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Diagnostic Microbiology and Infectious Disease 76 (2013) 133–137

Contents lists available at SciVerse ScienceDirect

Diagnostic Microbiology and Infectious Disease

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

Evaluation of 10 serological assays for diagnosing *Mycoplasma pneumoniae* infection ☆

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ARTICLE INFO

Article history:
Received 2 October 2012
Received in revised form 13 February 2013
Accepted 15 February 2013
Available online 26 March 2013

Keywords:
Mycoplasma pneumoniae
Serelogy
SeroMP
Liaison
Medac

ABSTRACT

In this study, the performance of 10 serological assays for the diagnosis of *Mycoplasma pneumoniae* infection was evaluated. A total of 145 sera from 120 patients were tested. They were obtained from patients who were serologically positive for *M. pneumoniae* infection as well as from patients who were infected with micro-organisms that may cause interstitial pneumonia. The following assays were utilized: SeroMP IgM and IgG, SeroMP recombinant IgM, IgA and IgG, Liaison *M. pneumoniae* IgM and IgG and *M. pneumoniae* IgM, IgA and IgG ELISA Medac. The SeroMP Recombinant and Liaison assays both showed low IgM specificity, and crossreactivity was mainly observed in groups of patients with acute cytomegalovirus and Epstein-Barr virus infections. For IgA, the Medac assay was less specific than the SeroMP Recombinant assay. Discrepancies between the four tests were observed in IgG analyses, and due to the lack of a gold standard, 22 results were removed prior to determining the sensitivity and specificity. Therefore, the overall performance of IgG assays may be overstated; nevertheless, the SeroMP assay demonstrated a lack of sensitivity. The seroprevalence of IgG appears to be very low, raising concerns regarding whether the serological techniques can detect IgG levels over time. Serology remains a biological tool of choice for diagnosing *M. pneumoniae* infection, but improvement and standardization of the assays are needed, particularly for the determination of IgG.

1. Introduction

Mycoplasma pneumoniae is a leading cause of bacterial community-acquired pneumonia (Cillóniz et al., 2012; Strålin et al., 2006), accounting for 15–20% of cases, and up to 40% in children. It may also be responsible for upper respiratory tract infections and extra-pulmonary manifestations. This infection is endemic, with epidemic peaks occurring every 4 to 7 years (Bébéar, 2007), as was observed in several European countries from 2010 to 2011 (Jacobs, 2012). Laboratory diagnosis was previously performed with cultures of the organism, but this technique is slow and less sensitive than serological or nucleic acid amplification assays (She et al., 2010). The nucleic acid amplification assays appear to be the most sensitive methods; however, those techniques cannot distinguish between asymptomatic and acute infections (Dorigo-Zetsma et al., 2001; Foy, 1993; Ieven and Goossens, 1997; Loens et al., 2003). Serology still has a place of choice in the diagnosis of *M. pneumoniae* infection, but this must rely on the analysis of two coupled sera taken at 2–3 weeks. Serological diagnosis can confirm a recent infection when there is an apparition of the IgG or when there is a significant increase in IgG levels between the two sera because upon reinfection, IgM may not be present (Waites and Talkington, 2004). Complement fixation has been replaced with a variety of commercially available techniques, specifically with the enzyme-linked immunosorbent assays (ELISA), which allow for a precise quantification of IgM, IgA or IgG (Bébéar, 2007). The aim of this work is to evaluate the performance of 10 serological assays for the diagnosis of *M. pneumoniae* infection.

2. Materials and methods

2.1. Sera

A total of 145 sera from 120 patients (54 women and 66 men) were evaluated. Fifty sera were coupled, and the interval between the two sera collections varied from 5 days to 5 months. The average age of the patients was 23.63 years (median: 15.5 years). Sera were sorted into different groups (Table 1): *M. pneumoniae* infections (n = 32), non-specific *M. pneumoniae* IgM (n = 25), other infections causing an

☆ Transparency declaration: The author declares no conflict of interest.

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http://dx.doi.org/10.1016/j.diagmicrobio.2013.02.027

Table 1
List of sera.

	Description	Criteria of selection	n
M. pneumoniae infection	Serologically confirmed *M. pneumoniae* infection	Apparition or significant augmentation of IgG between paired serum (with or without IgM and IgA)	26
	Possible *M. pneumoniae* infection	High amount of *M. pneumoniae* IgG, IgA and IgM	6
Non-specific IgM	Non-specific *M. pneumoniae* IgM	Persistent *M. pneumoniae* IgM over time without appearance of IgG	25
Other causes of interstitial pneumonia	*Chlamydia pneumoniae* infection	Seroconversion in anti-MOMP IgG (n = 2) or high levels of anti-LPS IgA and IgG (n = 5)	7
	Legionella pneumophila infection	Positive *L. pneumophila* antigen in urine (n = 2) or positive serology with immunofluorescence (n = 2)	4
	RSV infection	Positive RSV culture (n = 5) or positive RSV serology with complement fixation (n = 5)	10
	Adenovirus infection	Positive adenovirus culture (n = 4) or positive adenovirus serology with complement fixation (n = 5)	9
	Parainfluenza virus infection	Positive parainfluenza culture (n = 5) or positive parainfluenza serology with complement fixation (n = 5)	10
	Influenza A or B infection	Positive influenza A or B culture (n = 5) or positive influenza serology with complement fixation (n = 4)	9
	CMV infection	Positive CMV culture (n = 2) or positive CMV serology with Abbott Architect (n = 3) or both (n = 3)	8
	VZV infection	Positive VZV culture (n = 1) or positive VZV serology (n = 4) or both (n = 2)	7
	Measles infection	Positive measles culture on a respiratory sample (n = 1) or positive serology with evocative clinical examination (n = 8)	9
	Human metapneumovirus infection	Positive human metapneumovirus direct immunofluorescence on a respiratory sample (n = 4)	4
	Coronavirus 229E infection	Positive micro-array on a respiratory sample (n = 1)	1
	Aspergillus sp. infection	Positive galactomannan on a respiratory sample and serum (n = 4)	4
EBV acute infection	EBV acute infection	Positive EBV serology with evocative clinical examination and laboratory findings	6
		TOTAL	145

MOMP = major outer membrane protein; LPS = lipopolysaccharide.

interstitial pneumonia and positive Epstein-Barr virus (EBV) serology, which cross-reacts with *M. pneumoniae* serology (Beersma et al., 2005). Cases of *M. pneumoniae* infections were initially chosen in front of an apparition or a significant augmentation of IgG on paired sera using SeroMP (Savyon Diagnostics, Ashdod, Israel) which was in use for the routine analyses in our laboratory. The serological results of those samples were then compared to those obtained with the other techniques evaluated and finally the group of sera representing the *M. pneumoniae* infections was composed either from sera exhibiting an apparition or significant augmentation of IgG between paired samples with at least two of the evaluated techniques and sera with high amount of *M. pneumoniae* IgG, IgA and IgM with SeroMP Recombinant IgM, IgA and IgG (Savyon Diagnostics, Ashdod, Israel) and *M. pneumoniae* IgM, IgA and IgG ELISA Medac (Medac, Hamburg, Germany). Cases of Q fever would ideally have been included, but these were not available. Sera were selected from the serum bank at the Porte de Hal Laboratory, which performs serological analyses for four public university hospitals that are located in Brussels, Belgium.

2.2. Serological assays

- SeroMP IgM and IgG (Savyon Diagnostics, Ashdod, Israel): an ELISA test for the semi-quantitative detection of IgM and IgG antibodies against *M. pneumoniae* in human serum.
- SeroMP Recombinant IgM, IgA and IgG (Savyon Diagnostics, Ashdod, Israel): an ELISA test for the semi-quantitative detection of IgM, IgA and IgG antibodies against *M. pneumoniae* in human serum.
- LIAISON *M. pneumoniae* IgM and IgG (Biotrin International Ltd., Dublin, Ireland): a chemiluminescence immunoassay (CLIA) used for the qualitative (IgM) or semi-quantitative (IgG) determination of antibodies against *M. pneumoniae* in human serum or plasma, performed with the LIAISON analyzer.
- *M. pneumoniae* IgM, IgA and IgG ELISA Medac (Medac, Hamburg, Germany): an enzyme immunoassay for qualitative (IgM) or quantitative (IgA and IgG) determination of *M. pneumoniae* antibodies in human serum.

The results were classified as negative, equivocal or positive, according to the cut-off values that were specified by the manufacturers.

As there is no gold standard for *M. pneumoniae* serology, the sorting of the results into true or false positives or negatives was performed by matching the clinical data with the serological profile. If the interpretation was unclear, a paired sample was analyzed, if available. Despite our careful interpretation, some discrepant results could not be sorted and were not taken into account when calculating the performance of the assays. Of the 145 samples that were evaluated, this occurred for 3 IgM, 5 IgA and 22 IgG results.

3. Results

Table 2 shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) results for each assay. The PPV and the NPV are shown for informative purposes, as they depend on the disease prevalence in the population. For IgM, the specificity was recalculated after removing 25 sera with non-specific IgM results, as the

Table 2
Sensitivity, specificity, PPV and NPV of the different assays for IgM, IgA, and IgG.

	SeroMP	SeroMP Rec	Liaison	Medac	SeroMP Rec	Medac	SeroMP	SeroMP Rec	Liaison	Medac
	IgM	IgM	IgM	IgM	IgA	IgA	IgG	IgG	IgG	IgG
Sensitivity (%)	100	100	100	100	100	100	61.76	97.05	100	100
Specificity (Sp) (%)	75.22	68.93	71.68	80.58	98.33	88.13	96.42	100	97.75	100
Sp (without non-specific) (%)	92.04	81.48	82.95	91.56						
PPV (%)	50.87	47.54	47.54	59.18	90.9	58.82	87.5	100	94.44	100
NPV (%)	100	100	100	100	100	100	86.17	98.88	100	100
	n = 142(n = 117 without non-specific)				n = 140		n = 123			

Table 3
Discrepant results for IgG between assays.

SeroMP	SeroMP Recombinant	Liaison	Medac	n
+	-	-	+	7
-	-	+	+	4
-	-	-	+	4
-	-	+	+	2
+	-	-	-	2
+	-	+	+	1
+	-	+	-	1
+	-	-	Equivocal	1
			TOTAL	22

+ = positive result (IgG value over the cut-off defined by each manufacturer), − = negative result (IgG value under the cut-off defined by each manufacturer).

high proportion of those sera (25/145) could have a negative impact on the calculation. Table 3 shows the 22 IgG results that could not be categorized as true or false positives or negatives. Five sera were in the non-specific IgM group, 2 were in the *Chlamydia pneumoniae* group, 1 was in the respiratory syncytial virus (RSV) group, 3 were in the influenza group, 4 were in the acute EBV infection group, 4 were in the acute cytomegalovirus (CMV) infection group, 2 were in the varicella zoster virus (VZV) group, and 1 was in the measles group.

The group of sera that had a higher number of IgM false positive results from all of the assays (excluding the non-specific IgM group) included sera from patients with acute EBV infection; this cross-reactivity has been previously described (Beersma et al., 2005). Three out of 6 samples from patients with acute EBV infections had a false positive result with the SeroMP and Liaison assays, 2 false positives and one equivocal result were obtained using the SeroMP Recombinant assay, and 4 false positives and one equivocal result were obtained with the Medac test. The other group that had the highest number of IgM false positive results included patients with acute CMV infections, although this was not observed with all of the assays. The SeroMP assay had only 1 false positive result out of 8 samples that were tested, the Medac assay had 1 false positive result and 1 equivocal result, the SeroMP recombinant test had 5 false positives and 1 equivocal result, and the Liaison test had 6 false positive results. The only serum obtained from a Coronavirus 229E-infected patient showed false positive results for both of the Liaison and SeroMP Recombinant tests, but no conclusion could be drawn from a single sample. In the other groups, false positive results occurred less frequently and often with values close to the cut-off values. Table 4 shows the mean, the standard deviation (SD) and the median values of the false positive results for IgM, omitting the group with non-specific IgM results.

4. Discussion

4.1. Sensitivity

Each assay had a sensitivity of 100% for the detection of IgM, IgA, and IgG, except for the detection of IgG using the SeroMP and SeroMP Recombinant assays, which had a sensitivity of 61.76% and 97.05%, respectively. The SeroMP test was used in our routine practice, and in our experience, IgG disappear sometimes over time, which could explain the low sensitivity of this test. For the SeroMP Recombinant assay, only one sample had a false negative result in a 32-year-old patient with an acute *M. pneumoniae* infection. The control serum obtained one week later showed a high IgG titer. The three other assays showed a positive IgG result in the first serum sample.

Table 4
Mean, standard deviation (SD) and median values of the false positive IgM after exclusion of the group of non-specific *M. pneumoniae* IgM.

	SeroMP	SeroMP Recombinant	Liaison	Medac
Unit	BU/mL	COI	Index	Index
cut off	≥20	≥10	≥11	≥1.1
Mean	37.17	15.32	20.78	1.45
SD	28.33	6.46	13.16	0.58
Median	27	14	16.45	1.476
n	7	17	13	9

BU = arbitrary binding unit, COI = cut off index.

4.2. Specificity

The IgM tests had a higher specificity with the SeroMP and Medac assays compared to the two other tests, which can be partially explained by the lower number of false positive results that were obtained in the group of sera taken from patients with acute CMV infection. The Liaison and SeroMP Recombinant assays also produced a few more false positive results, distributed among the other groups of sera. When the group of non-specific *M. pneumoniae* IgM was taken into account the specificity of the assays is lower. The significance of those false positives IgM results could not be explained, but in 3 cases, these were obtained from patients undergoing polyclonal-based stimulation of the immune system, as confirmed by the numerous serological tests showing IgM perturbations. Three additional false positive samples were obtained from patients with *Streptococcus pyogenes* infections (positive for anti-streptolysin O or anti-*Streptococcus* deoxyribonuclease B); however, a clear link could not be drawn from these observations.

With IgA, the SeroMP test appears to be more specific than the Medac test. False-positive results were obtained with the Medac test, mainly in the group selected for its non-specific IgM results.

The IgG specificity is 100%, except for the SeroMP and Liaison tests, which produced a few false positive results very close to the cut off value.

4.3. Antibody kinetics and interest of the determination of IgA

IgM usually appear within 1 week of an initial infection and can persist for months or years following infection. Thus, a positive IgM result does not always implicate an acute infection. Upon reinfection, the IgM response can sometimes be absent (Thacker and Talkington, 2000).

IgG generally appear 2 weeks after IgM. They could be considered as the most important parameter in *M. pneumoniae* serology because the serological diagnosis is confirmed upon apparition or significant augmentation of IgG between the acute- and convalescent-phase sera taken within 2–3 weeks. This is particularly important in reinfection cases, as the IgM response may be absent.

IgA are produced in the early phase of the disease, rise quickly to peak levels and then decrease more rapidly than IgM or IgG. IgA are believed to be infrequently synthesized in children. In this work, 9 children less than 15 years of age were included in the group of serologically confirmed *M. pneumoniae* infections. Two 3-year-old children produced IgM, but not IgA, whereas the 7 others, including one 1-year-old child, produced IgA and IgM. The advantage of detecting IgA is that these antibodies usually appear in reinfections and thus could already help to suspect this state in an acute-phase serum sample that is positive for IgA and IgG without waiting for the results of the convalescent-phase serum that would show a rise in IgG levels (Table 5).

For financial and practical reasons, the determination of IgM and IgG alone is performed in our routine practice. Therefore, analysis of a convalescent-phase serum is required when an isolated IgG-positive result is obtained from acute-phase serum because this could incorrectly be interpreted as a serological scar whereas the patient is experiencing a reinfection.

Table 5
Suggestion of interpretation of *M. pneumoniae* serology.

	Acute serum				Control serum (2–3 weeks later)			
	IgM	IgA	IgG	Interpretation	IgM	IgA	IgG	Interpretation
1	-	-	-	Negative serology. Acute infection possible if serum was taken too early.	- +/−	- +/−	- +	Acute infection excluded. Confirmation of acute infection.
2	-	+	-	Non-specific IgA probable.	-	+/−	-	Confirmation of non-specific IgA.
3	+	-	-	Possibility of acute infection.	+/− +/−	- +/−	- +	Non-specific IgM probable. Confirmation of acute infection.
4	+	+	-	Possibility of acute infection.	+/− +/−	+/− +/−	- +	Non-specific IgM and IgA probable. Confirmation of acute infection.
5	-	-	+	Serological scar or reinfection.	- +/−	- +/−	= ↗↗	Serological scar. Confirmation of reinfection.
6	-	+	+	Reinfection probable.	- -	+/− +/−	= ↗↗	Serological scar. Non-specific IgA Confirmation of reinfection.
7	+	-	+	Acute infection or reinfection probable.	+/− +/−	- +/−	= ↗↗	Serological scar with non-specific IgM. Confirmation of reinfection.
8	+	+	+	Acute infection or reinfection probable.	+/− +/−	+/− +/−	= ↗↗	Serological scar with non-specific IgM and IgA. Confirmation of reinfection.

+ = positive result, − = negative result, +/− = positive or negative result, ↗↗ = significant augmentation of IgG, = : stable value of IgG. Determination of IgA is useful for differentiation of profile 5 and 6 on the acute serum.

4.4. Seroprevalence of IgG and performance of the IgG assays

As mentioned, of the 145 sera that were included in this work, 22 IgG results could not be classified due to the discrepancies between the assays that are reported in Table 3 and due to the lack of a gold standard for *M. pneumoniae* serology. In all of the discrepant cases, the SeroMP Recombinant assay yielded a negative result. Such discrepancies in IgG were also observed by other authors (Beersma et al., 2005; Talkington et al., 2004).

The seroprevalence of IgG was determined in the groups of sera, excluding *M. pneumoniae* infection and non-specific IgM cases. With the 88 sera remaining, the seroprevalence was different depending on the assay used, and there was an agreement among all 4 assays for only one sample (Table 6). The very low seroprevalence of IgG in general is very striking, particularly in patients over the age of 65 (9.09% for SeroMP and 0% for the 3 other assays, obtained from 11 samples from patients over the age of 65). These percentages are far from the 40–50% seroprevalence of IgG in adulthood and 60% over the age of 65, as described by Tuuminen et al. (2000). These observations raise several questions: (1) are the serological assays able to correctly detect *M. pneumoniae* IgG in acute phases and over time; (2) are the cut-offs of these assays too high; and (3) are the *M. pneumoniae* IgG lasting over time?

Table 6
Seroprevalence of IgG in the groups other than *M. pneumoniae* infections and non-specific IgM.

	SeroMP	SeroMP Rec	Liaison	Medac
Seroprevalence IgG	13/88 (14.77%)	2/88 (2.27%)	6/88 (6.81%)	13/88 (14.77%)
Seroprevalence IgG patients <15 yo	1/42 (2.38%)	0/42 (0%)	1/42 (2.38%)	3/42 (7.14%)
Seroprevalence IgG patients ≥15 yo	12/46 (26.08%)	2/46 (4.34%)	5/46 (10.86%)	10/46 (21.73%)
Seroprevalence IgG patients ≥65 yo	1/11 (9.09%)	0/11 (0%)	0/11 (0%)	0/11 (0%)

yo = year-old.

5. Conclusion

In this work, the performance of 10 serological assays for the diagnosis of *M. pneumoniae* was evaluated. For IgM, the SeroMP Recombinant and the Liaison tests obtained lower specificities, and cross-reactivity was mainly observed in sera from acute EBV and CMV infections. For IgA, the SeroMP Recombinant assay appears to be more specific than the Medac assay. The interest in determining IgA levels may be critical to early detection of reinfections because the IgM response may be absent. For IgG tests, the performances were comparable, except for the SeroMP test, which had several false negative results that led to a low sensitivity. However, 22 sera had to be excluded from the IgG results prior to the sensitivity and specificity calculations due to discrepancies between the assays, and thus, the performances may be overstated. These findings implicate the need for improving and standardizing the serological *M. pneumoniae* assays, particularly for IgG determination. A recent article reports the attempts to develop a blotting technique that improves the performance of the serological assays (Dumke et al., 2012).

Acknowledgments

The author acknowledges the serology team from the Porte de Hal Laboratory, and also Anne Dediste and Delphine Martiny for their comments and remarks.

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