

In vitro susceptibility test of Xiao'er Feire Kechuan Oral Solution to *Mycoplasma pneumoniae*

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Abstract

The aim of this study was to evaluate the inhibitory effect of antibiotics and Xiao'er Feire Kechuan Oral Solution on *Mycoplasma pneumoniae* (MP) clinical isolates.

Twenty clinical isolates containing A-to-G transition at position 2063 and 10 clinical isolates without mutations in 23S rRNA V regions were randomly selected. The international standard strain FH was chosen as control strain. The minimum inhibitory concentration (MIC) of macrolide, quinolones, tetracycline, and Xiao'er Feire Kechuan Oral Solution to MP clinical isolates were performed using broth microdilution method.

In vitro antibiotic susceptibility test of MP clinical isolates showed that MP showed high resistance to macrolide antibiotics (erythromycin and azithromycin); MIC of both were more than 64 µg/mL. The MICs of erythromycin and azithromycin for clinical isolates without mutations in 23S rRNA V regions were ≤0.5 µg/mL. The MICs of tetracycline and levofloxacin for all clinical isolated strains were ≤2.0 µg/mL and ≤1.0 µg/mL, respectively. The MIC of Xiao'er Feire Kechuan Oral Solution was 13.828~6.914 mg/mL.

In vitro, the drug resistance of MP to macrolide antibiotics is higher, MP clinical isolates are sensitive to tetracycline and levofloxacin, and Xiao'er Feire Kechuan Oral Solution also has a certain inhibitory effect on the macrolide-resistant MP.

Abbreviations: ATCC = American Type Culture Collection, CFU = colony-forming units, MIC = minimum inhibitory concentration, MP = *Mycoplasma pneumoniae*.

Keywords: antibiotic susceptibility test, *Mycoplasma pneumoniae*, Xiao'er Feire Kechuan Oral Liquid

1. Introduction

Mycoplasma pneumoniae (MP) is the smallest prokaryotic microorganism between bacteria and viruses that can grow in lifeless medium. It is one of the most important pathogens of community-acquired pneumonia in children and accounts for 10% to 40%.^[1] MP has no cell wall and is naturally resistant to

antibiotics that act on the cell wall; however, it is sensitive to antibiotics that inhibit or interfere with protein synthesis and DNA replication, such as macrolide antibiotics, quinolones, tetracyclines, and so on. Tetracycline drugs, such as minocycline and doxycycline, can cause tooth coloring and enamel dysplasia, may inhibit the growth of infants and children, and are prohibited in the late pregnancy and children younger than 8 years. Quinolones can cause abnormal cartilage development, are prohibited in pregnant women, breast-feeding women, and children, and should be used with caution in children younger than 18 years. Therefore, macrolides were the most commonly used drugs for the treatment of MP infection in children.

However, since the first isolation of MP-resistant macrolide antibiotics in Japan in 2001,^[2] China, France, the United States, and Germany have reported the isolation of MP resistant to macrolide antibiotics in clinical specimens of children and adult. The resistance rate of MP was 3% in Germany, increased from 5% to 30% in Japanese from 2002 to 2006, was >80% in children and was about 69% in adult of China.^[3–5] Recently, many studies have reported that the effectiveness of macrolide antibiotic-resistant MP infection has led to the decrease in the effectiveness of the original macrolide antibiotics, and the time of fever and coughing is longer than those of children with sensitive MP infection. In addition, it has been reported that MP is resistant to macrolides antibiotics, causing the increase of incurable or severe pneumonia.^[6–8]

To view on the current situation, especially in China, it is urgent to actively seek effective therapeutic drugs and treatment plans. Chinese herbal medicine has been used in the treatment of various infectious diseases for thousands of years. Furthermore,

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the multicenter clinical studies have also found that children with MP infection treated with Xiao'er Feire Kechuan Oral Solution had faster improvement of clinical symptoms compared with patients without traditional Chinese medicine.^[9,10] Xiao'er Feire Kechuan Oral Solution has been used for the treatment of MP pneumonia clinically in China for many years. In vitro, the drug resistance of MP to macrolide antibiotics is higher, MP clinical isolates are sensitive to tetracycline and levofloxacin, and Xiao'er Feire Kechuan Oral Solution also has a certain inhibitory effect on the macrolide resistant MP.^[5] The purpose of this article was to determine the minimum inhibitory concentration (MIC) of macrolides, tetracyclines, quinolones, and Xiao'er Feire Kechuan Oral Solution for clinical isolates of MP in vitro, which may provide data support for the treatment of MP infection, clinical rational use of drugs.

2. Materials and methods

2.1. Identification of MP clinical isolates

This study was approved by the ethics committee of Beijing Friendship Hospital, Capital Medical University, Beijing Tropical Medicine Research Institute and conducted according to the Helsinki Declaration. The MP clinical isolates were the strains stored in our laboratory in 2016. The international standard strain FH (ATCC15531), purchased from the American Type Culture Collection (ATCC) of United States, was chosen as the control strain. Sample DNA was extracted in strict accordance with the instructions of the genome extraction kit (Kangwei century biotechnology co., LTD. Universal type column genome extraction kit, CW2298S, 40137). Then, the sample DNA was amplified by the quantitative real-time polymerase chain reaction designed by the laboratory.^[11] The PCR-positive products were sequenced, completed by Yingweijie trading co., LTD., and then compared with the MP standard strain M129 gene sequence in NCBI database.

2.2. Drug sensitivity test

2.2.1. Culture medium configuration. PPLO basal medium (5) consists of 50 mL/L newborn calf serum, 10 g/L fresh yeast extract, 0.02 g/L phenol red indicator, 10 g/L glucose and penicillin 5×10^4 IU/100 mL.

2.2.2. Preparation of strain. The DNA samples amplified by MP clinical isolates were sequenced and analyzed. Twenty clinical isolates containing A-to-G transition at position 2063 and 10 clinical isolates without mutations in 23S rRNA V regions were randomly selected. Then, 0.2 mL bacteria solution were inoculated on the surface of solid culture medium and cultured in a humidified 5% CO₂ incubator at 37°C. The colony was observed under the microscope every day, and the colony-forming unit (CFU) was counted.

Preparation of drug macrolide was done with following: erythromycin (No.130307), azithromycin (No.130352), tetracycline (No. 130306); quinolone:levofloxacin (No.130455). All of the above drugs are purchased from the China Food and Drug Verification Research Institute. Xiao'er Feire Kechuan Oral Solution (drug approval Z10950080) was developed by Sunflower Pharmaceutical Group Co., Ltd. (Heilongjiang, China). A certain amount of antibiotics was diluted with medium into 2048 μg/mL, 1024 μg/mL, 1024 μg/mL, 512 μg/mL, 256 μg/mL, 256 μg/mL, 128 μg/mL, 64 μg/mL, 32 μg/mL, 16 μg/mL, 8 μg/mL. Furthermore, Xiao'er Feire Kechuan Oral Solution was also diluted with medium into 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512.

2.2.3. Broth microdilution. The experimental group consisted of 30 MP clinical isolates, including 20 MP mutant strains and 10 *Mycoplasma pneumoniae* clinical isolates without mutations in 23S rRNA V regions. The control group was standard strain FH. The MIC of the drug to MP clinical isolates was measured by broth microdilution. The bacterial liquid was diluted into 1×10^5 CFU/mL and cultured in a humidified 5% CO₂ incubator at 37°C for 2 hours. The experimental hole, positive control, negative control, and drug control were established. Two compound holes were set for each hole. The paraffin-sealed reaction plate was in a humidified 5% CO₂ incubator at 37°C for daily observation and recording results.

3. Result

The MP clinical isolates were determined as MP positive according to the amplification curve and dissolution curve (Figure 1), Ct value ≤ 38 , Tm value consistent with the positive control. The sequence of PCR-positive products was compared

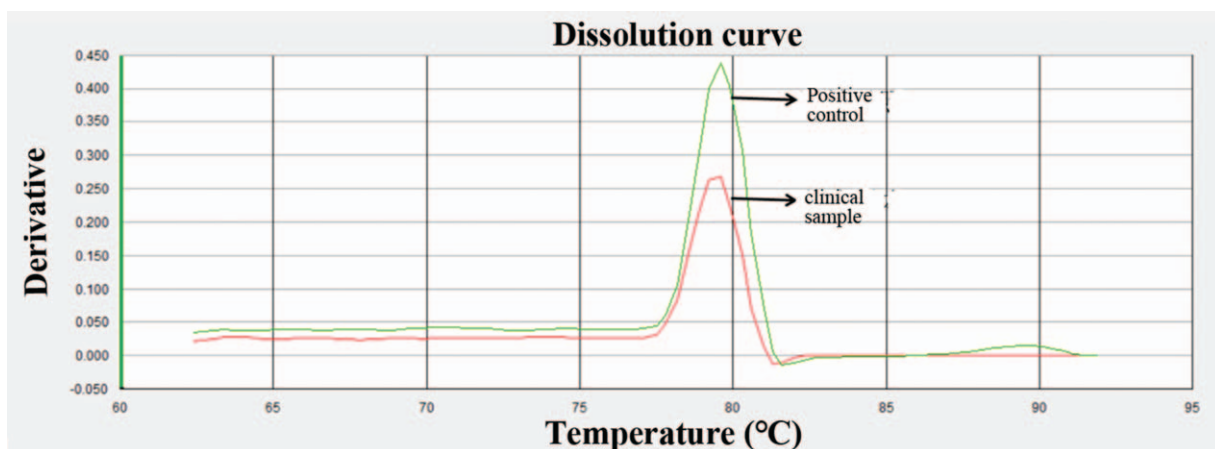


Figure 1. Dissolution curves of the clinical sample and positive control.

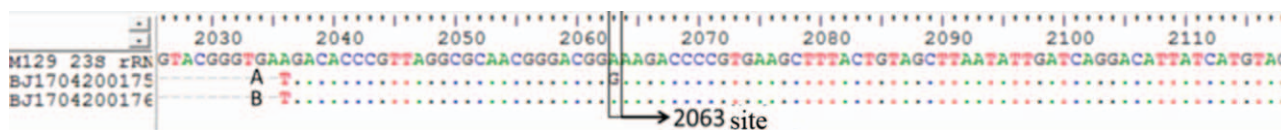


Figure 2. The schematic diagram of BLAST result. (A) Clinically isolated *Mycoplasma pneumoniae* strains (A2063G site mutation); (B) no site mutation in domain V of 23S rRNA.

with the 23S rRNA gene sequences of standard strains, which was already registered in NCBI database, as shown in Figure 2.

According to the criteria of resistance to clinical use of MP antibiotics (Table 1) that published by the Clinical and Microbiological Standardization Committee (CLSI) in 2011,^[12] the strain whether resistance to drug resistance was determined. MP standard strain FH was sensitive to macrolides, quinolones, and tetracycline antibiotics; the clinical isolates with A-to-G transition at position 2063 were resistant to erythromycin and azithromycin, and MP clinical isolates without mutations in 23S rRNA V regions were sensitive to erythromycin and azithromycin; MP clinical isolates were sensitive to tetracycline and levofloxacin. Children are sensitive to tetracycline and levofloxacin. The MIC of Xiao'er Feire Kechuan Oral Solution for MP clinical isolates and standard strains was 13.828~6.914 mg/mL. The drug sensitivity test in vitro was repeated 3 times; the results were shown in Table 2.

4. Discussion

MP infection occurs mostly in densely populated areas, and erupted once every 3 to 7 years. Its incidence is seasonal and occurs frequently in autumn. It has a slow onset, fever, paroxysmal irritating cough, and a small amount of mucous or mucopurulent sputum (occasionally bloody phlegm) and other symptoms. For MP patients, signs of the lungs are not obvious, but it is easy to cause multiple systems outside the lungs, which can also threaten life or death. MP occurs in children or adolescents, accounting for 15% to 30% of the total number of pneumonia, and the prevalence can be as high as 40% to 60%.^[13]

MP has special affinity for respiratory epithelial cells, is resistant to penicillin, and is highly sensitive to macrolides such as erythromycin. Drugs for treatment of MP infection mainly include macrolides, tetracycline, and fluoroquinolones. Among them, macrolide is the safe and effective antibiotic for the treatment of respiratory diseases. Erythromycin, as the first generation of macrolide antibiotics discovered in 1952,^[14] is widely applied and is the first choice for the treatment of mycoplasma infection. However, it has the disadvantage of being unstable to acid, incomplete oral absorption, and leading to gastrointestinal reactions. Then, in 1980s the second-generation macrolide antibiotics, represented by azithromycin, roxithromy-

cin, and clarithromycin, were developed.^[15] Azithromycin overcomes the deficiency of erythromycin to acid instability, improves the bioavailability, prolongs the half-life, reduces the dosage, and maintains good antibacterial activity.^[16] In addition, especially in China, Chinese herbal medicine has been used in the treatment of various infectious diseases for thousands of years. In this study, to provide data and ideas for the treatment of MP infection, the in vitro inhibition of MP by antibiotics and Xiao'er Feire Kechuan Oral Solution was discussed.

In this study, the MP clinical isolates with A-to-G transition at position 2063 were all resistant to erythromycin and azithromycin; the MP clinical isolates without mutations in 23S rRNA V regions were sensitive to erythromycin and azithromycin. In vitro antibiotic susceptibility test of MP clinical isolates showed that MP showed high resistance to macrolide antibiotics (erythromycin and azithromycin); MICs of both were >64 µg/mL, which were basically consistent with the previous reports.^[17,18] In this study, 30 clinical isolates of MP were sensitive to tetracycline and levofloxacin, which were also consistent with the previous studies that reported the emergence of MP clinical resistant strains to tetracycline.^[19]

The main components of Xiao'er Feire Kechuan Oral Solution include anemarrhena, honeysuckle, glycyrrhiza, and other traditional Chinese medicine. Among them, honeysuckle, astragalus, and forsythia can effectively inhibit the growth of pathogenic microorganisms and obstruct the synthesis of the bacterial protein; ophiopogon and anemarrhena have the effect of nourishing yin and moistening the lung, especially for the lung infection of the children; glycyrrhiza, ephedra, and almond can clear the lung heat and have the effect of spreading the lung and relieving asthma and removing evil spirits.^[20] In this study, the results showed that Xiao'er Feire Kechuan Oral Solution in children had the effect of inhibiting the growth of mutant and wild type of clinical isolates in vitro. Some multicenter clinical studies showed that Xiao'er Feire Kechuan Oral Solution has a

Table 1
Antimicrobial drug resistance standard of MP (µg/mL).

Antibacterial drug	Sensitivity (S)	Intermediates (I)	Resistant (R)
Erythromycin	≤0.5	0.5<I≤1.0	>1.0
Azithromycin	≤0.5	0.5<I≤1.0	>1.0
Tetracycline	≤2.0	—	—
Levofloxacin	≤1.0	—	—

MP = *Mycoplasma pneumoniae*.

Table 2
MIC of MP in vitro.

Drug	Clinical isolates		
	With A-to-C transition at position 2063	Without mutations in 23S rRNA V regions	Standard strain FH
Azithromycin, µg/mL	64–128	0.003–0.008	0.003
Erythromycin, µg/mL	64–256	0.008–0.0016	0.008
Levofloxacin, µg/mL	0.4–0.8	0.4–0.8	0.4
Tetracycline, µg/mL	0.1–0.2	0.1–0.2	0.2
Xiao'er Feire Kechuan Oral Solution, mg/mL	13.8–6.9	13.8–6.9	13.8–6.9

MIC = minimum inhibitory concentration, MP = *Mycoplasma pneumoniae*.

certain effect on MP infection, which is conducive to improving clinical symptoms and accelerating recovery.^[9,10]

In conclusion, in vitro, the drug resistance of MP to macrolide antibiotics is higher, MP clinical isolates are sensitive to tetracycline and levofloxacin, and Xiao'er Feire Kechuan Oral Solution also has a certain inhibitory effect on the macrolide-resistant MP.

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