J. Basic Microbiol. **44** (2004) 4, 275–279 DOI: 10.1002/jobm.200410385

(Department of Microbiology, School of Medicine, University of Granada; [1]Laboratorio Vircell; Granada; [2]Department of Surgery, School of Medicine, University of Granada, Spain)

A new microimmunofluorescence test for the detection of *Chlamydia pneumoniae* specific antibodies

F. FERNÁNDEZ, J. GUTIÉRREZ*, J. MENDOZA[1], J. LINARES[2] and M. J. SOTO

(Received 19 January 2004/Accepted 20 April 2004)

To evaluate a microimmunofluorescence (MIF) test (*Chlamydia pneumoniae* IgG, Vircell, Spain) that detects IgG against *Chlamydophila pneumoniae* (Cp), MRL Diagnostics MIF was used as reference test. Cross-reactivity against *Chlamydia trachomatis* (Ct) and *Chlamydophila psittaci* (Cps) was investigated. Eighty sera were analysed from 22 subjects with vascular disease, 38 with multiple sclerosis and 20 healthy individuals. Vircell and MRL MIF tests assessed 58.75% and 60% of the samples as positive, respectively, and their results coincided (positive/negative) in 98.75% of samples. One major (>1 IgG titre) and 32 minor (1 titre) discrepancies were observed. Correlation between tests was significant. Vircell MIF test demonstrated 97.9% sensitivity and 100% specificity. Differences in simultaneous reactivity to Ct and Cps between the tests were not significant. Vircell MIF test showed a good performance to detect the IgG against Cp.

Chlamydophila pneumoniae (Cp) is a strictly intracellular human respiratory pathogen associated with up to 20% of cases of bronchitis and community-acquired pneumonia (GRAYSTON 1992). Evidence has also been published relating persistent Cp infection to chronic diseases, including atherosclerosis (AT) and multiple sclerosis (MS) (SAIKKU *et al.* 1992, SRIRAM *et al.* 1999).

No reference test is yet available for the direct diagnosis of the infection, because of the complexity of the bacteria detection and the biological risks involved (APFALTER *et al.* 2001).

The presence of current or recent Cp' infection is tested through the demonstration of elevated IgG levels or a significant increase in level between serum samples taken at different times. This quantitative approach is adopted because of the high likelihood of antigenic contact during an individual's life, responsible for the progressive rise in the percentage of the healthy population with IgG against Cp as the population ages (GRAYSTON *et al.* 1990).

MIF is considered the reference test for the detection of antibodies (WANG and GRAYSTON 1994). It was developed to detect antibodies against *Chlamydia trachomatis* (Ct) (WANG and GRAYSTON 1970) and later against Cp. One type of MIF has been developed to detect antibodies against lipopolysaccharides (LPS) with epitopes common to gram negative bacilli and specific epitopes of the *Chlamydiaceae* family, and another to detect antibodies against LPS-purified proteins present in elemental bodies (EB) (BRADE *et al.* 1997). The latter type appears to be more specific. The existence of only one serotype in MIF tests (BECKER 1996) facilitates their application and guarantees their sensitivity (MESSMER *et al.* 2001).

The present work was designed to evaluate a new MIF for *Chlamydia pneumoniae* IgG (Vircell, Spain), using as gold standard the MRL MIF test (MRL Diagnostics, Cypress, CAL, USA), which has demonstrated adequate sensitivity and specificity to be validated for use in clinical diagnosis (PEELING *et al.* 2000, HERMANN *et al.* 2002).

* Corresponding author: Prof. Dr. José Gutiérrez; e-mail: josegf@ugr.es

 0233-111X/04/0408-0275

Materials and methods

The presence of IgG anti EB-Cp was investigated using two MIF tests (MRL Diagnostics *Chlamydia pneumoniae* IgG and Vircell *Chlamydia pneumoniae*) in serum samples diluted to 1:16 with PBS at pH 7.2. Samples that did not show an evident fluorescence at this dilution were considered negative. Positive samples were double-diluted and analysed successively to determine the maximum dilution at which a positive reaction was obtained. In order to reveal possible cross-reactions with Ct and *Chlamydophila psittaci* (Cps), IgG titres against EB of Ct and Cps were determined by a similar methodology.

The observations were made by a single researcher with previous experience and were carried out in duplicate to confirm their validity.

Clinical samples: In order to avoid shared antigenic reactivity, sera were studied from 80 subjects without respiratory or genital disease. Fourteen samples were from patients (66 ± 8 years) diagnosed at our hospital with AT of peripheral arteries according to the following established criteria: intermittent claudication, Fontaine stage II or III, and obliteration or stenosis of aortoiliac or femoral arteries, based on basal electrocardiogram, echo-Doppler, and clinical examinations (LABS *et al.* 1999); half of these patients presented clinical manifestations of the disease and half were chronic patients. Thirty-eight samples were from patients with MS (35 ± 10 years) diagnosed at our hospital according to previously established criteria (POSER *et al.* 1983). Twenty samples were from healthy blood donors (<35 years) and eight from patients (60 ± 3 years) undergoing varicose vein surgery and with no AT vascular disease. All 80 samples were kept at –20 °C until their analysis.

***Chlamydia pneumoniae* IgG of Vircell:** The test uses EB of Cp strain CM-1 (ATCC VR-1360) cultured in HEp-2 cells (ATCC CCL-23) in MEM medium (GIBCO, USA) with 10% foetal bovine serum (FBS) (KRAEBER, Germany). The strain was inoculated by centrifugation at 1500 g for 45 min and incubation at 37 ºC for one hour followed by addition of MEM with 5% FBS and 1 µg/ml cycloheximide. After 72 h, the cells were extracted using sterile glass pearls and inactivated by treatment with 1% formaldehyde at 4 ºC for 24 h. Inactivation control was carried out and, after sonication, the cells were centrifuged at 500 g for 10 min. The supernatant was washed three times with PBS of pH 7.2 at 39000 g for 30 min. The sediment was resuspended in PBS with 0.5% bovine albumin (EQUITECH, USA). The slides were fixed with cold acetone for 10 min. On the same slide were present LGV type II strain 434 (ATCC VR-902B) of Ct cultured in MCCOY cells and strain 6BC (ATCC VR-125) of Cps cultured in Hela 229 cells. For the MIF test, 5 µl of sera and controls were placed in the wells and incubated in humid chamber at 37 °C for 30 min. After washing the slide twice for 5 min with PBS at pH 7.2 and then for 5 min with distilled water, 5µl of fluorescein isothiocyanate-labelled goat anti-human IgG was added to each well. The above incubation and washing steps were repeated. Finally, the preparation was observed under fluorescence microscope (OLYMPUS BX40F4, Japan) at 400X. A minor discrepancy was defined as a difference in the IgG result of up to one titre dilution and a major discrepancy as a difference of more than one titre dilution.

MRL *Chlamydia pneumoniae* IgG MIF: This test was carried out as previously described by BENNEDSEN *et al.* (2002).

Statistical analysis: The Spearman correlation coefficient (r_{ho}) was used to compare IgG titres obtained by the tests and the chi-square test χ^2 to compare qualitative variables (SPSS 11.0).

Results

Table 1 shows the results obtained by the two MIF tests. The Vircell and MRL MIF tests assessed 58.75% (47/80) and 60% (48/80) of the samples as positive, respectively. In the global assessment of the samples as positive (IgG ≥ 1/16) or negative (IgG < 1/16), the tests coincided in 79 (98.75%) of the 80 samples (47 [59.5%] positive and 32 [40.5%] negative). The IgG titre determined by the tests was identical in 15 of the 47 positive samples (31.9%). There was a major discrepancy between the tests (1/32 by MRL and <1/16 by Vircell) in

Table 1
Results obtained in the samples analysed by the Vircell and MRL microimmunofluorescence (MIF) tests

Vircell	MRL MIF					
	<1/16	1/16	1/32	1/64	1/128	
<1/16	32	–	1	–	–	33
1/16	–	2	6	–	–	8
1/32	–	6	6	2	–	14
1/64	–	–	8	7	–	15
1/128	–	–	–	8	–	8
1/256	–	–	–	–	2	2
	32	8	21	17	2	80

only one sample (1.25%) and a minor discrepancy in 32 samples (24 with higher titres by Vircell and 8 with higher titres by MRL). A significant correlation was found between the two tests (r_{ho} de Spearman = 0.926; $p < 0.001$). The Vircell MIF demonstrated 97.9% sensitivity and 100% specificity in the global assessment of the samples as either positive (IgG ≥ 1/16) or negative (IgG < 1/16) (Table 1). With reference to each group, the sensitivity and specificity were always 100% except in the group with multiple sclerosis, for which the sensitivity was 95.8% and the specificity was 100%.

With respect to IgG against Ct, 72 of the samples were negative by both MIF tests with disparate results for the remaining eight (90% coincidence). For IgG against Cps, 73 samples were negative and one positive by both tests, with disparate results for the remaining six (92.5% coincidence).

Table 2 displays the results obtained in samples with ≥1/16 IgG titres against Cp, Ct and/or Cps. The MRL test detected ≥1/16 IgG titres against Ct in two samples and against Ct and Cps in one. The Vircell test detected ≥1/16 IgG titres against Cps in three samples and against Ct and Cps in one. There were no statistical differences between the tests in simultaneous immunologic reactivity against Ct and/or Cps (χ^2; p: n.s.), despite only coinciding in one sample.

Discussion

The results of two MIF tests that detect IgG against Cp were compared. They presented a very similar capacity to detect anti-Cp IgG and there was a good correlation between their results.

Only one report on the diagnostic ability of the Vircell MIF has been published to date (HERMANN *et al.* 2002), in a study of 11 tests for the detection of IgG anti-EB of Cp (including four MIF tests). Eighty serum samples from apparently healthy blood donors aged 22–57 years old were used. Using the criteria of the majority as the gold standard, the authors

Table 2
Samples with positive IgG against *Chlamydophila pneumoniae* and simultaneous reactivity against *Chlamydia trachomatis* and *Chlamydophila psittaci*

	Number of positive samples (%)		
	C. trachomatis	*C. psittaci*	*C. trachomatis* and *C. psittaci*
MRL	2/48 (4.17)	0 (0)	1/48 (2.08)
Vircell	0 (0)	3/47 (6.38)	1/47 (2.13)

found 71% of samples to be positive and 29% negative. The Vircell MIF showed the same specificity as the MRL test (100%) but a much lower sensitivity (66% vs. 100%). Both MIF tests showed a similar simultaneous reactivity, at least qualitatively, with Ct in five samples, Cps in two, and Ct and Cps in one, a higher number than in the present study (Table 2), although the difference between study populations should be taken into account. Moreover, no correlation between the Vircell and MRL tests was found ($r = 0.79$). This lack of correlation cannot be explained by the use of different strains (TW-183 and 2023), which have demonstrated a similar antigenic reactivity (WANG and GRAYSTON 1994). On the other hand, it has been proposed that EB cultured *in vitro* may express different proteins from those expressed in human infection, so that the results obtained do not specifically exclude some false negatives with the Vircell strain (MONNO *et al.* 2002).

In contrast to the above-mentioned study, we found an excellent correlation between the Vircell and MRL tests. The only apparent explanation is that the Vircell test applied by HERMANN *et al*. (2002) used strain 2023 (ATCC VR-1356), whereas the current test uses strain CM-1 (ATCC VR-1360).

Simultaneous positive reactions among *Chlamydiaceae* species were previously reported (LEINONEN *et al.* 1990, MAURIN *et al.* 1997). It is considered that at least one-third of the higher IgG titres determined by MIF may be due to epitopes shared by the species (WONG *et al.* 1999, MESSMER *et al.* 2001). Differences in findings for these reactions between the work by HERMANN *et al.* (2002) and the present study may be due to the use of distinct LPS purification protocols on strains that are themselves also different. In the present study, scant simultaneous positive reactions were found using the Vircell and MRL tests (Table 2), in neither case in more than 10% of the samples, much lower than the percentage reported by HERMANN *et al.* (2002). This discrepancy may again be due to differences between the study populations.

In conclusion, the current Vircell MIF test detected the presence of IgG anti-EB of Cp in the serum of our series of infected patients with a high degree of reliability.

References

APFALTER, P., BLASI, F. and BOMAN, J., 2001. Multicenter comparison trial of DNA extraction methods and PCR assays for detection of *Chlamydia pneumoniae* in endarterectomy specimens. J. Clin. Microbiol., **39**, 519–524.

BECKER, Y., 1996. Chlamydia. In: Medical Microbiology (4th ed.; Baron, S., ed.), pp. 503–513. Texas: Ed. Galveston.

BENNEDSEN, M., BERTHELSEN, L. and LIND, I., 2002. Performance of three microimmunofluorescence assays for detection of *Chlamydia pneumoniae* immunoglobulin M, G and A antibodies. Clin. Diagnost. Lab. Immunol., **9**, 833–839.

BRADE, H., BRABETZ, W., BRADE, L., HOLST, O., LÖBAU, S., LUKACOVA, M., MAMAT, U., ROZALSKI, A., ZYCH, K. and KOSMA, P., 1997. Chlamydial lipopolysaccharide. J. Endotoxin Res., **4**, 67–84.

GRAYSTON, J. T., CAMPBELL, L. A., KUO, C. C., MORDHORST, C. H., SAIKKU, P., THOM, D. H. and WANG, S. P., 1990. A new respiratory tract pathogen: *Chlamydia pneumoniae* strain TWAR. J. Infect. Dis., **161**, 618–625.

GRAYSTON, J. T., 1992. Infections caused by *Chlamydia pneumoniae*, strain TWAR. Clin. Infect. Dis., **15**, 757–763.

HERMANN, C., GRAF, K., GROH, A., STRAUBE, E. and HARTUNG, T., 2002. Comparison of eleven commercial tests for *Chlamydia pneumoniae*-specific immunoglobulin G in asymptomatic healthy individuals. J. Clin. Microbiol., **40**, 1603–1609.

LABS, K. H., DORMANDY, J. A., JAEGER, K. A., STUERZEBECHER, C. S. and HIATT, W. R., on Behalf of the Basel PAD Clinical Trial Methodology Group., 1999. Transatlantic Conference on Clinical Trial Guidelines in Peripheral Arterial Disease. Circulation, **100**, 75–81.

LEINONEN, M., LINNANMAKI, E., MATTILA, K., NIEMINEN, M. S., VALTONEN, V., LEIRISALO-REPO, M. and SAIKKU, P., 1990. Circulating inmune complexes containing chlamydial lipopolysaccharide in acute myocardial infarction. Microb. Pathog., **9**, 67–73.

MAURIN, M., EB, F., ETIENNE, J. and RAOULT, D., 1997. Serological cross-reaction between *Bartonella* and *Chlamydia* species implications for diagnosis. J. Clin. Microbiol., **35**, 2283–2287.

MESSMER, T. O., MARTINEZ, J., HASSOUNA, F., ZELL, E. R., HARRIS, W., DOWELL, S. and CARLONE, G. M., 2001. Comparison of two commercial microimmunofluorescence kits and an enzyme immunoassay kit for detection of serum immunoglobulin G antibodies to *Chlamydia pneumoniae*. Clin. Diagnost. Lab. Immunol., **8**, 588–592.

MONNO, R., SIBILIO, G., COSTI, A., D'APRILE, A., DE VITO, D., MARCUCCIO, P. and RIZZO, G., 2002. Comparison of two microimmunofluorescence tests for detecting antibodies to *Chlamydia pneumoniae*. N. Microbiol., **25**, 299–306.

PEELING, R. W., WANG, S. P., GRAYSTON, J. T., BLASI, F., BOMAN, J., CLAD, A., FREIDANK, H., GAYDOS, C. A., GNARPE, J., HAGIWARA, T., JONES, R. B., ORFILA, J., PERSSON, K., PUOLAKKAINEN, M., SAIKKU, P. and SCHACHTER, J., 2000. *Chlamydia pneumoniae* serology: interlaboratory variation in microimmunofluorescence assay results. J. Infect. Dis., **181**, 426–429.

POSER, C. M., PATY, D. W., SCHEINBERG, L., MCDONALD, W. I., DAVIS, F. A., EBERS, G. C., JOHNSON, K. P., SIBLEY, W. A., SILBERBERG, L. and TOURTELLOTE, W. W., 1983. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Ann. Neurol., **13**, 227–231.

SAIKKU, P., LEINONEN, M., TENKANEN, L., LINNANMAKI, E., EKMAN, M. R., MANNINEN, V., MANTTARI, M., FRICK, M. H. and HUTTUNEN, J. K. 1992. Chronic *Chlamydia pneumoniae* infection as a risk factor for coronary heart disease in the Helsinki Heart Study. Ann. Intern. Med., **116**, 273–278.

SRIRAM, S., STRATTON, C. W., YAO, S., THARP, A., DING, L., BANNAN, J. D. and MITCHELL, W. M., 1999. *C. pneumoniae* infection of the central nervous system in multiple sclerosis. Ann. Neurol., **46**, 6–14.

WANG, S. P. and GRAYSTON, J. T., 1970. Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. Am. J. Ophthalmol., **70**, 367–374.

WANG, S. P. and GRAYSTON, J. T., 1994. The similarity of *Chlamydia pneumoniae* (TWAR) isolates as antigen in the micro-immunofluorescence (MIF) test. In: Chlamydia infections. Proceedings of the Eighth International Symposium on Human Chlamydial Infection (Chantilly, France. Orfila, J., BYRNE, G. I., CHERNESKY, M. A., eds.), pp.181–184. Bologna, Italy: Societa Editrice Esculapio.

WONG, Y. K., SUEUR, J. M., FALL, C. H., ORFILA, J. and WARD, M. E., 1999. The species specificity of the microimmunofluorescence antibody test and comparisons with a time resolved fluoroscopic immunoassay for measuring IgG antibodies against *Chlamydia pneumoniae*. J. Clin. Pathol., **52**, 99–102.

Mailing address: Prof. Dr. JOSÉ GUTIÉRREZ, Departamento de Microbiología, Facultad de Medicina, Avda. de Madrid 11, E-18012 Granada, Spain
Tel: +34 958243550; Fax: +34 958246119
e-mail: josegf@ugr.es