden additives or irritant effects could lead to false-positive results (Anterasian and Geng, 2018). In addition, due to the personal characteristics of the pediatricians, discomfort often occurred during the process of skin testing, which led to the expansion of the redness area. Skin tests in pediatrics, similar to adult studies, show a high negative predictive value (NPV), but a positive result might prevent the use of drugs because some studies confirmed a higher rate of false positives (Macy and Ngor, 2013; Vyles et al., 2017b; Solensky et al., 2019). The positive result of a skin test was still used to diagnose anaphylaxis in clinical practice, despite some reports of a low PPV of skin tests in children (Caubet et al., 2011; Ibáñez et al., 2018; Plager et al., 2021). In addition, low-efficiency, resource-intensive, and painful methods may limit the use of skin tests in children (Arıkoğlu et al., 2022), as a study indicated that the prescription costs were much higher in patients with labeled penicillin allergies (Norton et al., 2018).

Although the negative predictive value (NPV) of skin tests is high in both children and adults, some patients can experience an anaphylactic reaction after a negative result (Kulhas Celik et al., 2020). Two studies (Ibáñez et al., 2018; Labrosse et al., 2020) investigated the mild immediate and nonimmediate reactions to amoxicillin in children. There was a significant false-negative rate with the standard penicillin skin test in children. In infants and young children, skin reactivity is poor, and false-negative results may occur. In addition, some drugs can suppress anaphylactic reactions, leading to false negative results. We need to make sure of our medication history before a skin test.

Relatively few studies have evaluated the sensitivity and specificity of cephalosporin skin tests in patients with allergic reactions to cephalosporins. The prediction value of the cephalosporin skin test before administration in anaphylaxis is not supported by sufficient evidence-based medical evidence (Romano et al., 2010). Although conventional skin testing before

administration of cephalosporins is not recommended, skin testing should be done in the following cases: Patients with a specific history of type I (immediate) allergy reactions to penicillin or cephalosporin, if it is necessary to use cephalosporins clinically for the patients, after obtaining the informed consent of the patient, should choose a cephalosporin with a side chain different from that of the allergy drug and the skin test results have certain reference values. Skin testing should be done when it is required in drug instructions (Kelkar and Li, 2001; Guéant et al., 2006; Brockow et al., 2013).

Skin testing is a painful method and difficult to interpret for children, especially infants. A false-positive result may increase the number of children suspected of having allergies to limit the use of antibiotics. The accuracy of skin tests in the allergic evaluation of suspected β-lactam allergic reactions has been highly debated recently (Moral and Caubet, 2017). In patients with suspected β-lactam antibiotic allergy reactions, non-β-lactam drugs, or desensitization are commonly used when alternative medicine is unavailable. Unfortunately, drug-resistant, resource-wasting, less effective, and more adverse reactions may occur when using alternative medicine or broad-spectrum antimicrobial agents, so all patients suspected of β-lactam allergy should be evaluated carefully.

5 More accurate allergy tests at present

5.1 Drug provocation test

Drug provocation test (DPT) is the method of administering a drug under controlled conditions to confirm whether there is an allergic reaction to the drug and whether the patient can tolerate the drug or not. The current data emphasize the accuracy of direct DPT in children with NIR and even potentially with IR, which is considered low risk. In some studies, only 3.4%–14% of children with a history of mild NIR had positive DPT and mild reactions. It is increasingly reported that direct DPT in children with a history of mild IR to β-lactam may be safe (Arıkoğlu et al., 2022). Accurate diagnosis of β-lactam anaphylactic reactions in children is often based on DPT (Garvey and Savic, 2019). In the last few years, direct DPT procedures without prior skin testing have gained acceptance as a safe and accurate strategy for patients (Caubet et al., 2011; Mill et al., 2016; Moral and Caubet, 2017; Macy and Vyles, 2018). According to the international consensus guidelines, skin testing is recommended as a first-line test for immediate reactions to drug allergies. If the result of the skin test is negative, DPT, as the current gold standard for diagnosis, is performed to confirm or exclude the presence of an allergy to the drug (Mirakian et al., 2015; Gomes et al., 2016; Romano et al., 2020), although no standardized protocols exist so far (Iammatteo et al., 2021).

Multiple studies supported the use of direct DPT without prior skin testing for pediatric and adult populations who were historically labeled with anaphylaxis to β-lactam antibiotics (Arıkoğlu et al., 2022). Serious adverse events due to DPT were also infrequent (Kuniyoshi et al., 2022). Some studies indicated that the false labeling of β-lactam anaphylactic reactions could be attributed to the virus infection (Caubet et al., 2011; Mori et al., 2015). Studies reported that direct DPT in children with a history of β-lactam anaphylaxis may be a safe and accurate strategy (Mill et al., 2016; Vyles et al., 2017a; Ibáñez et al., 2018; Labrosse et al., 2018). A study evaluated the frequency of severe adverse reactions after a direct DPT in patients with reported historical allergies to penicillin or other β-lactam antibiotics (Cardoso-Fernandes et al., 2021). The result of the study indicated that severe reactions due to DPT are infrequent and the superior safety of the DPT method supports its application in the diagnosis of penicillin anaphylaxis to contribute to ensuring the correct use of antibiotics, minimizing drug-induced risks, and improving clinical treatment outcomes.

However, DPT reproduces not only hypersensitivity symptoms but also any other adverse clinical manifestation. Some patients do not like to be re-exposed to the drug. Thus, DPT may be harmful and should only be considered after balancing the risk–benefit ratio for the individual patient (Bousquet et al., 2008). In addition, the PPV of DPT may be lower than expected. Thus, a second DPT is suggested to be performed within a few weeks or months. A study suggested that the allergic result should be confirmed with a second DPT within a few weeks or months to remove false labeling of allergies and ensure the safe use of drugs (Moral et al., 2022).

5.2 Oral provocation test

The oral provocation test (oral challenge) is the method to determine whether a patient is allergic to the drug or not. A systematic review found two studies reporting a positive predictive value of skin tests in children of 36% and 33%, respectively. A skin test could lead to an inaccurate diagnosis. An oral provocation test was finally needed to confirm tolerance in most of these children. In immediate and non-immediate reactions, the gold standard procedure to determine acute β-lactam tolerance was the oral provocation test. Oral challenge used a therapeutic β-lactam dose and at least 1 h of observation; it was costly and time-consuming Confino-Cohen et al., 2017. In the case of mild non-immediate reactions in children, skin tests were less commonly used, and oral provocation tests were a safe procedure (Moral and Caubet, 2017; Graham et al., 2018). The oral provocation test is formally contraindicated if there is a history of severe cutaneous adverse reactions (Felix and Kuschnir, 2020). In some studies, the evaluation of the direct oral provocation test was performed excluding high-risk patients (Iammatteo et al., 2019; Kuruvilla et al., 2019; Mustafa et al., 2019).

The oral provocation test is considered accurate with high positive and negative predictive values. A direct oral provocation test without a previous skin test has been increasingly used in patients, especially children with a history of mild, non-immediate reactions to β-lactam. In the case of mild non-immediate reactions in children, skin tests were less common and oral provocation tests were a safe procedure (Felix and Kuschnir, 2020). A study evaluated 119 children with a history of mild, non-immediate cutaneous reactions induced by β-lactam through direct oral provocation. Only four (3.4%) reacted with urticaria during oral provocation,

and there was no severe reaction (Vezir et al., 2016). Further studies, including those of various populations and age groups, are needed to enable a stronger recommendation in this regard.

6 Conclusion

β-lactam antibiotics, including penicillin and cephalosporin, are common causes of drug hypersensitivity reactions in children. The β-lactam antibiotic skin test is widely used to predict anaphylactic reactions before medication. However, multiple studies highlighted the suboptimal diagnostic value of skin tests in children; positive results of skin tests were more often encountered in pediatrics than in adults. In fact, most children with reported β-lactam allergies are not allergic, which leads to the use of broad-spectrum antibiotics, additional costs, and significantly increased drug resistance and complications.

Given the limitations of β-lactam antibiotic skin tests, drug provocation tests, and oral challenges, these were the current standards in the management of pediatric β-lactam allergies because there are no standardized protocols at present. Direct drug provocation tests and oral challenges by skipping skin tests in appropriate patients were gaining acceptance as delabeling strategies. These strategies would learn from skin tests in mutual complementarity.

Author contributions

LZ and WL provided ideas for the manuscript and reviewed the manuscript. CG consulted references and wrote the manuscript. BM provided advice for further modifications to this manuscript. All authors contributed to the article and approved the submitted version.

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

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Case Report: A case of spinal muscular atrophy with extensively drug-resistant *Acinetobacter baumannii* pneumonia treated with nebulization combined with intravenous polymyxin B: experience and a literature review

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Spinal muscular atrophy (SMA) is a neurodegenerative disease that results in progressive and symmetric muscle weakness and atrophy of the proximal limbs and trunk due to degeneration of spinal alpha-motor neurons. Children are classified into types 1–3, from severe to mild, according to the time of onset and motor ability. Children with type 1 are the most severe, are unable to sit independently, and experience a series of respiratory problems, such as hypoventilation, reduced cough, and sputum congestion. Respiratory failure is easily complicated by respiratory infections and is a major cause of death in children with SMA. Most type 1 children die within 2 years of age. Type 1 children with SMA usually require hospitalization for lower respiratory tract infections and invasive ventilator-assisted ventilation in severe cases. These children are frequently infected with drug-resistant bacteria due to repeated hospitalizations and require long hospital stays requiring invasive ventilation. In this paper, we report a case of nebulization combined with intravenous polymyxin B in a child with spinal muscular atrophy with extensively drug-resistant *Acinetobacter baumannii* pneumonia, hoping to provide a reference for the treatment of children with extensively drug-resistant *Acinetobacter baumannii* pneumonia.

KEYWORDS

polymyxin B, *Acinetobacter baumannii*, extensively drug-resistant, pneumonia, spinal muscular atrophy

1 Clinical data

1.1 General data

The child was a 4-year-old boy who was admitted to the hospital chiefly due to "postnatal hypotonia and intermittent asthmatic cough with dyspnea for 1 month." The child, G1P1, was born by cesarean section at full term with postnatal hypotonia and delayed gross motor development and was diagnosed with spinal muscular atrophy (type I) at the age of 10 months after completion of genetic testing. One year prior, the child underwent tracheal intubation and was connected to an invasive ventilator for treatment for 26 days. The tracheal cannula was removed, and the child was discharged without a ventilator and was treated for the original disease with risdiplam orally for 8 months. One month before admission, the child developed a paroxysmal cough without obvious inducement, with more white mucous sputum aspirated, with fever, wheezing, and labored breathing. The child was hospitalized in a local tertiary hospital, and sputum culture showed *Acinetobacter baumannii*. Piperacillin sodium and tazobactam sodium, cefoperazone sulbactam sodium, tigecycline for anti-infection, fluconazole, and micafungin for antifungal infection were given successively. On the second day of hospitalization, the child's dyspnea worsened. The dyspnea was relieved after the tracheal cannula was connected to an invasive ventilator for assisted ventilation. The child's dyspnea worsened after removal of the tracheal cannula and ventilator twice, and the child was reintubated and connected to ventilator-assisted ventilation. The parents refused tracheotomy and transferred the child to our hospital. The child was admitted to the hospital with "bronchopneumonia and spinal muscular atrophy."

The findings of the physical examination on admission were as follows: height 103 cm (P10–P25), weight 10 kg (<p3), respiration 40 bpm, heart rate 170 bpm, clear consciousness, slightly irritable, shortness of breath, positive nasal fan, and three depression signs. The patient had a bell-shaped thorax, coarse breath sounds in both lungs, scattered medium-fine moist rales, and wheezing sounds that were heard. There was no significant abnormalities in the cardiac and abdominal examinations; there was decreased muscle tone of the extremities, grade II muscle strength of the distal upper extremities, and grade I muscle strength of the proximal upper extremities and lower extremities. Tendon reflexes were not elicited, and both fingers were claw-shaped with contracture.

The routine blood results were as follows: WBC 8.21 × 10^9/l, N 36.1%, L 54%, HGB 110 g/l, PLT 557 × 10^9/l, CRP 2 mg/l. The blood gas analysis showed the following: P02 57.8 mmHg, PC02 44.1 mmHg, PH 7.413, HC03 27.6 mmol/l, S02 90.4%, and BE 3.1 mmol/l. The chest CT showed the following: there was a right lung upper lobe shadow, the bilateral lung lower lobe volume was decreased, a dense shadow was seen, a bronchial inflation phase was seen inside, there were bronchial gathering signs, there was an impression of pneumonia, and there was atelectasis of the bilateral lower lobes.

The child was admitted to the hospital with continuous invasive ventilator-assisted ventilation and was given cefoperazone sodium and sulbactam sodium ivgtt for anti-infection and intensive airway management. On the fourth day after admission, the bronchial lavage fluid was positive for *A. baumannii*, and sputum culture suggested a *S. aureus* and *A. baumannii* complex. Levofloxacin and tigecycline were added according to the drug susceptibility test. The child still had intermittent fever, and multiple bronchoalveolar lavage fluid and sputum bacterial cultures suggested the presence of the *Acinetobacter baumannii* complex, high- throughput gene detection tests on the bronchoalveolar lavage fluid suggested a high confidence level of the *Acinetobacter baumannii*. On the 44th day after admission, the child's temperature was normal and his respiration was stable. Therefore, the tracheal cannula was removed, and the patient was changed to non-invasive ventilator-assisted ventilation. After maintenance for 4 days, the child was again fevered and intubated and connected to invasive ventilator-assisted ventilation due to dyspnea. The bacterial culture taken from the tip of the tracheal cannula on the 49th day of admission showed the following: *A. baumannii* complex, colistin-sensitive, cefoperazone, and sulbactam; the remaining isolates were resistant (including to tigecycline). Table 1 shows our results. Tigecycline and levofloxacin were discontinued, intravenous polymyxin B combined with nebulization were added (intravenous administration: the first intravenous loading dose was 2.5 mg/kg (equivalent to 25,000 U/kg, 250,000 U/dose actually administered), and a maintenance dose at 1.25 mg/kg was given once after 12 h (equivalent to 12,500 U/kg/dose, 125,000 U/dose actually administered). For nebulization, 250,000 U/dose was dissolved in 5 ml saline; the solution was nebulized through a nebulizer device connected to a ventilator line, once/12 h) for anti-infectious treatment. The child's guardian agreed to the treatment and signed an informed consent form. In the early stage of nebulization of polymyxin B, the respiratory rate increased and the blood oxygen concentration decreased. Therefore, a β2 agonist was inhaled 20 min before nebulization to reduce airway complications and oxygenated nebulization. The late stage of treatment went smoothly. After 5 days of polymyxin B treatment, the sputum culture was negative on two consecutive occasions and the reexamination of chest imaging showed improvement. The tracheal cannula was successfully removed on the 61st day of admission, and the patient was changed to non-invasive ventilator-assisted ventilation. Polymyxin B was discontinued after 14 days of use, and sputum culture showed no extensively drug-resistant *Acinetobacter baumannii*, with rechecked routine blood results as follows: WBC 4.89 × 10^9/l, N 25.1%, L 62.4%, HGB 109 g/l, PLT 498 × 10^9/l, CRP 0.46 mg/l; renal function results showed the following: BUN 5.83 mmol/l, Cr 12.2 μmol/l. The child was discharged uneventfully after a 75-day hospital stay. Figure 1 illustrates the comparison of the patient's chest CT before and after treatment.

1.2 Literature review

The search terms "Polymyxin B," "Acinetobacter baumannii," "Extensively drug-resistant," and "Pneumonia" were searched in PubMed and Web of Science, and the search terms "Polymyxin B," "Acinetobacter baumannii," "Extensively drug-resistant," and "Pneumonia in children" were searched in the CNKI, SinoMed,

TABLE 1 Results of the bacterial culture taken from the tip of tracheal cannula on the 49th day of admission: *Acinetobacter baumannii* complex, solistin-sensitive, cefoperazone, and sulbactam; the remaining are resistant (including tigecycline).

Antibiotics	Results of the drug sensitivity tests	Minimal inhibitory concentration
Trimethoprim/sulfamethoxazole	Resistance	320
Piperacillin sodium/tazobactam sodium	Resistance	128
Tigecycline	Resistance	8
Cefoperazone/sulbactam	Intermediary	32 (≤16≥64)
Ticarcillin/clavulanic acid	Resistance	128
Colistin	Susceptible	0.5
Ceftazidime	Resistance	64
Ciprofloxacin	Resistance	4
Cefepime	Resistance	32
Doxycycline	Resistance	16 (≤4≥16)
Imipenem	Resistance	16
Levofloxacin	Resistance	8
Meropenem	Resistance	16
Minocycline	Resistance	16 (≤4≥16)
Tobramycin	Resistance	16

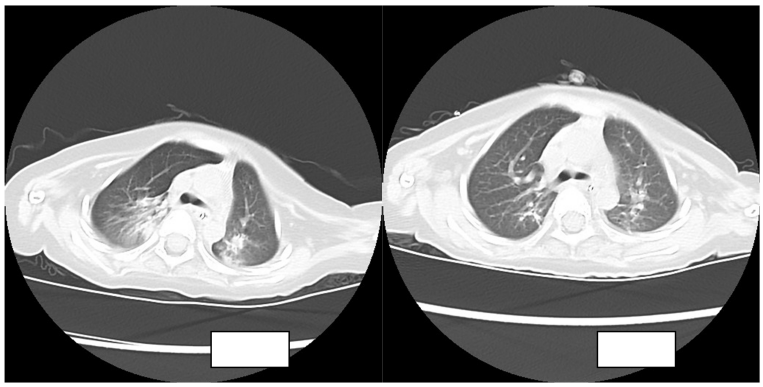

FIGURE 1
Comparison of the patient's chest CT before and after treatment.

and Wanfang databases, with the searching time being from database establishment until 01/09/2022.

The search did not include nebulization combined with intravenous polymyxin B for the treatment of childhood-associated pneumonia. Studies reporting nebulization of polymyxin B for the auxiliary treatment of pneumonia due to multidrug-resistant gram-negative infections have focused on adult patients. A recent prospective cohort study (Hasan et al., 2021) and a retrospective observational study in China (Zhou et al., 2021) showed that intravenous polymyxin B combined with nebulization therapy resulted in better clinical efficiency and microbial clearance, shortened the extubation time and ICU stay, and reduced the incidence of secondary infections without increasing the risk of renal damage. There are only a few clinical studies on the efficacy and safety of intravenous polymyxin B alone in the treatment of carbapenem-resistant gram-negative bacteria (CR-GNB) pneumonia in children, and these studies showed clinical efficacy rates of 47.8%–53.8%, microbial clearance rates of 30.8%–70.9%, in-hospital mortality rates of 7.3%–32.7%, and incidences of adverse events of 13.5%–27.3% (Liu et al., 2021; Jia et al., 2022; Wu et al., 2022).

2 Discussion

Spinal muscular atrophy (SMA) is a severe neuromuscular disorder due to a defect in the survival motor neuron 1 (SMN1)

gene (Mercuri et al., 2018). Children with spinal muscular atrophy are easily complicated with upper and lower respiratory tract infections due to reduced cough and impaired secretion clearance, and according to statistics, the proportion of Chinese patients with type 1–3 SMA hospitalized with pulmonary infections ranges from 24.7% to 61.5% (Ge et al., 2012). When SMA patients present with pulmonary infections, in principle, pathogenetic examinations and infection assessments should be performed on a comprehensive case-by-case basis (Guo et al., 2016). Our department has analyzed the clinical characteristics of children admitted with spinal muscular atrophy and pneumonia and published related articles. All six children with long-term tracheal intubation and tracheotomy developed ventilator-associated pneumonia, and all of them had multidrug-resistant bacterial infections that required long-term use and replacement with multiple antibiotics based on the culture and drug susceptibility results, including four cases of *Acinetobacter baumannii* infection (Guo and Cao, 2020). Active treatment of pulmonary infections is important to achieve the early withdrawal criteria in children with tracheal intubation, with the aim of achieving a better long-term survival.

Acinetobacter baumannii has a strong ability to acquire drug resistance and clonal transmission, and multidrug resistant, extensively drug-resistant, and fully resistant *Acinetobacter baumannii* is endemic worldwide and has become one of the most important pathogens of nosocomial infections in China (Peleg et al., 2008). The most common site of nosocomial *A. baumannii* infection is the lung, and it is an important pathogenic bacterium of hospital-acquired pneumonia (HAP), especially ventilator-associated pneumonia (VAP) (Chen et al., 2012). The results of the 2021 CHINET China bacterial drug resistance surveillance (Hu et al., 2021) suggest that *A. baumannii* ranks fifth in clinical isolates in China, second only to *Klebsiella pneumoniae*. The resistance rate of *A. baumannii* was 71.5% to imipenem and 72.3% to meropenem. The resistance rate of carbapenem-resistant *Acinetobacter baumannii* to polymyxin B was only 0.5%. The results of the 2020 pediatric bacterial drug resistance surveillance by the ISPED (He et al., 2021) showed that the overall detection rate of carbapenem-resistant *Acinetobacter baumannii* (CRAB) was 33.5% (382/1140) and the overall resistance rate to multiple antibiotics was > 60%. In the face of such serious drug resistance, there are very limited therapeutic drugs available in the clinic, and polymyxins with specific antibacterial mechanisms have returned to the clinic with renewed attention (Infectious Diseases Committee of China Pharmaceutical Education Association et al., 2021). Polymyxin B (PMB) is often used as the last line of clinical defense in the treatment of extensively drug-resistant gram-negative bacillary infections (Lim et al., 2016).

The Management of Adults With Hospital-acquired and Ventilator-associated Pneumonia: 2016 IDSA/ATS Clinical Practice Guideline (Kalil et al., 2016) recommends that HAP/VAP caused by carbapenem-resistant bacteria be treated with intravenous infusion of polymyxins (colistin or polymyxin B) (strong recommended, moderate quality evidence) supplemented with inhaled colistin (weakly recommended, low quality evidence). In recent years, several additional national and international guidelines and expert consensus have recommended adjuvant polymyxin nebulization with intravenous polymyxin for patients with hospital-acquired pneumonia (HAP) or ventilator-associated pneumonia (VAP) caused by suspected or confirmed multidrug-resistant (MDR) or extensively drug-resistant (XDR) bacterial infections (Committee of Critical Care Medicine, China Society of Research Hospitals et al., 2019; Tsuji et al., 2019; Infectious Diseases Committee of China Pharmaceutical Education Association et al., 2021; Infectious Diseases Group of the Chinese Medical Association and Respiratory Diseases Branch, 2021). The recommended dosage and administration of polymyxin B sulfate nebulization is as follows: 250,000 to 500,000 U is dissolved in 5 ml of distilled water and nebulized with a conventional device, and a β2 agonist is inhaled 20 min before nebulization twice a day. The use of a vibrating mesh nebulizer is recommended.

Current studies on polymyxin B nebulization for the adjuvant treatment of pneumonia caused by multidrug-resistant gram-negative bacterial infections have mainly focused on adult patients. In the field of pediatrics, there are only a few clinical studies on the efficacy and safety of intravenous polymyxin B alone in the treatment of pneumonia in children with carbapenem-resistant gram-negative bacteria (CR-GNB), and there is a lack of data on the safety and efficacy of polymyxin B nebulization in pediatric patients. In addition to the guidelines and expert consensus, some studies have shown that nebulization of polymyxin B is effective and safe in pneumonia caused by infections due to resistant gram-negative bacilli for which intravenous administration is not effective (Pereira et al., 2007). Polymyxin B nebulization can reduce the course of respiratory-associated pneumonia caused by multidrug-resistant gram-negative bacillus infections and has low nephrotoxicity (Abdellatif et al., 2016). The efficacy and safety of polymyxin B nebulization have also been reported negatively, and Demirdal et al. (2016) showed that the efficacy of polymyxin B nebulization in combination with intravenous administration for the treatment of hospital-acquired pneumonia or ventilator-associated pneumonia caused by gram-negative bacilli infection did not differ statistically significantly from the efficacy of intravenous administration alone. A case of acute respiratory failure secondary to nebulization of polymyxin B in an asthmatic patient has been reported in the literature (Ma, 1982). In addition, the Expert Consensus on the Rational Use of Nebulization Therapy (2019 Edition) (Chinese Medical Association Clinical Pharmacy Branch, 2019) does not recommend intravenous preparations for nebulization because it may induce asthma or increase the risk of lung infection.

The child in this case had an underlying disease of spinal muscular atrophy, recurrent pulmonary infections requiring tracheal intubation, and invasive ventilator support, and the child also had long-term hospitalizations with a variety of advanced antibiotic therapies. The current pulmonary lesions were serious, and it was difficult to withdraw the ventilator. The child had an extensively drug-resistant *Acinetobacter baumannii* infection, the drug susceptibility results showed colistin sensitivity, and the remaining isolates were resistant (including tigecycline). After the clinicians and pharmacists codeveloped the treatment regimen, the dosage and administration that were recommended were according to the 2021 Multidisciplinary Expert Consensus on

the Rational Clinical Use of Polymyxin Antibacterial Drugs in China. At present, there is no nebulization formulation of polymyxin B in China. In this case, the administration route of polymyxin B was nebulization in addition to an intravenous drip, and the treatment process went smoothly.

In this case, during there were no other complications, such as nephrotoxicity, hepatic damage, neurotoxicity, or skin damage, except for a mild decrease in transcutaneous oxygen saturation and increased heart rate at the initial stage of nebulization. The overall treatment was safe and effective, and the shortcoming of this case report is that the blood concentration of polymyxin B could not be monitored. The success of treatment in this case will provide a data reference for the optimal application of polymyxin B in pediatric patients with pulmonary infections. Please note that, since we only had one case, we were unable to confirm the effect of nebulization in the case, although the overall effect was good. Whether nebulization has an ideal effect remains to be discussed further.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Children's Hospital, Capital Institute of Pediatrics, Beijing, China. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

BC reviewed the literature and analyzed the case. LC supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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Conflict of interest

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

Publisher's note

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Antibiotic susceptibility and clonal distribution of *Staphylococcus aureus* from pediatric skin and soft tissue infections: 10-year trends in multicenter investigation in China

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Background: Skin and Soft Tissue Infections (SSTIs) Surveillance Network of *S. aureus* In Pediatrics in China was established in 2009 to routinely report epidemiological changes. We aimed to monitor the present antibiotic sensitivity and molecular characteristics of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) from SSTIs in children nationwide and track the changes over the past decade.

Methods: Patients diagnosed with SSTIs from the dermatology departments of 22 tertiary pediatric hospitals in seven geographical regions of China were recruited continuously from May 2019 to August 2021. *S. aureus* was isolated, and its sensitivity to 15 antimicrobials was evaluated using the broth microdilution method. The molecular characteristics of the MRSA isolates were determined through multilocus sequence typing (MLST) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing. The presence of the Panton–Valentine leukocidin gene (*pvl*) was determined.

Results: The detection rate of *S. aureus* was 62.57% (1379/2204), among which MRSA accounted for 14.79% (204/1379), significantly higher than the result in previous study in 2009-2011 (2.58%, 44/1075). Compared with previous study, the sensitivity to cephalosporins and fusidic acid decreased to varying degrees, while that to chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, penicillin, and tetracycline increased significantly. The sensitivity to mupirocin, trimethoprim/sulfamethoxazole (TRISUL), and rifampicin still maintained at a high level (97.90%, 99.35% and 96.66% respectively). The leading multidrug resistance pattern of MRSA and methicillin-sensitive *S. aureus* (MSSA) were erythromycin-clindamycin-tetracycline (55.84%; 43/77)

and erythromycin-clindamycin-chloramphenicol (27.85%, 44/158) respectively. 12 high-level mupirocin-resistant strains were detected, and notable differences in geographical distribution and seasonal variation were observed. The main types of MRSA were ST121 (46.08%, 94/204), followed by ST59 (19.61%, 40/204). SCC*mec* V (65.69%, 134/204) and SCC*mec* IV (31.86%, 65/204) were dominant epidemic types. ST121-V, ST59-IV, and ST22-V were the most prevalent clones nationwide. The detection rate of *pvl* had increased markedly from 9.09% (4/44) in 2009-2011 to 22.55% (46/204) in 2019-2021 (P<0.05).

Conclusion: The antibiotic sensitivity and molecular characteristics of *S. aureus* from pediatric SSTIs has changed significantly over the past decade. To standardize medical care, provide timely and reasonable clinical treatment, and effectively manage infection control, Chinese pediatric SSTIs guidelines are urgently needed.

KEYWORDS

SSTIs, *Staphylococcus aureus*, MRSA, antimicrobial sensitivity, molecular epidemiology, China

Introduction

Staphylococcus aureus is the main pathogen that causes skin and soft tissue infections (SSTIs), such as pustules, folliculitis, boils, and abscesses, in pediatric patients (Lowy, 1998) as well as fatal infections, such as necrotizing fasciitis and toxic shock syndrome (Burnham and Kollef, 2018). Methicillin-resistant *S. aureus* (MRSA) has attracted considerable attention owing to its drug resistance and virulence (Lee et al., 2018). Community-associated MRSA (CA-MRSA) mainly causes SSTIs in young and healthy people in the community (Turner et al., 2019). Over the past decade, CA-MRSA has been considered to be the main cause of the increased burden of MRSA diseases. Some CA-MRSA strains have been increasingly involved in nosocomial infections and have even become dominant strains in medical settings (Elston and Barlow, 2009). The resistance rate of CA-MRSA is increasing, not only to β-lactam antibiotics but also to non-β-lactam antibiotics (Guo et al., 2020). The spectrum of CA-MRSA invasive diseases is expanding and is increasingly becoming the focus of global infection (Hassoun et al., 2017). Epidemiological information on MRSA is important for clinical decision-making and public health monitoring. Furthermore, classification of MRSA is an important part of describing epidemiological trends and formulating infection control strategies (Mediavilla et al., 2012).

To our knowledge, few studies on the antibiotic sensitivity of *S. aureus* and molecular characteristics of MRSA from SSTIs in China have been conducted (Xiao et al., 2019; Zhao et al., 2022). Furthermore, results from different regions differ, and national studies related to children are rare. A national SSTIs surveillance network of *S. aureus* in pediatrics, established in 2009 and led by the Department of Dermatology, Beijing Children's Hospital, is the only available nationwide epidemiological surveillance network with regular investigation in China (Liu et al., 2016). Currently, 22 children's hospitals have joined. The present study aimed to track the changes in antibiotic sensitivity of *S. aureus* as well as the molecular characteristics and epidemiology of MRSA in children diagnosed with SSTIs in China.

Materials and methods

Patient enrollment

This was a multi-center, cross-sectional epidemiological study on children diagnosed with SSTIs in the dermatology departments of 22 tertiary pediatric hospitals. According to their geographical location, the hospitals were divided into seven groups: North, Middle, East, South, Northeast, Northwest, and Southwest China. All patients who met the criteria for SSTIs were recruited continuously from the dermatology departments from May 2019 to August 2021. The inclusion criteria were as follows: 1) no history of major congenital malformations or severe chronic diseases, 2) no history of surgery or hospitalization within the past year, 3) no history of dialysis or deep catheterization, and 4) no history of antibiotic use within the past week. Information, including sex, age, predisposing factors, disease type, specimen collection time, and basic medical history, was collected. Patients who could not provide general information were excluded. The enrolled patients were treated according to routine treatment, and the swab specimens of infection sites from the non-repetitive participants were collected continuously.

Strain identification

The isolated strains were identified using traditional microbial identification methods, coagulase and catalase tests, and latex slide agglutination test (Oxoid Ltd., Basingstoke, UK). All three tests were positive for *S. aureus* (Weist et al., 2006).

Identification of MRSA strains

In addition to resistance to cefoxitin and oxacillin, MRSA was further identified through polymerase chain reaction (PCR) amplification of the *nuc* and *mecA* genes according to the method described previously (Sahebnasagh et al., 2014). ATCC 29213 and ATCC35601 were used as a negative and positive control, respectively, for the *mecA* gene.

Susceptibility profiles

The antibiotic susceptibility profiles of the *S. aureus* isolates were determined using the Sensititre® Antimicrobial Susceptibility Testing System (Thermo Scientific, UK), following the manufacturer's instructions. The minimum inhibitory concentration (MIC) to 15 antibiotics (cefazolin, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fusidic acid, gentamicin, mupirocin, penicillin, rifampicin, tetracycline, TRISUL, and vancomycin) were detected using the broth microdilution method (Novy et al., 2014). *S. aureus* ATCC 29213 and ATCC 35601 were used for quality control. The antimicrobial sensitivity breakpoints were interpreted according to the current Clinical and Laboratory Standards Institute (CLSI) breakpoints for *S. aureus* (CLSI, 2019), while sensitivity to cefazolin, ceftriaxone, and cefuroxime was interpreted according to a previous version of CLSI (Clinical and Laboratory Standards Institute/NCCLS, 2005). An E-test ((bioMérieux, Marcy-L'Étoile, France) was further performed on the isolates classified as mupirocin-resistant through broth microdilution.

Epidemiological typing of MRSA

DNA was extracted for PCR using a bacterial genomic DNA extraction kit (Tiangen Biochemical Technology, China). Multilocus sequence typing (MLST) was performed on MRSA isolates using the method described previously (Enright et al., 2000). Sequences of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) were compared with known alleles from the MLST database (https://pubmlst.org/organisms/staphylococcus-aureus). Allelic profiles and sequence types (STs) were determined using the database.

The isolates were also subjected to staphylococcal cassette chromosome *mec* (SCC*mec*) typing, which is based on multiplex PCR with 10 primers (Omuse et al., 2016). SCC*mec* types I-V were assigned according to the combination of the cassette chromosome recombinase (*ccr*) type and *mec* class. Isolates that could not be assigned to any expected type were defined as non-typable.

Panton–Valentine leukocidin gene detection

pvl was amplified using PCR as described previously (Hesje et al., 2011) with minor modifications. MRSA N315 was used as a negative control, while ATCC 25923 was used as a positive control.

Statistical analysis

A database including the age, sex and disease patterns of the patients, antimicrobial resistance and molecular characteristics of the corresponding isolate was constructed in Microsoft Excel 2003. GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, United States) was used to create graphs. All susceptibility data were analyzed using WHONET software (version 5.6). JMP® 11 Statistical Discovery Software (S.A.S. Institute Inc., Cary, North Carolina) was used for statistical analysis. Categorical variables were analyzed using the chi-squared ($\chi 2$) or Fisher's exact test. Significance was set at $P < 0.05$.

Results

General information

The initial data of *S. aureus* and MRSA collection as well as the distribution of them are summarized here. From May 2019 to August 2021, 2204 patients with SSTIs were enrolled in 22 children's hospitals in 19 provinces of seven geographic regions. The overall positive rate of *S. aureus* was 62.57% (1379/2204). The detection rate of *S. aureus* was the highest at 75.77% (519/685) in South China and was the lowest at 46.49% (159/342) in North China. The proportion of MRSA was 14.79% (204/1379) nationally, with significantly different proportions across China ranging from 12.73% (21/165) in Middle China to 17.24% (5/29) in North West China. Single institutional prevalence ranged from 4.35% (1/23) to 30.77% (8/26) ($P < 0.05$). The distribution of enrolled patients and the number of positive *S. aureus* and MRSA isolates in each geographic region are shown in Figure 1.

The clinical features of patients from whom the strains were collected are analyzed as follows. The age of patients ranged from 3 days to 18 years old, with 77.16% (1064/1379) aged 1–6 years. Of the patients, 58.45% (806/1379) were males and 41.55% (573/1379) were females. Detailed clinical features are presented in Table 1.

Great changes on infection patterns had occurred during the past decade. The top three primary infections in this study were impetigo (39.38%; 543/1379), furuncles (19.87%; 274/1379), and folliculitis (12.76%; 176/1379). Compared with our study conducted in 2009-2011 (Liu et al., 2016), the distribution of deep infections such as folliculitis and furuncle in 2019-2021 increased significantly from 1.20% (21/1749) to 12.76% (176/1379) and from 0.57% (10/1749) to 19.87% (274/1379) with $P<0.001$, respectively. The distribution of superficial infections such as impetigo and staphylococcal scalded skin syndrome had decreased from 79.87% (1397/1749) to 39.38% (543/1379) and from 4.69% (82/1749) to 0.80% (11/1379) with $P<0.001$, respectively. The detailed composition of infections caused by *S. aureus* in 2009-2011 and 2019-2021 is shown in Figure 2.

Characteristics of resistance pattern of *S. aureus* and change trend

The results of the antimicrobial susceptibility test on strains collected in this present study are shown in Table 2. The resistance

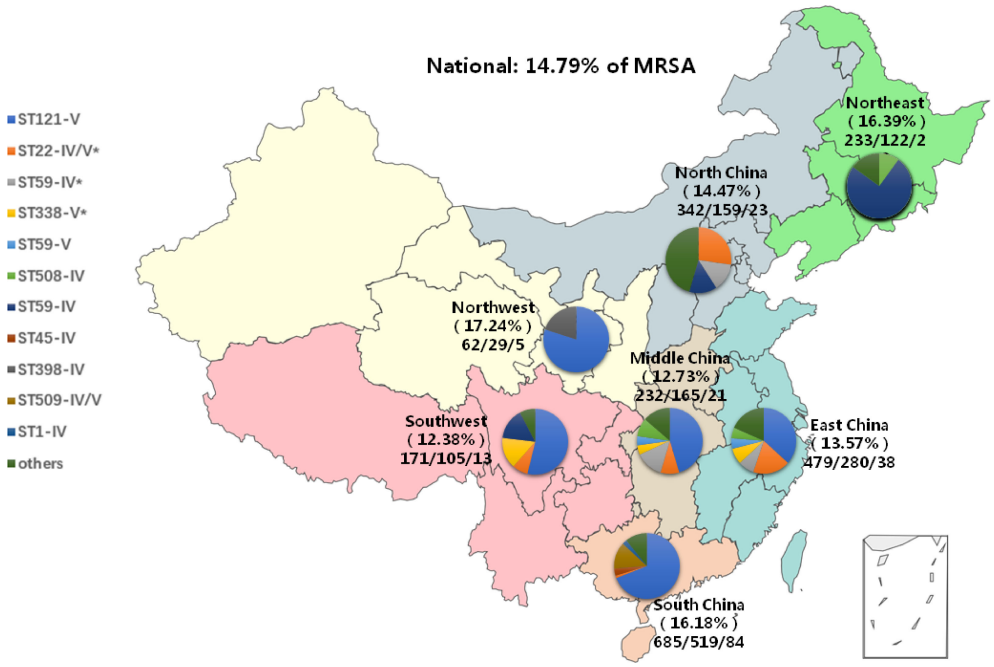

FIGURE 1
The distribution of specimens, *S. aureus* and MRSA as well as prevalent clones, by region. All seven geographical regions were included as follows: Northeast, green, North China, gray; East China, blue; Northwest, yellow; Middle China, khaiki; Southwest, pink; South China, orange. **pvl*-positive clones contained.

TABLE 1 Clinical features of patients from children with SSTIs in the present study.

Variable	Numbers (%)	Region	Hospital	Patients/SA/MRSA (n)
General information		**East China**		
Total patients	2204	Anhui province	AHCH	96/74/13
SA	1379 (62.57%)	Zhejiang province	HZCH	100/58/8
MSSA	1175 (85.21%)	Shandong province	JNCH	52/23/1
MRSA	204 (14.79%)	Jiangsu province	NJCH	36/21/2
Sex		Jiangsu province	XZCH	100/78/6
Male	806 (58.45%)	Zhejiang province	ZJCH	95/26/8
Female	573 (41.55%)	**Middle China**		
Ages		Hubei province	HBMCHH	34/18/2
0-28 days	11 (0.80%)	Hunan province	HNCH	98/61/12
1-12 months	167 (12.11%)	Henan province	ZZCH	100/86/7
1-3 years	401 (29.08%)	**North China**		
3-6 years	496 (35.97%)	Beijing	BJCH	140/50/10
6-12 years	278 (20.16%)	Inner Mongolia province	NMGCH	95/47/5
12-18 years	26 (1.89%)	Beijing	CIOPCH	107/62/8
Types of SSTIs	**SA/MRSA**	**North East**		
Primary infection	1059/155	Jilin province	CCCH	99/50/15
Impetigo	543/75	Liaoning province	DLCH	97/60/5

(Continued)

TABLE 1 Continued

Variable	Numbers (%)	Region	Hospital	Patients/SA/MRSA (n)
Furuncle	274/42	Heilongjiang province	HBCH	37/12/0
Folliculitis	176/28	**North West**		
Abscess	36/7	Shaanxi province	XACH	62/29/5
Paronychia	16/2	**South China**		
SSSS	11/1	Guangdong province	SZCH	332/281/48
Acne	2/0	Guangxi province	LZMCHH	94/60/7
Omphalitis	1/0	Guangdong province	GZCC	161/115/18
Secondary infection	320/49	Hainan province	HNMCHH	98/63/11
Secondary infection of eczema	177/19	**South West**		
Secondary infection of trauma	75/16	Sichuan province	CDCH	90/68/7
Secondary infection of herpes	36/8	Yunnan province	KMCH	81/37/6
Secondary infection of fungi	7/2			
Others	25/4			

MRSA, methicillin-resistant Staphylococcus aureus; MSSA, methicillin-sensitive S. aureus; SA, Staphylococcus aureus.
Dalian Children's Hospital of Dalian Medical University, DLCH; Children's Hospital of Changchun, CCCH; Harbin Children's Hospital, HBCH; Beijing Children's Hospital, BJCH; Children's Hospital Affiliated to Capital Institute of Pediatrics, CIOPCH; Inner Mongolia maternal and Child Health Hospital, NMGCH; Xuzhou Children's Hospital, XZCH; Children's Hospital Affiliated to Zhejiang University, ZJCH; Anhui children's Hospital, AHCH; Nanjing Children's Hospital, NJCH; Qilu Hospital Jinan children's Hospital, JNCH; Hangzhou Children's Hospital, HZCH; Xi'an Children's Hospital, XACH; Hubei Maternal and Child Health Hospital, HBMCHH; Hunan Children's Hospital, HNCH; Zhengzhou Children's Hospital, ZZCH; Chengdu Children's Hospital, CDCH; Kunming children's Hospital, KMCH; Guangzhou Women and Children's Medical Center, GZCC; Hainan maternal and Child Health Hospital, HNMCHH; Guangxi Liuzhou Maternal and Child Health Hospital, LZMCHH; Shenzhen Children's Hospital, SZCH.

rates of MRSA to ciprofloxacin, clindamycin, erythromycin, and tetracycline were significantly higher than those of MSSA (P<0.05).

S. aureus with resistance to three or more classes of antimicrobial agents were defined as multidrug-resistant (MDR). In this study, MDR was observed in 37.75% (77/204) of MRSA strains and 13.45% (158/1175) of MSSA strains. The predominant resistance patterns of MRSA to non-β-lactam antibiotics were erythromycin-clindamycin-tetracycline (55.84%; 43/77), followed by erythromycin-clindamycin-tetracycline-chloramphenicol (18.18%; 14/77). The resistance patterns of MSSA were very different from that of MRSA, the most common profiles of which were erythromycin-clindamycin-chloramphenicol (27.85%, 44/158) and erythromycin- clindamycin-tetracycline (22.15%, 35/158). Among different STs of MRSA strains, the proportion of MDR in ST121 was the highest (49.35%; 38/77), followed by ST59 (22.08%; 17/77) and ST338 (6.49%; 5/77).

A total of 12 high-level mupirocin-resistant (MuH) strains (MIC ≥ 512 μg/mL) were detected, including nine MSSA strains and three MRSA strains. The differences in ST distributions of MuH strains were irregular, while notable in the differences in geographical distribution and the seasonal variation. MuH strains mainly distributed in South China (66.67%, 8/12)、East China (16.67%, 2/12) and Middle China (16.67%, 2/12). Of the hospitals, the isolates were predominantly separated from Shenzhen Children's Hospital (41.67%, 5/12), which was geographically assigned to South China. The infection patterns were mainly secondary infection, including secondary infection of eczema (5/12), trauma (2/12) and herpes (1/12). The infections caused by MuH isolates mainly occured in autumn (8/12), followed by summer (4/12). The children mainly aged >3y (66.67%, 8/12).

The changes of resistance patterns of *S. aureus* collected in this study with strains collected in 2009-2011were analyzed. The current resistance rates of *S. aureus* to cefazolin, ceftriaxone, cefuroxime, and fusidic acid had increased significantly (P<0.05), and that to chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, rifampicin, tetracycline, and TRISUL decreased significantly (P<0.0001), while no significant difference was found in resistance to vancomycin and mupirocin. Besides, the resistance rate to penicillin decreased from 96.8% to 94.13% (P=0.0004). The comparison of the antimicrobial sensitivity of *S. aureus* isolates between 2009-2011 and 2019-2021 is shown in Figure 3.

Molecular characteristics of MRSA isolates

Overall, 25 STs were detected, of which ST121 accounted for 46.08% (94/204), followed by ST 59 (19.61%, 40/204) and ST22 (9.80%, 20/204). In SCC*mec* classification, 65.69% (134/204) were type V, and 25.98% (53/204) were type IV. ST121-V, ST59-IV, and ST22-V were the most prevalent clones nationwide. The molecular biological characteristics of MRSA isolates and the dominant MRSA clones by region are summarized in Table 3; Figure 1.

Major features of ST121 and ST59 strains

ST121 was the most prevalent type of MRSA strains, which all typed as SCC*mec* V and *pvl* negative. ST121 strains were mainly distributed in Northwest and South China with a positive rate of

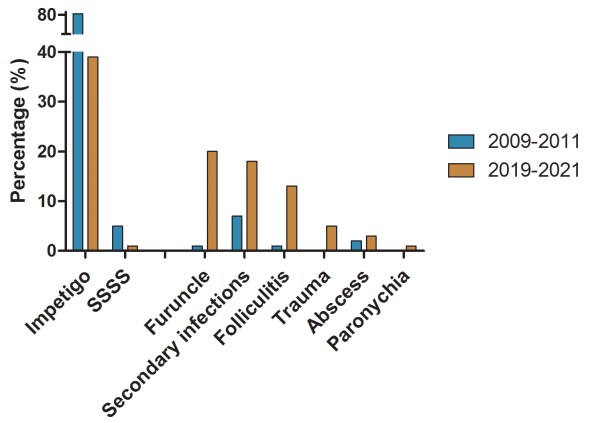

FIGURE 2
The comparation on distribution of major infection pattern caused by *S. aureus* in 2009-2011 and 2019-2021 respectively.

80.00% (4/5) and 69.05% (58/84), respectively, while they were not detected in Northeast China. Compared with non-ST121 isolates, ST121 isolates had a significantly higher resistance rate to clindamycin and lower resistance rates to cefazolin, cefuroxime, and ciprofloxacin. The detection rate of ST59 was second to that of ST121. ST59, which was opposite to ST121, was mainly distributed in Northeast and North China, with a positive rate of 80.00% (16/20) and 40.91% (9/22), respectively. Inconsistent with ST121, ST59 strains were mainly typed as SCC*mec* IV (77.50%, 31/40). The positive rate of *pvl* was 35.00% (14/40), significantly higher than that of ST121 (0.00%, 0/94). The resistance rate of ST59 isolates to ciprofloxacin, cefazolin, and cefuroxime was significantly higher than that of ST121 isolates ($P<0.05$), and there was no significant difference in the resistance rate to other antibiotics.

Clinical and molecular characteristics of *pvl*-positive MRSA strains

The detection rate of *pvl* had increased markedly from 9.09% (4/44) in 2009-2011 to 22.55% (46/204) in 2019-2021 (P<0.05). Infection patterns caused by *pvl*-positive and *pvl*-negative MRSA strains in this study are shown in Figure 4A. *pvl*-positive MRSA strains mainly caused furuncle (41.30%,19/46) and folliculitis (21.74%,10/46), higher than *pvl*-negative strains with P<0.0001 and P=0.073 respectively, while *pvl*-negative MRSA strains mainly caused impetigo (44.94%,71/158) and secondary infection (25.95%,41/158), higher than *pvl*-positive strains with P<0.0001 and P=0.067 respectively. Among the *pvl*-positive strains, ST22 (41.30%, 19/46), ST59 (30.43%, 14/46) and ST338 (15.22%, 7/46) were the most common types as shown in Figure 4B, which were significantly higher than the ratio of *pvl*-negative strains with P<0.05 respectively. In contrast, among *pvl*-negative strains, ST121 (59.49%,94/158) was the most prevalent ST, the ratio of which was significantly higher than that of *pvl*-positive strains (P<0.0001).

Discussion

This study was the second to conduct national multicenter epidemiological monitoring of *S. aureus* for SSTIs in pediatrics since the surveillance network was established in China in 2009. The epidemiological trends of SSTIs in 22 tertiary children's hospitals in seven geographical regions of mainland China were investigated. Compared with the study conducted 10 years ago, we presented four major findings: 1) the resistance profiles of *S. aureus* isolates had changed considerably; 2) the prevalence of CA-MRSA and its *pvl*-positive strains, increased significantly; 3) the proportion of deep infections increased significantly; and 4) ST121-V was the dominant clone, with the percentage increased.

In this study, MRSA accounted for 14.79%, significantly higher than in 2009-2011 (2.58%). It was reported that antimicrobial agents should be chosen to target MRSA and MSSA if MRSA accounts for >10% of *S. aureus* among SSTIs (David and Daum, 2017). Either clindamycin or TRISUL was recommended because of the low cost and activity against community-associated MRSA and MSSA strains of each of these drugs (Williams et al., 2011). According to previous studies, one of these antimicrobials should be used in addition to incision and drainage for a skin infection (Miller et al., 2015; Daum et al., 2017). In this study, we identified a low resistance rate of *S. aureus* to TRISUL (0.4%–2%). The long-term use of TRISUL remains a suitable option for treating complex hyperimmunoglobulin E syndrome and chronic granulomatous disease (Hashemi et al., 2017). Clindamycin was reported to be effective at treating infections caused by susceptible CA-MRSA isolates (Miller et al., 2015). However, in this study, though significantly decreased compared with that in 2009-2011, the resistance rate of clindamycin was still higher than 50%, indicating that it is not a good choice for treating SSTIs in children in China.

For empiric or targeted therapy for *S. aureus*, an anti-*staphylococcal* β-lactam drug was the most appropriate choice (David and Daum, 2010). Furthermore, it was reported that cephalosporins and penicillin are most commonly used in China (Li et al., 2016). Therefore, the MICs of the clinical strains to penicillin as well as cephalosporins was detected to track changes in sensitivity. The resistance rates of *S. aureus* to penicillin, though decreased significantly, remained at a high level (94.13%). On the contrary, the sensitivity to cephalosporins was maintained at a high level (90.65%–97.53%). According to this result, cephalosporins might be a suitable alternative to penicillin for empiric therapy.MDR to non-β-lactam antibiotics was detected in both MRSA and MSSA. The presence of MDR strains in outpatients with SSTIs can lead to persistent or recurrent MRSA infections (Lee et al., 2017).

Clinically, most SSTIs can be controlled only with topical antibiotics. Mupirocin is a topical antibiotic that has been extensively used for treating MRSA skin and soft-tissue infections, decreasing certain types of surgical site infections and eliminating nasal colonisation of MRSA among patients and medical staff. In the present study, the sensitivity to mupirocin was still high,

TABLE 2 Results of the antimicrobial sensitivity of the isolates in the present study.

Antimicrobial agent	MRSA (n=204)					MSSA (n=1175)				
	Sensitivity (%)	Resistance (%)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	MIC_{range} (μg/mL)	Sensitivity (%)	Resistance (%)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	MIC_{range} (μg/mL)
CEFAZO	88.24	11.76	2	>8	1~>8	99.15	0.85	1	1	≤0.25~>8
CEFTRI	49.51	50.49	16	64	6~>256	98.21	1.79	4	4	≤0.25~>16
CEFURO	46.08	10.78	8	32	≤4~>64	98.38	0.34	≤4	≤4	≤4~64
CHLORA	47.55	11.27	16	>32	≤4~>32	55.74	7.32	8	16	≤4~>32
CIPROF	93.14	4.41	≤0.25	0.5	≤0.25~>4	92.00	1.53	≤0.25	0.5	≤0.25~>4
CLINDA	31.86	66.18	>16	>16	≤0.5~>16	47.40	49.96	2	>16	≤0.5~>16
ERYTH	5.39	87.74	>64	>64	0.032>64	14.98	75.91	>64	>64	0.125>64
FUSIDA	97.06	2.94	≤0.125	0.25	≤0.125~>2	95.15	4.85	0.25	0.25	≤0.125~>2
GENTAM	97.55	1.47	≤0.125	0.25	≤0.125~>32	96.60	1.79	≤0.125	0.25	≤0.125~>32
MUPIRO	96.57	2.45	≤0.125	0.25	≤0.125~>512	98.13	1.19	≤0.125	≤0.125	0.06~>512
PENICI	0	100	>8	>8	0.12~>8	6.81	93.19	8	>8	≤0.125~>8
RIFAMP	93.63	0.49	≤0.015	1.5	≤0.015~4	97.19	0.43	≤0.015	0.5	0.002~>32
TETRA	65.20	34.80	≤0.5	32	≤0.5~32	90.04	9.36	≤0.5	2	≤0.5~>32
TRISUL	99.51	0.49	≤0.125	1	≤0.125~8	99.32	0.68	0.5	1	≤0.125~16
VANCOM	100.00	0.00	≤0.5	1	≤0.5~2	100.00	0.00	≤0.5	1	≤0.5~2

CEFAZO, Cefazolin; CEFTRI, cefatriaxone; CEFURO, Cefuroxime; CHLORA, Chloramphenicol; CIPROF, Ciprofloxacin; CLINDA, Clindamycin; ERYTH, Erythromycin; FUSIDA, Fusidic acid; GENTAM, Gentamicin; MUPIRO, Mupirocin; PENICI, Penicillin; RIFAMP, Rifampin; TETRA, Tetracycline; TRISUL, Trimethoprim-sulfamethoxazole; VANCOM, Vancomycin.

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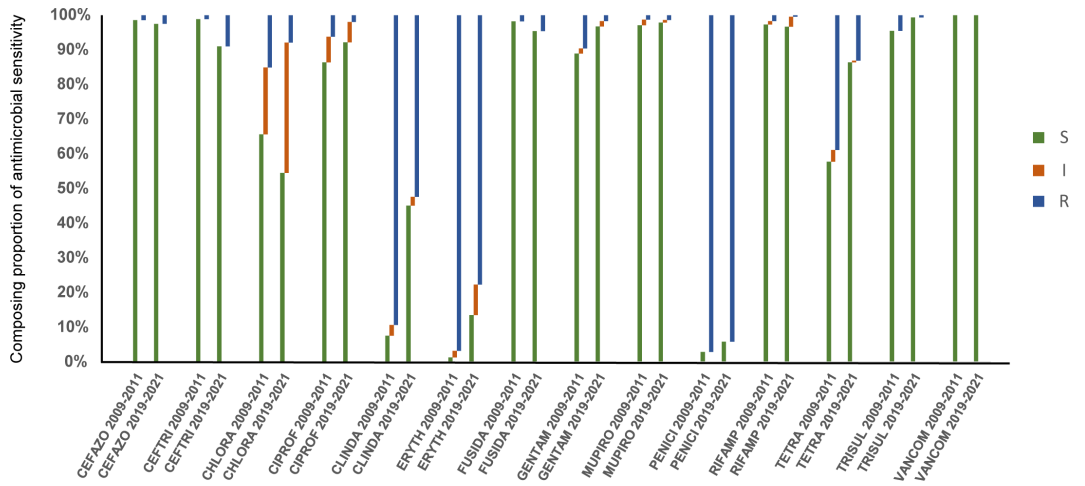

FIGURE 3
Comparison of antimicrobial susceptible, intermediate and resistant rates of *S. aureus* isolates in 2009-2011 and 2019-2021. S, susceptible; I, intermediate; R, resistant.

TABLE 3 Molecular biological characteristics of MRSA isolates in the present study.

CC	MLST	n	*pvl*		SCC*mec*		
			+	-	IV	V	NT
CC121	ST121	94		94		94	
	ST488	1		1		1	
CC59	ST59	40	14	26	31	6	3
CC22	ST22	20	19	1	5	15	
CC45	ST508	5		5	5		
	ST45	3		3	3		
CC1	ST1	3		3	3		
	ST834	1		1	1		
	ST4855	1		1	1		
CC8	ST72	1		1	1		
	ST630	1		1		1	
CC5	ST6	1		1	1		
CC30	ST30	1	1		1		
NT	ST509	11		11	6	5	
	ST338	7	7			7	
	ST88	2	1	1	1		1
	ST398	1		1		1	
	Others	11	4	7	6	4	1
Total		204	46	158	53	134	17

MLST, multilocus sequence typing; pvl, panton-valentine leukocidin gene; SCCmec, Staphylococcal cassette chromosome mec; NT, non-typable.
+, postive; -, negative.

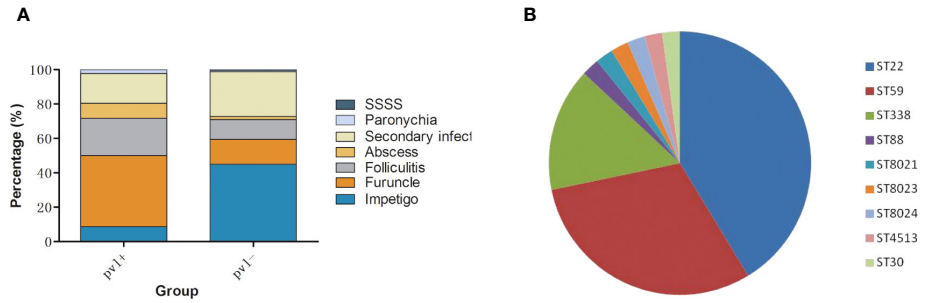

FIGURE 4
Clinical and molecular characteristics of *pvl*-positive MRSA strains. **(A)**, infection patterns caused by *pvl*-positive and *pvl*-negative MRSA strains. **(B)**, ST distribution of *pvl*-positive MRSA strains.

consistent with previous results (Liu et al., 2016; Chen et al., 2020). However, high-level mupirocin strains were detected in both MRSA and MSSA strains in this study. Dadashi et al. reported that the incidence of high-level mupirocin resistance in MRSA was the highest in Asia (12.1%), followed by Europe (8.0%) and the USA (5.9%) (Dadashi et al., 2020). In China, it was reported that *mupA* mainly accounted for high level mupirocin resistance (Jin et al., 2018; Guo et al., 2023). The *mupA* gene is typically located on mobile genetic elements and is plasmid mediated, which maybe the reasons for transmission of clones (Liu et al., 2010). In this study, high-level mupirocin resistant strains were mainly isolated from South China, which were generally with higher economical levels than others. Easy access to antibiotics without prescriptions, a high rate of antibiotic misuse, and the frequency of empiric treatment in these regions may lead to the situation. The results suggested that the rational use of mupirocin should be strengthened, and drug resistance should be further monitored.

Fusidic acid was also an important choice for SSTIs. The worldwide resistance rate of *S. aureus*, especially MRSA, to fusidic acid was reported to be 0.3%–64% (Gajdács, 2019). In the present study, the resistance rate of *S. aureus* to fusidic acid increased from 1.8% to 4.57%, which was still low, similar to that identified previously (Gu et al., 2016). The resistance rate in MSSA (4.85%) was higher than that in MRSA (2.94%), consistent with previous study (Zhanel et al., 2021). The increased detection rate of fusidic acid-resistant strains suggests that the drug should be used in moderation.

Based on the above results of antimicrobial sensitivity, we call for the timely introduction of guidelines for the treatment of SSTIs in children in China to develop scientific and effective diagnosis and treatment programs.

MRSA has been the focus of global SSTIs (Mediavilla et al., 2012). Recently, an upward trend in the incidence of *pvl*-positive MRSA was observed in Europe and Japan (Bouchiat et al., 2017; Nakaminami et al., 2020). Concern was raised as *pvl*-positive MRSA strains usually cause deep infections such as furuncles and abscesses (Shallcross et al., 2013). Compared with the study conducted 10 years ago, the detection rate of MRSA in the present study had increased by 5.65-fold (14.79% vs. 2.58%; $P<0.0001$), and the positive rate of *pvl* had increased by 2.3-fold (22.55% vs. 9.8%; $P<0.05$). In addition, the infection spectrum had changed, as deep infections including folliculitis, furuncle, and abscesses increased significantly, while superficial infections decreased. Therefore, according to our surveillance, there was an increasing trend in the prevalence of *pvl*-positive MRSA among SSTI isolates in Chinese children, which was probably connected with the increase in deep infections. Attention should be paid to the surveillance of *pvl*-positive MRSA in the future.

MLST is a universal method for understanding the molecular epidemiology of MRSA (Enright et al., 2000). Previous studies demonstrated that the most prevalent clones of CA-MRSA from SSTIs had unique geographic distribution, as ST8 was mostly reported in the USA (Otter and French, 2010), while ST80 was mainly in Europe, and ST59 was mainly in the Asia–Pacific region (Deurenberg and Stobberingh, 2008). In mainland of China, Taiwan, and Hong Kong, ST59 was reported as the most prevalent ST of MRSA strains from SSTIs (Yu et al., 2015), while ST121 was rarely dominant for clinical infections (Chuang and Huang, 2013; Wang et al., 2019). The epidemiological data hint that most ST121 strains were MSSA (Goering et al., 2008; Rao et al., 2015). However, in the present survey, ST121 (46.08%; 94/204) was the dominant ST in MRSA strains, followed by ST59, which was consistent with the results of our previous study (Liu et al., 2016). This was probably due to the differences in the population. In the present study, the enrolled children were at preschool age (1–6 years). It was reported that among preschool children, ST121 was the most prevalent clone in China (Fan et al., 2009). Besides, they were all outpatients who had no history of hospitalization. Therefore, the study was more representative of infections from the community.

In conclusion, tremendous changes in the antibiotic sensitivity of *S. aureus* from SSTIs in Chinese children had been observed compared with the results obtained 10 years ago. The incidence of MRSA as well as the positive rate of *pvl* had increased significantly, with ST121, ST59, and ST22 being the main epidemic types. With the significant changes, further research tracking sensitivity to antibiotics as well as the molecular epidemiological characteristics of MRSA is needed. Moreover, to standardize medical care, help clinicians make evidence-based treatment decisions, and effectively manage infection control, guidelines for SSTIs in pediatrics in China are urgently needed.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

Ethics statement

This project (2019-k-123) was approved by the Research Ethics Committee in Beijing Children's Hospital, China on May 21, 2019.

Author contributions

YL, KY, YY and LM designed the study. WS, YL, QW, LY and WG conducted the experiments. WS, YL and QW collected and analyzed the data, interpreted the results, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Severe problem of macrolides resistance to common pathogens in China

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With the widespread use of macrolide antibiotics in China, common pathogens causing children's infections, such as *Streptococcus pneumoniae*, *Streptococcus* (including *Group A streptococcus*, *Group B streptococcus*), *Staphylococcus aureus*, *Bordetella pertussis*, and *Mycoplasma pneumoniae*, have shown varying degrees of drug resistance. In order to provide such problem and related evidence for rational use of antibiotics in clinic, we reviewed the drug resistance of common bacteria to macrolides in children recent 20 years.

KEYWORDS

resistance, macrolides, bacteria, child, China

Introduction

Macrolides (MLs) are a diverse class of hydrophobic compounds characterized by a macrocyclic lactone ring that typically contains at least 12 elements and distinguished by variable side chains/groups. The 23S rRNA in 50S subunit in bacterial ribosome contains a peptidyl transferase (PTC) that catalyzes peptide bond formation to link amino acids into proteins. MLs interact with the nucleic acid in the domain V of the catalytic center of the enzyme, bind to the part between PTC and nascent peptide exit tunnel and then block the channel to inhibit the synthesis of bacterial proteins, which finally playing an antibacterial effect (Vázquez-Laslop and Mankin, 2018). In addition to antibiotic properties, MLs also have been shown to display antiviral, antiparasitic, antifungal, and immunosuppressive actions. So, they were widely used in clinical.

However, with the widespread use of these drugs, many bacteria are showing a tendency to become more resistant to the drugs. The mechanisms of ML resistance mainly include the following: 1.erm-mediated rRNA methylation: it is mainly caused by the double methylation of 23S rRNA in bacteria. Methylation can reduce the affinity of MLs to the ribosome site to one hundred thousand times of the previous, and is completed by erm enzymes (Farrell et al., 2003). 2. rRNA mutation: such as A2058U/A2058G in 23S rRNA in Streptococcus pneumoniae or A2064G/A2064G in 23S rRNA in Mycoplasma pneumonia (Farrell et al., 2004; Lu et al., 2020). 3. Efflux mediated by mef: mef encodes an efflux pump,

which can use MLs as a substrate to expel drugs from the bacteria by consuming energy, thereby reducing the sensitivity of bacteria to drugs (Farrell et al., 2003). 4. Ribosomal protein variants: the most important ones are L4 and L22 (Farrell et al., 2004).

In China, the resistance of many bacteria to MLs is on the rise. Here, we review the situation of drug resistance in China in the past 20-30 years. Search strategy: We searched PubMed, Wanfang database, Zhiwang of China, Google Scholar for articles published before 31 December 2022, by use of the terms: "*Streptococcus pneumoniae*", "*Mycoplasma pneumoniae*", "*Bordetella pertussis*", "*Staphylococcus aureus*", " *Group A Streptococcus/Streptococcus pyogenes",* " *Group B Streptococcus/Streptococcus agalactiae,"* AND "macrolide" AND "resistance", and reference lists of identified studies. Only articles written in Chinese and English were included. Finally, only the most relevant papers for this review were cited. The characteristics of the included literatures are shown in the Supplementary Table 1.

Streptococcus pneumoniae

SP is the most important pathogen in otitis, sinusitis, bronchitis, and community-acquired pneumonia (CAP), as well as a predominant cause of meningitis and bacteremia. The widespread use of MLs is associated with increased resistance. Reports of SP resistance to MLs first appeared in the 1980s–1990s globally and the first report of macrolides-resistant SP (MRSP) in China was also in 1980s (Ye et al., 1988).A total of 295 invasive SP strains were identified from 18 provinces of China during 1981-1983 in that study. Only one of them was resistant to erythromycin (ERY), with a resistance rate of 0.34% (1/295). Since then, domestic studies have found a significant increase in prevalence of MRSP. Li (Li et al., 1999) found that resistance to ERY of SP increased from 2% in the mid-1980s to 79% in the mid-1990s, and clarithromycin (CLA) from 2% in the mid-1980s to 76% in the mid-1990s. In 1997 (Yu et al., 2000), 76.8% of SP were found to be resistant to ERY and children who had taken the drug in the last month were more likely to carry ERY-resistant strains (39 vs 27%, RR 1.5, 95% CI 1.1-2.0). Then, the continuous monitoring in 1998-2000 showed that the ERY resistance rates of the SP in Beijing were 82.2% and 87.4%, respectively (Yu et al., 2001). In addition to Beijing, the increasing resistance rate were also reported in Shanghai (Zhang et al., 2000). The data from Europe for 1992-1998 showed that more than 40% of isolates from France and Italy were resistant to MLs, whereas less than 10% of isolates from Germany, the Netherlands, the Czech Republic, and Poland (Schito et al., 2000), which was lower than China in the same period.

At the turn of the century, Yang (Yang et al., 2002b) carried out drug sensitivity test of SP isolated from nasopharyngeal specimens of children with upper respiratory tract infection in Beijing, Shanghai, Guangzhou and Xi'an. They reported that the resistant rate of 624 isolates to ERY ranged from 75.4% to 96.7%, and 80-99% of the resistant isolates had minimum inhibitory concentration (MIC) ≥256μg/ml. At the same period, they also (Li et al., 2013) reported that 119 of 120 (99.2%) SP with serotype 19F showed resistant to ERY. Of the 119 ERY non-susceptible pneumococci, *ermB* and *mefA* were detected in 115 (96.6%) and 64 (53.8%) isolates, respectively. Both *ermB* and *mefA* were detected in 60 (50.4%) strains. Even more frustrating, 96.6% strains had MIC >256μg/ml. Subsequently, a large number of single and multi-center studies showed that the resistance rate of SP to MLs was in the range of 79%-100% (Liu et al., 2008; Wang et al., 2008; Zheng et al., 2009; Chen et al., 2010b; Xue et al., 2010; Xiong et al., 2012; Zhang et al., 2013c). Only a few single-center studies (Li et al., 2012; Jiang et al., 2013) have shown low rates of resistance to ERY (20%-21.7%). And the resistance rate was lower in adults (69.2%-73.3%) than in children over the same period (Li et al., 2003; Yao et al., 2005; Yang et al., 2008). The Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin (PROTEKT) data (1993-2003) also showed a global increase in Mls resistance of SP, from 31% in 1999 to 36.3% in 2003 (Schito and Felmingham, 2005). But, Felmingham collected 20,142 SP isolated from 151 centers in 40 countries between 2001 and 2004, and found there that was no significant temporal trend in the prevalence of MLs resistance in any country. The highest rates (~80%) were recorded among isolates collected in the Far East, followed by South Africa (~54%) and Southern Europe (~37%), whereas resistance was lowest in Latin America (~15%), Australia (~18%) and Northern Europe (~18%). And, telithromycin exhibited good antibacterial activity against SP. The most common macrolide resistance genotype among SP was *erm*(B) (~58%), followed by *mef*(A) (~30%). (Felmingham et al., 2007).

Since 2010, the resistance of MLs has increased even more, with many studies reporting a resistance rate of>92% (Zhao et al., 2017; Fu et al., 2018; Du et al., 2021; Liang et al., 2021; Liu et al., 2021b). One recent study (Zhou et al., 2021) even showed that more than 90% of the strains were resistant to azithromycin (AZM), CLA, and ERY. It is worth noting that MIC_{50} and MIC_{90} of AZM, CLA, and ERY were all>1024μg/ml. Only Shenzhen reported a slight downward trend in SP resistance to MLs (Li et al., 2019c). During this period of time, there was no significant difference in drug resistance between invasive SP and non-invasive SP strains, that is to say, both invasive and non-invasive strains showed high resistance to MLs (Huang et al., 2015b; Lyu et al., 2016; Wang et al., 2019a). Meanwhile, data from China Antimicrobial Surveillance Network (CHINET) (CHINET, 2023) since 2006 showed that both penicillin-sensitive and non-sensitive non-meningitis SP show high resistance rate to MLs (80.2%-100%). Furthermore, 143 of the 144 (99.3%) serotype 14 SP isolates resistant to ERY and the *ermB* gene was determined in all ERY resistant isolates (100%), and the *mefA* gene was positive additionally in 13 of them (9.03%) (He et al., 2015). Meanwhile, studies around the world have showed that macrolide resistance among SP is geographically variable but ranges from <10% to >90% of isolates (Pan et al., 2015; Xiao et al., 2015).

In a word, the onset time of macrolide resistance in SP is basically the same as that in other countries in China. In the past 30 years, the resistance of SP to MLs in children in China has shown an

increasing trend, especially in the 1990s, when a sharp upward trend occurred, and then maintained at a high level. *ermB* and *mefA* genes are major SP of resistant to MLs.

Staphylococcus aureus

SA is an aerobic or facultative anaerobic gram-positive coccus. With strong pathogenicity, it can cause a variety of infections including severe sepsis, pneumonia and wound infection. A growing number of studies have reported that the bacterium is resistant to a variety of antibiotics, especially MLs (Chen et al., 2022).

Resistance to MLs in SA was rarely reported in China before 2000. Huang (Huang and Liu, 1990) compared the resistance rate of SA between 1979-1984 and 1985-1988 in Chongqing, and earlier concluded that the drug resistance rate of ERY increased (P<0.05). Studies in several other southern provinces have also found significant increases in MLs resistance (from 72% to 100% in Guangdong, from 11.1% to 65.8% in Anhui) (P<0.05) (Zhang et al., 1998; Jin et al., 1999; Li and Xiong, 2001). In Beijing, Fan and Ma reported high rates of (79.7-90.3%) MLs resistance (Fan et al., 2000;Ma et al., 2000) and they also found that the resistance rate of methicillin-sensitive SA (MSSA) to ERY was significantly lower than that of MRSA (81% vs 97.1%) (Fan et al., 2000). Meanwhile, data from Guangdong showed that all the 58 MRSA isolated from 1990 to 1995 were resistant to ERY (Zhou, 1997). Prior to 2000, only SA from newborns in Shanxi province in 1995-1997 was reported to have a resistance rate of less than 50% to ERY (Guo et al., 2000). Many reports based on blood samples found that the resistance to ERY fluctuated between 63% and 76% (Duan et al., 2000; Huang and Chen, 2001; Xu and Shao, 2002). It has been reported that the detection rate of SA in sepsis children decreased from 35.4% in 1991 to 5.3% in 2000 (P<0.005) (Xu and Shao, 2002), but no decrease in the resistance of SA to MLs was found. In a word, it is clear that SA showed a significant upward trend in resistance to MLs before 2000. However, H.Westh studied 718 cases of bacteremia with MRSA, occurring between 1959 and 1988 and found that MRSA resistance to MLs occurred at a stable low level (13%) during the whole observation period and always had high MICs to ERY (Westh et al., 1991).

Since 2000, the number of studies on SA resistance to MLs gradually increased. Wang (Wang et al., 2008) monitored the resistance of 7835 SA isolated from children during 2000 to 2006, and found that the resistance rate of ERY was 66.39% and showed an increasing trend (P<0.001). Two studies bases on respiratory specimens in children showed that the resistance rates of SA to ERY was between 45.6%-58.1% (Wu J. et al., 2013) and 77-86.7% (Xia et al., 2012). Two other studies (Zhao et al., 2012a; Chen et al., 2017) found a decrease in the rate of resistance to ERY in SA (81.9-68.2% and 81.9%-54.7%), but the level of MIC was high (MIC90s ≥ 256ug/ml) (Zhao et al., 2012a). At the same time, some studies reported low resistance rate in some areas. For example, the resistance rate of SA to ERY from children in Hangzhou during 2001-2002 was 37.93% (Hua et al., 2003) and it was only 24.5% in Qinghai during 2009 to 2010 (Long and Wang, 2012). Moreover, the date from Zhejiang province from 2007 to 2010 showed low resistance rates of 11.1%, 20.0% and 25.9% (Sun, 2011). In this decade, there have been more studies to compare the resistance of MRSA and MSSA. Almost all studies have shown that the resistance of MRSA to ERY was significantly higher than that of MSSA, regardless of the type of specimen (Hu and Xia, 2009; Shi and Jian, 2010; Wang et al., 2011b; Wang, 2013; Zhang et al., 2013a). Similar findings were made for AZM resistance (Zhang and Jin, 2013). A deeper study (Wang et al., 2011a) found that the resistance rate of MRSA to ERY was as high as 97.9%, and MIC50 and MIC90 were both higher than 256ug/ml. They also found that MRSAs carrying type IV or V *SCCmec* often showed multiple resistance. In conclusion, from 2000 to 2010, the resistance rate of SA to MLs showed a trend of fluctuating increase and then decreasing. MRSA had a higher resistance rate to MLs than MSSA. In Belgium, Olivier Denis found the resistance of 455 clinical MRSA strains to ERY was 64% (Denis et al., 2004).

Since 2010, the research on the resistance of SA to MLs has been more extensive and in-depth. Continuous monitoring from several regions in China showed that the resistance of SA to MLs was on the rise (Huang et al., 2014; Li et al., 2016; Wu et al., 2019; Bao et al., 2021; Ding and Li, 2022). However, the data from CHINET (CHINET, 2023) showed that the resistance rates of MLs in MSSA and MRSA both showed a fluctuating downward trend. And some studies have shown a wide range of resistance rates, making it not easy to draw the conclusion that the drug resistance rate increases or decreases (Zhai et al., 2016; Chi et al., 2018). During this period, there were many studies on the resistance of MRSA and MSSA to MLs, and most studies showed that the resistance rate of MRSA (73.2%-100%) was significantly higher than that of MSSA (16.3%-66.1%) (Huang et al., 2015a; Li et al., 2016; He et al., 2017; Chi et al., 2018; Min et al., 2019; Zhao et al., 2020; Xiao et al., 2021; Zhou et al., 2022). Hu (Hu et al., 2016) reported that the resistance rate of CA-MRSA to ERY in Shenzhen in 2014 was 81.4% and that of HA-MRSA was 86.7%s. Chen (Chen et al., 2022) reported 60% resistance to ERY in CA-MSSA, 86% in CA-MRSA, 55% in HA-MSSA, and 82% HA-MRSA in 2015-2017. In addition to this, the data from the Chinese Pediatric Infectious Disease Surveillance Program (ISPED) (Fu et al., 2021) showed that the resistance rate of MRSA (n=11128) and MSSA (n=20667) to ERY from 11 children's hospitals during 2016-2020 was 78.2% and 51.9%, respectively. And the data of CHINET (CHINET, 2023) also showed that the resistance rates of MLs in MSSA ranged from 43.9% to 55.3%, while those in MRSA ranged from 93.4% to 73.2%. Antibiotic Resistance Monitoring in Ocular Microorganisms (ARMOR) reported 59.7% of SA isolates overall were resistant to MLs between 2009-2018 (Bispo et al., 2022), which was lower than China.

There have also been studies of resistance in SA from different sources of samples, but no significant differences have been found. Specimens from skin, soft tissue, pneumonia, pus, etc., all showed high (62.3%-98.5%) drug resistance rates (Deng et al., 2012; Deng et al., 2013; Ning et al., 2014; Zhang et al., 2014a; Ran et al., 2017). Some studies also analyzed resistance rates in both children and adults, but the results are not very clear. For example, a recent study (Li et al., 2020b) found lower rates of ERY resistance in adult than in

childhood (36.60% vs 46.31%, P<0.05). However, Ma (Ma et al., 2007) reported that the resistance rate of SA from adults to ERY was 89.8%, while that from children was 49.0%. Two other studies analyzed the causes of MLs resistance. Li (Li et al., 2020b) found that the resistance rate of Class I integron-positive isolates to ERY was higher than that of class I integron-negative isolates (50.85% vs39.29%, P<0.05). Chen (Chen et al., 2014a) reported that the *mecA* positive strain had a higher resistance rate to ERY than that of negative strain (80.30% vs 35.0%).

From the literature above, we can see that the research on the resistance of SA to MLs first experienced a rapid rising and then showed a decreasing trend of volatility, with certain regional differences in China. And, the resistance rate of MRSA was higher than MSSA.

Group A streptococcus

GAS, alternatively termed *Streptococcus pyogenes*, is a Gram-positive pathogen that routinely causes a variety of non-invasive (pharyngitis, impetigo, cellulitis, etc) and invasive infections (necrotising fasciitis, sepsis, etc). MLs is an important treatment for GAS infections. In the present study, we investigated trends in GAS resistance to MLs in China.

In 1968, a strain of GAS resistant to ERY isolated from a throat swab in a child was reported for the first time in the world (Sanders et al., 1968). In Europe, the ML resistant-GAS strain first appeared in Spain in the 1990s, and despite this, studies in Spain since then have shown that GAS has a resistance rate of 0% to ERY (Ardanuy et al., 2010). The first drug susceptibility test in mainland China was conducted by Su (Su et al., 2003), who collected 620 strains from Guangzhou, Jilin, Hubei and Chongqing during 1988-1994. The resistance rate of GAS to ERY was 35.2%. Later, Dong (Dong et al., 1999) collected strains from various provinces and cities during 1993-1994 and found that the resistance rate of ERY was different in different regions: 75.26% in Jilin, 22.22% in Sichuan, 1.1% in Hubei, and 27.19% in Guangzhou. In addition to differences in drug resistance between different cities, Dong (Dong et al., 2001) found that there were also differences between urban and rural areas: during 7 consecutive years of surveillance, GAS resistance to ERY in rural children in Guangzhou (21.4%-67.4%) was much more severe than that in urban children (4.4%-23.8%). Lin (Lin et al., 2010) reported that the resistance rate of GAS to telithromycin also showed an increasing trend, from 20.37% during 1993-1994 to 87.93% during 2005-2008. Similarly, the resistance rate of Josamycin (JOS) increased from 84.8% in 1993-1994 to 98.1% in 2005-2008 and the CLA resistance rate of GAS in Beijing area during 1993-1994 was 79% (Wu et al., 2014). In a word, the GAS resistance rate of MLs in China before 2000 was relatively high, although there were regional differences.

Deng (Deng et al., 2008) collected 47 strains in children with impetigo from 2005 to 2007 in Beijing, and the resistance rate of ERY was 47.1%. In 2009, a total of 265 GAS strains were collected from 14 hospitals, of which 82.1% were resistant to ERY (Wang et al., 2010). During the same period, many multi-center studies showed that the resistance rate of ERY in Beijing, Shanghai, Liaoning, Sichuan, Shenzhen were all above 90% (Liang et al., 2008; Liu et al., 2009; Chang et al., 2010; Chen et al., 2012; Ji et al., 2012; Zhou et al., 2014a). Meanwhile, the drug resistance rate of AZM in Beijing, Tianjin and other areas were also higher than 95% (Liang et al., 2008; Liu et al., 2009; Chang et al., 2010; Ji et al., 2012; Yin et al., 2018). Other kind of MLs were also at high drug resistance levels. For example, the resistance rates of GAS from Beijing, Shanghai, Chongqing, Tianjin to CLA were all >90% (Liang et al., 2008; Ma et al., 2008; Liu et al., 2009; Chang et al., 2010; Feng et al., 2010a; Ji et al., 2012; Wu et al., 2014; Yin et al., 2018) and the resistance rates of roxithromycin and doxomycin are also ≥90% (Han et al., 2007; Ma et al., 2008; Feng et al., 2010a). In conclusion, the data from 2000-2010 in China show that the resistance rate of GAS to MLs is on the rise, especially after 2005. However, a decline in the rate of resistance to ERY in GAS in several abroad regions was reported after 2005 (<10%) (Ardanuy et al., 2010; Montes et al., 2014; Berbel et al., 2021).

After 2010, the resistance rate of GAS to MLs remained high. Wang (Wang et al., 2013a) reported that 71 strains collected in Beijing in 2011 were all resistant to ERY (100%). Also in Beijing, Zhu (Zhu et al., 2021) collected a total of 234 strains from 2013 to 2019, and the resistance rate of ERY was as high as 98.29%. Sun (Sun et al., 2022) compared the ERY resistance rate of 50 strains in Shenzhen from 2016 to 2020, and it was all>96% in the other years except for 92% in 2017. In 2017, the resistance rate of 66 strains to ERY in a hospital in Guangzhou was as high as 96.97% (Tan et al., 2019). It has been reported that 35 GAS strains cultured from throat swabs of children in Beijing were 100% resistant to mediamycin, and the resistance rate to acetylspiramycin was 97.14% (Yang et al., 2015a). In Asia, in addition to China, Japan also has a high rate of MLs resistance (Tatara et al., 2020; Ikebe et al., 2021).

There are few studies on GAS resistance genes in China. The earliest strains related to GAS resistance genes in mainland China came from 2003 (Chang et al., 2010). Of 91 MLs resistant isolates, 77 (84.6%) had the *ermB* gene, while 14 isolates (15.4%) had the *ermA* gene. Ji (Ji et al., 2012) collected 52 strains from children with impetigo from 2003 to 2008 in Beijing, among which 92.3% carried *ermB* and 7.7% carried *ermA*. In addition, *ermB* genes accounted for more than 90% of the strains from Beijing, Shanghai, Shenzhen, and Guangzhou before 2010 (Deng et al., 2008; Ma et al., 2008; Feng et al., 2010a; Feng et al., 2010b). A study from Shandong after 2010 showed that the *ermB* gene accounted for up to 100% of all strains (Liu et al., 2015).

To sum up, the resistance of GAS to MLs in China shows an obvious upward trend, with some fluctuations, and the resistance varies in different regions. *ErmB* gene is an important gene for GAS resistance to MLs, and it is dominant in China.

Group B Streptococcus

GBS, also known as *Streptococcus agalactiae*, is a β-hemolytic Gram-positive bacterium that colonizes the lower genital tract as an asymptomatic microbe. However, it can be highly pathogenic if it was established in other host niches. During pregnancy, ascending GBS infection is associated with preterm birth, stillbirth, and fetal

injury. In addition, fetuses and neonates are uniquely susceptible to GBS infections, which most commonly include sepsis, pneumonia, meningitis, and encephalopathy. MLs are one of the first-line treatments for GBS. In the past decades, resistance to MLs continue to rise all over the world.

The earliest article on the resistance of GBS to ERY in China was published in 1989 (Cao et al., 1989). They obtained vaginal secretions of pregnant women and skin wipes of newborns from Beijing. A total of 10 strains of GBS were cultured and no ERY resistant strains was found. Only 2 years later, Zhang (Zhang et al., 1995) reported 53 strains also from pregnant women and newborns, and the resistance rate to ERY was as high as 66.04%. However, a susceptibility test of the GBS strain in Australian in 1999 showed that the ERY resistance rate was only 2.8% (Stylianopoulos et al., 2002). Shen (Shen et al., 2005) reported that the resistance rate of GBS to ERY was 8% in Beijing in 1998, increased to 16% in 1999, and 45% in Guangzhou in 1999, which was significantly higher than that in Beijing ($p<0.01$). Of the 45 ERY-resistant isolates, 44% contained *ermB* and 29% contained *mefA*, 13.33% contained both *ermB* and *mefA* genes. Yang (Yang et al., 2002a) compared the resistance of 113 GBS from Beijing (n=69) and Guangzhou (n=31), China, and St. Petersburg (n=13), Russia between 1996 and 1999. Forty-six percent of the isolates from China were ERY resistant while the isolates from Russia were all susceptible. The *ermA* gene was detected only in Beijing strain. Thirty-four strains (30.1%) carried *ermA* and/or *ermB*, of which 33 (97.1%) strains were resistant to ERY, no *ermC* gene was found. However, 53 strains carrying *mreA*, 18 strains carrying *mefA*, and 1 *ermA* positive strain were still sensitive to ERY. Two resistant strains did not carry any detected genes (*mreA*, *mefA* and *ermA/B/C*). In Canada, a study of 32 ERY-resistant GBS strains were genotyped and 88% were found to be *erm* positive (54% constitutive) and 12% *mef* positive (De Azavedo et al., 2001).

It was reported that only 8.6% of 46 strains of GBS isolated from maternal cervical secretions and neonatal pharyngeal during 2003-2006 were resistant to ERY (Zhao et al., 2007). Among 193 strains isolated from throat swabs of children and vaginal secretions of pregnant women in Jingdezhen between 2004 and 2008, the ERY resistance was also only 15% (Lin et al., 2015). However, Guo (Guo et al., 2012) reported that the resistance rate of ERY in Zhejiang Province was as high as 86.2% in 2006, 86.1% in 2007, and 84.8% in 2010. A number of studies (Guo et al., 2012; Li et al., 2015; Lin et al., 2015; Zhang et al., 2015b; Lin et al., 2022a) comparing the changes in GBS resistance rates in different years suggested that the high ERY resistance rate in the 10 years from 2001 to 2010, most of which fluctuated between 41% and 93.3%. The study (Chen et al., 2010a) from Guangzhou also showed that *ermB* gene expression accounted for 71.1%, *mefA* 52.2% and *mefE* 68.9% in ERY resistant strains.

There has been an increase in research on GBS resistance since 2010. Studies in children mainly focused on invasive infections in neonates, and most of these studies have been based solely on blood specimen (Wu X. et al., 2013; Chen et al., 2014b; Huang et al., 2018; Wang et al., 2018; Yu and Hu, 2018; Zhan et al., 2018; Liang and Wang, 2019; Liu et al., 2019; Xie and Liu, 2020; Liu et al., 2021a; Lin et al., 2022a; Qu et al., 2022), with a few studies on other specimens, such as cerebrospinal fluid (CSF) and sterile body fluid (Huang et al., 2016; Li et al., 2019a). These studies have shown that the rate of ERY resistance among invasive GBS strains has fluctuated between 43.33% and 100% (Wu X. et al., 2013; Chen et al., 2014b; Huang et al., 2018; Wang et al., 2018; Yu and Hu, 2018; Zhan et al., 2018; Liang and Wang, 2019; Liu et al., 2019; Xie and Liu, 2020; Liu et al., 2021a; Lin et al., 2022a; Qu et al., 2022), among which only two studies (Huang et al., 2018; Liang and Wang, 2019) from Shanghai and Zhejiang province showed that GBS resistance to ERY was less than 50%. A multi-center continuous monitoring in southern China (Li et al., 2019a; Li et al., 2020a) showed a fluctuating upward trend in the resistance rate of ERY (66.7 in 2013 to 78.6% in 2016). They also found that 48 of 56 (85.7%) ERY resistant strains carried the *ermB* gene and two (3.6%) strains carried both *ermB* and *mefA*. However, it should be noted that 100% of the 17 intermediate strains carried *ermB* and 5.9% also carried *mefA*. In the United States, ERY resistance rates are also high in the GBS strains that cause invasive adult and neonatal disease, at 54.8% and 44.8%, respectively (Francois Watkins et al., 2019; Nanduri et al., 2019).

In addition to neonates, studies (Lei et al., 2015; Wang et al., 2015a; Zhang et al., 2015a; Zhong et al., 2015; Li et al., 2018c; Lin et al., 2019; Wang et al., 2019b; Li et al., 2020a) on the resistance of GBS strains isolated from infants and young children were also mainly derived from invasive infection strains. Among these studies, only one study (Zhang et al., 2015a) from Shenzhen reported that the resistance rate of ERY was 41% and the intermediate rate was 25%. Other studies all showed that the resistance rate of ERY was higher than 75%, the highest was 92.5% (Wang et al., 2015b) and MIC50-90 was higher than 256μg/ml. Similarly, most AZM resistance rates have been above 95% since 2010 (Wang et al., 2015a; Wang et al., 2015b; Wang et al., 2020). The earliest study on the resistance rate of CLA can be traced back to 2008. Wang (Wang et al., 2015a) collected 40 strains in Shenzhen and Beijing from 2008 to 2013 and found that 92.5% of the strains resistant to CLA. So far, studies on the CLA resistance rate of GBS from different regions in China are all lower than this value, but the overall resistance rate is still high. For example, Zhang (Zhang et al., 2018) reported that the resistance rate of GBS to CLA in Kunming, Yunnan Province was 42.5%, which is the lowest reported level in China. The study on the resistance rate of GBS to telithromycin was relatively late and rare. It was reported that the resistance rate of 40 strains during 2008-2013 was 0 (Zhong et al., 2015), and 48 strains isolated during 2013-2014 was 30.56% (Wang et al., 2015a). Studies on the detection of resistance genes increased during this decade, with the *ermB* gene accounting for more than 80% in Guangzhou, Beijing, Shanghai and Jiangxi province (Li et al., 2018a; Li et al., 2018b; Nie et al., 2018; Li et al., 2019a; Du, 2022). In Sub-Saharan Africa, a meta analysis shows the resistance rate of ERY was only 25.11% (Wadilo et al., 2023).

In conclusion, the resistance rate of GBS to MLs has remained at a high level in China, with the *ermB* gene dominant.

Bordetella pertussis

BP is the responsible pathogen of pertussis, an acute respiratory infectious disease that occurs in children usually and causes paroxysmal, spasmodic cough. MLs represented by ERY have been the first choice for the prevention and treatment of pertussis. The first ML-resistant BP (MRBP) strain was reported in 1994 in a 2-month-old infant in Yuma, Arizona, US (Lewis et al., 1995). Since then, countries such as the United Kingdom, France, Iran and even East Asian and Southeast Asian countries including Japan, Vietnam and Cambodia, have reported the appearance of MRBP, but the prevalence in these countries is generally low (0.5% to 18.2%) (Korgenski and Daly, 1997; Wilson et al., 2002; Bartkus et al., 2003; Guillot et al., 2012; Shahcheraghi et al., 2014; Kamachi et al., 2020; Yamaguchi et al., 2020; Koide et al., 2022).

The earliest study on the drug resistance of BP in mainland of China was published in Jun 2008 (Ou, et al, 2008). In this study, 16 strains of BP were collected from 2000 to 2007 in Beijing and 4 strains were from 1970s. The results showed that all strains were ML-sensitive, and the MICs of ERY, AZM and CLA were very low. There were few studies on the resistance of BP in the following period in China, but combining the research conducted abroad of the same period (Korgenski and Daly, 1997; Wilson et al., 2002; Bartkus et al., 2003; Guillot et al., 2012);, we concluded that the incidence of ML-resistant BP was low in this period globally.

Then, in 2011, a cross-sectional study of BP seropositivity and carriage among healthy adolescents in Shandong Province, 2 ERY-resistant strains were identified with MIC>256μg/ml, and both clinical isolates had the A2047G mutation in the 23S rRNA (Zhang et al., 2013b). In 2012, 4 strains of ML-resistant BP were found in Xi'an, with A2047G mutation and all MICs of ERY, CLA and AZI were all >256ug/mL (Wang et al., 2013b). Several other studies have also shown that the molecular mechanism of ERY resistance is mainly related to the A2047G mutation in the V domain of 23S rRNA (Guillot et al., 2012; Wang et al., 2013b). A study in Xi'an (Wang et al., 2014) included 16 strains from 2012-2013, of which 87.5% (14/16) were resistant to ERY and all had A2047G mutation. Meanwhile, of the 100 samples positive for 23S rRNA PCR, 85 (85.0%) were found to have the A2047G mutation by sequencing. About the same time, data from Beijing showed that 91.9% (91/99) strains were resistant to MLs (MIC>256μg/ml), and all but one ERY-resistant strain contained the A2047G mutation (Yang et al., 2015b). Further studies conducted in Xi'an showed that the drug-resistant pertussis rate in Xi'an was 75.86-100% during 2012 to 2020, and the MIC50s of the involved bacteria to ERY, AZI and CLA were all >256μg/ml. However, some studies from south China such as Guangdong, Zhejiang, Hunan and Shanghai showed a relatively lower resistance rate (48.6%-77.1%) (Hua et al., 2019; Yan, 2019; Zhe et al., 2019; Zhang et al., 2020; Lin et al., 2022b), while it was generally higher in northern China (79.3%-100%) (Wang et al., 2014; Li et al., 2018; Li et al., 2019b; Li et al., 2019d; Juan et al., 2022).

Due to the high difficulty of pertussis culture, many regions do not carry out pertussis culture and susceptibility tests. Therefore, there is relatively little literature on pertussis resistance in China. Nevertheless, from the few studies at present, we can still see that the probability of BP resistant to MLs has increased significantly since 2010, although there are some regional differences. In order to overcome the difficulty of BP culture and avoid the missed diagnosis of drug-resistant BP, some studies also recommend the use of PCR-based sequencing for rapid detection of possible antimicrobial resistance (Wang et al., 2014; Zhang et al., 2017). Given that pertussis is clearly on the rise in China, concern about pertussis resistance is an ongoing and important issue.

Mycoplasma pneumoniae

MP is one of the main pathogens of CAP in children and adolescents. MLs are the main drugs for the treatment of MP infection. From 1968 to 1999, ML-resistant MP (MRMP) has been reported in only a few countries (Niitu et al., 1970; Stopler and Branski, 1986; Critchley et al., 2002; Pereyre et al., 2007). However, since 2000, the resistance rate has increased significantly and continuously (Okazaki et al., 2001; Morozumi et al., 2008).

The detection of drug resistance of MP started late in China but high rates of resistance were found at the outset. It was not until 2003 that Chinese scholars first reported that MP was 100% sensitive to AZM and roxithromycin (ROX), but all of them were resistant to ERY (Guo and Mai, 2003). To our knowledge, this is the first report of MRMP in China. In 2005, Xin reported (Xin et al., 2005) that 80% (4/5) of MP isolated in Beijing from 2003 to 2004 were resistant to ERY, ROX, CLA and AZM. Subsequently, they continued reported (Xin et al., 2006; Xin et al., 2009) the resistance rate of MLs was 69.2% (9/13) to 92% (46/50). The MICs of ERY, AZM and JOS were 512->1024μg/ml, 16-64 and 8-64 μg/mL, respectively. Meanwhile, they found all the drug-resistant strains were accompanied by 23S rRNA mutations, including A2063G (80%), A2063C (2%) and A2064G (10%) (Xin et al., 2009). Chen reported that the resistance rate to ERY in Hangzhou from 2006 to 2008 was 54.5-60.7% and it reached 78.9% (15/19) in 2009 (Chen et al., 2009). In a word, the MLs resistance of MP in China discovered late, but showed high resistance as soon as it was discovered.

Studies from 2010 onwards also showed high levels of resistance. Many studies showed that the resistance rate of MP to ERY in Beijing is relatively high and most of which were above 80% (Dong et al., 2013; Tian et al., 2013; Yin et al., 2013). Even in the study of 235 strains showed that the resistance rate of ERY reached 87% (Zhou et al., 2014b) in 2014. Similar trend was also found in other cities in Zhejiang province, and the drug resistance rate was relatively high (70.59%-100%) (Zhou et al., 2015; Chen et al., 2018; Lin et al., 2021). The study conducted in Guangdong showed that the resistance rate of ERY was 66.47-69.46% during 2014 to 2018. It's relatively more sensitive to AZM (9.79%), ERY (5.34%), erythromycin istolate (1.59%), ROX (3.13%) and CLA (2.99%), but the resistance rate of JOS(36.44%) and acetylspiramycin (48.17%) to MP is higher (Chen et al., 2019). In subsequent reports, the resistant rate has gradually increased, fluctuating between 24% and 77% (Lu et al., 2020; Hung et al., 2021). According to the studies in other provinces and regions, the overall trend of MLs resistant MP in China is on the rise, but the

reported resistance rate in Guangdong (5.34%), Yunnan (1.08%-5.64%) and Shanxi (64%) is lower than that in other regions (Ye et al., 2013; Chen et al., 2019; Jia et al., 2022; Lin et al., 2022c). In addition, some other studies conducted in Guangdong also showed that the rate of ERY in Guangdong (42.15-63%) was lower than that in Tianjin(92%), Shanghai (85.7%). (Liu et al., 2010; Xu et al., 2013; Ma et al., 2014; Du et al., 2017; Du et al., 2020). The distribution of MRMP is not even in the world. The resistance rate of MLs in East Asian countries represented by China is relatively high, while that in European and American countries is relatively low. Since the beginning of reporting, the drug resistance rate in Japan has shown an upward and then downward trend, where in 2009-2011, the rate was the highest at 72%. The reporting rate of European and American countries is relatively low, ranging from 0 to 27% (Wang et al., 2022).

Previous studies have revealed that there are 2 types of clinical isolates (type 1 and type 2), differing significantly in their P1 gene sequences (Su et al., 1990), and the resistance rate of type 2 isolates is relatively low (Zhao et al., 2012b; Xue et al., 2014; Shi et al., 2017; Qu et al., 2019). Before 2012, the epidemic strain in Beijing was type 1 for the P1 gene, and the genotype shift from type 1 to type 2 began in 2013 (Waites et al., 2008), which may explain the decrease in the proportion of MRMP in recent studies in Beijing. Several studies have shown that the A2063G mutation in the 23S rRNA gene domain V is the most common in MRMP isolates in China. In addition, A2064G, A2063C/T, C2617G/A, A430G, T279C, T508C, etc. are associated with the resistance (Zhang et al., 2014b; Jiang et al., 2021). A study in Zhejiang showed that (Zhou et al., 2015), A2063G channels are responsible for the inhibition of 14- and 15-member ring MLs (ERY MIC: 128->256μg/ml and AZM MIC: 32->64μg/ml, etc.), while retaining activity against 16-member ring MLs (JOS 1-8μg/ml).

From the current study, it can be seen that the incidence of MRMP is increasingly high in China, and only relatively low in a few areas. Although the drug resistance of MP cannot enhance the virulence of the pathogen, it will increase the difficulty of clinical treatment and limit the choice of antibiotics, and will cause more complications if the treatment is not timely (Zhou et al., 2014b). Therefore, it is necessary to continue to promote the monitoring of resistance.

Conclusion

In conclusion, various common pathogens such as SP, GAS, GBS, SA, BP and MP, have shown high resistance rates and high resistance level to MLs in Chinese children. The drug resistance of some pathogens occurs suddenly (such as MP and BP), which is a high drug resistance rate and high drug resistance level. There are regional differences in the resistance level of these pathogens. Mutations in the 23S rRNA site and/or carrying the *ermB* gene are currently common causes of resistance in these pathogens.

Author contributions

XM and LL contributed to conception and design of the study. JL, JG, HZ, XW and MX organized the database. LL and XM wrote the first draft of the manuscript. JL, HZ and JG wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

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

The reviewer WZ declared a shared affiliation with the authors JL, HZ, JG to the handling editor at the time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1181633/full#supplementary-material

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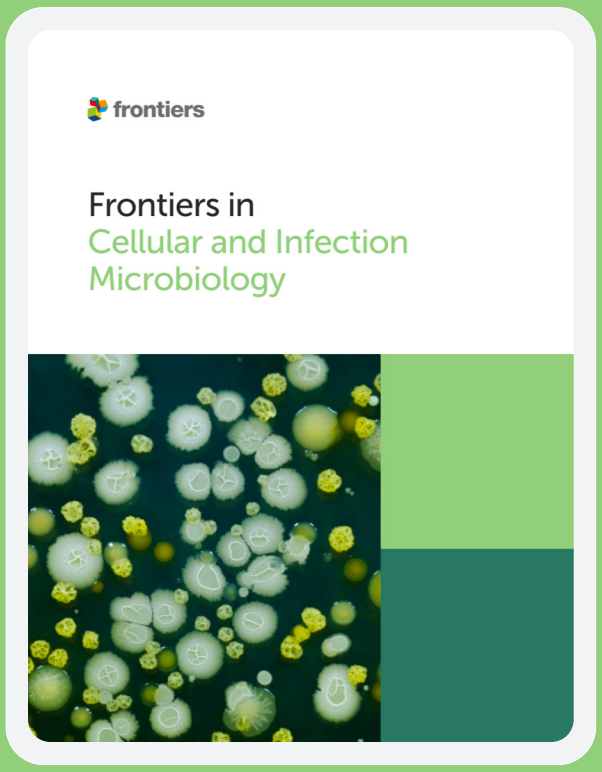

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