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Evaluation of 12 Commercial Tests and the Complement Fixation Test for *Mycoplasma pneumoniae*-Specific Immunoglobulin G (IgG) and IgM Antibodies, with PCR Used as the "Gold Standard"

Matthias F. C. Beersma,[1]* Kristien Dirven,[2] Alje P. van Dam,[1] Kate E. Templeton,[1] Eric C. J. Claas,[1] and Herman Goossens[1,2]

Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands,[1] and Department of Microbiology, University of Antwerp, Antwerp, Belgium[2]

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Serology and nucleic acid amplification are the main diagnostic tools for the diagnosis of *Mycoplasma pneumoniae* infection. Since no reference standard is generally accepted, serologic assays for *M. pneumoniae* have not been evaluated on a broad scale. In this study, 12 commercially available serologic assays (for immunoglobulin G [IgG] and IgM) and the complement fixation test (CFT) were evaluated by using *M. pneumoniae* DNA detection by real-time PCR as the "gold standard." The assays tested were Platelia EIA (Bio-Rad), SeroMP EIA (Savyon), Serion classic EIA (Virion/Serion), Biotest EIA (Biotest), Ridascreen EIA (r-Biopharm), AniLabsystems EIA (Labsystems), Novum EIA (Novum Diagnostica), Diagnosys EIA (MP products), Genzyme/Virotech EIA, ImmunoWell EIA (Genbio), ImmunoCard EIA (Meridian), and Serodia-MycoII microparticle agglutination (Fujirebio). Serum samples ($n = 46$) from 27 PCR-positive patients with a known first day of disease and sera ($n = 33$) from PCR-negative controls were obtained from prospective studies of acute lower respiratory tract infections. Additionally, control sera ($n = 63$) from patients with acute viral or bacterial respiratory infections other than those caused by *M. pneumoniae* were tested. The results showed low specificities for both the Novum and the ImmunoCard IgM assays. The IgM assays with the best performances in terms of sensitivity and specificity were AniLabsystems (77% and 92%, respectively), SeroMP (71% and 88%, respectively), and CFT (65% and 97%, respectively). Good receiver operating characteristic areas under the curve were found for CFT (0.94), the Platelia assay (0.87), and the AniLabsystems assay (0.85). We conclude that there are few commercial serologic assays for the detection of *M. pneumoniae* infections with appropriate performances in terms of sensitivity and specificity and that PCR has become increasingly important for the diagnosis of *M. pneumoniae* infections in defined groups of patients.

Mycoplasma pneumoniae is a common cause of upper and lower respiratory tract infections (LRTIs) in humans. The clinical picture is that of a slowly progressing tracheobronchitis with malaise and nonproductive cough (4). In 5 to 10% of patients with *M. pneumoniae* infection, especially young adults, the infection may cause atypical pneumonia (17). Reinfections with *M. pneumoniae* do occur, but it is unclear whether persistent carriage of mycoplasmas in an immune subject occurs (5, 8, 13). As *M. pneumoniae* lacks a cell wall, the commonly described β-lactam antibiotics are not effective and adequate laboratory diagnosis is important.

Diagnosis of *M. pneumoniae* infection in routine clinical practice has been based on serology, since bacterial culture of this organism is slow and lacks sensitivity (6, 14). The serologic assays that have been used in the past are immunofluorescence, the complement fixation test (CFT), and the microparticle agglutination (MAG) assay, which are based on antigens derived from crude culture extracts that contain large amounts of cross-reactive glycolipids (12, 14, 27, 29). Therefore, the conventional serologic tests for the diagnosis of *M. pneumoniae* infections are considered nonspecific and may cross-react with other *Mycoplasma* species or gram-negative bacteria. In the past 10 years, a number of commercial microtiter enzyme immunoassays (EIAs) for the detection of *M. pneumoniae* have been developed. These assays use either whole-cell lysates, which contain glycolipid antigens, or protein extracts without glycolipid antigens (15). Furthermore, enrichment for the cytadhesin protein P1 and short synthetic peptides has been used to further improve the performances of the *M. pneumoniae* antibody EIAs (11, 25, 29). Despite their availability, the performances of the serologic tests have not been compared on a large scale, and the studies that have been performed on this subject used different clinical parameters or the consensus of the results of serologic assays (1, 22, 26, 27, 33). Recently, the detection of *M. pneumoniae* DNA in throat swab specimens by PCR has been found to be a highly sensitive and specific diagnostic technique for the diagnosis of acute *M. pneumoniae* infection (7, 26, 30). Therefore, the detection of *M. pneumoniae* DNA in throat swab specimens by PCR may provide an improved standard for the diagnosis of acute *M. pneumoniae* infection (18). In the present study, 11 commercially available EIAs, the CFT, and a MAG assay widely used for the diagnosis of *M. pneumoniae* infection were evaluated by using serum samples from 27 cases with a known time of disease onset. This first study of a very large number of *M. pneumoniae* serologic assays with samples from patients with acute LRTIs, with PCR used as a "gold standard," shows considerable differences between their performances.

* Corresponding author. Mailing address: Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Phone: 31 71 526 3646. Fax: 31 37 524 8148. E-mail: M.F.C.Beersma@lumc.nl.

MATERIALS AND METHODS

Patients and sera. Patients with acute *M. pneumoniae* infection were obtained from two prospective studies on LRTIs in Leiden, The Netherlands, and Antwerp, Belgium. Acute-phase and convalescent-phase sera as well as a throat swab specimen for PCR were collected from 27 patients. The ages of the patients ranged from 4 to 74 years (median age, 43 years). All but two patients were older than 20 years of age. For all patients the first day of illness was recorded during anamnesis at the first visit. The range of time between the collection of acute- and convalescent-phase sera was 7 to 48 days (mean, 15.8 days). A total of 46 serum specimens (19 paired serum specimens and 8 single serum specimens) were collected from patients with a PCR-confirmed diagnosis of *M. pneumoniae* infection. All but one of the single serum specimens were acute-phase samples, three of which had been drawn within 7 days after the onset of disease. Ten serum specimens (seven acute-phase serum specimens and three convalescent-phase serum specimens) had a restricted volume and were omitted only for the ImmunoCard test because of the large serum volume (500 μl) that is required for this test. Control sera ($n = 96$) were obtained from 20 patients (33 serum specimens) with acute LRTIs that were due to various bacterial or viral agents but that were negative for *M. pneumoniae* by PCR and from 61 patients (63 serum specimens) with LRTIs due to various well-documented infections caused by bacteria or viruses other than *M. pneumoniae*. Patients infected with cytomegalovirus, Epstein-Barr virus (EBV), *Bartonella henselae*, *Rickettsia conorii*, and *Treponema pallidum* had no clinical signs of an LRTI. All control sera were collected during the acute phase of infection.

Real-time PCR. The real-time PCR detection technique used in this study has been described recently (26). Briefly, throat swab specimens (which were obtained with cotton-tipped swab samples) were obtained during the first visit of the patient and were stored at −70°C. The medium used for the transport and storage of the throat swab specimens was SP4 broth (30). Nucleic acids were extracted with a QiaAmp DNA kit (QIAGEN, Hilden, Germany). Primers and molecular beacon sequences were selected from the P1 cytadhesin sequences of *M. pneumoniae*. Amplification was carried out with platinum Supermix (Invitrogen, Paisley, United Kingdom) in an I-cycler IQ real-time PCR platform (Bio-Rad, Veenendaal, The Netherlands). Negative controls were included repeatedly at every fifth position in each run. Each sample was spiked with 10^3 copies of a phocine herpesvirus internal control that was coextracted and coamplified with the sample to check for proper DNA isolation and inhibition, as described previously (26).

Commercial assays. Twelve commercial immunoassays (10 microtiter EIAs, one rapid membrane EIA, 1 MAG assay, and the CFT) were evaluated. All microtiter EIAs except for the Diagnosys EIA separately detected immunoglobulin G (IgG) or IgM; the Diagnosys EIA detects IgA and IgM combined. Serum samples were tested in a single run for each particular IgG or IgM assay. Repeat testing was not performed. All tests were performed according to the manufacturer's instructions. Preadsorption of IgG rheumatoid factor was performed if it was so indicated by the manufacturer. Distinct kit vials always shared the same lot or batch number. Extinction ratios were calculated on a Titertek Multiscan MCC/340 instrument (Merlin Diagnostic Systems).

Calculation of cutoff values and interpretation of the results was performed in accordance with the instructions of the manufacturers. A significant increase in the IgG titer in paired serum samples was defined as either seroconversion or a threefold increase in the IgG titer, unless stated otherwise by the manufacturer.

The AniLabsystems EIA (Labsystems, Helsinki, Finland; purchased from Labsystems, Tilburg, The Netherlands) is a microtiter EIA for *M. pneumoniae*-specific IgG or IgM antibodies. The antigen used is enriched for cytadhesin protein P1. It is recommended that IgG and IgM tests with samples from older patients be performed in conjunction with an IgA test. Results can be expressed in enzyme immunounits.

The Biotest EIA (Biotest; purchased from Biotest Seralc, Soest, The Netherlands) is a semiquantitative microtiter EIA for the detection of *M. pneumoniae*-specific IgG, IgM, or IgA antibodies. The assay is based on purified homogeneous antigen (whole-cell lysates). The results are expressed in arbitrary units/ml on the basis of a two-point standard curve. Pretreatment of sera with the IgG rheumatoid factor absorbent from Biotest is recommended.

CFT was performed with a commercially available *M. pneumoniae* whole-cell preparation of strain ATCC 10119 (Virion/Serion, Würtzburg, Germany). The assay detects antibodies to *M. pneumoniae* glycolipids. Titers of ≥1:64 were interpreted as positive.

The Diagnosys EIA (purchased from MP Products, Amersfoort, The Netherlands) is a semiquantitative microtiter EIA for the detection of specific *M. pneumoniae*-specific IgG or IgM-IgA antibodies. The test uses *M. pneumoniae* membrane proteins that are purified from lysates and that contain P1 protein.

The ImmunoCard EIA (Meridian Bioscience, Cincinnati, Ohio; purchased from Meridian Bioscience Europe, Boxtel, The Netherlands) is a rapid card-based membrane EIA for the detection of *M. pneumoniae*-specific IgM antibodies. The test can be performed in 5 min. The *M. pneumoniae* antigens used in the test are not specified. The test result consists of a visual color change. The test is approved for clinical use by the Food and Drug Administration (FDA).

The ImmunoWell EIA (GenBio; purchased from Biomedical Diagnostics, Bruges, Belgium) is a semiquantitative microtiter EIA for the detection of *M. pneumoniae*-specific IgG and IgM. The microtiter plates are coated with purified glycolipid extract (obtained with chloroform-methanol) of *M. pneumoniae* strain FH (ATCC 15531). A "low-positive" result is considered a borderline result. The test is approved for clinical use by FDA.

The Novum EIA (Novum Diagnostica, Dietzenbach Germany; purchased from Orange Medicals, Tilburg, The Netherlands) is a semiquantitative microtiter EIA for the detection of *M. pneumoniae*-specific IgM and IgG antibodies. The test is based on an ether-extracted antigen.

The Platelia EIA (Pasteur; purchased from Bio-Rad Laboratories, Veenendaal, The Netherlands) is a semiquantitative microtiter EIA for the detection of *M. pneumoniae*-specific IgG or IgM antibodies. The IgM test is a double-sandwich EIA that captures serum IgM antibodies. IgG results (in absorbance units/ml) and IgM results (as ratios) can be calculated manually or automatically by using a calibration curve. The antigen production process includes a selective enrichment of P1 cytadhesin and some other cytoadherence-associated proteins.

The Ridascreen EIA (r-biopharm, Darmstadt, Germany; purchased from Bipharma) is a semiquantitative microtiter EIA for the detection of *M. pneumoniae*-specific IgG, IgM, or IgA antibodies. Detection of IgA is recommended to detect possible colonization. The results are expressed in U/ml on the basis of a calibration curve and a lot-dependent correction factor.

The Serion classic EIA (Virion/Serion, Würzburg, Germany) is a quantitative EIA for the detection of *M. pneumoniae*-specific IgG or IgM antibodies. The use of the IgA EIA is recommended as an essential complement to the IgG and IgM EIA, especially in cases of reinfections. To compensate for normal test variations, a reference serum sample is used in each individual test run. The result is given in U/ml on the basis of a standard curve and an evaluation table included with the test kit. Automatic test evaluation can be performed with 4PL evaluate software. The antigen is enriched for the partially purified P1 major antigen.

The SeroMP EIA (Savyon Diagnostics, Ashdod, Israel; purchased from Bipharma, Weesp, The Netherlands) is a semiquantitative indirect microtiter EIA for the detection of *M. pneumoniae*-specific IgG or IgM antibodies. The result is expressed in arbitrary binding units/ml on the basis of a standard curve with three calibrators. The antigen used is a membrane preparation of *M. pneumoniae* that contains the P1 membrane protein. The manufacturer recommends the detection of IgA, in addition to the detection of IgG and IgM.

SerodiaMycoII (Fujirebio Inc, Tokyo, Japan) is a quantitative MAG assay for the detection of *M. pneumoniae* antibodies. The test exclusively detects *M. pneumoniae*-specific IgM and is based on a crude mixture of *M. pneumoniae* antigens (Mac strain). The results are expressed as titers. The cutoff titer recommended by the manufacturer is 1:80.

The Virotech EIA (Genzyme Virotech, Russelsheim, Germany) is a semiquantitative microtiter EIA for the detection of *M. pneumoniae*-specific IgG or IgM antibodies. The EIA is based on a defined antigen that includes P1, P100 (P116), and P30. The results are expressed in Virotech units/ml.

Statistics. Diagnostic sensitivity was defined as the fraction of the patients correctly identified as having *M. pneumoniae* infections (on the basis of the results of PCR with throat swab specimens). Analytical sensitivity was defined as the number of serum samples for which the results were correctly identified. The clinical accuracies of the serologic assays were examined by using receiver operating characteristic (ROC) plots with SPSS software (version 10.0.7). ROC plots were calculated by expressing the cutoff relation between the fraction "correctly identified to be positive" and the fraction "falsely identified to be positive." The area under the curve (AUC) is a measure that shows how well the assay separates "true positives" from "true negatives." AUC values range between 0.5 (no separation) and 1.0 (full separation) (21).

RESULTS

Sensitivities and specificities of the IgM assays. Thirteen serologic assays for *M. pneumoniae* (11 microtiter EIAs, 1 MAG assay, and the CFT) were evaluated by using a panel of 46 serum specimens from 27 PCR-positive cases with *M. pneu-*

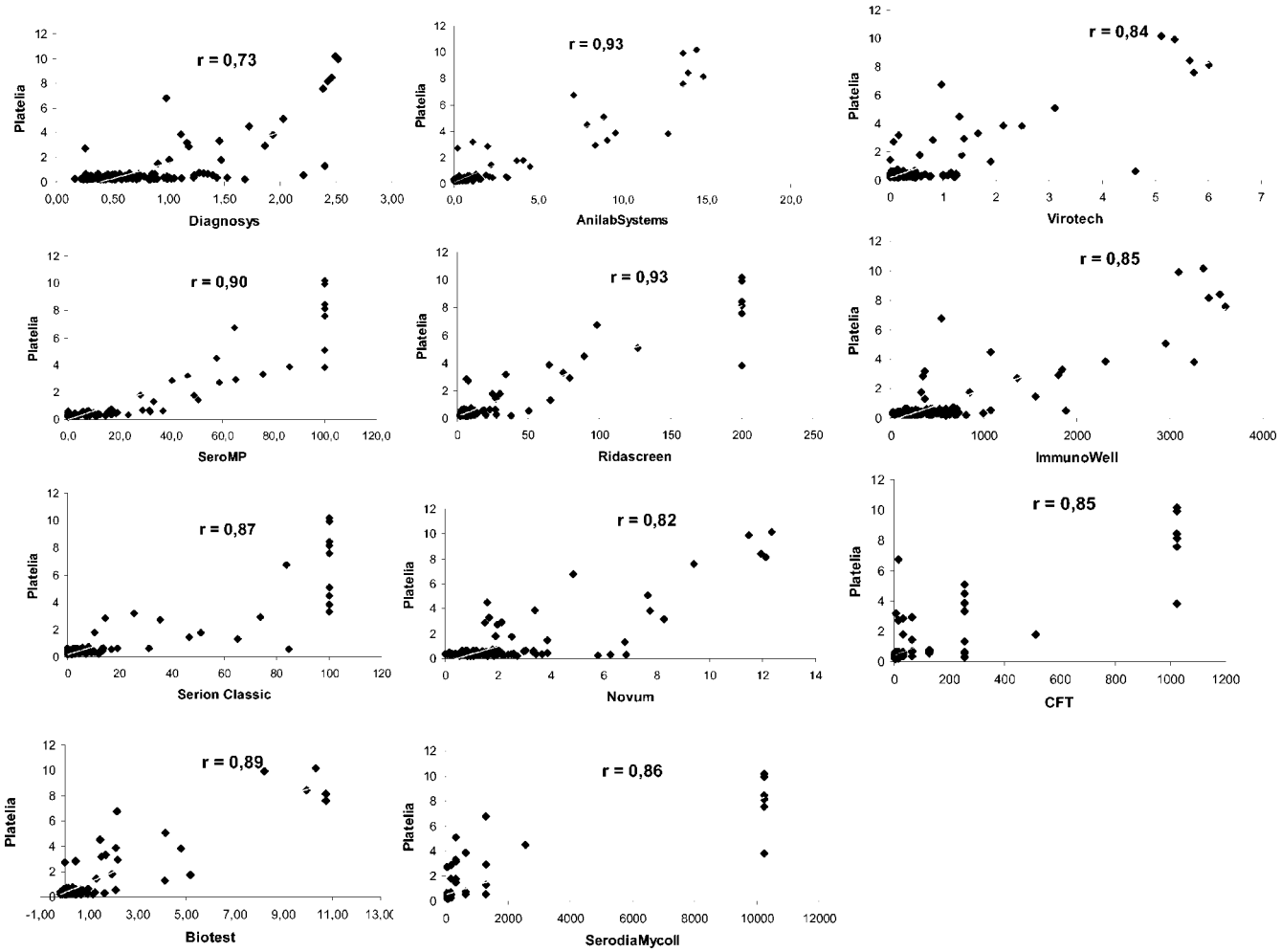

FIG. 1. Spearman correlation of *M. pneumoniae* IgM titers obtained by various IgM assays in relation to those obtained by the Platelia IgM test. All correlations were significant ($P < 0.001$).

moniae infection and 96 control serum specimens. To examine the distribution of the IgM levels, the IgM results obtained by the various assays were arbitrarily compared to those obtained by the Platelia μ-capture IgM test. The results showed a normal distribution in a bimodal fashion and significant correlations between the results of the various tests and the Platelia IgM test results (Fig. 1). The diagnostic sensitivities of the IgM assays for the cases is shown in Table 1. Twenty of the 27 cases (74%) were positive for IgM by at least 1 of the 13 assays evaluated. In seven cases no IgM was detected by any of the tests. The diagnostic sensitivities of the IgM assays with acute-phase sera ranged from 16% (ImmunoWell and Virotech assays) to 42% (Novum and AniLabsystems assays), and on analysis of convalescent-phase sera, the sensitivities for the detection of IgM ranged from 32% (Ridascreen assay) to 84% (AniLabsystems).

To evaluate the analytical sensitivities of IgM assays with case sera, sera were grouped according to the duration of disease: early phase, 1 to 6 days after the onset of disease symptoms; middle phase, 7 to 15 days after the onset of disease symptoms; and late phase, ≥16 days after the onset of disease symptoms. The sensitivities of the IgM EIAs and those of the MAG assay and CFT ranged from 7 to 25% for the early-phase samples, 31 to 69% for the middle-phase samples, and 33 to 87% for the late-phase samples (Fig. 2). To calculate the overall analytical sensitivities of the IgM assays, the results for the early-phase sera were discarded and only those for the middle- and late-phase sera were used. The overall analytical sensitivities ranged from 35% to 77%. The AniLabsystems, Novum, and SeroMP assays revealed the best analytical sensitivities (77%, 71%, and 71%, respectively) (Table 2).

The IgM results for the control sera are shown in Table 2. The specificities of the IgM assays ranged from 49% to 100%. The assays with the best specificity values were the Ridascreen and Platelia assays and CFT, followed by the Virotech and ImmunoWell assays. Most of the assays showed a high rate of false-positive results with sera from patients with acute EBV infection (Table 3).

PPVs and NPVs of the IgM assays. The positive predictive values (PPVs) ranged from 31% to 100% (Table 2). Most of the assays tested had PPVs between 60% and 80%. The best PPVs were for the Ridascreen and Platelia assays and the CFT. The negative predictive values (NPVs) ranged from 83% to 94%. The best NPVs were for the AniLabsystems and SeroMP

TABLE 1. Diagnostic sensitivities of *M. pneumoniae* IgM and IgG assays

Assay	No. (%) of serum specimens positive for:				IgG increase	
	IgM		IgG			
	Acute phase (n = 19)	Convalescent phase (n = 19)	Acute phase (n = 19)	Convalescent phase (n = 19)	No. (%) of paired serum specimens	No. (%) of IgM-negative paired serum specimens
AniLabsystems	8 (42)	16 (84)	17 (89)	19 (100)	9 (64)	2/3 (67)
Biotest	5 (26)	10 (53)	17 (89)	19 (100)	10 (53)	6/9 (67)
CFT	6 (32)	15 (79)				
Diagnosys	6 (32)	13 (68)	14 (74)	17 (89)	5 (26)	3/6 (50)
ImmunoCard	5 (33)[a]	7 (44)[b]				
ImmunoWell	3 (16)	7 (37)	8 (42)	16 (83)	6 (32)	6/12 (50)
Novum	8 (42)	15 (79)	11 (58)	17 (90)	11 (58)	2/4 (50)
Platelia	6 (32)	9 (47)	15 (79)	18 (95)	9 (47)	5/10 (50)
Ridascreen	4 (21)	6 (32)	8 (42)	17 (85)	12 (63)	9/13 (64)
Serion classic	4 (21)	10 (53)	10 (53)	8 (95)	13 (68)	6/9 (67)
SerodiaMycoII	7 (37)	15 (79)				
SeroMP	7 (37)	15 (79)	7 (37)	7 (85)	11 (58)	2/4 (50)
Virotech	3 (16)	10 (53)	8 (42)	7 (90)	10 (53)	4/9 (44)

[a] n = 15.
[b] n = 16.

assays and the CFT. The accuracies of the assays ranged from 54% to 89%. The highest accuracy results were found for the CFT (89.0%), the AniLabsystems assay (88.1%), and the Platelia assay (86,6%).

Calculation of ROC AUC for the IgM assays. Analysis of the agreement between the results of the IgM assays and the PCR results for the classification of negative and positive cases revealed rates of concordant results of between 89.0% and 54.3% (Table 2). The EIAs with the most concordant results were CFT (89.0%), the AniLabsystems assay (88.1%), the Patelia assay (86.6%), and the Diagnosys assay (85.0%). The SerodiaMycoII assay results also showed good agreement with those of PCR (81.9%). To further evaluate the diagnostic performances independently from the cutoff values, ROC AUC values were calculated for the individual assays (Table 4). The EIAs with the highest AUCs were the Platelia assay (0.87) and the AniLabsystems assay (0.85). Two other EIAs with AUCs above 0.8 were the Serion classic assay (0.81) and the SeroMP assay (0.8). The non-EIAs (CFT and the SerodiaMycoII MAG test) had AUC values of 0.94 and 0.83, respectively.

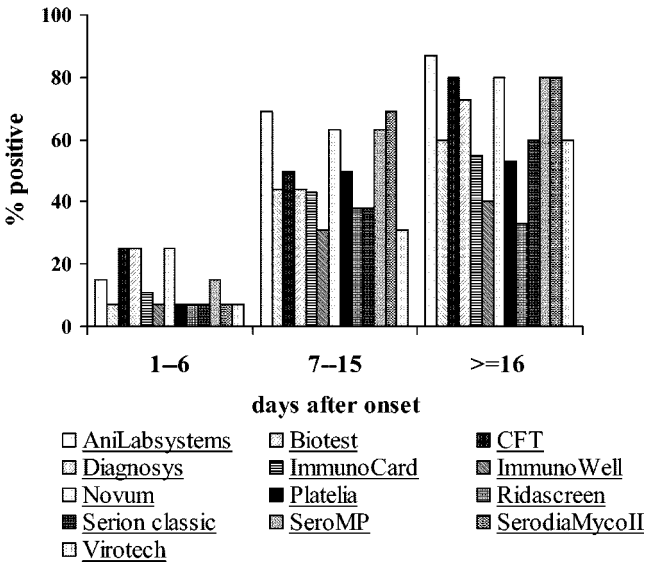

FIG. 2. Analytical sensitivities (percent) of IgM assays in relation to the duration of illness: 1 to 6 days, n = 15; 7 to 15 days, n = 16; ≥16 days, n = 15.

IgM discrepancy analysis. Seven PCR-positive patients (10 serum specimens) had a negative outcome for IgM by all assays, including the CFT. For four of these patients, only a single serum sample had been obtained in the first week after the onset of disease, which could explain the negative outcome. For three patients, the IgM result was negative with both the acute- and the convalescent-phase samples. None of these three patients had detectable IgG in the acute-phase serum, suggesting the absence of infection in the past. Two patients (both of whom were more than 40 years old) showed IgG seroconversion in the convalescent phase. The third patient (age, 25 years), however, developed no IgG response in the convalescent phase (33 days). Equivocal results occurred frequently by the AniLabsystems EIA (32 of 145 specimens), the SerodiaMycoII EIA (29 of 144 specimens), and the Novum EIA (20 of 144 specimens). The other assays had less than 7% equivocal results.

Five control serum specimens showed IgM reactivity by at least four of the IgM assays tested. All of these serum specimens were from patients with acute EBV infection, and some of them tested positive for heterophilic antibodies (Table 3). The clinical data for these patients revealed no evidence of recent respiratory disease. None of the other control serum specimens revealed positive IgM results by more than four different assays. All IgM assays with low specificities provided positive results for a broad range of control sera that were not confined to certain specific pathogens.

Antigen composition and IgM test performances. Since the ROC AUC presents a good parameter for the diagnostic power of an individual test, we looked for associations between the antigenic compositions of the assays and the ROC AUC outcome. The tests that used crude or ether-extracted whole-cell antigen, including glycolipids, were CFT and the Serodia-

TABLE 2. Analytical sensitivities, specificities, and predictive values of IgM detection in relation to PCR results[a]

Assay	No. (%) of serum specimens		PPV (%)	NPV (%)	% Agreement
	Case sera testing positive (n = 31)[b]	Control sera testing negative (n = 96)			
AniLabsystems	24 (77)	88 (92)	24/32 (75)	88/95 (93)	88.1
Biotest	16 (52)	91 (95)	16/21 (73)	91/106 (86)	84.3
CFT	20 (65)	93 (97)	20/23 (87)	93/104 (89)	89.0
Diagnosys	18 (58)	90 (94)	18/24 (75)	90/103 (87)	85.0
ImmunoCard	12 (48)[c]	76 (79)	12/32 (38)	76/89 (85)	72.7
ImmunoWell	11 (35)	92 (96)	11/15 (73)	92/112 (82)	81.1
Novum	22 (71)	47 (49)	22/71 (31)	47/56 (84)	54.3
Platelia	16 (52)	94 (98)	16/18 (89)	94/109 (86)	86.6
Ridascreen	11 (35)	96 (100)	11/11 (100)	96/116 (83)	84.3
Serion classic	15 (48)	91 (95)	15/20 (75)	91/107 (86)	83.5
SeroMP	22 (71)	84 (88)	22/34 (65)	84/93 (92)	83.5
SerodiaMycoII	20 (65)	84 (88)	20/32 (63)	84/95 (88)	81.9
Virotech	14 (45)	92 (96)	14/18 (78)	92/109 (84)	83.5

[a] Equivocal results were considered negative.
[b] More than 6 days after disease onset.
[c] n = 25.

MycoII and ImmunoWell assays. The assays that used P1-enriched antigen were Diagnosys, Biotest, AniLabsystems, Serion classic, and the Platelia assays. The Virotech assay used recombinant proteins (p1, p100, or p30). Three tests did not further specify the antigen used. All EIAs that used crude antigen or that did not specify the antigen had AUCs below 0.7. However, CFT and the SerodiaMycoII assay, which also used crude antigen preparations, had AUC values ≥0.8. EIAs that used P1-enriched or recombinant proteins all had AUC values >0.7.

IgG seroprevalence and agreement. The prevalence of IgG in the control sera ranged from 36% (Virotech assay) to 93% (AniLabsystems assay). No data on the specificities of the IgG tests for the control sera could be obtained, since it is unknown whether the patients had previously contracted *M. pneumoniae* infections. However, an assessment of the distribution of the IgG results by the different tests was obtained by comparing the IgG results obtained by the assays with those obtained by the Platelia IgG test (Fig. 3). All correlations between the Platelia IgG test and the other IgG assay results were highly significant (Spearman correlation, $P < 0.001$). The prevalence of IgG antibodies in early-phase samples (obtained <6 days after the onset of disease) varied between 14% (Virotech assay) and 80% (AniLabsystems and Biotest assays) (data not shown). The prevalence of IgG in acute-phase sera from paired serum samples varied between 37% (SeroMPl IgG) and 89% (AniLabsystems and Biotest IgGs) (Table 1). Almost all IgG assays had more than 85% positive IgG results with the convalescent-phase sera (Table 1).

In the control group, 19% (18 of 96) of the serum specimens were negative for *M. pneumoniae* IgG by all assays. Thirty-five percent (34 of 96) of the control serum specimens had no

TABLE 3. False-positive results for sera from controls previously diagnosed with infections other than *M. pneumoniae* infection

Virus or bacterium	No. of serum specimens tested	No. of serum specimens with false-positive results by the following serologic assay[a]:												
		Ani	Bio	CFT	Dia	ImC	ImW	Nov	Pla	Rid	SeC	SeM	SII	Vir
None	2		1					2				1	1	
Influenza A virus	4							4						
Influenza B virus	4	2			1	2		2				2	2	
Parainfluenza virus	6		1		1	2		4				4		
Respiratory syncytial virus	3													
Adenovirus	2	1				1	2							
Cytomegalovirus	6					2		5					1	
Ebstein-Barr virus	10	2	3	2	3	1	1	8	2		3	6	6	1
B. henselae	9					2		5				2		1
Bordetella pertussis	5							2				1	1	
Coxiella burnetii	4					3		4				2		
Chlamydia pneumoniae	4					3		2						
Legionella pneumophila	10			1	1	1		4						1
R. conorii	1													
Streptococcus pyogenes	10	3				3		3			1	3	1	
T. pallidum	6						1	4			1	4		
Total	96	8	5	3	6	20	4	49	2	0	5	25	12	3

[a] Ani, AniLabsystems; Bio, Biotest; Dia, Diagnosys; ImC, ImmunoCard; ImW, ImmunoWell; Nov, Novum; Pla, Platelia; Rid, Ridascreen; SeC, Serion classic; SeM, SeroMP; SII, SerodiaMycoII; Vir, Virotech.

TABLE 4. ROC AUC[a] values of IgM assays[b]

Assay	ROC AUC (SE)[c]	95% CI[d]
AniLabsystems	0.85 (0.056)	0.74–0.96
Biotest	0.76 (0.059)	0.64–0.87
CFT	0.94 (0.033)	0.87–1.00
Diagnosys	0.75 (0.065)	0.63–0.88
ImmunoCard	0.64 (0.068)	0.50–0.77
ImmunoWell	0.67 (0.063)	0.54–0.79
Novum	0.66 (0.069)	0.53–0.80
Platelia	0.87 (0.043)	0.79–0.96
Ridascreen	0.76 (0.062)	0.64–0.88
Serion classic	0.81 (0.055)	0.70–0.92
SeroMP	0.80 (0.058)	0.69–0.92
SerodiaMycoII	0.83 (0.053)	0.73–0.93
Virotech	0.73 (0.066)	0.60–0.86

[a] ROC estimate of the area under the curve.
[b] Total number of sera tested, 142.
[c] SE, standard error.
[d] CI, confidence interval.

agreement between the various tests. Forty-six percent (44 of 96) of the control serum specimens were positive for IgG by all assays.

IgG sensitivity and specificity. By using either seroconversion or a threefold increase in the ratio or arbitrary units as a criterion, a significant rise of IgG was detected in 26 to 68% of the cases (Table 1). The increase in diagnostic reliability by detecting both IgM and IgG in paired serum specimens compared to the diagnostic reliability of IgM detection without IgG detection ranged from 5% (1 of 19 specimens; Novum assay) to 47% (9 of 19 specimens; Ridascreen assay) (mean, 22%) (Fig. 4). Furthermore, an increase in IgG titers in paired serum specimens in which no IgM was detected occurred in 44% (Virotech assay) to 67% (AniLabsystems, Serion classic, and Biotest assays) of the serum specimens (Table 1). Since the ImmunoCard assay and the SerodiaMycoII MAG test do not detect IgG, whereas the CFT cannot differentiate between immunoglobulin classes, any effect of IgG testing was not examined for these assays.

DISCUSSION

In the present study, 13 serologic assays for *M. pneumoniae*-specific IgG and IgM antibodies were evaluated with sera prospectively collected from predominantly adult patients with acute LRTIs, with PCR used as a reference standard. The reliability of nucleic acid amplification techniques for *M. pneumoniae* detection in throat swab specimens has been addressed in a number of studies that were recently reviewed by Loens et al. (18). These studies showed that these PCR assays had high sensitivities and specificities for the detection of *M. pneumoniae* in children and adults with acute LRTIs. Some studies reported on the detection of *M. pneumoniae* in specimens from healthy subjects, suggesting that transient asymptomatic carriage may occur in immunocompetent individuals (13). However, their numbers in outbreak-free periods were reported to be very low (≤0.1%) (16, 28, 31). In our study, the convales-

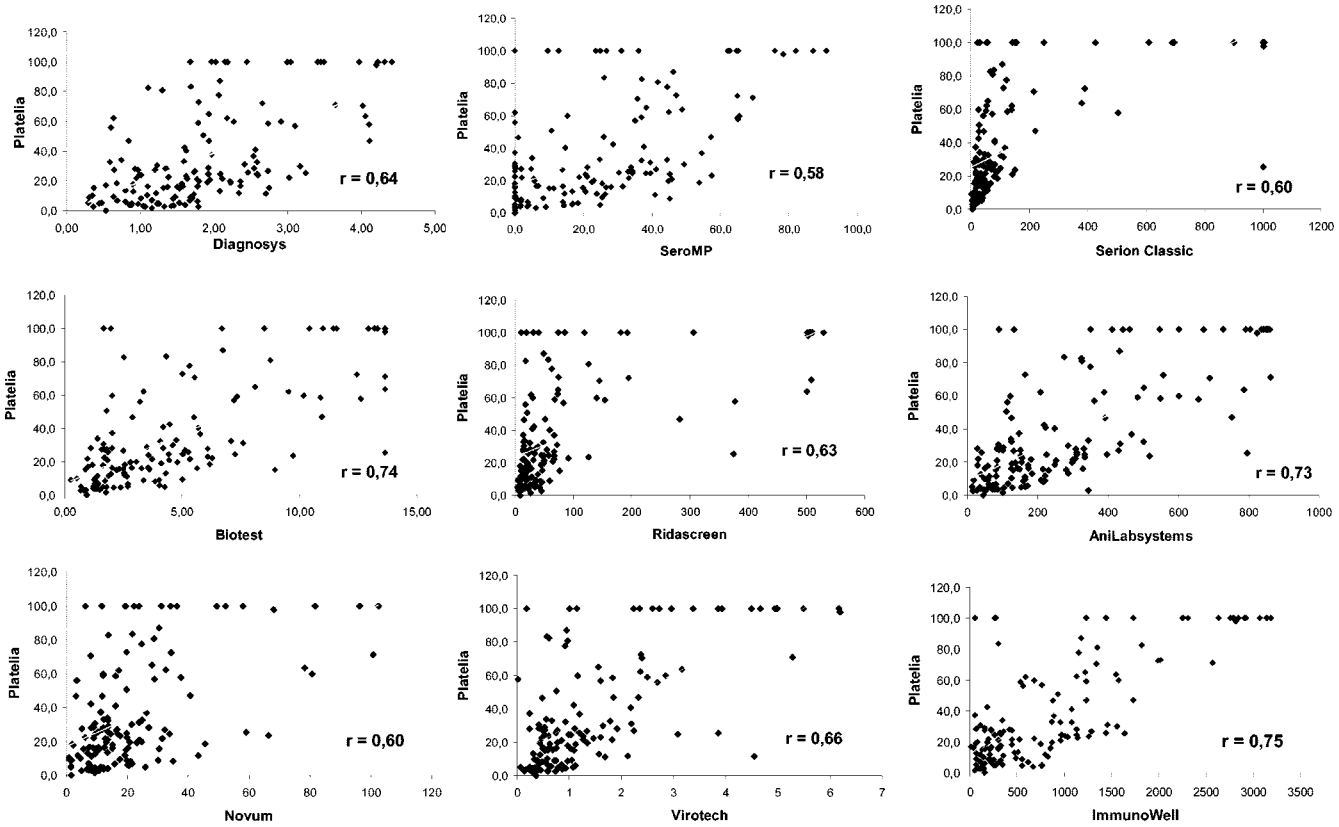

FIG. 3. Spearman correlation of *M. pneumoniae* IgG titers obtained by different assays in relation to those obtained by the Platelia IgG test. All correlations were significant ($P < 0.001$).

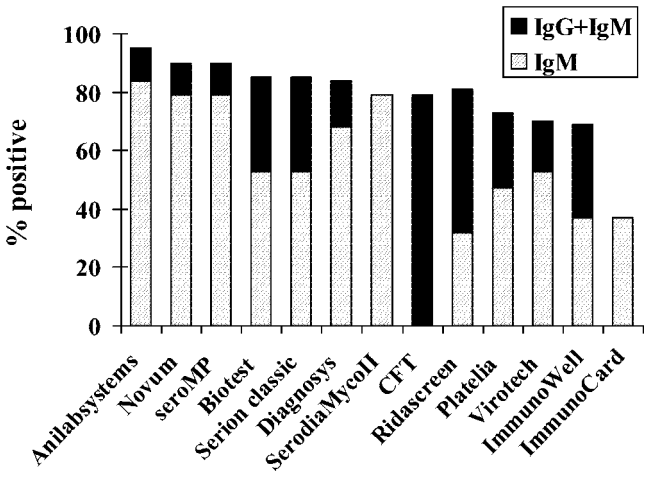

FIG. 4. Increase in diagnostic yields by testing for IgG antibodies in addition to IgM antibodies in paired serum samples for different assays ($n = 19$).

cent-phase sera of 18 of the 19 PCR-positive patients for whom paired serum samples were available had significant IgM or IgG responses. The results thus confirm the value of PCR as an improved reference standard for the diagnosis of *M. pneumoniae* infection, but they also stress the need for further studies on this subject. To prevent false-positive results, PCR tests require extensive validation and quality control of the procedures. The internally controlled real-time PCR assay that was used in the present study was clinically validated, and positive results were previously confirmed by 16S rRNA nucleic acid sequence-based amplification, with 100% agreement (19, 26).

The results of evaluation of the IgM assays showed that the diagnostic sensitivities of the IgM assays with paired serum samples were comparable and ranged from 32% to 84% (Table 1). The best sensitivities (>75%) of the IgM assays were found for the AniLabsystems, SerodiaMycoII, Novum, and SeroMP assays. For evaluation of the analytical sensitivity for IgM detection, information on the illness on the first day proved essential, since our results showed the absence of IgM in the first week after the onset of symptoms of illness for all assays in more than 80% of the early-phase serum specimens (3). As a whole, the sensitivity results for the IgM assays in our study tended to be below those reported in the literature (29, 30, 32). This difference might be explained to some extent by the predominance of adults in our population, but it may also relate to the use of a highly sensitive gold standard, i.e., PCR. In a recent study with adults cases, which also used PCR as the gold standard, similar sensitivities ranging from 38% to 77% were reported (22).

Evaluation of the specificities of the IgM antibody assays by testing control sera from patients with other acute viral or bacterial infections showed good specificities (>95%) for the majority of the assays (the Serion classic, Platelia, Biotest, Ridascreen, Virotech, and ImmunoWell assays and CFT) (Table 2). This is in agreement with the results of studies in the literature that reported good specificities for CFT and the Platelia, Biotest, and ImmunoWell assays (10, 22, 32). IgM assays with specificities below 80% (the SeroMP, Novum, and ImmunoCard assays) showed reactivities to a wide range of control sera, which may be due to improper assay optimization. Several assays, notably, the Novum, SeroMP, and SerodiaMycoII assays (Table 4), showed false-positive results for EBV IgM-positive sera, which was not clearly related to the presence or absence of heterophile antibodies. The increased reactivity of *M. pneumoniae* IgM in sera from patients with acute EBV infection has been reported previously and may be relevant for clinical practice (9).

The evaluation of the ROC AUCs of the various IgM assays, including CFT, allowed comparison of their diagnostic powers without dependence on the chosen cutoff values. The highest ROC AUC values (more than 0.8) were found for CFT and the Platelia, AniLabsystems, Serion classic, SerodiaMycoII, and SeroMP assays (Table 3). Of interest, the best ROC AUC result was found for the CFT (0.94). Whereas the CFT has always been considered a standard for *M. pneumoniae* serology, several studies reported that it had a lower sensitivity and a lower specificity than the EIAs (14, 23). Our findings, however, are in agreement with the recent results of Dorigo-Zetsma et al. (10), who compared PCR and CFT in a large study. From their results, a good sensitivity and a good specificity for the CFT (71% and 99%, respectively) can be calculated if the PCR results from their study were used as a standard. The high ROC AUC values for the P1-enriched AniLabsystems IgM EIA confirmed previous studies from Tuuminen and colleagues (25, 29), who reported on the superiority of assays with the P1-enriched antigen on the basis of a comparison of the strengths of the immunoresponses. Since the Anilabsystem EIA revealed good test optimization as well (both good sensitivity and good specificity results), this test eventually showed the best overall test performance. Good ROC AUC values were also found for the SerodiaMycoII MAG assay, which is used in many laboratories because of its practical advantages. This assay was reported to be slightly more sensitive than CFT (2, 14). However, our results revealed that the assay had a moderate specificity (88%), and since the assay exclusively detects IgM, it may be of limited use with samples from adult patients, who are known to develop poor IgM responses. Another IgM assay with a good ROC AUC was the Platelia assay, which is an EIA based on the μ-capture technique. Although our results showed a very good specificity (98%) for the assay, we found that it had only moderate sensitivities. Other studies also reported a high specificity for the Platelia EIA as well as good sensitivity with samples from children but low sensitivity with samples from adults (38%) (22, 32). Suni et al. (25) reported a high sensitivity and a high specificity (100% and 97%, respectively) for the Platelia assay. Our results showed only moderate sensitivity, specificity, and ROC values (<0.65) for the ImmunoCard assay, which is a 10-min rapid test approved by FDA for use with samples from children. These findings confirm the findings of others (27, 33), who reported low performances of the ImmunoCard assay with samples from adults, but they are in contrast to the findings of studies by Alexander et al. (1) and Matas et al. (20), who reported more than 90% sensitivity. The latter study reported an even superior sensitivity for the ImmunoCard assay with samples from adults compared to that of SerodiaMycoII MAG test or the CFT, but they did not include control groups to evaluate the specificity.

The evaluation of the performances of IgG assays is complicated by the lack of information on *M. pneumoniae* infections in the past. However, detection of high percentages (>70%) of positive IgG results by some assays with early acute-phase sera (<6 days illness) from cases suggested some false-positive reactivities for those assays. Since the EIA ratios were low, optimization of the assays by changing the cutoff levels may be considered. Evaluation of the IgG specificity by the use of immunoblot assays may be required to further address the specificities of the IgG assays.

Since adult patients in particular are known to develop weak IgM responses during primary infection or reinfection (5, 24), it was important to examine the diagnostic yields of testing for IgG and IgM combined for the individual assays. The results showed substantially higher diagnostic yields with paired serum specimens of assays for IgG and IgM combined compared with those for assays for IgM alone, especially for those assays with a low sensitivity for IgM detection (Table 1; Fig. 4). The results thus emphasize the diagnostic value of testing of paired serum samples for IgG and IgM combined and show good overall diagnostic yields for specific tests, such as the AniLabsystems assay (95%), the Serion classic and Biotest assays (both 85%), and the Diagnosys assay (84%). In our study, a possible additive role of testing for IgA in single or paired serum specimens was not addressed, although one of the assays that we tested (Diagnosys) actually detects IgA and IgM combined. The performance of this assay for IgA and IgM combined did not differ from the those of other tests. Some manufacturers state that testing for IgA should be regarded as an essential complement of the IgG and IgM EIAs. The manufacturers of the AniLabsystems, Biotest, Ridascreen, SeroMP, and Diagnosys assays supply IgA tests, in addition to IgG or IgM assays. To our knowledge, PCR-controlled studies for IgA have not been published (33).

In summary, the results of the present study demonstrate that all tests evaluated except the Novum test have good specificities for the detection of IgM. Most of the IgM assays showed inaccurate sensitivities, although good diagnostic sensitivities (>75%) were obtained with the AniLabsystems assay; CFT; and the Novum, SerodiaMycoII, and SeroMP assays. However, because of the lack of specificity, the Novum test is inaccurate for the routine detection of *M. pneumoniae* IgM. Good IgM ROC AUC values (>0.8) were found for the CFT and the Platelia, AniLabsystems, SerodiaMycoII, Serion classic, and SeroMP assays. When all parameters are considered together, the AniLabsystems IgM EIA eventually showed the best performance. Our study demonstrated that most IgG assays showed good overall sensitivities for the detection of significant increases in IgG titers. Hence, testing for IgG in addition to IgM in paired serum specimens generated better diagnostic yields than those obtained by CFT and the MAG assay.

Serology remains a practical and undemanding method for the diagnosis of *M. pneumoniae* infection, particularly in young patients with a time of disease onset of more than 1 week earlier. However, given the low sensitivities of IgM assays, particularly for adult patients, who are known to develop weak antibody responses, and the need for paired serum specimens with a rise in IgG antibody titer for the diagnosis of *M. pneumoniae* infection, nucleic acid amplification methods might become the preferred diagnostic procedures for the diagnosis of *M. pneumoniae* infections, provided that the quality of the procedures is guaranteed.

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