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Detection of *Chlamydophila pneumoniae* IgG in paired serum samples: comparison of serological techniques in pneumonia cases

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Serological diagnosis of *Chamydophila pneumoniae* is usually undertaken by complement fixation test (CFT) or by microimmunofluorescence (MIF). A number of commercial methods for detecting *C. pneumoniae*-specific IgG have been developed. The aim of this study was to compare the performance characteristics of six methods for the diagnosis of pneumonia due to *C. pneumoniae*, including CFT (in house), MIF (Vircell, Spain), and four ELISAs (Medac, Germany; Savyon, Israel; Serion, Germany; and DRG, Germany). ELISA-Medac, ELISA-Savyon, ELISA-DRG and MIF use *C. pneumoniae* antigens while ELISA-Serion and CFT use *Chlamydophila* genus-specific antigen. Acute and convalescent samples from 85 pneumonia patients were studied. Using CFT, cases were initially classified as due to *Chlamydophila* (43 cases); to other agents (23 cases) (influenza A and B, *Mycoplasma pneumoniae*, respiratory syncytial virus, adenovirus and *Legionella pneumophila*); or as negative (19 cases). Cases were considered positive if they showed seroconversion, a significant rise in titer or high titer; and were finally classified as positive if they gave a positive result in at least three assays. Sensitivity values ranged from 87% to 97.8%; and specificity from 84.6% to 97.4%. In conclusion, the assays compared appear to be useful tools for the diagnosis of pneumonia due to *Chlamydophila.*

Key words: *Chlamydophila*; Serology; ELISA; Microimmunofluorescence; Complement fixation; IgG.

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Chlamydophila pneumoniae (formerly *Chlamydia pneumoniae*) (1) is a frequent cause of respiratory infection. Since it is an intracellular parasite, it can only be isolated by inoculation in cell cultures, which requires prolonged incubation periods in order to obtain results. Isolation does not provide a high degree of sensitivity and is not a practical method for diagnosis. In recent years genomic amplification methods have been developed for diagnosing pneumonia due to *Chlamydophila*; meanwhile, these techniques have revealed lack of sensitivity as a method of diagnosis (2, 3) and have not been sufficiently assessed in regular clinical laboratory practice (4). The use of suitable serological tests has led to the recognition of *C. pneumoniae* as responsible for between 7.5% and 21% of cases of community-acquired pneumonia (5–8). For this reason it is currently recognized that serology is the most suitable diagnostic method. Microfluorescence (MIF), originally designed to detect antibodies against *Chlamydia trachomatis* (9), provides suitable sensitivity values (10). Amongst currently available techniques of serological diagnosis, the most widely used is MIF.

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However, as this assay is technically complex, interpretation of results is subjective, and diagnostic criteria have not yet been harmonized (11), it is only used in highly specialized laboratories. In comparison, the complement fixation test (CFT) offers a practical, low-cost approach, since it permits screening of samples for many different agents within one single assay, though it is time-consuming, difficult to standardize and must be performed by highly qualified personnel with strict quality control. In recent years, a number of commercial assays, including both MIF and enzyme immunoassays (ELISA) for detecting antibodies to *C. pneumoniae*, have been developed. There is very limited information about the performance characteristics of these assays for diagnostic purposes. The aim of this paper is to compare four commercial ELISAs, one commercial MIF, for detecting specific IgG, and one in-house CFT for total specific antibodies for the diagnosis of *C. pneumoniae* pneumonia in paired serum samples.

MATERIAL AND METHODS

One hundred and seventy serum samples (acute and convalescent), taken 2–3 weeks apart, from 85 patients were included in the study. The samples were received in our laboratory (as reference laboratory) for serological diagnosis of pneumonia. No data from direct diagnosis (bacterial or viral isolation, or nucleic acid testing) were available. In 64 cases the age was available (mean 57.6 years, standard deviation 17.6, range 4 months to 94 years). Etiological diagnosis of patients was initially based on either detection of seroconversion (SC) (defined as a change from negative to positive) or a significant rise (defined as a four-fold or higher greater change in the titer), obtained by CFT, against a panel of antigens, including influenza A and B viruses (IAV, IBV), adenovirus (AV), respiratory syncytial virus (RSV), *Mycoplasma pneumoniae*, and *Chlamydophila*, or by indirect immunofluorescence for *Legionella pneumophila* (12). Initially, 43 cases were considered to have been caused by *Chlamydophila*; 23 cases were due to other respiratory pathogens, including IAV (4 cases), IBV (4 cases), *M. pneumoniae* (4 cases), RSV (4 cases), AV (4 cases), and *L. pneumophila* (3 cases). Finally, 19 cases were serologically negative to all the pathogens studied.

All samples were tested in parallel using the following methods:

1. *Chlamydia pneumoniae*-IgG-sELISA Medac (Medac Diagnostika, Hamburg, Germany) (ELISA-Medac). This method uses a purified *C. pneumoniae* antigen. The samples were tested at a 1:50 dilution. Cut-off was calculated as the mean of two determinations of a negative control+0.380. For each sample a ratio was set for the cut-off. Samples were positive if the ratio was ≥1.1. Cases were classified as positive if SC was observed, if the convalescent sample ratio was at least 1.5 times higher than the acute sample ratio, or if acute or convalescent sample gave a ratio≥2.5.
2. SeroCP Quant IgG (Savyon Diagnostics Ltd., Ashdod, Israel) (ELISA-Savyon). The method uses purified elementary bodies of *C. pneumoniae.* Samples were tested at a 1:100 dilution. Results were calculated against three calibrators set in binding units (BU)/ml. The BU/ml values obtained in each sample were converted to MIF equivalent end-point titers (EEPT). Thus, results were finally expressed as such. Cases were considered as positive if, first, they showed SC or, secondly, if four-fold or greater changes in EEPTs were observed, or thirdly, if EEPTs were ≥1:256 either in acute or convalescent samples.
3. SERION ELISA *classic Chlamydia* IgG (Institut Virion\Serion, Wurzburg, Germany) (ELISA-Serion). This method uses a genus-specific antigen and samples were tested at 1:20. The results were calculated using SERION *easy base* 4PL-Software/SERION *evaluate*-Software, by using two determinations of a batch-specific calibrator, and expressed as units/ml. Samples were positive if they gave values≥15 u/ml. Cases were classified as positive if SC was observed, if the convalescent sample ratio was at least 1.5 times higher than the acute sample ratio, or if a result ≥100 u/ml was observed for either acute or convalescent samples.
4. *Chlamydia pneumoniae* IgG ELISA (DRG Instruments, Germany) (ELISA-DRG). The method uses the *Chlamydophila pneumoniae* antigen. The samples were tested at a 1:100 dilution. Results are expressed as enzyme immunounits (EIU), referring to a batch-specific calibrator, tested twice. Cases were classified as positive if SC was observed, if the convalescent sample ratio was at least 1.5 times higher than the acute sample ratio (in samples with EIU values below 130) or 1.3 (in samples with EIU values above 130), or if a result ≥200 EIU was seen in either acute or convalescent samples.
5. MIF, using commercially available slides (Vircell, Granada, Spain), containing *C. pneumoniae* and *C. psitacci* and *Chlamydia trachomatis* antigens in separate wells. Samples were titrated from 1:16 to 1:1024, by using four-fold dilutions. The isotype IgG response was detected by means of an anti-human IgG FITC-conjugated (Dako, Denmark), diluted at 1:400. The reading was done by at least two different people. Cases were classified as positive if, first, SC or a four-fold or higher rise in

TABLE 1. *Summary of results obtained by the compared methods*

	Reference	
	Positive (n=46)	Negative (n=39)
ELISA-Medac		
Positive (n=45)	42	3
Negative (n=40)	4	36
ELISA-Savyon		
Positive (n=51)	45	6
Negative (n=34)	1	33
ELISA-Serion		
Positive (n=43)	40	3
Negative (n=42)	6	36
ELISA-DRG		
Positive (n=46)	40	6
Negative (n=39)	6	33
MIF-Vircell		
Positive (n=42)	41	1
Negative (n=43)	5	38
CFT-In-house		
Positive (n=43)	42	1
Negative (n=42)	4	38

titer was seen or, secondly, if a titer≥1:256 was seen in either acute or convalescent sample.

6. CFT followed a standard procedure, as described (13), using commercially available antigens (Institute Virion, Switzerland). Samples were titrated from 1:8, by using two-fold dilutions. All positive cases included in the study showed SC or a significant rise in titer, to the corresponding antigens.

All commercial kits were used following the manufacturer's instructions. Samples were stored at −20°C until used in the different assays.

RESULTS

Cases were classified on a consensus basis: a positive result in three or more assays was finally considered to be positive. On the basis of this criterion 46 cases were classified as positive for *Chlamydophila* and 39 as negative. All six methods showed agreement in 31 positive and 24 negative cases.

Table 1 summarizes the results obtained by all the assays, as compared with the reference criterion. Table 2 shows agreement, sensitivity and specificity, and positive and negative predictive values (PPV, NPV) for the methods compared. Agreement ranged from 85.9% (ELISA-DRG) to 94.1% (CFT); sensitivity from 87% (ELISA-Serion and ELISA-DRG) to 97.8% (ELISA-Savyon); and specificity from 84.6% (ELISA-Savyon and ELISA-DRG) to 97.4% (CFT and MIF). The corresponding figures for PPV ranged from 87% (ELISA-DRG) to 97.7% (CFT), and for NPV from 84.6% (ELISA-DRG) to 97.1% (ELISA-Savyon).

Tables 3 and 4 show either cases where there was a discrepancy between the results obtained and the reference criterion, or false negatives (Table 3), or false positives (Table 4). Amongst the 19 cases initially characterized as negative for the pathogens studied using CFT, three were finally classified as positive, since either three (cases N14 and N15) or four (N18) positive results were obtained (Table 3). Seven cases showed a single discrepant negative result, two by ELISA-Medac (C42 and C43), one by ELISA-Serion (C35), two by ELISA-DRG (C6 and C26), and two by MIF (C31 and C32). All cases with false-negative results in MIF showed *C. pneumoniae*-specific IgG but at a very low level (≤1/64). In cases which were positive for pathogens other than *Chlamydophila*, one case, positive for AV, was also finally classified as positive for *C. pneumoniae* as well (AV20, Table 3).

In contrast, a single false-positive result was obtained in 10 cases (Table 4): one by ELISA-Medac (IA3), 3 by ELISA-Savyon (N2, N13 and L22), one by ELISA-Serion (N6), 4 by ELISA-DRG (N8, N17, IA2 and RSV4), and one by CFT (C18). The remaining five cases gave a positive result in two assays.

TABLE 2.*Agreement, sensitivity, specificity and positive and negative predictive values of the compared methods*

	Agreement	Sensitivity	Specificity	PPV	NPV
ELISA-Medac	91.8% (78/85)	91.3% (42/46)	92.3% (36/39)	93.3% (42/45)	90% (36/40)
ELISA-Savyon	91.8% (78/85)	97.8% (45/46)	84.6% (33/39)	88.2% (45/51)	97.1% (33/34)
ELISA-Serion	89.4% (76/85)	87% (40/46)	92.3% (36/39)	93% (40/43)	85.7% (36/42)
ELISA-DRG	85.9% (73/85)	87% (40/46)	84.6% (33/39)	87% (40/46)	84.6% (33/39)
MIF-Vircell	92.9% (79/85)	89.1% (41/46)	97.4% (38/39)	97.6% (41/42)	88.4% (38/43)
CFT-In house	94.1% (80/85)	91.3% (42/46)	97.4% (38/39)	97.7% (42/43)	90.5% (38/42)

TABLE 3.*Discrepant results (false negatives according to reference criterion)*

Case	ELISA-Medac		ELISA-Savyon		ELISA-Serion		ELISA-DRG		MIF-Vircell		CFT-In house	
C6	1.4	Pos	256	Pos	25	Pos	4	Neg	16	Pos	<8	Pos
	3.1		>512		114		12		64		16	
C7	1.4	Pos	64	Neg	61	Pos	48	Neg	64	Neg	<8	Pos
	4.1		128		525		36		64		16	
C11	3.5	Pos	128	Pos	50	Neg	136	Neg	256	Pos	<8	pos
	4.5		>512		35		138		256		16	
C22	0.1	Neg	<64	Pos	3	Pos	19	Neg	<16	Pos	<8	Pos
	0.9		64		16		27		64		128	
C23	0.9	Pos	<64	Pos	10	Neg	30	Pos	16	Neg	32	Pos
	1.4		64		10		50		16		128	
C26	2	Pos	128	Pos	10	Pos	149	Neg	64	Pos	<8	Pos
	2.8		>512		105		180		1024		256	
C31	1.9	Pos	128	Pos	76	Pos	63	Pos	64	Neg	<8	Pos
	3.2		256		115		284		64		32	
C32	2.4	Pos	128	Pos	116	Pos	117	Pos	64	Neg	<8	Pos
	2.9		256		316		154		64		16	
C35	2.3	Pos	256	Pos	19	Neg	118	Pos	16	Pos	<8	Pos
	2.9		256		30		217		64		64	
C42	1.4	Neg	128	Pos	6	Pos	48	Pos	16	Pos	32	Pos
	1.8		256		16		186		64		256	
C43	1.7	Neg	64	Pos	8	Pos	85	Pos	<16	Pos	<8	Pos
	1.9		256		18		206		64		16	
N14	0.4	Neg	<64	Pos	35	Neg	17	Pos	16	Pos	<8	Neg
	0.8		256		36		60		64		<8	
N15	2.4	Pos	128	Pos	3	Neg	55	Pos	64	Neg	<8	Neg
	2.9		256		4		299		64		<8	
N18	4.2	Pos	>512	Pos	14	Neg	377	Pos	256	Pos	<8	Neg
	3.4		>512		14		456		1024		<8	
AV20	2.3	Pos	256	Pos	176	Pos	76	Neg	256	Pos	<8	Neg
	2.6		>512		171		93		256		<8	

DISCUSSION

In this assessment the reference criterion used was that of a majority of results from the six compared assays, although MIF is recognized as the best technique for the detection of antibodies against *C. pneumoniae* (10). Following this criterion, the different assays have, in general, produced acceptable sensitivity and specificity results, which were over 85%.

The four ELISA methods studied gave sensitivity values between 87% and 97.8%, and specificity values between 84.6% and 92.3%. These differences do not appear to have been caused by the dilution used in each case, which varies between 1:20 and 1:100. In fact, the assay which analyzes the samples at a lower dilution (1:20) (ELISA-Serion) is not the most sensitive, and shows higher specificity values. Another possible reason for the differences obtained could be the antigen used in each assay. This was obtained from *C. pneumoniae* in three cases (ELISA-Medac, ELISA-Savyon y ELISA-DRG), or was genus specific (ELISA-Serion). Nevertheless, the last assay showed very high specificity, with results lower only than those for MIF and CFT.

For this evaluation we have considered in ELISA and MIF as positive the cases showing not only SC but high titer as well. This serological approach has proved useful for the diagnosis of other bacterial diseases such as pertussis (14, 15). In the case of *C. pneumoniae* infection, however, this approach should be used with caution (10), since background titers to the bacteria are high in the healthy population although they may be present only at low titers. Thus, additional studies on serum samples from the general population are required, especially carried out using ELISA tests.

Earlier studies have suggested that CFT sensitivity is very low when compared with other

TABLE 4.*Discrepant results (false positives according to reference criterion)*

Case	ELISA-Medac		ELISA-Savyon		ELISA-Serion		ELISA-DRG		MIF-Vircell		CFT-In house	
C18	0	Neg	<64	Neg	9	Neg	1	Neg	<16	Neg	<8	Pos
	0.2		<64		6		13		<16		32	
N2	1.1	Neg	<64	Pos	23	Neg	50	Neg	64	Neg	<8	Neg
	1.3		64		31		66		64		<8	
N6	2	Neg	128	Neg	43	Pos	62	Neg	64	Neg	<8	Neg
	2.1		128		110		81		64		<8	
N8	2.3	Neg	128	Neg	13	Neg	45	Pos	64	Neg	<8	Neg
	1.8		128		11		88		64		<8	
N11	1.7	Neg	128	Neg	7	Pos	328	Pos	64	Neg	<8	Neg
	1.2		64		20		231		64		<8	
N13	1.1	Neg	<64	Pos	54	Neg	30	Neg	16	Neg	<8	Neg
	1.2		64		45		31		16		<8	
N17	0	Neg	<64	Neg	3	Neg	396	Pos	64	Neg	<8	Neg
	0		<64		2		217		64		<8	
IA2	0.7	Neg	<64	Neg	6	Neg	23	Pos	64	Neg	<8	Neg
	0.8		<64		10		67		64		<8	
IA3	1.8	Pos	64	Neg	12	Neg	117	Neg	16	Neg	<8	Neg
	2.4		128		16		92		16		<8	
IA4	1.8	Neg	256	Pos	22	Neg	205	Pos	64	Neg	<8	Neg
	2.2		256		5		178		64		<8	
M9	2.7	Pos	256	Pos	12	Neg	150	Neg	64	Neg	<8	Neg
	3.4		>512		14		88		64		<8	
RSV14	0.2	Neg	<64	Neg	10	Neg	12	Pos	<16	Neg	<8	Neg
	0.1		<64		5		133		<16		<8	
AV18	1.3	Neg	256	Pos	31	Pos	40	Neg	64	Neg	<8	Neg
	1.7		128		115		44		64		<8	
L21	3	Pos	128	Neg	52	Neg	151	Neg	256	Pos	<8	Neg
	2.7		128		50		187		256		<8	
L22	1	Neg	<64	Pos	8	Neg	141	Neg	64	Neg	<8	Neg
	1.2		64		12		78		64		<8	

diagnostic markers (16). However, in this study it was the technique which showed the highest agreement value. This may in part be due to the fact that the initial selection of cases was done using that technique. Moreover, it is recognized that it is a technique with a certain lack of specificity, mainly due to the cross-reactivity produced by certain enteric bacteria (17). In fact, C18 (Table 4) showed a single positive result using CFT. This did not occur with any other method. The main disadvantage of CFT is the difficulty of standardization, although recently an automated assay was undertaken which proved to be applicable for serological diagnosis of acute respiratory infection from viruses and atypical bacteria, particularly *C. pneumoniae* (18).

PPV ranged from 87% to 97.7%, and for NPV from 84.6% to 97.1%. It is important, meanwhile, to point out that such figures were obtained for a theoretical prevalence about 54% (46 cases from *C. pneumoniae* infection of a total of 85 cases studies), a figure higher than those obtained in pneumonia prevalence studies (5–8).

Some recent studies evaluating the application of commercial kits of ELISA, CFT and MIF have been reported (19, 20). ELISA-Savyon and ELISA-Medac were compared with MIF in their application of *C. pneumoniae* diagnosis, showing values of sensitivity, specificity, and NPV and PPV similar to those obtained here (20). No previous reports on the performance of ELISA-Serion and ELISA-DRG are available.

In conclusion, the assays compared here have proven to be effective for serological diagnosis of pneumonia from *Chlamydophila* and their use in clinical laboratories will improve knowledge of the real incidence of this bacterium as responsible for community-acquired pneumonia.

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