Author's response to reviews

Title: Accuracy of IgM antibody testing, FQ-PCR and culture in laboratory diagnosis of acute infection by Mycoplasma pneumoniae in adults and adolescents with community acquired pneumonia

Authors:

Jiuxin Qu (qujiuxin@163.com)
Li Gu (guliangel@yahoo.com.cn)
Jiang Wu (wj2102@sohu.com)
Jianping Dong (djp1970111@sina.com)
Zenghui Pu (puzenghui80055@163.com)
Yan Gao (gaoyan6384@163.com)
Ming Hu (wadmq@sina.com)
Yongxiang Zhang (zyx1915@yahoo.com.cn)
Feng Gao (nswj@sohu.com)
Bin Cao (caobin1999@gmail.com)
Chen Wang (cyh-birm@263.net)

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Author's response to reviews: see over
Dear the editor:

Enclosed, please find the revised version of our manuscript entitled "Accuracy of IgM antibody testing, FQ-PCR and culture in laboratory diagnosis of acute infection by Mycoplasma pneumoniae in adults and adolescents with community acquired pneumonia". In your letter, you encouraged us to revise our manuscript and address the issues raised by the reviewers.

We have carefully revised the manuscript according to the reviewer’s queries and a point-by-point reply to their comments is provided below.

We are very grateful to the reviewers for the time they spent reviewing the manuscript and to Editorial Team for reconsidering this work for publication in “BMC Infectious Diseases”.

Yours faithfully,

Bin Cao, M.D.

Point-by-point response to reviewer’s comments:

Reviewer 1:

This paper compares different methods for the diagnosis of Mycoplasma pneumoniae. In this article, the question is well defined and title and abstract are adequate. Methods are appropriate but description of some of them is too concise. Results are clearly exposed. Some of them are unexpected that could be explained by the limited sample. This limitation is commented by the authors themselves. Discussion and conclusions are appropriate and supported by the data. The writing is acceptable.

Discretionary revisions:

Considering that in the article diagnostic techniques are compared, it is recommended that authors describe in more detail the PCR kit used, eg primers and thermocycler program applied.

Answer: Thank you for your nice suggestions. In the revised version, more detailed information of PCR kit, eg: target genes and thermocycler program has
been added in “methods”. Because primers are commercially confidential, we have no more data.

It would be very interesting to compare the results of culture compared to PCR and analyze agreement between three techniques (IF, culture and PCR).

Answer: Thank you for your nice suggestions. In the revised version, we described the discordant results among evaluated methods, and analyzed the possible reasons in discussion as followings.

In the Results section, we added data as following:

Among the discordant results, 8 patients showed fourfold or greater increase in IgG titer, but had negative results in IgM testing, PCR or culture methods. 15 patients were negative by paired sera while positive by other methods. Among them, 4 were positive by both FQ-PCR and culture, 1 was positive by FQ-PCR and IgM test, 1 was positive by culture and IgM test, 6 were positive by FQ-PCR, and 3 were positive by IgM test.

In the Discussion section, we tried to explain the discordance between different methods:

The possible reasons contributing to discordant results between FQ-PCR or culture and IgG serology have been explored. 4 patients (1 positive by FQ-PCR and culture, and 3 positive by FQ-PCR) may represent asymptomatic carriage of *M. pneumoniae* is a result of persistence from a previous infection, since whose CAP etiology has been identified as *influenza virus A*, human *metapneumovirus*, human *rhinovirus* and *adenovirus* respectively. 5 patients (3 positive by FQ-PCR and culture, and 2 positive by FQ-PCR or culture and IgM test) displayed 2.5-3.8 fold increase of IgG response, which could be explained by inadequate time interval between acute and convalescent sera, as the mean interval was 17 days. The remaining 3 patients (only positive by FQ-PCR) were 69, 73 and 74 years old, who failed to develop IgG antibody response in paired sera, which could be explained by a deterioration of the immune response due to ageing. These cases highlight the limitations of a serological diagnosis, since which could be affected by the timing of specimen collection and the age of the patient.
A low bacterial load resulting from previous antimicrobial treatment or dilution of the sample in the throat swabs below the limit of detection could explain the negative results of PCR and culture. The detection limit of FQ-PCR kit is stated as 10 copies per µl in the manual, lower than 0.1-1 copies per µl of other commercial kits as reported. Besides, it is found that the efficiency of PCR assay could be influenced by sample type, and sputum could be superior than other respiratory samples included throat swab.

Minor Essential Revisions:
In third paragraph of discussion, it should be corrected last name of the author (Martínez and not Maria).
Answer: Thank you for your nice suggestions. In the revised version, we have made correction.

Reviewer 2:
The manuscript of Qu JX et al. describes the diagnostic values of IgM serology, FQ-PCR and culture for early diagnosis of community-acquired *M. pneumoniae* pneumonia in adults and adolescents, with a fourfold or greater increase of IgG antibody titers of paired sera as the “gold standard”. The authors found that specificity and negative predictive values of all three methods were high, around 90% in specificity and around 80% in NPV, but sensitivity was low.

Major comments:
1. The authors found that IgM serology is not suitable for the diagnosis of *M. pneumoniae* pneumonia and FQ-PCR and culture are still needed to be improved. Because of the low sensitivity and non-rapid diagnostic methods, many researchers are using the clinical diagnostic methods. Recently, from China, Yin Y-D et al. demonstrated the good sensitivity and specificity of scoring system for the early presumptive diagnosis of *M. pneumoniae* pneumonia (Respirology 2012; 17: 1131-1136). Thus, the authors should include and discuss the clinical diagnosis.
Answer: Thank you for your nice suggestions. In our revised manuscript, we have added the data of clinical scoring system. In our cohort, we proved that with a fourfold or greater increase of IgG antibody titers of paired sera as the “gold standard”, the sensitivity and specificity was 63% and 59.2%. The sensitivity decreased to 61.9% and specificity increased to 74.7% when use either positivity by PCR, culture, IgM, or fourfold increase by IgG as the diagnosis for *M. pneumoniae* pneumonia.

2. How many patients determined the causative pathogens in patients without *M. pneumoniae* pneumonia. The authors should enroll and compare the pneumonia patients who were determined causative pathogens.

Answer: Thank you for your comments. Except for patients with *M. pneumoniae*, there were 23 patients had causative pathogens. Among these patients, 1 was positive by *parainfluenza virus* type 1, type 2 and *influenza virus* A; 1 was positive by *influenza virus* A and *Pseudomonas aeruginosa*; 1 was positive by *influenza virus* A and *Klebsiella pneumoniae*; 9 were positive by *influenza virus* A; 3 were positive by *adenovirus*; 3 were positive by *parainfluenza virus* type 1; 1 was positive by human *metapneumovirus*; 1 was positive by human *rhinovirus*; 1 was positive by *parainfluenza virus* type 2; 1 was positive by *parainfluenza virus* type 3.


Answer: Thank you for your comments. Indeed, the prevalence of macrolide-resistant *M. pneumoniae* is 69% among adults in China, the highest all
over the world. And an increase rate of macrolide-resistant *M. pneumoniae* has been observed in our study.

We do agree with you that it is necessary to determine the *M. pneumoniae* as the causative pathogen in community-acquired pneumonia in both adults and children. When talked to diagnosis of macrolide-resistant *M. pneumoniae*, minimum inhibitory concentrations (MICs) assay is the traditional method and gold standard. Recently, there are several reports on the molecular diagnostic method based on the association between macrolide-resistance and gene mutation in 23S rRNA gene, including real-time PCR, high-resolution melt analysis (Bernard J, et al. Antimicrobial Agents Chemother 2008, 52: 3542–3549), and allele-specific-PCR (LI Shao-li, et al. *Chinese Medical Journal* 2012;125:2671-2676).

4. The included number of patients characterized as *M. pneumoniae* pneumonia are low to clarify the conclusions. The authors should enroll more patients.

**Answer:** Thank you for your comments. Low number of patients is one of our limitations. This study is part of a 1000 case cohort which we prospectively collected between 2010 till 2012. It is very difficult to get a convalescent serum 2-4 weeks apart since most patients recovered within a week and would not like to come back. To our knowledge, the number of patients in our study is larger than other relevant reports. For example: (1) Martínez et al. (*Journal of medical microbiology* 2008, 57:1491-1495) conducted a study on adult CAP patients, and 21 out of 27 patients have paired sera. (2) Thacker WLet al (*ClinDiagn Lab Immunol* 2000, 7:778-780) reported 64 adults suspected outbreaks of respiratory infections with paired sera. (3) Nilsson AC et al (*BMC microbiology* 2008, 8:93) enrolled 164 patients aged 2 to 82 years old, with acute respiratory symptoms, there were 96 patients have paired sera.

5. The authors should shortened the Discussion section.

**Answer:** Thank you for the suggestions. In the revised version, the discussion has been edited to make it more concise.

Minor comments:
6. As the authors noted in the manuscript, it is well known that IgM antibody usually does not appear within 7 days. Please include the days after onset at the IgM antibody testing in Table 1.

Answer: Thank you for the comments. In the revised version, we have added the days after onset at the IgM testing in Table 1. Moreover, in the manuscript, we stated that “after infection by M. pneumoniae, IgM antibodies appear during the first week of the illness, and reach peak titers during the third week”, and “in some adult patients, IgM antibodies are constantly negative or produced 15 days after the onset, most likely as a result of multiple previous infections”.

Reviewer 3:

Here is the review of Manuscript Titled ‘Accuracy of IgM antibody testing, FQ-PCR and culture in diagnosis of acute infection by Mycoplasma pneumoniae in adults and adolescents with community-acquired pneumonia’:

Major Concern:

This manuscript collected 125 CAP patients’ paired sera to perform prospectivestudy, to compare the three ways for evaluating acute infection of Mycoplasma pneumoniae. However, the scientific significance of this investigation lacks enough novelty, which is known before that culture is the best among the three measurement methods for MP acute infection.

Answer: Thank you for your comments. Several similar studies have been previously published examining the differences between serology and/or culture and/or molecular detection for this organism but this is the first to describe the use of the FQ-PCR test.

And there was disagreement on the use of culture for the diagnosis of Mycoplasma pneumonia. She et al found that culture was unacceptably insensitive and had an extremely low yield in M. pneumoniae infection diagnosis [JCM She, 2010]. However, in our study, we have revealed that culture had a good agreement between positive rate of MP cultivation of throat swabs and
acute *M. pneumoniae* infection (positive likelihood ratio is 10.9), which is different from previous reports.

Moreover, the patient amount is small, not enough to get this conclusion.

Answer: Thank you for your comments. Low number of patients is one of our limitations. This study is part of a 1000 case cohort which we prospectively collected between 2010 till 2012. It is very difficult to get a convalescent serum 2-4 weeks apart since most patients recovered within a week and would not like to come back. To our knowledge, the number of patients in our study is larger than other relevant reports. For example: (1) Martínez et al. (*Journal of medical microbiology* 2008, 57:1491-1495) conducted a study on adult CAP patients, and 21 out of 27 patients have paired sera. (2) Thacker WL et al (*ClinDiagn Lab Immunol* 2000, 7:778-780) reported 64 adults suspected outbreaks of respiratory infections with paired sera. (3) Nilsson AC et al (*BMC microbiology* 2008, 8:93) enrolled 164 patients aged 2 to 82 years old, with acute respiratory symptoms, there were 96 patients have paired sera. Furthermore, we believe that our data are solid, and conclusion is reasonable.

The relationship and time lasting between the antibody of IgG and IgM for MP is not explained clearly.

Answer: Thank you for your comment. It is known that after infection by *M. pneumoniae*, IgM antibodies appear during the first week of the illness, and reach peak titers during the third week. After peak titers, IgM antibodies decrease rapidly. The level of specific IgG antibodies increases slowly in the course of the illness, reaching peak titers 5 weeks after the onset, and the high seroprevalence of IgG antibodies that persist for long periods in persons with a history of *M. pneumoniae* infection. Thus, the detection of IgM in the acute serum can be used as the early serologic diagnosis, and a fourfold or greater increase of IgG antibodies in paired sera with time interval of 2-4 weeks indicates a current infection.
Minor Concern:

1. The quality of English writing of this manuscript need improved, especially in the abstract.

Answer: Thank you for your nice suggestions. In the revised version, we have carefully modified our manuscript, especially for the abstract.

2. The title is not accurate and suit for the study.

Answer: Thank you for your comment. In our revised version, the title has been modified as “Accuracy of IgM antibody testing, FQ-PCR and culture in laboratory diagnosis of acute infection by *Mycoplasma pneumoniae* in adults and adolescents with community acquired pneumonia”.

Reviewer 4:

The authors describe a study comparing the performance of IgM, culture and FQ-PCR (“fluorescence quantitative” PCR) for *M. pneumoniae* diagnosis. Several similar studies have been previously published examining the differences between serology and/or culture and/or molecular detection for this organism but this is the first to describe the use of the FQ-PCR test. However, it is not clear to what extent this FQ-PCR commercial test differs from real-time PCR and in what way it is quantitative as the results are expressed only qualitatively.

Answer: Thank you for the comments and questions. The FQ-PCR kit is real-time PCR based. The gene target of the kit 16S rRNA genes, and the results are expressed as qualitative. In our revised version, more detailed information of PCR kit, eg target gene and thermocycler program has been added in the “methods”.

Comparison is made between the three test methods and the “gold-standard” of a 4-fold titre rise in IgG between acute and convalescent sera, however
nosatisfactory evidence or explanation is given as to why this is the gold standard. Contrary to what is stated in the introduction, Mandell et al 2007 and Waites and Talkington 2004 do not actually say or infer that the use of IgG in this way is “the most reliable method”.

Answer: Thank you for the comments and questions. Actually, Mandell et al 2007 and Waites and Talkington 2004 do not point out that paired IgG serology is “the most reliable method”. In another two papers, Thacker et al (ClinDiagn Lab Immunol 2000, 7:778-780) revealed that it is advisable to test simultaneously for both IgM and IgG in paired specimens collected 2 to 3 weeks apart for the most accurate diagnosis of recent or current M. pneumoniae infection, especially in adults. She et al. (Journal of clinical microbiology 2010, 48(9):3380-3382) reported that paired IgG serology results would have offered a more reliable diagnosis of recent infection. Therefore, in the revised version, we have corrected our citations and stated that “paired sera showing a fourfold increase in IgG antibody titer has been considered as more reliable method for the diagnosis of current M. pneumoniae infection” in the introduction section.

The patient population is an adolescent/adult CAP cohort but no results of any other microbiological test are given. This would help in interpreting positive results from the three test methods where the gold-standard is negative.

Answer: Thank you for your comments. Except for patients with M. pneumoniae, there were 23 patients had causative pathogens. Among these patients, 1 was positive by parainfluenza virus type 1, type 2 and influenza virus A; 1 was positive by influenza virus A and Pseudomonas aeruginosa; 1 was positive by influenza virus A and Klebsiellapneumoniae; 9 were positive by influenza virus A; 3 were positive by adenovirus; 3 were positive by parainfluenza virus type 1; 1 was positive by human metapneumovirus; 1 was positive by human rhinovirus; 1 was positive by parainfluenza virus type 2; 1 was positive by parainfluenza virus type 3. In the revised version, we described the discordant results among the evaluated methods, and analyzed the possible reasons in discussion. There is no
specimen positive by all three methods but negative by the gold standard. Third, among the discordant results, there are 4 patients (1 positive by FQ-PCR and culture, and 3 positive by FQ-PCR) have other CAP etiology as Influenza virus A, human metapneumovirus, Adenovirus and human rhinovirus. Thus these cases may represent asymptomatic carriage of M. pneumoniae is a result of persistence from a previous infection.

The discussion needs to be edited to make it more concise and the whole manuscript needs an English language review for grammatical errors.

Answer: Thank you for the suggestions. In the revised version, the manuscript has been edited, especially for discussion section.

Finally, the authors have not always properly referenced statements made in the article, for example, citing review articles rather than going back to the original source, and in some cases citations appear to be incorrect.

Answer: Thank you for the nice suggestions. Incorrect citations may be a result of software errors of “Endnote X4”. In the revised version, all references are cited correctly from original source.

Major compulsory revisions:

What is the nature or principle of the FQ-PCR test – is it real-time PCR-based?

Do the authors know the gene target for the FQ-PCR? Results are expressed as positive or negative, but the test is described as quantitative. FQ-PCR is stated as “more rapid, convenient and sensitive” however the two supporting articles cited (Loens et al 2003, Morozumi et al 2004) do not use FQ-PCR. If the authors mean PCR in general they should clarify this.

Answer: Thank you for the comments and questions. The FQ-PCR kit is real-time PCR based. The gene target of the kit 16S rRNA genes, and the results are expressed as qualitative. In our revised version, more detailed information of PCR kit, eg. target gene and thermocycler program has been added in “methods”. In lines 212-214 of revised version, we clarify that PCR, especially for real-time PCR is “more rapid, practicable and sensitive”.


“False positives” which were negative by the gold standard but positive by one of the three other methods tested are noted in Table 2 but not described in the text. Were any specimens positive by all three methods but negative by the gold standard? Was there another microbiological explanation for CAP? If not, perhaps these are true positives, given that there is really no gold-standard single diagnostic method for *M. pneumoniae*. This should be discussed further in the discussion section.

*Answer: Thank you for the nice suggestions. First, in the revised version, we described the discordant results among the evaluated methods, and analyzed the possible reasons in discussion. Second, there is no specimen positive by all three methods but negative by the gold standard. Third, among the discordant results, there are 4 patients (1 positive by FQ-PCR and culture, and 3 positive by FQ-PCR) have other CAP etiology as *Influenza virus A*, human *metapneumovirus*, *Adenovirus* and human *rhinovirus*. Thus these cases may represent asymptomatic carriage of *M. pneumoniae* is a result of persistence from a previous infection.*

It is unclear if the statement “although *M. pneumoniae* had been found to asymptomatically colonise the respiratory tract” refers to the present study; all 125 patients were symptomatic with CAP.

*Answer: Thank you for the comments. Asymptomatically colonization of *M. pneumoniae* could be found in 4.6%-13.5% of healthy adults (Foy, 1993,*Clin Infect Dis, 17 Suppl 1*, S37-46.), not to our study. The statement may cause misunderstanding. Thus, in the revised version, we have deleted the statement.*

Can the authors state the reason for excluding patients with an onset of more than 7 days?

*Answer: Thank you for the comments. In our study, we tried to understand the diagnosis of acute infection by *M. pneumoniae*. Actually, many patients recovered within a week after onset of illness.*
We also followed the WHO-SARI (Severe acute respiratory infection) definition in which SARI was defined as an acute respiratory illness with onset during the previous 7 days requiring overnight hospitalization that includes: history of fever or measured fever of ≥ 38°C, AND cough, AND shortness of breath or difficulty breathing. (WHO Regional Office for Europe Guidance for Influenza Surveillance in Humans 2009). http://www.euro.who.int/__data/assets/pdf_file/0020/90443/E92738.pdf.


Rapid culture is mentioned in the discussion but the reference cited (Liu et al 2010) does not use this method. Therefore the correct reference should be cited. Furthermore, in the last line of the discussion the authors comment that “rapid culture method could be potentially clinically applicable” but have not actually used it in the present study and do not provide any more information about it. Could they comment further on this or remove the reference to it.

Answer: Thank you for the comments. First, when rapid culture has been discussed, the wrong reference has been cited. In the revised version, we have cited the correct reference (Ma et al 2010 and Li et al 2011). Second, in the revised version, we have removed “rapid culture method could be potentially clinically applicable” in discussion section, since rapid culture has not been evaluated in our study.

Minor essential revisions

Introduction:

Cao et al 2010 is referenced for the finding that 20% CAP in adults is due to M. pneumoniae. However, Cao et al actually describe a 29.3% prevalence and
referin their own paper to a study by Liu et al 2009 in which there was a prevalence of 20.7%. The authors should correct this.

Answer: Thank you for the nice suggestions. In the revised version, “the incidence is from 20-30% in China” has been supported by reference Cao et al 2010 (describe a 29.3% prevalence) and Liu et al 2009 (describe a 20.7% prevalence).

Methods:
The authors use "throat swab" and "throat wash" interchangeably in the manuscript although they are two different specimen types – could they clarify which is the one they used in this study?

Answer: Thank you for the nice suggestions. Specimen type we used in our study is “throat swab”, and all “throat wash” phases have been corrected.

What was the volume used for extraction for FQ-PCR and which Qiagen manual extraction kit was used?

Answer: Thank you for the comments. In the study, 200 µl sample has been used for extraction, and the Cat. No. of Qiagen manual extraction kit is 51306.

The authors cite Cao et al 2010 for the method of confirming culture positives by PCR, however Cao et al cite a method published in a book without any modifications. It would be better to cite the original source in the present manuscript.

Answer: Thank you for the nice suggestions. Culture method used in the study is same as the method published in book (Waites KB, Nolte FS: Laboratory diagnosis of mycoplasmal infections. Washington, D.C.: ASM Press; 2001), and in the revised version, the book has been cited.

Were the U/ml cut-off values for the interpretation of the serology based on manufacturer’s recommendations or on other criteria?

Answer: Thank you for the comments. U/ml cut-off values for the interpretation of the serology are based on manufacturer's recommendations, which has been added in “method” in the revised version.

Results:
What were the co-morbidities noted? These should be described in the methods section.

**Answer:** Thank you for the nice suggestions. Recorded comorbidities in the study include diabetes, heart diseases, cerebral vascular disease, chronic lung and renal disease. In the revised version, the descriptions of comorbidities have been added in “method”.

Discussion

The second sentence in the first paragraph appears to be almost identical to the wording of the abstract from the von Baum et al 2009 paper cited. The essence should be summarised in the author’s own words.

**Answer:** Thank you for the nice suggestions. In the revised version, the sentence has been carefully modified in our own words.

In the second paragraph is a reference to “produced 15 days after the onset” – this is straight out of Martinez et al 2008 as cited but actually refers to other studies described in that paper, rather than Martinez’s own data. Their own data describes some patients with IgG seroconversion but no IgM however there is no analysis of timing in respect of onset.

**Answer:** Thank you for the nice suggestions and corrections. In the revised version, we have cited the correct references as Moule et al 1987 and Jacobs et al 1993.

When referring to a paper, use the author’s surname not forename e.g.

“Martinez et al” not “Maria et al”.

**Answer:** Thank you for your nice suggestions. In the revised version, we have made correction.

Loen et al 2010 is cited as evidence of a high re-infection rate in China, however this review paper quotes only one (paediatric) study with an incidence of 7.1%, therefore I think the authors have cited the wrong reference here.

**Answer:** Thank you for your nice suggestions. In the revised version, we have cited the right references as Liu et al 2009 and Cao et al 2010.
The sensitivities of 5 commercial kits assessed in Touati et al 2009 are actually 61.9-97.6% which rounds up to 62-98% rather than the 61-97% quoted by the authors.

Answer: Thank you for your careful checking. In the revised version, we have made correction.

Touati et al 2009 states detection limits in ng DNA/uL but the authors quote 2-5 copies/uL. The authors should clarify how they have converted this.

Answer: Thank you for your comments. Dumke R et al 2009 reports that 1 M. pneumoniae DNA copy/µl equals with 5.3×10⁻⁶ ng DNA/µl, and Touati et al 2009 describes detection limits from 4.3×10⁻⁶ ng DNA/µl to 4.3×10⁻⁷ ng DNA/µl, which equals with 0.1~1 copy/µl.

The third paragraph of the discussion quotes a study by Ieven and Goosens 1997 giving 61% culture sensitivity, however the original source of this figure is the 1996 paper by Ieven et al cited in Ieven and Goosens 1997.

Answer: Thank you for your careful checking. In the revised version, we have made correction.

Table 1:
ECG abnormality is listed but not explained in the methods section (as for co-morbidities). What definition have the authors used for this? (NB: 25/125 is 20.0% not 19.2% as in the table).

Answer: Thank you for your comments. There is no association between ECG abnormality and M. pneumoniae infection, therefore, in the revised version, we delete ECG in table 1. And, the wrong percentage of 19.2% is a mistake in calculating, we have correct 19.2% with 20.0%.

Similarly no further information is given about antibiotics prior to admission in the methods section although it is in the table; how many days prior to admission was this taken from, and was the class of antibiotic recorded?

Answer: Thank you for your good suggestions. Unfortunately, the length of antibiotics usage before admission has not been recorded. The class of antibiotics
includes β-lactams, macrolides and fluoroquinolones, and which is described in the last but one paragraph of discussion.