Molecular typing of Mycoplasma pneumoniae and its correlation with macrolide resistance in Henan of China

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Abstract

Background/purpose To date, molecular typing studies of Mycoplasma pneumoniae are limited in Henan. We researched the molecular types of Mycoplasma pneumoniae in pediatric patients in Henan in 2020. Methods M. pneumonia was detected by SAT-MP kit. The domain V of their 23S rRNA were sequenced for detection of macrolide-resistant point mutations. Molecular typing with multiple locus variable-number tandem repeat analysis (MLVA) was done for both macrolide-susceptible and macrolide -resistance M. pneumoniae samples. Results M. pneumoniae was detected in 9.8% (121/1237) respiratory samples in 2020. Among the M. pneumoniae-positive samples, 96% (116/121) had macrolide-resistant genotypes, and all of them were A2063G mutation. 105 macrolide-resistant strains and 4 macrolide-susceptible strains fulfill MLVA typing. MLVA 4-5-7-2 was the most frequent type (60/109, 55%), followed by 3-4-6-2 (49/109, 45%). There was no association between MLVA types and macrolide resistance (p >0.05). Conclusion The percentage of macrolide resistance M. pneumoniae was high (96%) in pediatric patients in 2020 in Henan, and A2063G was the dominant point mutation. MLVA types was independent to macrolide resistance.

Introduction

Mycoplasma pneumoniae is one of the most common causes of acute respiratory tract infection(ARTI) and community-acquired pneumonia(CAP) especially in children. About 20%˜40% of CAP in pediatric department is infected by *M.pneumoniae* .[1, 2] The infection is transmitted through close contact with infected patients, which may leads to epidemics in family and community. Global *M.pneumoniae*epidemics occur every 3 to 7 years with various incidence rates. Studies show that an epidemic has been spreading in many countries since 2010.[3-6] Upper and lower respiratory tract infections are often mild and self-limited; however, sometimes occasional complications in other organs may develop to death.

M. pneumoniae naturally lacks a cell wall as the action position, it is resistance to β-lactam antibiotics such as penicillin and cephalosporin. Macrolides are usually the first pharmaceuticals to treat children with CAP caused by *M. pneumonia* . However, long time excessive use of macrolides provided condition for macrolide-resistant*M. pneumoniae* (MRMP) strains in the world. Since 2000,the first case of MRMP strains was reported , MRMP has spread rapidly worldwide, especially in East Asia.[7, 8]It was reported that the resistant rates reach to about 90% in China and Japan. Several point mutations on the 23s RNA account for the macrolide-resistant of *M. pneumoniae* . The most common mutation sites were A2063 andA2064 which usually induce high-level macrolide-resistant, the mutation site at C2617 was related to low-level macrolide-resistant.[7]

For a long time, the PCR product length analysis of the P1 gene has been the most common molecular typing method. However, this method can only classified

M. pneumonia strains into two groups. In 2009, D'egrange et al. published a new method—multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA). This method remarkably increases the typing groups for *M. pneumoniae*and provides a new powerful tool for the epidemicology of *M. pneumoniae* infections. [9]In 2011,Dumke et al. developed the MLVA method.[10] The original project was based on VNTR at five loci on the 23s RNA. One loci named Mpn1 was finally excluded because of the instability problem in recent proposal project. [10-12]

The aim of this study is to explore the relation between MLVA types and macrolide-resistant of *M. pneumonia* in children in Henan province, which might provide some interesting information for studying the emergence of MRMP in Henan province and other regions.

Method

Sample collection

This study was proceeded from January, 2020 to December, 2020 in Henan province of China. Samples were collected from Henan Children's Hospital, Zhengzhou, China. A total of 1237 samples were collected from patients 14 years old or younger preliminarily confirmed with CAP on the clinician's decision. The exclusion criteria were as follows: (a) few cells in the specimen or insufficient amount of nucleic acid, (b) respiratory tract infection with a precise etiological diagnosis. This study was approved by the Ethics Committee of the Zhengzhou Children's Hospital (Approval number:2021-K-073). Informed consent was taken from parents or guardians.

Total nucleic acid extraction and detection of*M.pneumoniae*

Total nucleic acids were extracted using a throat swab extraction kit (Health Gene Technologies, China) according to the manufacturer's instruction.*M. pneumonia* was detected by SAT-MP kit (Shanghai Rendu Biotechnology Co, Ltd). Primers were targeted to 16s rRNA:MP-1(5'AATTTAATACGACTCACTATAGGGAGACACCGC

TCCACATGAAATTCCAAAACTCCC3') and MP-2(5'CGGTAATACATAGGTCGCAAGC3').The probe is FAM-5'CGGACUAUUAAUCUAGAGUGUGUCCG3'-DABCYL. The detection limit of this assay was 1000 DNA copies/ml regarding the respiratory specimens.

Detection of the macrolide resistance

If the specimen is positive for *M. pneumoniae* , macrolide resistance was detected by amplification of the 1997-2707 nucleotides position of 23S rRNA, followed by DNA sequencing for mutation loci analysis (A2063 G/C, A2064 G/C,C2617 G/A).

MLVA typing

MLVA typing was completed based on a culture-independent method by Chao Yan et al. with slight modifications.[13] Briefly, PCR was done using primers targeting the four loci containing tandem repeat sequence (Table 1). The PCR was completed on a Verity thermal cycler (Thermo Fisher Scientific Inc) following this methodology: step 1, 94°C for 10 min; step 2, 94°C for 1min, 58°C for 1min, and 72°C for 1min and 30 s. The second step was iterated for 35 cycles. The label PCR products were separated by capillary electrophoresis using an 3500DX Genetic Analyzer platform (Thermo Fisher Scientific, USA),and the data were analyzed using GeneMapper software (version 5.0;Applied Biosystems).

Statistical analysis

SPSS (version 22th)was used for statistical analysis. Independence test was used for the relationship of MLVA types and macrolide-resistant mutation. A p value of <0.05 was considered significant.

Results

A total of 1237 specimens from children with CAP in the year of 2020 were tested. We detected *M. pneumoniae* by real-time qPCR in 121 (9.8%) of all specimens. Fig. 1 shows the testing results in different months

of the year. The number of *M. pneumoniae* -positive specimens fluctuated from 1 to 49 in each month. Of all*M.pneumoniae* -positive strains,116 (96%) were macrolide resistant by detecting point-mutations in 23S rRNA. All of 23S rRNA mutations were A2063G, one of them was heterozygous mutation.

All of positive specimens, including 116 macrolide resistant *M. pneumoniae* (MRMP) and 5 macrolide-susceptible *M. pneumoniae*(MSMP), were further analyzed by MLVA typing. However, there was 11 MRMP and 1 MSMP samples unable to fulfill MLVA typing, and it was probably because the levels of *M.pneumoniae* nucleic acid were too low. Finally they were excluded in analysis.

As a result, the majority (60/105, 57%) of strains in the MRMP group could be matched to MLVA type 4-5-7-2 by the 4-loci typing scheme,45 strains in the MRMP group were 3-4-6-2. On the other hand, all of strains in the MSMP group were 3-4-6-2(Table 2). The independence test showed that MLVA types had no relationship with macrolide-resistant mutation (P>0.05).The distribution of MLVA types among MRMP and MSMP is showed in Fig 2.

Discussion

MLVA type 4-5-7-2 was a dominant stain of *M. pneumoniae* in many countries in Asia, Europe, America .[14-17] Our results showed a similar result in Henan province of China. The relationship between MLVA types and macrolide-resistant mutation was also shown in our study. MLVA types have no relationship with macrolide-resistant mutation.

The relationship between MLVA types and macrolide resistance mutation have been reported before. In 2015, Xue et al. reported that increasing MRMP in China was linked to the increase of *M. pneumoniae* strains typed 4-5-7-2,[18] and the similar finding was also reported in Hongkong and Japan.[19, 20] However, MLVA type 4-5-7-2 is not always related to macrolide-resistance mutation. For example, 4-5-7-2 was also the predominant type in the United States of America, European, Australia, and Thailand,[21-24] where the prevalence rate of MRMP was low. The percentage of MRMP among MLVA typed 4-5-7-2 may change with time. In Japan, the percentage of A2063G mutation in M. pneumonia strains typed 4-5-7-2 was only 0.9% between 2004 and 2010, but increased rapidly to 83.8% between 2011 and 2014.[19]

In this study, the A2063G mutation was the primary mutation, accounting for 100%, no other mutations such as 2064 or 2617 were found. Beside this, our study found mixed infection of wild strain and mutation strain in one sample. Several mixed infections were reported as case reports before.[25-27] In these reports, patients were first infected by the macrolide-sensitive *M. pneumoniae* . After a period of treatment (usually longer than a week), the macrolide-resistance mutation on 23s rRNA was found. However, whether these patients had received macrolide antibiotics before specimen collection was difficult to confirm. So, these patients might have developed macrolide resistance during treatment, or mixed strains might have infected them before specimen collection. More research is needed to do in this area.

Macrolides were the first line pharmaceutics for children with *M. pneumoniae* infection. Some mutations(A2063 and A2064)are associated with high-level resistance to macrolides,[7] widespread use of macrolides for *M. pneumoniae* with these mutations is not only useless but also dangerous to induce more MRMP. Delayed usage of appropriate antibiotics against MRMP is responsible for poor clinical response and prolonged clinical course.[28]As a result, severe CAP caused by MRMP will increase. To break this vicious circle, reasonable usage of antibody is important not only in treating the patient but also in preventing further increase of MRMP.

There was a limitation in our study. All samples were from one center in one year. The results of this study should be interpreted cautiously due to possible selection bias.

In conclusion, this is the first study about MLVA profile and its correlation with macrolide resistance in pediatric patients in Henan province. In 2020, *M. pneumoniae* was responsible for 9.8% pediatric patients with CAP. Macrolide resistance genes was detected in 96% strains in Henan province in 2020. All the MRMP strains carried A2063G mutation (100%). The most common type was 4-5-7-2 ,followed by 3-4-6-2. MRMP was independent to MLVA types.

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Declarations of competing interest None.

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