

# Laboratory diagnosis of *Mycoplasma pneumoniae* infections: data analysis from clinical practice

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## SUMMARY

An etiological diagnosis of respiratory infections caused by *Mycoplasma pneumoniae* is particularly challenging due to the lack of a definite standard test.

This study aimed to analyse the correlation and combination of diagnostic results obtained from direct and indirect assays (*Mycoplasma pneumoniae* DNA by PCR and serology) in use at a first level diagnostic laboratory. Samples from patients with respiratory infections tested for *M. pneumoniae* during routine clinical practice were retrospectively analysed. In pediatric patients <15 years old, we documented a significantly higher proportion of IgM positive results (23.6% versus 3.9% in ≥15-year-old patients,  $p < 0.0001$ ) but a lower IgM specificity (false positive IgM 34.8% versus 12.2% in ≥15 years old patients,  $p 0.01$ ), as verified by seroconversion. A small percentage (4%) of respiratory samples were positive for *M. pneumoniae* DNA regardless of age and type of sample. Assuming IgM positivity as the reference standard, PCR showed a total lack of sensitivity in patients <15 years old and 20% sensitivity in children <15 years old; specificity was 95% in both age groups. Agreement between PCR and IgM serology was slight (Cohen's kappa 0.09). According to our data, no single diagnostic test could be considered optimal for *M. pneumoniae* detection and improved assays are required.

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## INTRODUCTION

Community-acquired pneumonia (CAP) is a commonly observed infectious illness, but it is frequently treated empirically with possible antibiotic overuse and complications on managing therapeutic failures. An etiological diagnosis is especially difficult to obtain when CAP and other respiratory infections are caused by atypical bacteria, such as *Mycoplasma pneumoniae*.

It is well known that historical use of serum cold agglutinins lacks diagnostic sensitivity and that classic microbiology culture from respiratory specimens is not suitable for this fastidious bacterium (Loens and Ieven, 2016, Holzman and Simberkoff, 2015; Waites *et al.*, 2017). Up-to-date, serological tests and DNA detection by polymerase chain reaction (PCR) are usually employed as diagnostic methods for *M. pneumoniae* infections, but they both have limitations: serology can be negative, mainly in the early disease stages, and the sensitivity of DNA detection by PCR is reported to vary in relation to the type of sample (e.g. nasopharyngeal swabs, nasopharyngeal aspirate, sputum or bronchoalveolar lavage)

(Holzman and Simberkoff, 2015; Waterer, 2017; Parrot *et al.*, 2016; Loens and Ieven, 2016; de Groot *et al.*, 2017, Loens *et al.*, 2009). Instead, false positive results can also be obtained by DNA detection in healthy carriers, mainly in the pediatric population (Loens and Ieven, 2016; de Groot *et al.*, 2017).

The concomitant combination of different assays has been evaluated to increase sensitivity and specificity (Loens and Ieven, 2016), but a gold standard or a standardized laboratory flow chart for *M. pneumoniae* diagnosis have not yet been defined.

The aim of the present study was to analyse the correlation and combination of diagnostic results obtained from direct and indirect tests (PCR and serology) in use at a first level diagnostic laboratory. Observed data in this clinical practice are discussed to highlight critical issues and to speculate on the possible optimal diagnostic approaches.

## MATERIALS AND METHODS

The results of *M. pneumoniae* DNA assays performed using PCR on respiratory samples and specific IgG/IgM assays results from patients with suspected respiratory infections, from January to December 2016, were retrospectively extrapolated from the database of the Microbiology Unit of Siena Hospital, Siena, Central Italy. *M. pneumoniae* DNA was detected by real time PCR, as previously described (Dumke *et al.*, 2003). *M. pneumoniae* IgG and IgM were detected by Liaison Mycoplasma kit (Biotrin DiaSorin, Ireland Ltd.). According to manufactur-

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er's indications, IgM and IgG were considered to be positive with a titer higher than or equal to 10 AU/ml.

Where available, results from paired longitudinal sera collected at least 10 days apart were also included in the analysis. A diagnostic seroconversion was defined as:

- seroconversion from a *M. pneumoniae*-specific IgM positive/IgG negative serum to a *M. pneumoniae*-specific IgG positive serum, regardless of IgM result in the second serum sample, or
- $\geq 3$ -fold increase in the *M. pneumoniae*-specific IgG titer over the first serum sample.

A false positive result of *M. pneumoniae*-specific IgM positivity was defined as:

- *M. pneumoniae*-specific IgM positive/IgG negative in the first serum sample with an IgG negative result in the second serum sample, or
- *M. pneumoniae*-specific IgM and IgG positivity in the first serum sample without a  $\geq 3$ -fold IgG increase in the second serum sample.

Since the number of patients with paired sera during the pre-analytic phase appeared to be limited (below 7% on the total enrolled patients) and since PCR was available in only 3 patients with defined seroconversion, IgM positivity was assumed as the reference standard in the subsequent comparison analyses. With the aim of increasing the specificity of this test, IgM were assumed to be positive with a titer  $>14$  AU. When a single serum sample was available:

- a positive IgM result was assumed to be indicative of acute infection, regardless IgG result, and
- a positive IgG result with negative IgM was assumed to exclude acute infection.

Results from both serology and PCR were analyzed separately and in combination, selecting assays performed on samples collected on the same day or at maximum of  $\pm 10$  days apart. We evaluated percentages of positive results with each technique and agreement of PCR results with *M. pneumoniae*-specific serology. A sub-analysis was performed in the pediatric population under the age of 15 years.

**Statistical analysis.** Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of PCR were evaluated in selected patients with paired contemporary available serology results, assuming IgM results as the reference standard. Statistical coefficient Cohen's kappa was used to analyze agreement between PCR and serology. Categorical variables were

compared using a chi-square test or Fisher's exact test, as appropriate. Differences between groups were considered significant at the conventional p level  $<0.05$ .

## RESULTS

During 2016, data from 2,592 patients were retrospectively collected from the laboratory database, with at least one serum sample for *M. pneumoniae*-specific IgM and/or IgG detection: 28 (1.1%) were tested only for IgM and 18 (0.7%) only for IgG. Patients with only IgG detection and without a further serum sample to evaluate seroconversion (n=15) were excluded from further analysis. Therefore, serological results from 2,577 (99%) patients were included in the analysis. At least two paired sera collected  $\geq 10$  days apart were available in 170 (6.6%) of these patients.

Overall, 1,427 subjects (55.4%) were males and 1,150 (44.6%) were females, with a median age of 64.6 years (IQR 41.58-80.15). Of the total, 382 (14.8%) were  $<15$  years old (median age 5.02 years, IQR 2.9-9.4).

**Serological results.** Complete serological results are summarized in Table 1. *M. pneumoniae* IgM were detected in 176/2,577 patients (6.8%). Overall, 170 patients had available paired sera. Among these, 26 IgM results (15.3%) were considered false positives due to the absence of seroconversion in the subsequent serum sample. Seroconversion was observed in 12/170 cases (7%). A negative serology for acute infection, including false positive IgM, was therefore detected in 2,416 (93.7%) patients. Regarding the subgroup of pediatric subjects ( $<15$  years old), *M. pneumoniae* IgM were detected in 90/382 patients (23.6%). Overall, 23 pediatric patients had available paired sera. Among these, 8 IgM results (34.8%) were considered false positives due to the absence of seroconversion in the subsequent serum sample. Seroconversion was observed in 3/23 cases (13%). Compared to  $\geq 15$ -year-old individuals, pediatric patients showed a significantly higher percentage of IgM positives (23.6% vs 3.9%,  $p < 0.0001$ ) with a significantly higher rate of false positive IgM results (34.8% vs 12.2%,  $p 0.01$ ), as verified by seroconversion. A negative serology for acute infection, including false positive IgM, was therefore detected in 297 (77.7%) of all pediatric patients (Table 1).

**PCR results.** Results of *M. pneumoniae* DNA detection by PCR were obtained from 590 patients: a positive result in at least one respiratory sample was obtained in 24 cases

**Table 1 - Serological results of 2,577 patients and comparison by age groups\*:**

382 patients  $<15$  years-old (23 patients with at least two sera) and 2,193 patients  $\geq 15$  years-old (147 patients with at least two sera).

Serological profile	N°/total tested (%)			p value
	All	$<15$ years-old	$\geq 15$ years-old	
POSITIVE IgM	176/2,577 (6.8)	90/382 (23.6)	86/2,193 (3.9)	$<0.0001$
FALSE POSITIVE IgM	26/170 (15.3)	8/23 (34.8)	18/147 (12.2)	0.01
SEROCONVERSION	12/170 (7)	3/23 (13)	9/147 (6.1)	0.2
NEGATIVE for acute infection**	2,416/2,577 (93.7)	297/382 (77.7)	2,117/2,193 (96.5)	$<0.0001$

\*Age was not available for two patients.

\*\*Including false positive IgM.

(4.1%). A total of 629 samples were analysed: pharyngeal swabs (PS, n=443, 70.4%), sputum, broncho-alveolar lavage (BAL) or bronchial aspirate (BAS) (n=120, 19%), oropharyngeal lavage (OPL) (n=60, 9.5%) and pleural fluid (n=6, 0.95%). On the overall tested samples, 25 of 629 (4%) were positive for *M. pneumoniae* DNA: 5.8% of cumulative sputum, BAS and BAL samples, 3.8% of PS and 1.6% of OPL. A negative result was obtained from all 6 samples of pleural fluid. No significant difference in the proportion of positive *M. pneumoniae* DNA results was observed comparing the different respiratory specimen types and between age groups ( $\geq$  vs <15-year-old patients, data not shown).

**Correlation and combination of serological and PCR results.** Both serological and PCR results were available for 97 patients. Overall, 7 (7.2%) patients had a positive PCR result in a respiratory sample: 2 BAL and 1 OPL (all from  $\geq$ 15-year-old patients) and 4 PS (all from <15-year-old patients). Detailed results are shown in Table 2.

With the aim of performing a correlation analysis between PCR and serology results, seroconversion was not suitable as a serological reference standard due to the lack of a significant number of patients with paired sera and concomitant PCR. Therefore, even with the limitations of possible false results, IgM positivity was assumed as the reference standard. PCR showed an overall sensitivity of 11.8% and a specificity of 95.1%, with a PPV of 33.3% and NPV of 83.9% and an accuracy of 80.8%. A slight agreement was obtained between IgM positivity and PCR (Cohen's kappa 0.09). A substantially equivalent specificity was observed in the two different age groups, but a total lack of sensitivity of PCR was observed in patients  $\geq$ 15 years old. Details of data on PPV, NPV, accuracy and agreement are shown in Table 3.

In the 97 patients undergoing both *Mycoplasma* serology and PCR, 19 (21.1%) had one positive result in at least one

assay. Among them, 12 (24.5%) were <15 years old and 7 (14.8%)  $\geq$ 15 years old (p=0.33).

## DISCUSSION

Etiological diagnosis of respiratory infections caused by *M. pneumoniae* is a constantly challenging issue due to the lack of a rapid, sensitive and specific diagnostic gold standard. Results from our study confirm the need for an improvement in diagnostic tests for *M. pneumoniae* infections in routine clinical practice.

We observed a very limited use of paired sera in our clinical practice: no more than 6% both in the general study population and in the subgroup of <15-year-old subjects had at least two subsequent sera tested. The limited number of patients with paired sera and biological samples to apply both direct and indirect diagnostic techniques is certainly one of the limitations of our study, although this observation seems to reflect the "real world" practice. A second limitation is related to the retrospective collection data methodology and sample analysis: we could not access detailed clinical patient information, which prevented us from evaluating the timing of sample collection with respect to symptoms onset or clinical history and, therefore, to evaluate possible reinfections.

IgM antibodies can be considered a possible alternative diagnostic method in the absence of paired sera (Holzman and Simberkoff, 2015), but they can result in false negative results in children, during the early phase of a primary infection and during reinfections and, on the other hand, a high prevalence of *M. pneumoniae* IgM has also been reported in healthy children (Loens and Ieven, 2016; de Groot *et al.*, 2017). Moreover, cross-reactions are widely described (Miyashita *et al.*, 2013; Busson *et al.*, 2013). We similarly documented a lack of specificity with a false positive IgM result, as verified by the absence of sero-

**Table 2** - Results of *M. pneumoniae* DNA detection by PCR in patients with different serological profiles (N. = 97).

Patient's serological profile	N° positive PCR/total tested (%)			p value
	All	<15 years-old	$\geq$ 15 years-old	
POSITIVE IgM	2/17 (11.1)	2/10 (20)	0/7 (0)	0.48
FALSE POSITIVE IgM	0/4 (0)	N/A	0/4 (0)	N/A
SEROCONVERSION	1/5 (20)	0/2 (0)	1/3 (33.3)	1
NEGATIVE for acute infection*	4/82 (4.9)	2/40 (5)	2/42 (4.8)	1

\*Including false positive IgM.

N/A: Not Applicable.

**Table 3** - Sensitivity, specificity, PPV, NPV, accuracy of PCR and its agreement by Cohen's kappa with IgM positivity as reference standard.

	Total population		Patients <15 years old		Patients $\geq$ 15 years old	
	%*	95% CI	%*	95% CI	%*	95% CI
Sensitivity	11.8	1.46-36.44	20	2.52-55.61	0	0-40.96
Specificity	95.1	87.98-98.66	95	83.08-99.39	95.2	83.84-99.42
PPV	33.3	9.04-71.54	50	13.78-86.22	0	
NPV	83.9	81.28-86.16	82.6	77.56-86.72	85.1	84.23-85.94
Accuracy	80.8	71.66-88.03	80.4	66.28-89.97	81.6	67.98-91.24
Cohen's kappa	0.09	N/A	0.19	N/A	N/A	N/A

\*Values are expressed as % except for the Cohen's kappa value; PPV: positive predictive value, NPV: negative predictive value; N/A: Not Applicable.

conversion, in >34% of patients under 15 years of age, a significantly higher rate when compared to ≥15-year-old subjects.

In the past, culture was considered the gold standard for diagnosis of *M. pneumoniae* infections and, indeed, it shows a 100% specificity in case of acute infections, but it is also a time-consuming procedure and requires experienced laboratories to achieve the best sensitivity performance (Waites *et al.*, 2017). PCR assays can now be considered the new reference test for direct diagnosis, although most of the clinically used PCR-based assays have never been comprehensively validated against culture and comparison studies yielded miscellaneous results (Waites *et al.*, 2017). Moreover, the high sensitivity of PCR (<100 CFU/ml) may overestimate the etiologic role of *M. pneumoniae*, especially in the pediatric population where a high carriage rate is present (Waites *et al.*, 2017).

Surprisingly, we found a low proportion of positive results of *M. pneumoniae* DNA detection by PCR on respiratory samples (4%), regardless of age. The unknown timing of sampling with respect on clinical onset could represent a bias and partly justify this observation: a negative PCR result could be due to the antibiotic therapy administered before sampling (Waites *et al.*, 2017). Interestingly, however, in our population the respiratory sample type did not affect the probability of detection, suggesting that pharyngeal swabs can be used as well as samples from the lower respiratory tract (e.g. sputum, BAL). This observation is in agreement with Xu *et al.* who compared PCR results from nasopharyngeal swab and from BAL and suggested a nasopharyngeal swab as the respiratory specimen of choice in pediatric patients (Xu *et al.*, 2011).

In our population, assuming IgM positivity as the reference standard, PCR revealed a total lack of sensitivity in patients ≥15 years old and a low sensitivity (20%) in children <15 years old. The assumption of IgM positivity as the reference standard represents a potential limitation when comparing the results of serology with PCR. In fact, a single measurement of IgM has not been considered fully reliable (Waites *et al.*, 2017), but the lack of a sufficient number of paired sera in our population made it impossible to use the results of seroconversion as the reference standard. Indeed, in practical terms, it is cumbersome to collect paired sera in many cases, particularly in pediatric patients, and the time needed to obtain seroconversion results is not suitable to guide the therapeutic choice: observed data are therefore a reflection of everyday clinical practice. On the other hand, assuming IgM positivity as the reference standard, a specificity of 95% was observed for PCR in both age groups, which is partially unexpected, considering the high rate of healthy carriers in the pediatric age group (Loens *et al.*, 2016; de Groot *et al.*, 2017). Agreement between PCR and IgM serology was indeed only slight in the overall studied population.

No single test has ever been considered the optimal diagnostic tool for *M. pneumoniae*: neither serology nor PCR, either qualitative and quantitative, nor culture are able to distinguish between an asymptomatic carrier status and active infection, therefore the need to combine different methods to improve sensitivity and specificity has been highlighted (Thurman *et al.*, 2009; Spuesens *et al.*, 2013; Qu *et al.*, 2013; de Groot *et al.*, 2017; Loens and Ieven, 2016). Based on our data, combining the two methodologies, serology and PCR, positive detection

rates were significantly increased ( $p < 0.0001$ ) compared to results obtained by only IgM or PCR assays: over 20% of the patients whose samples were tested using both direct and indirect techniques, could obtain an etiological diagnosis, with at least one test positive. The known possible false negative results of both PCR and serology, mainly due to the sampling time (Waites *et al.*, 2017), together with our observations in clinical practice, seem to suggest that the current best algorithm to improve sensitivity and specificity for acute *Mycoplasma* infections is a combination of PCR and serology. To improve the etiological diagnosis, we suggest both PCR and IgM be performed within the first week after clinical onset. Since IgG are usually detected within weeks from onset and that during reinfections IgM response may be blunted (Waites *et al.*, 2017), confirmation serology in paired sera should be considered in selected cases. Until a definite diagnostic standard is established, continuation of empiric therapy against *M. pneumoniae* infections should be considered in case of suggestive clinical features, even with negative *Mycoplasma* diagnostic assays and in the absence of an alternative microbiological diagnosis.

Recently, the combination of short-term paired *M. pneumoniae* specific-IgM determinations and paired cold-agglutinin determinations has been proposed to improve diagnostic performance in the acute stage (Lee *et al.*, 2016). This proposal, however, doesn't overcome the problem of repeated blood sampling, with all its major limitations, particularly in the pediatric age group.

Many technological developments have been proposed to improve the overall sensitivity and specificity of *M. pneumoniae* laboratory diagnosis, such as immunochromatographic assays targeting a region of the P1 gene or the ribosomal protein L7/L12 of *M. pneumoniae*, but their results were not univocal when applied to clinical specimens (Loens and Ieven, 2016). A possible stimulating diagnostic application could come from the use of *M. pneumoniae* recombinant chimeric antigens that have been demonstrated to improve the sensitivity and diagnostic performance of enzyme-linked immunosorbent (ELISA) assays with respect to an existing assay (Montagnani *et al.*, 2010). The "chimeric approach" has recently been proposed in ELISA assays to improve the diagnosis of other *Mycoplasma* species, which also suffer similar methodological limitations and which can cause severe genital infections in reproductive age patients (Saadat *et al.*, 2018; Wang *et al.*, 2017; Martin, 2015).

In summary, a rapid, accurate and definite diagnostic method for the etiological diagnosis of *M. pneumoniae* infection is still a long way off. Further experimental improvements and analyses of their application to clinical practice are required to achieve this goal.

#### Conflict of interest

FM has received non financial support from Angelini and Astellas, outside the submitted work. She has done contract research for Novartis Vaccine and Diagnostic S.r.l. (now GSK Vaccine S.r.l.) on behalf of the University Hospital of Siena; she is Infectious Diseases Consultant for GSK (consultancy fee on behalf of University of Siena). BR received consultant fees from Janssen, ViiV Healthcare, Abbvie, MSD and Gilead Sciences, all outside the submitted work.

AV Nothing to declare.

MGC Nothing to declare.

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