Macrolide Susceptibility and Molecular Characteristics of *Bordetella Pertussis*

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Research

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Abstract

Objective

To compare the macrolide resistance and molecular characteristics of clinical isolated *Bordetella pertussis*, and explore the relationship between the macrolide-resistance and genotypes.

Methods

Erythromycin–azithromycin and clarithromycin susceptibility of clinical isolates during 2018-2020 was determined by E-test. The A2047G of the 23S rRNA genes was sequenced for drug-resistance mutation. Multilocus antigen sequence typing (MAST)–Multiple Locus Variable-number tandem repeat Analysis (MLVA) and Pulsed field gel electrophoresis (PFGE) methods were employed to do molecular typing for the strains.

Results

58 strains were isolated in this study, 46 of them were macrolide-resistant and 12 sensitive. All macrolide-resistant strains carried a genetic mutation at the A2047G site, genotype was *prn1/ptxP1/ptxA1/fim3-1/fim2-1*, the MLVA types were identified as MT195–MT55 and MT104, PFGE profiles were classified into BPSR23 and BpFINR9 types. However, no mutations were found in all macrolide-sensitive strains whose genotypes were (*prn9* or *prn2*)/*ptxP1/ptxA1/fim3-1/fim2-1* and MT27, and PFGE classified other profiles.

Conclusions

The clinical isolated *Bordetella pertussis* has serious resistance to erythromycin and began to spread to other macrolides. There were differences between macrolide-resistant and -sensitive *Bordetella pertussis* in genotypes. The acquisition of macrolide resistance may be associated with change of specific molecular types.

Introduction

Pertussis is an acute respiratory disease of infants caused by *Bordetella pertussis*. Macrolide antibiotics, represented by erythromycin, have always been the first choice for the prevention and treatment of pertussis. In the past decade, erythromycin resistance of pertussis strains has been reported in many countries around the world, but it is still rare in Europe and America. However, high resistance has been reported in Tianjin^[1], Shanghai^[2] and other areas in China, and the resistance may be related to mutation of adenine (A) to guanine (G) at 2047 site in 23S rRNA. Moreover, our previous studies showed the erythromycin-resistance began to spread to other macrolide antibiotics, like azithromycin and clarithromycin.

Adaptive changes of vaccine-associated genes in circulating pertussis strains has been reported in many countries over the past 10 years^[3]. For example, the current pertussis toxin A (Pertussis toxin–PtxA) was mainly ptxA1, different from the vaccine strain which harbored *ptxA2*. At the same time, the *B. pertussis* MT27 strain has gradually replaced the MT83 vaccine strain and been prevalent in Europe^[4]. However, few researches in this field were found in China.

Studies shown the antimicrobial resistance phenotypes of *Candida albicans* and *Staphylococcus aureus* were associated with their genotypes^[5,6], yet there have been few reports about *Bordetella pertussis* in this respect. A study from Shanghai showed the *ptxP1* pertussis strains were significantly more resistant to erythromycin than *ptxP3* strains^[2]. To explore the macrolide susceptibility of *Bordetella pertussis* and the correlation with its genotype, compare the genotypes of strains with different macrolide-resistant phenotypes, 58 clinical *B. pertussis* isolates from 2018 to 2020 were analyzed by E-test to three macrolide antibiotics, A2047G mutation sites detection, and three genotyping methods including multilocus antigen sequence typing (MAST), Multiple Locus Variable-number tandem repeat Analysis (MLVA), and pulsed-field gel electrophoresis (PFGE).

Materials And Methods

1.1 Strains

Nasopharyngeal swabs (NPSs) were collected from 1200 patients aged ≥ 1 years hospitalized with suspected pertussis infection in Xi'an Children's Hospital during 2008-2020. Bacterial DNA was extracted from the NPSs using the QIAamp DNA Mini Kit (Qiagen, Dusseldorf, Germany) and subjected to real-time PCR to assess the expression of *IS481* and *ptxP* (Mabsky, Shenzhen, China). The NPSs positive for both *IS481* and *ptxP* were cultured on charcoal agar (OXOID, UK) plates supplemented with 15% sheep blood at 37 °C for 3-5 days. A total of 58 isolates were positive for culture, biochemical testing, and slide agglutination with specific anti-sera to *B. pertussis* (BD, Sparks, MD, USA). strains were stored at -70°C with 30% glycerin for later use.

1.2 Antimicrobial susceptibility testing and mutation site detection

Minimum inhibitory concentrations (MICs) of three macrolide antibiotics, including erythromycin, azithromycin and clarithromycin were determined via E-Test methods. We subcultured bacterial suspension with 0.5 McFarland turbidity standard on 20 mL Bordet-Gengou agar containing 10% sheep blood and cultured at 36°C. We then measured MICs and inhibition zone sizes when incubated for 5 days. Reference strains of *B. pertussis* (ATCC9797) and *Staphylococcus aureus* (ATCC29213) were used as controls. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) do not yet provide breakpoint criteria for antimicrobial susceptibility for *B. pertussis*, we determined MIC $\leq$ 0.12mg/L as resistant reference to reports before^[7].

Primers were designed to amplify and sequence the A2047G of 23S rRNA gene of 58 isolates^[8]. Compared the sequences with the standard X68323 sequence and the allele of Chinese vaccine strain (CP002695) in GenBank. Mutant and sensitive strains preserved in laboratory were used as control.

1.3 Multilocus antigen sequence typing (MAST)

MAST was performed for antigenic genes *prn*, *ptxP*, *ptxA*, *fim2* and *fim3* according to the methods described previously^[9,10]. The different alleles mentioned above were determined by sequencing and comparing at <https://pubmlst.org/>. The *B. pertussis* standard strains ATCC 9797 and reference strain 13038 with known alleles kept in our laboratory were included as positive controls.

1.4 Multiple Locus Variable-number tandem repeat Analysis (MLVA)

For MLVA, the variable number of tandem repeats in six loci (VNTR1, VNTR3a, VNTR3b, VNTR4, VNTR5, and VNTR6) was determined as described previously [11,12]. After the allele of every variable number tandem repeat (VNTR) locus was obtained, the MLVA types (MTs) were assigned to the reference database at <http://www.mlva.net/>. The *B. pertussis* ATCC 9797 (18323) with known MT was included as a reference strain in each run.

1.5 Pulsed field gel electrophoresis (PFGE)

Isolates were analyzed according to the standardized recommendations for typing *B. pertussis* using *XbaI* as a restriction enzyme [13]. The nomenclature was based on the profiles already defined for Finland (BpFINR) Sweden (BpSR) and China (BpCHR). Furthermore, isolates with new profiles were designated as BPX01XA001 and BPX01XA002, respectively, in the series. The *B. pertussis* standard strain ATCC 9797 and *Salmonella* standard strain H9812 were included as reference strains. BioNumerics 7.6 software was used for cluster analysis of the electrophoresis band.

Results

2.1 Antimicrobial susceptibility testing and mutation site detection

E-test results showed that 44 of the 58 isolates were resistant to erythromycin, azithromycin and clarithromycin (MIC≥128mg/L), 2 isolates were resistant to erythromycin and azithromycin, but sensitive to clarithromycin. 12 isolates were sensitive to erythromycin, azithromycin and clarithromycin (MIC < 0.016mg/L). A2047G mutation was detected in 46 macrolide-resistant strains, while no mutation was found in 12 sensitive strains. Fig1 E-test of Bordetella pertussis isolates to macrolides antibiotics.

2.2 MAST types of pertussis isolates and its relationship with macrolide-resistance

In this study, all isolates harbored the *ptxA1/fim2-1/fim3-1* allele. Three *prn* alleles were detected: *prn1*, *prn2*, and *prn9*, and *ptxP* identified two alleles: *ptxP1* and *ptxP3*. According to the alleles of *prn* and *ptxP*, three antigenic genotypes were obtained: *prn1/ptxP1/ptxA1/fim3-1/fim2-1*, *prn9/ptxP3/ptxA1/fim3-1/fim2-1* and *prn2/ptxP3/ptxA1/fim3-1/fim2-1*. The antigenic genotypes of all the macrolide-resistant strains were *prn1/ptxP1/ptxA1/fim3-1/fim2-1* (46/58, 79.3%), while the sensitive strains were identified as (*prn9 or prn2*)/*ptxP3/ptxA1/fim3-1/fim2-1* (12/58, 20.7%). The results suggested that the antigenic genotypes of macrolide-resistant *B.pertussis* are different from those of macrolide-sensitive *B.pertussis*. Table1 shows the macrolide susceptibility and genotypic distribution of 58 *B. pertussis* isolates.

Table 1. Macrolide susceptibility and genotypic distribution of 58 B. pertussis isolates

E-test (mg/L)			A2047G	Allele types					MTs @	PFGE	Frequency (n,%)
EM [*]	AZ [#]	CH [^]		prn	ptxP	ptxA	fim2	fim3			
R	R	R	G	1	1	1	1	1	195	BpFINR9	15(25.9%)
R	R	R	G	1	1	1	1	1	195	BpSR23	4(6.9%)
R	R	R	G	1	1	1	1	1	104	BpFINR9	2(3.4%)
R	R	R	G	1	1	1	1	1	104	BpSR23	10(17.2%)
R	R	R	G	1	1	1	1	1	55	BpFINR9	11(19.0%)
R	R	R	G	1	1	1	1	1	55	BpSR23	2(3.4%)
R	R	S	G	1	1	1	1	1	104	BpFINR9	1(1.7%)
R	R	S	G	1	1	1	1	1	104	BpSR23	1(1.7%)
S	S	S	A	2	3	1	1	1	27	BpCHR23	2(3.4%)
S	S	S	A	2	3	1	1	1	27	BpCHR16	1(1.7%)
S	S	S	A	9	3	1	1	1	27	BPX01XA001	2(3.4%)
S	S	S	A	9	3	1	1	1	27	BPX01XA002	1(1.7%)
S	S	S	A	9	3	1	1	1	27	BPX01XA003	3(5.2%)
S	S	S	A	9	3	1	1	1	27	BPX01XA004	3(5.2%)

*EM: erythromycin; #AZ: azithromycin; ^CH: clarithromycin; @MTs MLVA types

2.3 MLVA types of pertussis isolates and its relationship with macrolide-resistance

For MLVA, 4 MTs were obtained from the 58 strains: MT195 (19, 32.4%) MT104 (14, 26.5%) MT55 (13, 23.6%) and MT27 (12, 17.6%). It's worth noting that MT195, MT104 and MT55 were detected from macrolide-resistant strains, while 12 susceptible strains were identified as MT27. The results may suggested that the MLVA types of macrolide-resistant B.pertussis are radically different from the sensitive isolates.

2.4 PFGE profiles of pertussis isolates and its relationship with macrolide-resistance

In this study, eight PFGE profiles (BpFINR9/BpSR23/BpCHR23/BpCHR16/BPX01XA001/BPX01XA002/BPX01XA003/BPX01XA005) were retrieved. The two most common PFGE profiles were BpFINR9 (29, 50.0%) and BpSR23 (17, 29.3%). BpFINR9 and BpSR23 were obtained from macrolide-resistant strains, while 12 susceptible strains were identified as other profiles. The results may suggested that the PFGE types of macrolide-resistant B.pertussis differs the sensitive. Fig.2 shows the dendrogram of PFGE profiles and its association with [molecular characteristics](#)

Discussion

Erythromycin, as the preferred antibiotic for treatment and prevention of pertussis, has been reported in many countries for its resistance to pertussis, especially in China^[8,14]. In our previous studies, we found the clinical isolated *Bordetella pertussis* has serious resistance to erythromycin and began to spread to other macrolides, like azithromycin and clarithromycin, etc. In this study, the resistance rates of the strains to erythromycin and azithromycin were 79.3% (46/58), and the rate to clarithromycin were 75.9% (44/58), indicating that the macrolide-resistance of the pertussis strains was quite high in China. A2047G site mutation of 23S rRNA was detected in macrolide-resistant strains, while no mutation sites were found in susceptible strains. The results were consistent with previous researches^[15], and provides evidence that A2047G mutation may be the main mechanism of pertussis resistance to macrolide antibiotics.

It has been reported that the conserved sequence of 25S rDNA transposable introns is different between the fluconazole-resistant and -susceptible *Candida albicans* strains^[5]. Moreover, specific virulence factors also showed certain aggregation distribution in methicillin-resistant and -sensitive *Staphylococcus aureus* strains, suggested that the acquisition of drug resistance in *S.aureus* may be accompanied by changes of specific virulence characteristics^[6], however, there have been few reports on pertussis in this respect. In this study, all the prn1 strains were resistant to macrolides, while prn9 and prn2 strains were sensitive. In addition, all the ptxP1 strains were resistant to macrolides, while ptxP3 strains were susceptible. This also explains why there is no prevalence of macrolide-resistant strains in Europe, that is, the prevalent strain is ptxP3. The results suggested that the acquisition of macrolide resistance in *B. pertussis* may be related to the change of antigen-related genes, especially ptxP and prn genes.

In our study, 4 MTs were obtained: MT195, MT104, MT55 and MT27. The isolation rate of MT27 strain has been increasing continuously in Europe in the past 20 years and more than 80% of the isolates were reported to be MT27 at present^[16]. However, detection rate of MT27 strains was still relatively low in China, and a total of 12 strains (20.7%) were detected in this study. There were reports declared that great heterogeneity of MLVA types was identified among erythromycin-resistant and erythromycin-sensitive pertussis isolates^[17]. In our study, MT195, MT104 and MT55 were detected from macrolide-resistant strains, while MT27 were identified from susceptible strains. The results corroborates the opinion stated above, suggesting that acquisition of macrolide-resistance may be associated with change of MLVA types.

Studies have shown that there is a certain correlation between the drug-resistance phenotype and the PFGE genotype in *Escherichia coli*^[18]. In our study, BpFINR9 and BpSR23 were obtained from macrolide-resistant pertussis strains, while susceptible strains were identified as other profiles (fig2). The results may suggested that there is a correlation between macrolide-resistant and the PFGE profiles in *B.pertussis* as well.

The high resistance rate to macrolide in this study suggested that the monitoring of antibiotic resistance in pertussis strains should be paid attention to. In addition, the MIC value of ptxP1 strain to macrolide was significantly higher than that of ptxP3 strain, the MIC of other MT strains was also higher than MT27 strain suggesting the molecular characteristics of pertussis strains should also be noted. All macrolide-resistant strains carried a genetic mutation at the A2047G site, pertussis toxin (ptxP) and adhesin (prn) were ptxP1 and prn1, the MLVA types were identified as MT195, MT55 and MT104, PFGE profiles were classified into BpSR23 and BpFINR9 types. However, no mutations were found in all macrolide-sensitive strains whose genotypes were (prn9 or prn2)/ptxP1/MT27, and PFGE classified other profiles. The results suggested that the acquisition

of macrolide resistance may be associated with change of specific molecular characteristics. This study provides a reference for improving prevention and treatment of pertussis.

As outbreaks and drug resistance of pertussis have been reported in many parts of the world, many foreign reports have conducted in-depth studies on the genomic diversity of pertussis strains, while few reports in China^[19]. The data released in this study provided a reference for the related research on genomic diversity of *Bordetella pertussis* in China. However, the results of this study have certain limitations due to the small number of strains used and the collection of strains were only from Shaanxi Province and its surrounding areas. A large number of strains are still needed to be verified in future studies.

Declarations

Ethics approval and consent to participate

This study was approved by the Human Ethics Committee of Xi'an Center for Disease Control and Prevention and was conducted in accordance with the Declaration of Helsinki. The samples used in this study were throat swab specimen, which was non-invasive. Therefore, informed consent was obtained in an oral confirmation form. Written informed consent was obtained from each participant's parents or [legal guardian](#) prior to the study. These samples were only used in this study with no commercial objectives. The parents of the children involved in the sample collection approved the preparation of the manuscript.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors report no personal, political, commercial, financial, or academic conflicts of interest. All authors have approved the final article.

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Authors' contributions

ZJS wrote the manuscript; ZDQ revised the manuscript; WXQ, LH, WXG did the data analysis; ZYW, LY, LF, HRJ did the data collection. All authors read and approved the final manuscript.

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Figures

Figure 1

E-test of *Bordetella pertussis* isolates to macrolides antibiotics. A) erythromycin-resistant; B) erythromycin-susceptible; C) azithromycin-resistant; D) azithromycin-susceptible; E) clarithromycin-resistant; F) clarithromycin-susceptible;

Figure 2

Dendrogram of PFGE profiles and its association with molecular characteristics. all isolates carried the ptxA1/fim2-1/fim3-1 alleles