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ELISA test to detect *Chlamydophila pneumoniae* IgG

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A new ELISA test (*Chlamydophila pneumoniae* IgG, Vircell, Spain) to detect *Chlamydophila pneumoniae* IgG was evaluated. The micro-immunofluorescence (MIF) test was used as reference method. *Chlamydia trachomatis* and *Chlamydophila psittaci* elementary bodies were also assayed. Two hundred and sixteen sera were included in the study: 66 from patients with peripheral arterial occlusive disease (Panel 1), 68 from adults with pneumonia (Panel 2), 44 from healthy adults (Panel 3) and 38 from patients with a sexuality transmitted disease by *C. trachomatis* (Panel 4). In Panel 1, 51 sera (77%) had antibody titres between 32 and 128; 4 out of 15 sera with IgG titres <32 were positive by ELISA test and 2 sera with 32 IgG titres were uncertain by ELISA; the remaining 60 sera were correctly classified, giving a 91% concordance between the techniques. In Panel 2, 55 sera (81%) had IgG titres between 32 and 512; 2 out of 13 sera with IgG titres <32 were positive by ELISA and 2 sera with 32 titres were uncertain by ELISA; the remaining 64 sera were correctly classified, giving a 97% concordance. In Panel 3, 22 sera (50%) had IgG titres between 32 and 64; only 1 out of 22 sera with IgG titres <32 was positive by ELISA, giving a 97% concordance between the techniques. In Panel 4, there were 24 (63%) negative, 10 (26%) uncertain and 4 (10%) positive results by ELISA, giving an 86% concordance. The *C. pneumoniae* ELISA test demonstrated 100% sensitivity and 85% specificity. The IgG ELISA test demonstrated a good concordance with the MIF test without the drawbacks associated with the latter assay. We conclude that the ELISA test could be an alternative to the MIF test.

Chlamydophila pneumoniae is a human respiratory pathogenic bacterium that has been associated with pneumonia, chronic bronchitis, bronchial obstruction and arteriosclerosis (GUTIERREZ *et al.* 2000, 2001a and b, GRAYSTON *et al.* 1990, 1992, KUO *et al.* 1995, LINARES-PALOMINO *et al.* 2001). Direct detection of bacteria is very difficult and has a low success rate. The diagnosis is mainly focused on specific antibody detection (BARNES *et al.* 1989, GRAYSTON *et al.* 1993), because PCR and culture are more complicated techniques. Several antigens have been studied for this purpose, including lipopolysaccharide (LPS) from the outer membrane (BRADE *et al.* 1997) and various proteins. LPS has an active role in complement fixation (BERDAL *et al.* 1991, FONSECA *et al.* 1994) and has been used in ELISA tests (VERKOOYEN *et al.* 1998, BRADE *et al.* 1994). However, it is a genus-specific antigen that does not differentiate between antibodies against different species of *Chlamydiceae*. The complement fixation test is technically difficult and is in declining use in the laboratory. Although LPS has been used in ELISA tests to detect IgG, IgA or IgM, alone or in combination, there is no definitive consensus on its clinical utility (VERKOOYEN *et al.* 1998, KUTLIN *et al.* 1997). Serological studies carried out with elementary body proteins have mainly employed major outer membrane proteins (MOMPs), using the micro-immunofluorescence (MIF) test, the gold standard technique in spite of its difficulty (FREIDANK *et al.* 1993, GRAYSTON *et al.* 1986, GONEN *et al.* 1993). The MIF test requires complex and subjective interpretation and its automation in the laboratory is impossible. ELISA

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tests with elementary bodies have been studied (Biendo *et al.* 1994, Kishimoto *et al.* 1996, Numazaki *et al.* 1996) and some are commercially available. However, they must be evaluated against MIF before they can replace it in the clinical microbiology laboratory.

We performed a clinical evaluation of a new ELISA test (*Chlamydophila pneumoniae* IgG, Vircell, Spain) to detect IgG to *C. pneumoniae*. Its concordance with MIF test was analysed in different populations with high infection risk. Our objective was to determine whether the concordance between these assays is adequate for the MIF test to be replaced by the ELISA test.

Materials and methods

Clinical samples: 216 serum samples were studied. They were stored at $-20\,°C$ and divided into four groups:

Panel 1: included 66 samples from patients with peripheral arterial occlusive disease of the lower extremities, studied at our hospital.

Panel 2: included 68 samples from 34 episodes of pneumonia studied at our hospital.

Panel 3: included 44 samples from healthy adult subjects.

Panel 4: included 38 samples from HD Supplies (UK) and Hospital Son Dureta (Palma de Mallorca, Spain) that had anti-*Chlamydia trachomatis* IgG but not anti-*C. pneumoniae* IgG by MIF test.

C. pneumoniae IgG was investigated in all samples by two methods: the MIF test (as method of reference) and ELISA. Inter-assay discrepancies for *C. trachomatis* and *C. psittaci* IgG were also analysed.

MIF: the test was performed according to the recommendations of Grayston (1990). The samples were diluted, beginning with a 32 dilution (Kutlin *et al.* 1997). All samples were read by the same person.

ELISA (*Chlamydophila pneumoniae* IgG, Vircell): A commercial ELISA employing *Chlamydia* outer membrane complexes (COMC) was used. Briefly, *C. pneumoniae* strain 2023 (ATCC 1356 VR) was grown in HEp-2 cells with 1 µl/ml of cycloheximide. They were prepared from purified elementary bodies by treatment with 10 mM sodium phosphate containing 2% sarcosyl, 1.5 mM EDTA and 0.14 mM NaCl as described previously (Caldwell *et al.* 1981). 96-well plates (Nunc Maxisorb, Denmark) were coated with COMC at 0.2 µg/well in PBS 8.5 mM. All assays included a positive control, a cutoff control (in duplicate) and a negative control provided in the kit. 100 µl of sera diluted 1/20 were incubated at 37 °C for 45 minutes. In a second step, 100 µl of a goat anti-human IgG peroxidase conjugate was added and incubated at 37 °C for 30 minutes. 100 µl of tetramethyl-benzidine was used as substrate and the reaction was stopped after 20 minutes with 50 µl of 0.5 M sulphuric acid. Absorbances were measured at 450/620 nm. Results were expressed as indexes by dividing the absorbance of the sample by that of the cut-off. Indexes <0.9 were scored as negative, $0.9-1.1$ as uncertain, and >1.1 as positive. Uncertain results were repeated and the new result was taken as valid. For greater precision, the process was automated with the use of a sample dilutor (Tecan Megaflex, Austria) and plate processor (Dade-Behring, BEP III, Germany). Before the results evaluation, 10% of the samples were retested and similar results were obtained.

Statistical analysis: Pearson correlation coefficient between ELISA indexes and MIF titres was stated (SPSS, 9.0). For this purpose, uncertain ELISA results were omitted.

Results

In Panel 1 (Table 1), 51 sera (77%) had antibody titres between 32 and 128; 4 out of 15 sera with IgG titres <32 were positive by ELISA test and 2 sera with 32 IgG titres were uncertain by ELISA; the remaining 60 sera were correctly classified, giving a 91% concordance

between the techniques. In Panel 2 (Table 1), 55 sera (81%) had IgG titres between 32 and 512; 2 out of 13 sera with IgG titres <32 were positive by ELISA and 2 sera with 32 titres were uncertain by ELISA; the remaining 64 sera were correctly classified, giving a 97% concordance. In Panel 3 (Table 2), 22 sera (50%) had IgG titres between 32 and 64; only 1 out of 22 sera with IgG titres <32 was positive by ELISA, giving a 97% concordance between the techniques. In Panels 1 to 3, no antibodies against *C. trachomatis* or *C. psittaci* were detected. In Panel 4 (Table 3), there were 24 (63%) negative, 10 (26%) uncertain and 4 (10%) positive results by ELISA, giving an 86% concordance. The *C. pneumoniae* ELISA test showed 100% sensitivity and 85% specificity when compared with the MIF test. Initially uncertain samples were repeated and the same results were obtained. There was a significant direct correlation (r: 0.436; p = 0.007) between ELISA indexes and MIF titres.

Table 1
Relationship between the results obtained with MIF and ELISA tests to detect IgG against *C. pneumoniae* in panels 1 (patients with peripheral arterial occlusive vascular disease) and 2 (patients with pneumonia)

MIF	ELISA							
	Panel 1				Panel 2			
	Negative	Uncertain	Positive	Total	Negative	Uncertain	Positive	Total
<32	11		4	**15**	11		2	**13**
32		2	8	**10**		2	21	**23**
64			21	**21**			17	**17**
128			20	**20**			14	**14**
512							1	**1**
Total	**11**	**2**	**53**	**66**	**11**	**2**	**55**	**68**

Table 2
Relationship between the results obtained with MIF and ELISA tests to detect IgG against *C. pneumoniae* in panel 3 (healthy subjects)

MIF	ELISA			
	Panel 3			
	Negative	Uncertain	Positive	Total
<32	16	5	1	**22**
32		1	11	**12**
64			10	**10**
Total	**16**	**6**	**22**	**44**

Table 3
Relationship between the results obtained with MIF test for IgG against C. *trachomatis* and ELISA test for IgG against *C. pneumoniae* in panel 4 (samples without IgG against *C. pneumoniae* by MIF)

MIF *C. trachomatis*	ELISA *C. pneumoniae*			
	Panel 4			
	Negative	Uncertain	Positive	Total
32	14	6	1	**21**
64	9	2	3	**14**
128	1	1		**2**
512		1		**1**
Total	**24**	**10**	**4**	**38**

Table 4
Relationship between titres in MIF and ranges of absorbances in ELISA

MIF titre	ELISA absorbance							
	<0.9	1.2–2	2.1–3	3.1–4	4.1–5	5.1–6	>6.1	Total
<32	62	7	1	1		2		**73**
32		17	10	9	4			**40**
64		6	23	2	8	5	4	**48**
128		1	14	3	2	5	9	**34**
512				1				**1**
Total	**62**	**31**	**48**	**16**	**14**	**12**	**13**	**196**

Figure 1 shows the correspondence between absorbances obtained in the ELISA test and MIF titres. A significant direct relationship (p = 0.007, Microsoft Excel) was found.

Discussion

Assessment of a test for the diagnosis of an infection should take account of the type of antibodies investigated, the specificity of the epitopes that induce the antibodies, and the technical complexity of the test.

In primary infection by *C. pneumoniae*, IgM tends to appear first but cannot always be detected. In re-infections, the presence of IgM is uncommon but IgG levels increase rapidly.
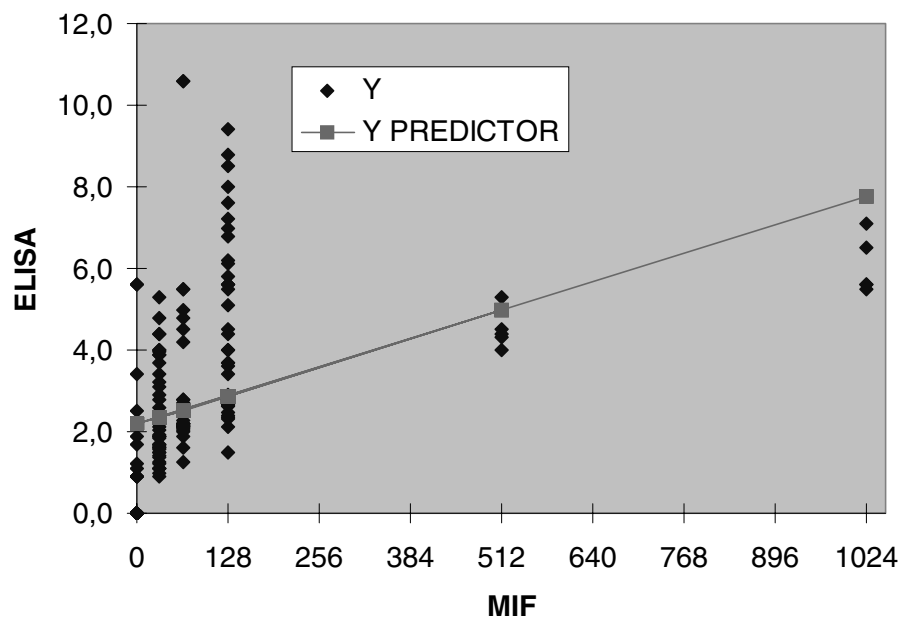

Fig. 1
Relationships between IgG ELISA and MIF tests

Since primary infections occur in the first years of life (GRAYSTON *et al.* 1993), most patients suffer re-infections or reactivations of previous infections. These are accompanied by an increase in IgG and an absence of IgM.

The advantages of ELISA over MIF tests are the objectivity of their results, the possibility of their automation, and their utility to detect IgG or IgM.

In *Chlamydia,* the major genus epitopes locate in the LPS and are responsible for most of the serological cross-reactions. There are genus antigens, such as the 60 kDa protein. When used in immunoblots (CAMPBELL *et al.* 1990a and b; PÉREZ *et al.* 1994, 1993), the 60 kDa protein appears as an important genus antigen. However, this protein is poorly present in the elementary body. On the other hand, the 98 kDa protein, an abundant constituent of elementary body MOMP, shows an important species-specific reactivity in immunoblot. Therefore, the antigens of the elementary bodies are essentially species-specific, presenting a negligible unspecific reactivity.

The ELISA test to detect antibodies against *C. pneumoniae* is recent and has been hardly used in the clinical microbiology laboratory (BIENDO and ORFILA 1994, KISHIMOTO *et al.* 1996a and b, NUMAZAKI *et al.* 1996). Based on the specificity data detailed above, the insoluble sarcosyl fraction of the elementary bodies was employed for the ELISA test. In all cases, a good correlation with the MIF test was found. The main discrepancies have been described in individuals with *C. trachomatis* infection (BIENDO and ORFILA 1994). We also found false positives in samples with high quantities of IgG against *C. trachomatis*, although the proportion was lower than that reported by BIENDO and ORFILA (1994) (48% specificity). This difference may be due to the fact that they did not use samples negative for anti-*C. pneumoniae* IgG in the MIF test, which were included in the present study. The greater number of positives in the ELISA test versus the MIF test can be explained by the greater sensitivity of ELISA. This problem could be avoided by employing the ELISA test for the diagnosis of the current disease when an important increase in IgG between two samples is demonstrated. We have automated the ELISA test by using a sample dilutor and a plate processor (BEP III). This system showed a good repeatability of results and the uncertain samples were not reclassified after retesting. We conclude that the ELISA test has a good correspondence with the MIF test and presents fewer drawbacks. ELISA tests from the elementary body could be an alternative to MIF tests in the clinical microbiology laboratory.

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