

Measurement of *Chlamydia pneumoniae*-Specific Immunoglobulin A (IgA) Antibodies by the Microimmunofluorescence (MIF) Method: Comparison of Seven Fluorescein-Labeled Anti-Human IgA Conjugates in an In-House MIF Test Using One Commercial MIF and One Enzyme Immunoassay Kit

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For the serological diagnosis of acute *Chlamydia pneumoniae* infection, the microimmunofluorescence (MIF) test is the most commonly used method and also the “gold standard” for the measurement of immunoglobulin G (IgG) and IgM antibodies. The role of IgA antibodies in diagnosis has not been established. Commercially available fluorescein-labeled anti-human IgA conjugates have not been systematically compared to each other, and this situation may cause considerable variations in IgA results. Therefore, we tested 261 serum samples from 122 patients with pneumonia for IgA antibodies by using six α -chain-specific anti-IgA conjugates in our in-house MIF test, one commercial MIF test, and one enzyme immunoassay (EIA). Interfering IgG antibodies were removed with Gullorsorb reagent before the measurement of IgA antibodies. Altogether, 14 significant IgA antibody increases in serum samples between the acute phase and the convalescent phase were detected by at least one of the conjugates in the MIF test, while no increases were found in the IgA EIA. Only one patient showed a significant IgA antibody increase with all of the fluorescein-labeled conjugates. Five significant titer changes were detected by at least two conjugates, and in nine instances, the titer increase was detected by one conjugate only. The titer agreement indicated by kappa coefficients was very good or good for all of the fluorescein-labeled conjugates and the EIA with low antibody titers but decreased with increasing titers.

The diagnosis of acute *Chlamydia pneumoniae* infection is usually based on the demonstration of at least a fourfold increase in immunoglobulin G (IgG) antibody levels in serum samples between the acute phase and the convalescent phase or the presence of IgM antibodies in any serum sample. The microimmunofluorescence (MIF) test is considered the “gold standard” for the measurement of chlamydia species-specific antibodies (4, 5, 11). The role of IgA antibodies in the diagnosis of acute-phase infection has not been definitely established (4), and these antibodies are not measured in all laboratories. However, the measurement of IgA antibodies has been shown to increase diagnostic findings in some studies (5, 20). Commercial fluorescein-conjugated anti-human IgA antibodies have not been standardized, and the use of different conjugates may cause considerable variations in IgA results (29). Thus, a comparison of different commercial conjugates may help laboratories to make choices and may lead to more standardized MIF antibody findings.

The removal of IgG antibodies before the measurement of IgM antibodies is generally recommended to avoid false-positive IgM findings due to the presence of IgM rheumatoid factor (7). Furthermore, IgG antibodies with a high affinity may also interfere with the measurement of IgM antibodies in

the immunofluorescence method (10). It was shown earlier that the removal of IgG antibodies before the measurement of IgA antibodies to *C. pneumoniae* by MIF makes IgA antibody reactivity easier to interpret, the prozone effect disappears, and titers increase, especially in serum samples with high IgG titers (9).

In the present study, we compared seven commercial fluorescein-conjugated anti-human IgA antibodies by using our in-house MIF test, one commercial MIF kit, and one commercial enzyme immunoassay (EIA) kit for the measurement of IgA antibodies in serum samples obtained from adult and child patients with pneumonia. For easier interpretation, we removed interfering IgG antibodies with Gullorsorb reagent before the measurement of IgA antibodies by MIF.

MATERIALS AND METHODS

Altogether, 261 serum samples from 90 adults and 32 children with pneumonia (collected in different pneumonia studies) were originally tested with the in-house MIF test for *C. pneumoniae*-specific antibodies. Paired serum samples obtained at a median interval of 21 days (range, 5 to 224 days) were available from all of the patients, and a third set of samples was obtained later (range, 14 to 28 days) from 17 patients.

The serum samples were tested by using seven different fluorescein isothiocyanate (FITC)-conjugated α -chain-specific anti-human IgA antibodies in the in-house MIF test and one commercial MIF kit (Labsystems, Helsinki, Finland). IgA antibodies were measured by the in-house MIF test (30) with purified elementary bodies of *C. trachomatis* strain L2 and *C. pneumoniae* strain K6 (5) as antigens. The seven commercial FITC-conjugated α -chain-specific anti-human IgA antibodies used were designated as follows: Dako (Dako, Glostrup, Den-

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TABLE 1. IgA increases obtained with FITC conjugates in the in-house MIF test compared to the reference method

Patient	Fold IgA increase obtained with the following test or conjugate:									Total no. of increases detected ^a	Days between serum sample collection	Test with which earlier diagnosis was made (fold IgA increase)
	Reference	MIF kit	Jackson	Sigma	Zymed	Lab-systems	Dako	Caltag	EIA kit			
1	4									1	28	
2	4	4	16	32	4	4	8	4		8	25	IgG MIF (4)
3	4									1	35	
4	4									1	28	IgG in-house MIF (4)
5	4		4							2	Missing	IgG Labsystem EIA (1.5)
6	8									1	21	Medac IgA EIA (2.8)
7		4	4			4				3	28	Savyon and Labsystem IgM EIAs
8		4								1	29	IgG in-house MIF (4) and Savyon IgG EIA (1.4)
9		16								1	27	
10		4		4						2	24	
11			4	4	8					3	29	
12							8			1	27	
13								4		1	24	
14		4								1	25	

^a Out of a possible 9.

mark), Kallestadt (Sanofi Diagnostics Pasteur), Zymed (Zymed, South San Francisco, Calif.), Jackson (Jackson ImmunoResearch), Caltag (Caltag, Burlingame, Calif.), Labsystems (Labsystems), and Sigma (Sigma, St. Louis, Mo.). Before comparison, optimal dilutions for the anti-human IgA conjugates were determined by using 10 IgA-positive serum samples at a 1/20 dilution in phosphate-buffered saline (PBS), and 2% Amido Schwartz counterstain was used for all of the conjugates. In the commercial MIF kit slides, the elementary body antigens of *C. trachomatis* and *C. pneumoniae* were treated to remove genus-specific lipopolysaccharide, while the elementary bodies of *C. psittaci* were not treated. The serum samples were tested after treatment with Gullorsorb (Gull Laboratories, Salt Lake City, Utah) in accordance with the instructions of the manufacturer to remove interfering IgG antibodies (9, 27). The serum samples were tested in serial twofold dilutions, starting at 1:10, in 0.25% PBS-egg yolk sac (Labema, Kerava, Finland), except that the commercial MIF kit had its own sample diluent.

The patient serum samples for daily use were thawed and diluted in sufficient volumes to allow simultaneous testing of all conjugates. Positive and negative control serum samples were included in all test series. The commercial MIF kit included the sample diluent, the conjugate, and the controls for the test. The serum samples were incubated on Teflon-coated slides (Knittel, Braunschweig, Germany) for 60 min in a moisture chamber at 37°C. The incubation time for serum dilutions was longer with the in-house MIF test (1 h) than with the commercial MIF kit (30 min). Slides were washed by being lifted and lowered about 20 times in four dishes with PBS (pH 7.4) and in two dishes with distilled water. After the washing and drying steps, the IgA conjugates were added and incubated in the moisture chamber for 30 min at 37°C. Mounting fluid for setting coverslips on the slides contained glycerol and Veronal buffer (0.1 M 5,5-barbituric acid, 0.15 M NaCl [pH 8.5]) (3:2). All of the slides were read by one experienced reader (M.P.) using a Leitz Aristoplan fluorescence microscope with a ×40 objective on the same day as that on which the slides were prepared or on the next day. The serum samples for the EIA were diluted (1/101) according to the manufacturer's (Labsystems) instructions without removing IgG with Gullorsorb reagent. The EIA analysis was performed in Helsinki, Finland, by the manufacturer, blinded to the results of the other tests.

The sensitivity of each test for the detection of *C. pneumoniae* seropositivity at different cutoff points (twofold serial dilutions from 1/10 to 1/160) were calculated with the reference MIF conjugate as the gold standard. The strength of agreement between the in-house MIF test with the Kallestadt conjugate versus the other conjugates was analyzed by calculating the kappa values for each titer. The strength of agreement for the kappa values was as follows: <0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; and 0.81 to 1.00, very good.

RESULTS

Among the paired serum samples from 122 patients with pneumonia, a total of 14 seroconversions for IgA antibodies were demonstrated by at least one of the anti-IgA conjugates (Table 1); all of these seroconversions were demonstrated in

adult patients. Five fourfold increases and one eightfold increase (patients 1, 2, 3, 4, 5, and 6) were detected by the reference MIF method. One of the six increases detected by the reference method (patient 2) and five new seroconversions (patients 7, 8, 9, 10, and 14) were identified by the MIF kit. Four seroconversions (patients 2, 5, 7, and 11) were demonstrated by the Jackson conjugate; two of them (patients 2 and 5) were also detected by the reference method. Three seroconversions (patients 2, 10, and 11) were found by the Sigma conjugate, and two (patients 2 and 11) were found by the Zymed conjugate; one of these (patient 2) was also found positive by the reference method. Significant titer changes in our in-house MIF test were detected for patients 2 and 7 with the Labsystems conjugate, for patients 2 and 12 with the Dako conjugate, and for patients 2 and 13 with the Caltag conjugate. Only one patient (patient 2) had an IgA antibody increase demonstrated by all of the conjugates (Table 1).

Five (36%) of the significant titer changes were demonstrated by at least two conjugates. In nine instances, the conjugates reacted alone: the Dako and Caltag conjugates in one each, the MIF kit in three, and the Kallestadt conjugate in four. The IgA EIA did not detect any of the 1.3- or 1.5-fold IgA changes in the serum samples. An acute-phase infection had been diagnosed earlier in 6 (43%) of these 14 patients (patients 2, 4, 5, 6, 7, and 8) by at least two *C. pneumoniae* tests when the serum samples were tested with two different MIF tests and three commercial EIAs for IgG, IgA, and IgM antibodies (data not shown; M. Paldanius et al., unpublished data). An earlier diagnosis had been made in four patients by the reference method and in two patients by the commercial MIF kit. Two of the acute-phase infections had been detected by the IgG and IgM EIAs.

The IgA seropositivity rates determined by the commercial EIA (54%) and MIF test (46%) at a cutoff point of 1/10 were significantly higher than those determined by the in-house MIF test (38%) (95% confidence intervals for the difference between the rates were 13 to 19 for EIA and 5 to 11 for MIF) (Tables 2 and 3). With the different FITC conjugates, agreement varied from 81 to 96% (Table 4). The best agreement with the reference method was obtained at the cutoff point of

TABLE 2. IgA seropositivity rates at different titer cutoff points

IgA cutoff	% IgA seropositivity determined with the following test or conjugate:								
	Reference	Sigma	Labsystems	Dako	Jackson	Caltag	Zymed	Labsystems MIF kit	Labsystems EIA kit
≥10	38	32	31	39	36	29	33	46	54
≥20	26	23	22	25	23	18	21	28	36
≥40	26	23	21	25	22	18	21	28	20
≥80	17	12	10	14	12	7	11	15	4
≥160	10	5	3	11	6	3	8	8	

1/10 with the Sigma conjugate (94%), and the lowest was obtained by the EIA (81%). The agreement was ≥90% with all FITC conjugates in the in-house MIF test at any cutoff point and the lowest with the commercial EIA kit (range, 81 to 90%) and MIF kit (range, 87 to 94%). The agreements between the IgA titers obtained with the different fluorescein-labeled conjugates and the EIA were also compared by calculating kappa values. The kappa values were classified as very good or good when titers of 1/10, 1/20, and 1/40 in the serum samples were used as the cutoff points for all of the FITC conjugates and the EIA (Tables 2 and 3). Higher titers were not found by the EIA because the serum samples in which the absorbances were higher than in the positive control were not retested at a higher dilution (as recommended by the manufacturer). Although the strength of agreement declined at high titers even with the FITC conjugates, the kappa values remained good, except with the Caltag conjugate, for which the values dropped from good to moderate (Table 3).

With the different FITC conjugates, the sensitivity varied from 68 to 97% at low titers (≤1/40). The highest sensitivities compared with the reference method were obtained at the cutoff point of 1/10 with the Labsystems MIF kit (95%) and EIA kit (97%), and the lowest sensitivity was obtained with the in-house MIF test with the Caltag conjugate (76%). With the Dako conjugate and the EIA kit, the highest sensitivity compared with the reference method was obtained at the cutoff point of 1/20. When cutoff points of 1/40 and 1/80 were compared, sensitivity dropped more rapidly with the EIA than with the MIF tests. The highest sensitivities were obtained with the Dako conjugate (90%) at the cutoff point of 1/40 and with the commercial MIF kit (79%) at the cutoff point of 1/80. With the FITC conjugates, sensitivity varied from 32 to 79% at the cutoff point of 1/80. At the highest cutoff point, 1/160, sensitivity was highest with the Dako conjugate and lowest with the Caltag conjugate (Table 4). The specificities were ≥93% with all of

the FITC conjugates in the in-house MIF test at any cutoff point and lowest with the commercial EIA kit (range, 71 to 100%) and MIF kit (range, 83 to 98%).

DISCUSSION

In the present study, we compared the IgA titers and seroconversions obtained with six FITC-conjugated anti-IgA preparations and two commercial kits to those obtained with an in-house MIF test with a reference conjugate in paired serum samples from patients with pneumonia. Altogether, 14 seroconversions with at least one of the conjugates used were detected among the paired serum samples obtained from 122 patients with pneumonia, and all of these IgA responses were found in adult patients. Surprisingly, different conjugates demonstrated IgA seroconversions in different patients, and only one of the seroconversions was detected with all of the conjugates used. In most of the cases, only one of the conjugates produced a significant titer increase, and in only one-third of the cases were significant titer changes detected with at least two conjugates. In 6 of the 14 patients showing an IgA antibody response in paired serum samples, an acute-phase *C. pneumoniae* infection had been diagnosed earlier on the basis of IgG and/or IgM antibodies by different methods. Accepting a fourfold titer increase as diagnostic for acute-phase infection would significantly increase the number of diagnostic findings in the present study, but several anti-IgA conjugates must be used to detect all significant responses. It was shown earlier, during a *C. pneumoniae* epidemic in Finland, that when a fourfold change in the IgA titer alone was accepted as a diagnostic criterion for acute-phase infection, the diagnostic findings increased by one-fourth (5). On the other hand, Persson and Boman (20) did not detect any additional cases of acute-phase infections with their IgA MIF test; instead, additional IgA seroconversions (3 to 7%) were detected by three com-

TABLE 3. Agreement with the reference method, determined by measurement of kappa values and 95% confidence intervals (CI) for all of the samples

IgA cutoff	Agreement for the following test or conjugate:															
	Sigma		Labsystems		Dako		Jackson		Caltag		Zymed		Labsystems MIF kit		Labsystems EIA kit	
	κ	CI	κ	CI	κ	CI	κ	CI	κ	CI	κ	CI	κ	CI	κ	CI
≥10	0.87	0.81–0.93	0.85	0.78–0.91	0.83	0.76–0.90	0.83	0.76–0.90	0.78	0.70–0.86	0.85	0.78–0.92	0.74	0.66–0.82	0.63	0.54–0.71
≥20	0.88	0.81–0.94	0.82	0.74–0.90	0.89	0.83–0.95	0.88	0.81–0.94	0.76	0.66–0.85	0.81	0.73–0.89	0.77	0.68–0.86	0.68	0.59–0.78
≥40	0.88	0.81–0.94	0.82	0.74–0.90	0.87	0.80–0.94	0.86	0.79–0.94	0.77	0.67–0.86	0.82	0.74–0.90	0.77	0.68–0.85	0.72	0.62–0.82
≥80	0.76	0.65–0.88	0.70	0.57–0.83	0.79	0.69–0.90	0.76	0.65–0.88	0.52	0.37–0.67	0.76	0.64–0.87	0.79	0.68–0.89	0.30	0.14–0.45
≥160	0.61	0.42–0.79	0.50	0.30–0.71	0.67	0.52–0.82	0.61	0.42–0.79	0.46	0.25–0.67	0.64	0.47–0.81	0.64	0.47–0.81		

TABLE 4. Sensitivity (S) and agreement (A) at different cutoff points for all of the samples

IgA titer	Result (%) obtained with the following test or conjugate:															
	Sigma		Labsystems		Dako		Jackson		Caltag		Zymed		Labsystems MIF kit		Labsystems EIA kit	
	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A
≥10	85	94	83	93	91	92	87	92	76	90	85	93	95	87	97	81
≥20	86	95	78	93	90	96	86	95	68	92	77	93	86	91	93	86
≥40	85	95	78	93	90	95	84	95	69	92	78	94	87	91	69	90
≥80	67	94	58	93	77	95	67	94	40	90	65	93	79	94	21	87
≥160	48	95	36	94	76	94	52	94	32	93	60	94	60	94		

mercial EIAs. Also, Bennedsen et al. (1) compared three MIF assays, which showed about the same abilities to detect *C. pneumoniae* IgA antibodies, but some variations were found in the antibody levels demonstrated.

In 8 out of 14 cases in the present study, IgA seroconversions were detected in patients without any diagnostic findings of IgG or IgM antibodies. We can speculate that these cases represent not acute reinfection but reactivation of chronic infection. Animal experiments have suggested that chronic *C. pneumoniae* infection can be reactivated by immunosuppression (13, 16). Interestingly, it has also been shown that coronary angioplasty induces increases in both the prevalence of *C. pneumoniae*-specific antibodies and the corresponding titers, a finding also seen for IgA antibodies (24). It is possible that cytokine production during any infection in pneumonia patients may reactivate chronic latent *C.pneumoniae* infection; actually, *C. pneumoniae* has been shown to be present in the lung tissues of patients with chronic pulmonary disease and in those of healthy adults (31). This possibility has been speculated on recently in studies showing that an influenza vaccine protects elderly people from cardiac events (18).

All of the IgA responses in the present study were demonstrated in adult patients. IgA antibodies to *C. pneumoniae* measured by MIF tests seem to be rare in children (unpublished data), and low IgA prevalences have also been found when antibodies to chlamydial lipopolysaccharide have been measured by EIAs (12), even in children found positive for *C. pneumoniae* by culturing or PCR. Tuuminen et al. recently reported very high IgA antibody prevalences in Finnish children over 4 years old by using a commercial EIA method for *C. pneumoniae*, but their method has not been validated by comparison to MIF tests performed in an experienced laboratory (26). It is possible that small children do not frequently produce IgA antibodies as a response to primary upper respiratory tract infections and that IgA responses are generally more common in reinfections, which are more common in adults (5, 22).

The serum samples used for the comparison of different anti-IgA conjugates were tested simultaneously at similar dilutions by using the same microscope and interpreted by the same experienced reader (M.P.). Furthermore, the antigen slides were all prepared by one person at the same time, guaranteeing the same antigen densities and quality for all experiments. The optimal concentration of elementary bodies is important when in-house tests are used, and Gnarpe et al. (6) increased the sensitivity of IgA antibody detection by using a high antigen concentration. We also removed IgG antibodies

before testing with Gullorsorb reagent, making the interpretation of the findings easier, and all seroconversions were confirmed by new titrations at the end of the study by the same reader. Thus, the obvious differences in antibody titers and IgA seroconversions obtained with the different conjugates cannot be due to methodological errors. San-Pin Wang, the founder of the MIF method, has pointed out that the drawback in the measurement of IgA antibodies is the considerable variation in commercial anti-IgA conjugates, a problem which hampers the accurate detection of IgA antibodies (29). In our in-house MIF test, we used 1 h of incubation with serum samples, whereas the incubation time used in the commercial MIF and EIA kits was only 30 min. Gnarpe et al. (6) studied the effect of incubation time on IgA antibody levels and found increased sensitivity with overnight incubation.

The agreement between the different conjugates and methods was better for antibody-negative serum samples and for low titers rather than for high titers. For the different FITC conjugates, agreement varied from 81 to 96%. The strength of agreement measured by kappa coefficients was also very good or good for all FITC conjugates and the EIA at low cutoff points. At cutoff points of 1/80 or more, the strength of agreement varied from very good to moderate for all FITC conjugates; the EIA kit was an exception, but one possible reason for this result is that the samples were not retested at a higher dilution when the absorbances were higher than that of the positive control. The sensitivities of all FITC conjugates and the commercial MIF and EIA tests were the lowest at the highest titers compared to that of the reference method at the highest cutoff points.

The presence of elevated levels of serum IgA antibodies to *C. pneumoniae* has been suggested to be a better marker of chronic infections than that of IgG antibodies (14, 21) due to the short life of IgA antibodies (17). The results from the prospective Helsinki Heart Study have shown that chronic *C. pneumoniae* infection, as indicated by an elevated IgA titer, is an independent risk factor for the development of coronary heart disease, especially when present continuously for a prolonged period (21; T. Huittinen, M. Leinonen, L. Tenkanen, H. Virkkunen, M. Mänttari, T. Palosuo, V. Manninen, and P. Saikku, submitted for publication). In addition, stable, elevated levels of IgA antibodies as markers of chronic *C. pneumoniae* infections have been found to be associated with lung cancer in regular smokers (14). The presence of serum IgA has been associated with the production of interleukin-10 or the predominance of a Th2 type of immune response (15), a factor which may be associated with the development of chronic

infections caused by an intracellular pathogen. The independent association between elevated *C. pneumoniae* IgA antibody levels and fibrinogen levels further indicates that chronic infections could be of importance for disease activity (25). However, several studies have failed to show any association between *C. pneumoniae*-specific IgA antibodies and chronic lung or cardiovascular diseases (2, 3, 23, 26, 28). The present results clearly indicate that there are large variations in the abilities of different commercial anti-IgA FITC conjugates to detect IgA antibodies in serum. Moreover, the sensitivity of the EIA used for detecting low-titer patient sera and its inability to detect high titers in routine titrations partially offset the difference between chronically infected patients and controls. The lack of a true gold standard for the diagnosis of persistent infections hampers efforts to validate potential serological markers (4, 8). The methods used to measure *C. pneumoniae*-specific antibodies vary from laboratory to laboratory (19), and there is thus a need for improvement, simplification, and standardization of the methods to be used in *C. pneumoniae* studies (7).

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