Evaluating Laboratory Diagnoses of Children’s Mycoplasma Pneumoniae Infection Reveal Effective Clinical Approaches

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Research article
Abstract

This study aims to evaluate laboratory methods for the diagnosis of Mycoplasma pneumoniae. Ninety-three children infected with Mycoplasma pneumoniae were tested, and large sample size makes the results representative. The pharyngeal swabs and serum samples were mostly collected from the first week of the disease, or from the second to third week of the disease in recovery children. The pharyngeal swabs from healthy children were cultured and two of them were positive for the bacterium. The accuracy of laboratory diagnostic methods for Mycoplasma pneumoniae were evaluated against serum test with 4-fold increase or decrease in the titers of antibody in two serum samples. The specificity of culture method was high, which suggested that the method was suitable for clinical research. In acute phase, the sensitivities of single serological test or simultaneous amplification and testing of Mycoplasma pneumoniae (SAT) were 71.7% and 85%, respectively.

Introduction

*Mycoplasma pneumoniae* (MP) is an important pathogen of community acquired pneumonia (CAP) in children. The incidence of MP accounts for about 10–40% of CAP. The severity of the disease varies greatly and may develop into severe and refractory MP that endangers patient's life or leads to serious sequelae. It is believed that early diagnosis and timely as well as effective treatment are the key to reduce severe infections. The clinical symptoms and signs of MP infection are often unspecific, and laboratory diagnosis is becoming increasingly crucial.

At present, the commonly used diagnostic methods in laboratory include bacterial culture method, serological diagnosis, and molecular diagnosis. However, these methods have not been extensively verified for their clinical values. The sensitivity of bacterial culture method is low (60%, 4) and the culture needs special culture medium as well as other specific laboratory conditions. The method requires a long growth cycle, which is not suitable for rapid clinical diagnosis. However, the method has high specificity, which is employed in either academic or clinical researches. Molecular diagnosis method is sensitive and fast, which is less affected by the immune status of the body. It is specifically essential for infants and is a preferable laboratory diagnosis method. Evaluation shows that the fluorescence quantitative PCR method developed in our laboratory is more rapid and accurate than the nested PCR as well as PCR with commercial kits. Using dye method, the specificity of this method is the same as the probe method of commercial kits, but it is more cost-effective and more sensitive than other alternatives. RNA real-time fluorescence thermostatic amplification technology (RNA-SAT) diagnoses the infection by detecting MP RNA, which is a better indicator of the pathogen presence. Wang et al found that SAT and PCR have higher sensitivity than serology test, which are consistent with fluorescence quantitative PCR in the sensitivity and coincidence rate. MP carriers have been shown to be 0.1% - 56% in healthy people. The specificity of molecular biology diagnosis method needs to be further evaluated somehow.

Serological test is a commonly used method for the MP infection diagnosis. Four-fold increase or decrease in the titer of MP specific antibodies in double serum samples is recognized as the standard for
MP infection\textsuperscript{10,11,12}. Talkington et al. found that positive rates were 14\% to 45\% using 8 commercial kits, and 39\% to 88\% when combined with test of two serum samples\textsuperscript{13}. However, this approach requests to collect blood samples at a 2 week interval, which therefore makes it less practically suitable for rapid clinical diagnosis. Furthermore, the collection rate of the two serum samples is low and may be less than 10\% based on report from Taiwan\textsuperscript{14}.

In this study, we collected pharyngeal swab samples from healthy children and serum samples from hospitalized children, and analyzed the samples for MP infection using different methods. The results were used to evaluate the diagnosis values of these methods and provided recommendations for efficient laboratory MP diagnosis methods.

**Materials And Methods**

**Participants**

349 healthy children from Chaoyang kindergartens, Beijing, China were enrolled as possible MP carriers. The inclusion criteria were (1) no symptoms of respiratory tract infections such as fever and cough within one month; (2) normal height and weight with normally developed facial organs, hearing, version and month; and (3) no immune deficiency and other systemic diseases. Ethics committee of Beijing Friendship Hospital approved this study.

93 MP children that hospitalized at Shengjing Hospital, Shenyang, China between 2016 and 2017 were included. To date, and to our knowledge, this is the largest MP infection target sample size so far. They were clinically diagnosed to have MP infection with symptoms such as fever (uncertain fever type or no fever), cough (persistent severe cough as the main manifestation, no or less phlegm), or with dyspnea and wheezing. They had rough pulmonary auscultation or dry and wet rales, who were positive for one of the three tests: serum MP-IgM antibody test, MP-PCR, or MP culture. They also had significant pulmonary X-ray signs, such as enhanced image of hilum of lung, bronchitis, interstitial pneumonia, homogeneous consolidation, atelectasis\textsuperscript{15}. Patients were excluded if they had one of the following symptoms: multiple system and organ damage; severe liver, kidney, cardiovascular and hematopoietic diseases, immune diseases and psychosis.

**Sample collection**

Pharyngeal swab samples were collected from the healthy and MP children. Double blood samples were collected at acute and recovery phase of the MP children.

**MP culture**
The bacteria were inoculated in pleuropneumonia-like organism (PPLO) culture medium, and were incubated in incubator. The indicator color change was used to judge the MP growth\(^\text{16}\). The reference strain was FH international standard strain (ATCC15531), which was purchased from American Type Culture Collection, Virginia, USA.

**Fluorescence quantitative PCR**

Genomic DNA was extracted using a universal columnar genome extraction kit (CW2298, Century Biotechnology, Jiangsu, China) following the supplier’s instructions. MP DNA was detected according to previously study.

**Isothermal amplification of RNA and real-time fluorescence detection**

Isothermal amplification of RNA was carried out using MP RNA detection kit (RNA-SAT, YZB/Guo3229–2013, Rendu Biotechnology, Shanghai) according to supplier's instructions.

**ELISA**

Detection of MP-specific IgM antibody was performed using Serion ELISA classic for MP IgM antibody quantitation kit (cat. no. SCG.AO, Virion/ Serion) according to the standard process.

**Serological test**

Passive agglutination assay was used to measure the titer of MP-specific serum antibodies via SERODIA MYCO II (cat no. YZB/JAP8033–2013, Fuji Rebi, Japan) following the manufacturer's instructions. Four-fold increase or decrease in the titer was used as a diagnosis cut-off\(^\text{9,10,11}\).

**Statistical analysis**

The measurement data were expressed as means ± standard error, which were analyzed using SPSS15.0 statistical software. Data in percentage were compared using Chi-square or Fisher test. \(P<0.05\) was considered to be statistically significant.

**Results**

**MP infection in asymptomatic carriers**

Fluorescence quantitative PCR (RT-PCR) and bacterial culture showed that out of 349 samples that tested, 66 (18.9\%) and 2 (0.06\%) samples were positive, respectively.
MP infection in diseased patients

Serological test showed that out of 42 (45.2%) boys and 51 (54.5%) girls (aged 3–14 years), 59.5% and 68.6% of them were MP positive, respectively. The infection rates were similar between genders. When the results were analyzed based on age (under 3 years, between 3 and 5 years and older than 5 years), no difference in the infection rates were found between different age groups (p = 0.612, 0.444 and 0.913, respectively), and the infection rates were 23.08%, 60.98% and 82.05%, respectively (Figure 1a). The difference of infection rates between the three groups was statistically significant (p < 0.05, P = 0.017, P = 0.037) by Chi-square or Fisher test, suggesting that the infection rate of MP increases with age (Table 1).

60 (64.5%), 39 (41.9%), 68 (73%) and 59 (63.4%) children were diagnosed to have MP infection based on the Serological test, bacterial culture, RT-PCR and SAT, respectively. Compared with the gold standard, the sensitivity and specificity of RT-PCR were the highest (93.3%) and low (63.6%), respectively, suggesting a higher rate of false positive. The specificity and sensitivity of bacterial culture were the highest (100%) and low (65%), respectively. Since the culture period was about 14–21 days or longer, the method could effectively help improve early diagnosis precision. SAT had high sensitivity and specificity (85% and 75.8%, respectively). The combined diagnosis of single acute serum serological test with RT-PCR generated a sensitivity of 100% with the unchanged specificity. The combined diagnosis of single acute serum serological test with SAT increased the sensitivity to 95% with unchanged specificity (Figure 1b). This method had the highest coincidence rate, Yoden index and Kappa value. The ROC curves of various methods compared with gold standard are shown in Figure 2, and the sensitivity and specificity are shown in Table 2 and Figure 1c. These data suggest that combined SAT with single acute serum serological test is the most suitable diagnostic method.

Discussion

MP is an important pathogen of community-acquired pneumonia in children. Its incidence is high and clinical manifestations are not specific. Laboratory diagnosis has become an important complementary approach to determine the pathogens for medication. Diagnostic methods that provide early, rapid and accurate results are highly demanded though they are hardly available. This study evaluates several commonly used laboratory methods in order to provide guidance for clinical diagnosis.

Bacterial culture is highly specific, which is used as the golden standard for MP diagnosis. In our study, its specificity reaches 100%. However, the sensitivity is relatively low (65%). It is noteworthy that culture of pharyngeal swab samples from 349 healthy children generated two positive samples, suggesting that they might be asymptomatic carriers of MP. Partly due to the amount of bacteria carried and the immune status of the body, the children were not affected. Asymptomatic MP carriers have also been reported previously. For instance, Jiang found that the MP infection might be related to the bacterial load. It is believed that high fever is more likely to occur when the MP load is greater than $10^7$ copies/µl. However, the relationship between low MP load and pathogenesis is not clear. In present study, the growth cycle of MP was 14 to 21 days, which is too long to meet the needs of early and rapid clinical diagnosis. Report
showed that adding catalase to culture medium could accelerate MP growth \(^{18}\), but the results still need to be further verified.

Serological methods are widely used in clinic. However, there is still controversy about the diagnosis value based on single serum sample from acute phase. It was reported that the sensitivity of single serum sample from acute phase is 32\%\--35\% \(^{19}\). In our study, the sensitivity reached 71.7\%. This might be because the serum samples were taken from hospitalized children at about one week of disease. In addition, Miyashita et al. assayed serum samples from 200 healthy adults using ELISA and found 61 were positive \(^{20}\), Csango et al. tested serum samples from 102 healthy volunteers using four commercial kits, IgM and IgA were positive \(^{21}\). Previous infections could also produce false positive. Therefore, positive serum result needs to be confirmed based on clinical data. Lin et al. found that IgA in acute serum is of significance for the diagnosis of diseases. The combination of IgM and IgA can improve the sensitivity of detection and can be used as a choice for clinical diagnosis \(^{22}\). However, Nrita et al found that IgA is less useful for the diagnosis of MP \(^{23}\). Experts in China agree that MP infection may be diagnosed if the titer of MP specific antibody is > 1:160 in single serum sample \(^{24}\). According to the ROC curve drawn in this study, the threshold value is slightly higher than the best diagnostic point and thus may result in missed diagnosis. Considering the number of samples used in this study, larger study is needed to confirm the conclusion.

Molecular methods are rapid and sensitive. The sensitivity of RT-PCR protocol developed in our laboratory is 93.3\%, but the specificity is low. The reasons might be due to asymptotic MP carriers. The detection rate of MP in swab samples of healthy children was 18.9\% and were identified as MP by sequencing. It is reported that the carrying rate of MP in the respiratory tract of healthy people is 0.1\%\--56\%. In addition, MP DNA can be detected continuously for 7 weeks to 7 months after infection \(^{25}\), although the detection of DNA cannot be diagnosed as MP infection. Li et al showed that SAT has high sensitivity and specificity in detecting MP infection, and has a high coincidence rate with RT-PCR (Kappa = 0.97) \(^{26}\). In this study, the sensitivity of SAT was slightly lower than RT-PCR, probably because RNA is easier to degrade than DNA, and is more susceptible to the influence of sampling time and storage methods. In this study, samples were collected from children hospitalized for about a week, and most of them had already be treated with antibiotics. These may affect the detection rate of MP. In this study, the specificity of RNA is higher than that of DNA, which may be related to the fact that RNA can better reflect the presence of pathogens than DNA. However, total RNA, not messenger RNA were detected by the kit used. Therefore, the results cannot be used to distinguish between asymptomatic infection and healthy carriers. Based on our data, it is clear that at early disease phase, molecular detection is sensitive, rapid and suitable for the clinical needs. However, due to asymptomatic carrier of MP, it may lead to over diagnosis if it is solely based on molecular method. MP infection still needs to be confirmed with clinical data.

Joint diagnosis of serological and molecular methods for detection of MP infection is highly recommended \(^{27,28}\). Our RT-PCR protocol has a high sensitivity for detecting MP. Once combining with serological method, the sensitivity was further improved (up to 100\%), while the specificity remained
unchanged, leading to less missed diagnosis. However, this approach cannot solve the problem of low specificity caused by asymptomatic carrying. In the future, we will continue to investigate the relationship between DNA load and pathogenesis in order to address this problem. The sensitivity of detecting MP RNA in acute phase single serum samples combined with SAT was greatly improved (up to 95%). Because RNA is easy to degrade, its specificity is greater than DNA in MP detection, the accuracy of diagnosis is better than that of a single method. This combined method is the best diagnostic method tested in this study.

**Conclusion**

The current laboratory testing methods have their own limitations, their results should be verified based on clinical data for MP diagnose. In our study, the sensitivity of bacterial culture which required 14–21 days to complete is 65%, therefore, it is not appropriate for rapid clinical diagnosis. In the future, it is important to investigate how to improve the sensitivity and shorten the culture time. Molecular biology diagnostic methods have high sensitivity (93.3%, 85%). Because of the asymptomatic carrying of MP, the specificity is relatively low (63%, 75%). The issue may be solved by optimizing the relationship between MP load and pathogenesis. The sensitivity of diagnosis based on single acute stage serum sample is high and may be used as reference for clinical diagnosis. Defining the optimal titer threshold in single acute stage serum sample may help improve the sensitivity of this method. When using single serum test, positive results should be checked against previous infection, the immune situation of children, blood collection time and use of antibiotics to rule out false positive. Taken together, our results suggest that joint diagnosis of single acute stage serum sample and molecular method can improve the accuracy and is recommended to use in connection with clinical data for MP detection.

**Declarations**

**Abbreviations**

SAT: simultaneous amplification and testing of *Mycoplasma pneumoniae*

MP: *Mycoplasma pneumonia*

CAP: community acquired pneumonia

RNA-SAT: RNA real-time fluorescence thermostatic amplification technology

PPLO: pleuropneumonia-like organism

AUC: Area under the curve

**Ethics approval and consent to participate**
Ethics committee of Beijing Friendship Hospital approved this study. Patients or their parents signed the informed consent.

**Consent for publication**

Compliance with Ethical Standards.

**Availability of data and material**

Data would be available upon requests.

**Competing interests**

None declared.

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**Authors’ contributions**

Liangyu Wang, Xiaohua Han, Ran Wei, Lina Han, Dongxing Guo, Xijie Liu, Jingyi Li, Haiwei Dou, Zhaoyong Wu, Dan Li, Xiujun Tian, and Shaogang Li performed the analyses, wrote the manuscript and participated in study design. Deli Xin and Kunling Shen conceived and supervised the study and wrote the manuscript.

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Tables

Due to technical limitations, Tables 1 - 3 are only available for download from the Supplementary Files section.

Figures
Figure 1a. Prevalence of patients across all the ages.

Figure 1b. Comparison of molecular biological detection method and the method combined with serological detections.

Figure 1c. Sensitivity and specificity comparisons for four detection methods.

Figure 1

a. Prevalence of patients across all the ages. b. Comparison of molecular biological detection method and the method combined with serological detections. c. Sensitivity and specificity comparisons for four detection methods.
Figure 2

ROC curves of various methods compared with gold standard. Numbers in the brackets denote the AUC values.

Supplementary Files

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