Clinical Epidemiology and Global Health 16 (2022) 101084

Contents lists available at ScienceDirect

Clinical Epidemiology and Global Health

journal homepage: www.elsevier.com/locate/cegh

Original article

Evaluation of real-time PCR with serology for diagnosis of community acquired pneumonia caused by *Mycoplasma pneumoniae*

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ARTICLE INFO

Keywords:
CAP
ELISA
IgM
M. pneumoniae
Real-time polymerase chain reaction

ABSTRACT

Objective: To evaluate the utility of real-time polymerase chain reaction (PCR) against serology (IgM) for the diagnosis of community acquired pneumonia (CAP) caused by *Mycoplasma pneumoniae (M. pneumoniae)*.
Methods: Hospitalized children from 1 month to 18 years of age with symptoms and findings consistent with CAP as per World Health Organization (WHO) criteria were enrolled. Mycoplasma IgM serology (Acute phase) was done in all children using enzyme-linked immunosorbent assay (ELISA). Real-time PCR was done using a commercial FDA approved Multiplex Real-time PCR kit for *M. pneumoniae*.
Results: M. pneumoniae was confirmed in 15% (10 out of 66 patients) of the hospitalized children with CAP. Of those with confirmed Mycoplasma infection, 90% (9 out of 10) patients were serology reactive while PCR was positive in only 40% (4 out of 10). Both IgM and PCR were positive in 30% (3 out of 10 patients) and only one real-time PCR positive patient was negative for IgM.
Conclusion: In our study, real-time PCR had a lower sensitivity compared to serology. However, this test has the advantage of detecting early infection prior to the development of an immune response (presence of IgM). Clinicians could consider real-time PCR testing if the patient presents early after onset of symptoms and prior to the use of empirical antibiotics and serology should be the test of choice at late presentations.

1. Introduction

Community acquired pneumonia (CAP) is a common cause of morbidity and mortality among children.[1] *Mycoplasma pneumoniae* is an important cause of human respiratory disease, accounting for 15–20% of total CAP.[2] Clinical diagnosis of *M. pneumoniae* is difficult because it simulates the clinical picture of viral and other pneumonias.[3] In a typical clinical setting, due to relative unavailability of rapid, sensitive and specific laboratory tests, an etiologic diagnosis is not established, thereby necessitating the use of empirical therapy. An etiologic diagnosis will help avoid empiric therapy, reduce antibiotic misuse and allow early initiation of specific antibiotics effective against *M. pneumoniae*.

The clinical course of mycoplasma infections in humans is thought to be mediated by host immune and inflammatory responses, rather than direct cytopathological effects initiated by mycoplasmal cell components.[4] Classically, the symptoms are worse than the signs would suggest.[5]

Although the main burden of infection is found in school-age children (5 years and above), *M. pneumoniae* has also been noted as a significant cause of respiratory tract infection in children under the age of five.[6,7] Due to the lack of a cell wall, all mycoplasma species are innately resistant to beta-lactams and glycopeptides. Fluoroquinolones have been shown to be bactericidal for *M. pneumoniae*, whereas macrolides and tetracyclines are primarily bacteriostatic.[7,8]

Serology is the mainstay of laboratory diagnosis of *M. pneumonia.*[8] Commercial enzyme immunoassays (EIA) for IgM detection are employed in tertiary care centers. In specialized centers, real-time PCR can also be performed for diagnosis; it is useful especially in early presentation where typical symptoms may not manifest[8,9] and has been considered as a gold standard test for diagnosis of Mycoplasma infections.[10,11]

The present study evaluated the utility of a real-time PCR against serology for the diagnosis of *M. pneumoniae* and describes the frequency

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https://doi.org/10.1016/j.cegh.2022.101084
Received 27 April 2022; Received in revised form 25 May 2022; Accepted 30 May 2022
Available online 3 June 2022

of *M. pneumoniae* in children with CAP comparing clinical presentation with the results of Real Time PCR and serology.

2. Methodology

2.1. Study design

It is a prospective cross sectional observational study performed in children between 1 month and 18 years of age. The overall study population included children who were hospitalized for symptoms and findings consistent with CAP, as per WHO criteria - pneumonia with fast breathing and/or chest indrawing warranting admission and oral antibiotics or severe pneumonia with any danger signs (e.g., lethargy, inability to drink, persistent vomiting, stridor or convulsions) requiring injectable therapy. The population excluded children hospitalized in the past 14 days for any other illness. Baseline investigations were done for all. Chest radiography was done where required; results were interpreted according to WHO categorization - Class I = consolidation/pleural effusion, Class II = interstitial pattern/infiltrate, Class III = no consolidation/infiltrate/effusion, Class IV = radiograph quality not sufficient for reading.

2.2. Serology

Mycoplasma IgM serology (Acute phase) was done in all children using Nova Lisa- *M. pneumoniae* IgM ELISA, (NOVATEC Immunodiagnostica, GmbH, Germany). This is an indirect IgM ELISA intended for the qualitative determination of IgM antibodies against *M. pneumoniae*.

2.3. Real-time PCR

Nasopharyngeal (NP) swab was collected from all enrolled subjects in viral transport medium (VTM). Total nucleic acids were extracted from NP samples using the Versant 1.0 Reagent kit (Siemens, Belgium). Automated extraction (VERSANT kPCR, Siemens) was done if load was more than 10 patient samples. Real-time PCR was done using Fast Track Diagnostics (FTD) kit, Respiratory pathogen 21 plus kit (FTD Jung linster, Luxembourg) which is a multiplex real-time PCR and can detect 20 viral and 5 bacterial pathogens. Real-Time PCR was used as the gold standard test for this study as paired sera was not available and culture was not feasible.

Informed consent was obtained from the parents of the children or the children themselves (as per age), who were included for the study. The study commenced in January 2020 and continued till March 2020. Subsequently, enrollment had to be halted due to the COVID-19 pandemic. Enrollment was re-initiated a year later in March 2021 and continued till May 2021. This study was cleared by the Institutional Ethics Committee (IEC) for its scientific content and ethics.

2.4. Statistical analysis

Statistical analysis was performed by the statistical software STATA 11.0. Continuous variables were represented as Mean (SD), and categorical variables were represented as Frequency (percentage). Chi-square test or Fisher's exact tests were used to assess differences in categorical data. Mann Whitney *U* test were used for differences in means of independent data. Sensitivity and specificity were measures of a test's ability to correctly classify a person as having a disease or not having a disease.

3. Results

This study enrolled 66 children diagnosed with CAP. Ten patients were positive for *M. pneumoniae* (Table 1). Nine of 10 were positive for IgM and four were positive by real-time PCR. Both IgM and real-time PCR were positive in three out of 10 patients and only one patient was serology negative but real time PCR positive.

Table 1
Demography of subjects enrolled, *M. pneumonia* positive patients and *M. pneumoniae* negative patients.

	Subjects enrolled N = 66 (%)	*M. pneumoniae* positive patients N = 10 (%)	*M. Pneumoniae* negative *patients* N = 56 (%)
Age:			
Mean (±SD)	3.8 (±3.6) years	6.2 (±3.5) years	3.43 ± 3.45
Median (Range)	2.6 years (1 month–13 years)	6.6 years (14 months–11 years)	1.95 years (1 month–13 years)
Age distribution:			
≤1 year	18 (27.27)	0 (0%)	18(32.14%)
>1 to ≤ -5 years	28 (42.42)	3 (30)	25(44.64%)
>5 to ≤ 10 years	14 (21.21)	5 (50)	9(16.07%)
>10 to <18 years	6 (9.09)	2 (20)	4(7.14%)
Gender:			
Male	41 (62.12)	5 (50)	36 (64.29%)
Female	25 (37.88)	5 (50)	20 (35.71%)
Symptoms:			
Short duration fever	35 (53.03)	2 (20)	33 (58.92)
High grade fever	36 (54.54)	7 (70)	29 (51.78)
Preceding URI[a]	34 (51.51)	2 (20)	32 (57.14)
Sore throat	3 (4.54)	1 (10)	2 (3.57)
Cough <7 days	48 (72.72)	6 (60)	42 (75)
Non-Productive cough	55 (83.33)	10 (100)	45 (80.35)
Cough severity	15 (22.72)	3 (30)	12 (21.42)
Systemic symptoms	32 (48.48)	3 (30)	29 (51.78)
Signs:			
Tachypnea	41 (62.12)	4 (40)	37 (66.07)
Dyspnea	19 (28.78)	2 (20)	17 (30.35)
Hypoxia	17 (25.75)	0 (0)	17 (30.35)
Increased work of breathing	30 (45.45)	4 (40)	26 (46.42)
Systemic signs of consolidation	18 (27.27)	5 (50)	13 (23.21)
Other respiratory signs	42 (63.63)	4 (40)	38 (67.85)
Laboratory and radiological investigations:			
Elevated WBC counts	31 (46.96)	3 (30)	28 (50)
Neutrophilic predominance	49 (74.24)	8 (80)	41 (73.21)
WHO categorization of chest radiography			
Class I	37 (56.06)	7 (70)	30 (53.57)
Class II	12 (18.18)	1 (10)	11 (19.64)
Class III	11 (16.66)	2 (20)	9 (16.07)
Duration of hospitalization	5.93 ± 3.59	7.1 ± 3.95	5.73 ± 3.51

[a] Upper Respiratory Tract Infection.

The age and gender distribution among the overall enrolled population and in the subset of *M. pneumoniae* positive patients is provided in Table 1. The mean and median age of the M. pneumoniae positive patients (6.2 and 6.6 years) was observed to be higher than the overall population (3.8 and 2.6 years respectively). In the youngest (≤1 year) age group, no child was positive for *M. pneumoniae*, but as age advances, the percentage of children positive for *M. pneumoniae* becomes higher than children negative for *M. pneumoniae*.

Most of the children were not critical at presentation and did not require intensive care. The typical presentation of Mycoplasma was prolonged high-grade fever (70%) associated with non-productive, short duration cough (60%), not associated with tachypnea, dyspnea or

persistent vomiting. Other systemic findings were not prominent. Of the 10 confirmed cases of *M. pneumoniae*, more than half the patients (70%) presented with Class I WHO categorization of chest radiography suggestive of consolidation/pleural effusion. Other investigations were suggestive of normal white blood cell count with neutrophilic predominance. The duration of hospitalization was approximately 7 days in majority of the patients.

Table 2 presents the comparison of real-time PCR versus serology (Acute IgM) for Mycoplasma among the 66 cases. Using real-time PCR as the "gold standard" the sensitivity and specificity of IgM were estimated to be 75% (3 out of 4) and 90.32% (56 out of 62), respectively. Positive predictive value of IgM was 33.3% (3 true positive out of 9 overall positive) and negative predictive value was 98.25% (56 true negative out of 57 overall negative).

4. Discussion

M. pneumoniae is an important pathogen causing CAP. Although literature shows the prevalence of Mycoplasma to be up to 40%,[4] the usual range is between 15 and 20%.[2] Likewise, this study enrolled 66 children, of which 10 were confirmed positive for *M. pneumoniae* (an incidence of 15%).

Literature suggests that *M. pneumoniae* has a peak incidence at 5 years and above [1,2] Consistent with that, we observed that the mean and median age of the *M. pneumoniae* positive patients (6.2 and 6.6 years) was higher than the overall population (3.8 and 2.6 years) afflicted with CAP.

The clinical features of *M. pneumoniae* range from inapparent infection to upper respiratory-tract disease to bronchopneumonia.[12] The most common clinical symptoms are cough (non-productive at the start), fever, chills, sore throat, headache, hoarseness, myalgia and general malaise.[13] Consistent with literature, in our study too, we had symptoms with prolonged high-grade fever and non-productive cough, not associated with tachypnea, dyspnea or persistent vomiting. Other systemic findings, though present in few, were not prominent. More than half of the patients (N = 7/10; 70%) infected with Mycoplasma had radiological evidence of lobar consolidation and all except one, were in the age group from 5 to 10 years which corresponds with the data from other studies.[13]

Confirmation of *M. pneumoniae* infection is clinically challenging. Culture of this pathogen is difficult to perform and time consuming. Detection of antibody conversion or four-fold rise in antibody titers (IgG) in two consecutive sera samples collected 2 weeks apart indicates an acute infection. However, this is generally infeasible, since many patients do not return for follow-up, and even in positive cases, it provides only retrospective confirmation of diagnosis.[10] To allow prospective diagnosis in our study we performed serology as well as real-time PCR analysis for all cases of CAP. Real-time PCR was considered the 'gold standard' against which serology was compared, an approach used in earlier studies as well[10,11]

Among the 10 confirmed *M. pneumoniae* cases, 9 were positive by serology and 4 were positive by real-time PCR. In the 4 real-time PCR positive cases, 3 were positive by serology too (75%). Six of the serologically diagnosed patients were negative by real-time PCR. The reason for this could be that *M. pneumoniae* had decreased to undetectable levels in the throat at the time of sampling, possibly either due to initiation of antibiotics or the sampling was done more than a week or 10 days after the onset of symptoms due to late presentation. Molecular tests perform best when samples are collected within a week after onset of illness. Other possibilities include presence of unrecognized PCR inhibitors. An error in sample collection (i.e., insufficient scraping of the pharyngeal wall) is also possible, however, this is unlikely since collection was performed by well-trained nurses. All patients who were positive by real-time PCR for *M. pneumoniae* were investigated around 7–8 days of illness and were not administered prior antibiotics. In contrast, patients negative by real-time PCR for *M. pneumoniae* but positive by serology had a more delayed presentation, about 9 days and beyond after the onset of symptoms. These findings correlate with other studies.[13] This emphasizes that the methodology of identifying Mycoplasma by real-time PCR will be effective when done during early infection period and when the patient has not had any prior antibiotics.

Table 2
Comparison of NP swab real-time PCR vs serology (acute IgM).

Acute IgM	Real Time PCR		
	Positive	Negative	Total
Positive	3	6	9
	75%	9.68%	13.64%
Negative	1	56	57
	25%	90.32%	86.36%
Total	4	62	66
	100%	100%	100%

Literature suggests that in order to diagnose an acute *M. pneumoniae* infection, a combination of PCR and IgM serology is the most sensitive and convenient method. However, in our study, 6 (60%) of the 10 patients with *M.pneumoniae* infection were identified on the basis of serology alone. Without serology the diagnostic yield would have been meagre. Most of the studies done for comparing real time PCR with serology also had a sensitivity range of serology from 62% to 78%.[1,2,10,11] Studies have also compared PCR and serological tests for the diagnosis of *M. pneumoniae*.[10,11] Hsin-Yu Chang et al.[10] evaluated the sensitivity and specificity of IgM keeping PCR as the gold standard which was found to be 62.2% and 85.5%. Our results were in line with these earlier reports.

Both serology and real-time PCR have their pros and cons. The key advantages of serology are: specialized equipment is not necessary, small volumes of serum is adequate,[8] and *M. pneumoniae*-specific IgM elevation is adequate evidence of acute infection.[9] IgM antibody typically appears within 1 week of infection[9] and is a reliable indicator of infection in pediatric patients older than 5 years and those with a disease duration of 1–2 weeks.[11]

On the other hand, the key advantages of real-time PCR are: potential for rapid testing (it can be completed in 1 day),[1] it may be positive earlier during infection than serology, and is perhaps more sensitive (detection of even a single organism may be possible).[7,8] Limitations with PCR include: the need for rigorous specificity testing to ensure that nonpathogenic commensal mycoplasma species in the respiratory tract do not affect the test.[8] Another key limitation is that PCR may be negative when empiric antibiotics are started prior to etiologic confirmation, a consequence of indiscriminate use of antibiotics.

Study related limitations include inability to use nasopharyngeal aspirate in lieu of NP swab. One may therefore argue that NP swab real-time PCR assay may not be the ideal gold standard due to the limitations cited above. Of concern was, majority of our study patients presented to us during late infection and/or were administered empirical antibiotics elsewhere and referred here. Lastly, our sample size was relatively small, and further research with larger sample size is needed to validate our findings.

Taken together, it is important to establish etiologic diagnosis of CAP, to ensure judicious use of antibiotics. However, early and indiscriminate antibiotic use vitiates the assessment of etiologic diagnosis. Notwithstanding these issues, clinicians should endeavor to confirm the etiology of CAP soon after presentation and in cases with suspected *M. pneumoniae* infection, serology or real-time PCR should be chosen as per the time of assessment for confirmation of diagnosis.

Clinicians must prefer real-time PCR as the first line investigation when there is suspicion of *M. pneumoniae* in patients with CAP who presents early without prior use of empirical antibiotics and serology should be opted when they present at a later stage. Confirmation of diagnosis helps ensure appropriate management and avoid emergence of resistant organisms.

Funding sources

Authors acknowledged intramural funding support from The CHILDS Trust Medical Research Foundation (CTMRF), Chennai, Tamil Nadu, India.

Ethical approval

The study was approved by the Institutional Review Board (IRB) Ethics committee of Kanchi Kamakoti CHILDS Trust Hospital & The CHILDS Trust Medical Research Foundation (KKCTH-CTMRF: IEC- 36/ July 2019 (IRB Min. dt. July 18, 2019).

Declaration of competing interest

None.

Acknowledgments

Dr.K.Dhanalakshmi, Dr.Sumanth Amperayani and Dr.P.Sulochana Putli Bai for providing clinical and laboratory support data. Mr.Robinson Peter for technical assistance in performing the laboratory tests.

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