

The species specificity of the microimmunofluorescence antibody test and comparisons with a time resolved fluoroscopic immunoassay for measuring IgG antibodies against *Chlamydia pneumoniae*

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Abstract

Aims—To examine the species specificity of the microimmunofluorescence test (MIF) and assess a time resolved fluoroscopic immunoassay (TRIA) for measuring IgG antibodies to *C pneumoniae*.

Methods—Sera from 1020 subjects were tested by MIF for IgG, IgM, and IgA antibodies to *C pneumoniae*, *C trachomatis*, and *C psittaci*; 501 serum samples were also tested by TRIA for IgG antibodies to *C pneumoniae*.

Results—*C pneumoniae* antibody titres as measured by MIF were correlated with those for *C psittaci* and *C trachomatis*. It was estimated that on average, one third of the twofold dilution steps that make up the final *C pneumoniae* antibody titre may be due to cross reacting genus specific antibody. The results of TRIA correlated well with those of MIF. In 75% of cases, the TRIA result predicted a three titre range within which the actual MIF result would fall.

Conclusions—MIF does not appear to be as species specific as claimed. TRIA is unlikely to be as specific but as it is completely objective, easier to perform, amenable to automation, and gives reproducible results, it is a rapid and useful method for comparing populations.

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Keywords: microimmunofluorescence test; time resolved fluoroscopic immunoassay; chlamydia

Chlamydia pneumoniae was first described in a seminal study in 1986,¹ in which it was shown to be a cause of respiratory tract infections. Since then it has been associated with several diseases including asthma, sarcoidosis, bronchial carcinoma, and particularly atherosclerosis.² As it is difficult to culture from clinical material, most of the evidence for these associations has come from serological studies. The microimmunofluorescence test (MIF) of Wang and Grayston³ was first validated for the measurement of *C trachomatis* antibodies and is now the gold standard serological test for antibody to chlamydia. In expert hands, it is generally considered to be a species specific test, as it is possible to discriminate visually between the ring-like specific fluorescence caused by antibody to outer envelope proteins and the diffuse

genus specific staining of antibody to lipopolysaccharide.⁴ However, some investigators have questioned this specificity.^{5,6} Undoubtedly, the biggest problem of MIF is that it is subjective and tedious when used to screen large numbers of sera. Enzyme immunoassay is more suitable for large scale screening, being both objective and relatively simple to perform. However, it not thought to be as species specific as MIF and its results are linear only over a relatively narrow range of antibody concentration. Time resolved fluoroscopic immunoassay (TRIA), using europium as a labelling agent, offers linearity over a greater range⁷ so that for screening purposes it may be sufficient to test for antibody at a single serum dilution only. In this study, we have used MIF to measure specific antibody to *C pneumoniae*, *C trachomatis*, and *C psittaci* in sera from 1020 subjects. TRIA was also used to measure IgG antibodies to *C pneumoniae* in 501 of these sera and the results compared.

Methods

SUBJECTS

Our study sample comprised 1020 healthy men (n = 632) and women (n = 388) aged 60 to 75 years, who took part in an earlier study of the association between fetal and infant growth and adult *Helicobacter pylori* infection.⁸ Aliquots of serum taken at that time (1992 to 1994) were stored at -80°C until used for the current study. The subjects were selected on the basis that they had been born and still lived in east or north west Hertfordshire and agreed to take part.

MICROIMMUNOFLUORESCENCE TEST

Indirect MIF assay was performed by the Biobanque De Picardie Laboratory (Amiens, France) using as antigen either *C pneumoniae* (IOL-207), *C psittaci* (Loth), or *C trachomatis* (LB₁ and the serovar L₂) prepared from acetone fixed preparations of infected egg yolk sacs.⁹ Non-infected egg yolk sacs were used as negative controls. Serial twofold dilutions of each serum sample (1:16 to 1:2048 for IgG, 1:16 to 1:512 for IgA, and 1:12 to 1:48 for IgM) were tested. Specific antibody was detected with 1:100 fluorescein isothiocyanate (FITC) conjugated antihuman immunoglobulin (Tago). For the detection of IgM, serum samples were pretreated with rheumatoid factor absorbant (Behringwerke AG) according to the manufacturer's instructions. Where

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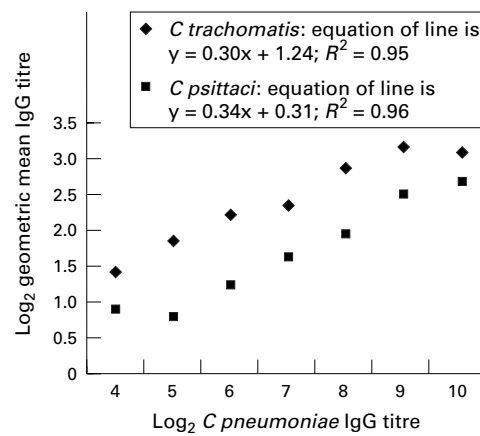


Figure 1 Correlation between *C pneumoniae*, *C trachomatis*, and *C psittaci* microimmunofluorescence IgG titres.

control preparations were positive, chlamydial antigen prepared from organisms grown in tissue culture (Hela 229 cells) was used instead. For IgG and IgA, we considered that species specific antibodies were absent if titres were less than 16 but present if titres for one species were at least two dilution steps greater than for the other species, or if titres for the other species were absent. Similar criteria were used for IgM except that titres less than 12 were considered negative.

TIME RESOLVED FLUOROSCOPIC IMMUNOASSAY

We coated 96-well plates (Nunc Maxisorb) with whole, purified *C pneumoniae* (VR1310) organisms (2 µg in 100 µl 1% sodium azide/phosphate buffered saline solution) at room temperature overnight. We then blocked the plates with 200 µl of 10% normal goat serum and sequentially incubated them with 100 µl of 1:100 serum, 100 µl of 1:1000 biotin

labelled goat antihuman IgG immunoglobulin (Kirkegaard and Perry Laboratories), and 100 µl of 1:1000 Europium labelled streptavidin (Wallac Oy). All incubations were done at 37°C. The first two incubation stages were for two hours and the final two for one hour. Sera, antihuman immunoglobulin, and europium labelled streptavidin were diluted in a solution containing 150 mM NaCl, 25 mM Tris, 0.005% Tween 40, 0.25% bovine serum albumin, 0.023% bovine γ globulin, 10 µmol diethylenetriaminepentaacetic acid, and 0.025% sodium azide at pH 7.8. We washed the plates between each incubation with a solution containing 0.1 M NaCl, 0.025 M Tris, and 0.05% Tween 20 at pH 8. Finally, we added 100 µl of Delfia® enhancement agent (Wallac Oy) to each well and determined the fluorescence count with a 1234 Wallac fluorometer. Eight control sera were included in each plate. We tested each serum sample in duplicate without knowledge of the MIF result.

Results

SPECIFICITY OF THE MIF TEST

Valid results were obtained for 947 subjects for IgG, 985 for IgA, and 950 for IgM. For 71 IgG samples, 33 IgA samples, and 68 IgM samples, control preparations from egg yolk were reported as positive. Antigen preparations from tissue culture grown organisms were used for 54 of these subjects (IgG only), giving a total of 1001 IgG results. For two subjects, no result was available because of insufficient serum.

For IgG, there was an almost linear association between *C pneumoniae* titres and geometric mean titres for *C trachomatis* and *C psittaci* (fig 1). Such an association was not seen for IgA and IgM, probably because of the small number of samples with high titre values. Table 1 shows the number of samples with specificity for one chlamydial species and the number of subjects seropositive for *C pneumoniae*, *C trachomatis* and *C psittaci*.

Table 1 Number (percentage) of subjects with specific antibody to *C pneumoniae*, *C trachomatis*, and *C psittaci* by microimmunofluorescence

	<i>C pneumoniae</i>	<i>C trachomatis</i>	<i>C psittaci</i>
IgG titre (n = 1001*)			
Genus specific antibody	154	346	276
<1:16	218 (25.7)	616 (94.0)	724 (99.9)
1:16	42 (5.0)	6 (0.9)	0
1:32	60 (7.1)	5 (0.8)	0
1:64	146 (17.2)	7 (1.1)	1 (0.1)
1:128	167 (19.7)	7 (1.1)	0
1:256	110 (13.0)	10 (1.5)	0
1:512	75 (8.9)	2 (0.3)	0
1:1024	22 (2.6)	2 (0.3)	0
1:2048	6 (0.7)	0	0
1:4096	1 (0.1)	0	0
IgA titre (n = 985*)			
Genus specific antibody	20	28	15
<1:16	622 (64.5)	947 (99.0)	970 (100)
1:16	94 (9.7)	3 (0.3)	0
1:32	102 (10.6)	5 (0.5)	0
1:48	2 (0.2)	0	0
1:64	71 (7.4)	1 (0.1)	0
1:128	53 (5.5)	0	0
1:256	16 (1.7)	1 (0.1)	0
1:512	5 (0.5)	0	0
IgM titre (n = 950*)			
Genus specific antibody	14	16	8
<1:12	895 (95.6)	928 (99.4)	942 (100)
1:12	30 (3.2)	5 (0.5)	0
1:24	6 (0.6)	1 (0.1)	0
1:48	5 (0.6)	0	0

*The denominator used in calculating the percentage of subjects with species specific antibody was the total number of subjects with species specific antibody for that immunoglobulin class.

COMPARISON OF MIF AND TRIA IN MEASURING IgG ANTIBODIES FOR *C PNEUMONIAE*

Five hundred and one serum samples were assayed by TRIA in duplicate, with intra- and interassay coefficients of variation of 4.7 and 18.2, respectively. However, only 484 samples had IgG antibodies for *C pneumoniae* measured by both MIF and TRIA (294 male, 190 female, average age 67 years), of which only 389 had species specific MIF results. Table 2 shows a comparison of results for the MIF and TRIA tests for these 389 subjects. Comparison is limited by the fact that MIF titres are categorical variables whereas TRIA fluorescence counts are continuous variables. Nevertheless, for any fluorescence count, a range of possible MIF titres can be predicted within which most values will fall and which consist of the modal MIF titre ±1 titre. Furthermore, the mean fluorescence count for a particular population has a linear relation with the log₂ geometric mean MIF titre for that population (fig 2). However, some sera clearly fall outside the predicted MIF titre—57 serum samples (14.7%) had MIF titres that were lower than predicted

Table 2 The correlation between time resolved fluoroscopic immunoassay and the microimmunofluorescence test for measuring IgG antibodies to *C pneumoniae*

TRIA fluorescence count (arbitrary units)	Mean fluorescence count	MIF <16	MIF 16	MIF 32	MIF 64	MIF 128	MIF 256	MIF 512	MIF 1024	MIF 2048	Geometric mean MIF titre*	Subjects within predicted MIF range†
<200 000	96 571.3	81 (89.0%)	2 (2.2%)	1 (1.1%)	5 (5.5%)	1 (1.1%)	0	1 (1.1%)	0	0	1:12.1	83 (91.2%)
200 000–399 999	296 961.2	15 (30.6%)	4 (8.2%)	8 (16.3%)	12 (24.5%)	6 (12.2%)	4 (8.2%)	0	0	0	1:35.2	26 (53.0%)
400 000–599 999	507 878.8	7 (9.7%)	2 (2.7%)	8 (11.1%)	20 (27.8%)	22 (30.6%)	8 (11.1%)	5 (6.9%)	0	0	1:79.3	50 (69.5%)
600 000–799 999	711 326.5	1 (1.2%)	1 (1.2%)	6 (7.0%)	20 (23.3%)	22 (25.6%)	23 (26.7%)	10 (11.6%)	3 (3.5%)	0	1:142.5	65 (75.6%)
800 000–999 999	896 996.2	3 (3.8%)	0	1 (1.3%)	8 (10.0%)	14 (17.5%)	23 (28.8%)	21 (26.3%)	8 (10.0%)	2 (2.5%)	1:247.2	58 (72.6%)
1 000 000–1 999 999	1 030 441.4	0	0	0	0	1 (9.1%)	2 (18.2%)	5 (45.5%)	2 (18.2%)	1 (9.1%)	1:512	9 (81.9%)

*To allow calculation of the geometric mean MIF titre, samples with a titre < 16 were given a titre value of 1. †The predicted MIF range for the corresponding TRIA range is the modal MIF titre \pm 1 titre (highlighted cells).

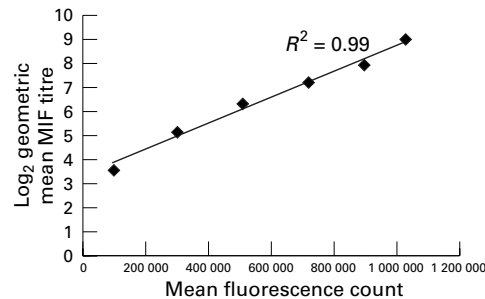


Figure 2 Linear correlation between mean time resolved fluoroscopic immunoassay fluorescence count and Log_2 geometric mean microimmunofluorescence (MIF) antibody titre.

and 41 (10.5%) had titres that were higher. Similar findings were obtained when MIF was compared with TRIA using all 484 samples that had IgG antibodies measured by both techniques and not just those that had species specific MIF results.

Discussion

The MIF test is the classic and time honoured method for measuring antibody to chlamydia but it involves fluorescence microscopy which inevitably has a large subjective component.¹⁰ Although MIF uses crude whole chlamydial antigen which inevitably cross reacts with antibody to other bacteria, the strength of the test is that species specific antibody can be distinguished from other antibody by the pattern of fluorescence. Such distinctions are difficult to standardise, require considerable experience, and observer errors can occur. Also, the chlamydial strains used and the method of antigen preparation vary between different laboratories and this may contribute to differences in results.¹¹ In this large study, MIF was performed by a national reference laboratory that achieved excellent results in a multicentre interlaboratory comparison of MIF results.¹¹ However, a strong association between antibody titres to the three chlamydial species was still seen, indicating that even with a great deal of experience, genus specific antibody still influences the results. Nevertheless, our findings for this population were as expected—that is, antibodies to *C pneumoniae* were more common than antibodies to *C trachomatis*, which in turn were more common than antibodies to *C psittaci*. One possibility is that genus specific antibody occasionally produces a homogeneous ring fluorescence. Figure 1 shows the geometric mean

C trachomatis and *C psittaci* IgG titres for the corresponding *C pneumoniae* IgG titre. If MIF is species specific then the geometric mean IgG titres should be the same no matter what the IgG titre for *C pneumoniae*. However, there is clearly a linear increase in the log_2 geometric mean titre for *C psittaci* and *C trachomatis* with increasing log_2 *C pneumoniae* titre, the rate of increase (gradient of the equations) being about the same for both *C trachomatis* (30%) and *C psittaci* (34%). This may indicate that on average, 30–34% of the log_2 IgG titre for *C pneumoniae* results from the effect of genus specific antibody. The log_2 IgG titre is essentially the number of twofold dilution steps. Therefore, on average, approximately one third of the dilution steps that make up the final titre may be due to genus specific antibody. Whereas the gradient of the respective equations are the same, the intercepts are different and represent the geometric mean titres for *C trachomatis* and *C psittaci* when there is no *C pneumoniae* IgG antibody and presumably no cross reactive antibody (in this study, the starting titre was 16). One approach widely used to reduce these problems of cross reactivity is to consider that species specific antibodies in MIF are only present if their titre is at least two dilution steps greater than that for the other species. This should improve specificity, but in samples where there are antibodies to more than one species, sensitivity will be reduced. Thus in this study, while 1001 IgG results were available, it was not possible to assess 154 samples (15.4%) for *C pneumoniae* because titres were within two dilution steps of the other two species. For *C psittaci*, it was not possible to assess 276 samples (27.6%). Another problem encountered with MIF was that a significant number of control preparations from egg yolk were positive, especially for measurement of IgG (7%). This probably results from the presence of serum antibodies against egg yolk antigen.

In this study, we also compared TRIA with MIF. In comparing a “new” test with an established method, the new test should be at least as good as the old one in terms of specificity and sensitivity, or offer some other significant advantage. There is no doubt that TRIA is much easier to perform and does not require expert interpretation. Also, it gives reproducible results and is amenable to automation. In 75% of samples we were able to predict the MIF \pm 1 titre from the fluorescence count. Moreover, the accuracy of MIF itself is unlikely to be better than \pm 1 titre. Agreement between TRIA and MIF was the best for those sera

which had the lowest or highest fluorescence counts (table 2) and this may reflect the fact that MIF is most accurate when antibodies are absent or are present in very high quantities. Other studies have compared enzyme immunoassay tests with MIF^{12 13} but TRIA using europium as a labelling agent has the advantage that it gives results over a much wider and more linear range⁷ but the disadvantage that specialised hardware is required. However, time resolved fluorometers are often available in chemical pathology laboratories. We did not use TRIA to assay for antibodies to *C trachomatis* or *C psittaci* and therefore cannot compare its species specificity with that of MIF, although it is unlikely to be as good. However, if the specificity of TRIA were much worse than that of MIF, we would expect its results to overestimate MIF titres when in fact it was just as likely to underestimate them.

CONCLUSIONS

We have confirmed that MIF cannot be considered a truly species specific test, even in expert hands, and we have attempted to quantify this disparity. TRIA has the advantages of being more objective and easier to perform and it can predict MIF titres well. We believe it is a rapid, useful method for comparing populations, although obviously no test can be better than the comparative gold standard. The increasing suspicion that *C pneumoniae* and perhaps other members of the Chlamydiaceae may cause chronic disease means that there is an urgent need for the development of truly species specific tests for chlamydial antibody based on defined antigens.

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- 1 Grayston JT, Kuo CC, Wang SP, et al. A new *Chlamydia psittaci* strain, TWAR, isolated in acute respiratory tract infections. *N Engl J Med* 1986;315:161–8.
- 2 Saikku P, Mattila K, Nieminen MS, et al. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart-disease and acute myocardial-infarction. *Lancet* 1988;ii:983–6.
- 3 Wang SP, Grayston JT. Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. *Am J Ophthalmol* 1970;70:367–74.
- 4 Domeika K, Brade L, Mardh PA, et al. Characterization of serum antibody response to chlamydiae in patients with sexually acquired reactive arthritis. *FEMS Immunol Med Microbiol* 1997;19:191–202.
- 5 Kern DG, Neill MA, Schachter J. A seroepidemiologic study of Chlamydia pneumoniae in Rhode-Island—evidence of serologic cross-reactivity. *Chest* 1993;104:208–13.
- 6 Ozanne G, Lefebvre J. Specificity of the microimmunofluorescence assay for the serodiagnosis of Chlamydia pneumoniae infections. *Can J Microbiol* 1992;38:1185–9.
- 7 Suonpää M, Markela E, Ståhlberg T, et al. Europium-labelled streptavidin as a highly sensitive universal label. *J Immunol Methods* 1992;149:247–53.
- 8 Fall CHD, Goggin PM, Hawtin P, et al. Growth in infancy, infant feeding, childhood living conditions, and Helicobacter pylori infection at age 70. *Arch Dis Child* 1997;77:310–14.
- 9 Eb F, Orfila J. Serotyping of Chlamydia psittaci by the micro-immunofluorescence test—isolates of ovine origin. *Infect Immun* 1982;37:1289–91.
- 10 Schachter J. Human Chlamydia psittacci infection. In: Oriol D, Ridgway G, Schachter J, et al, eds. *Chlamydial infections*. New York: Cambridge University Press, 1986:311–20.
- 11 Peeling RW, Wang SP, Grayston JT, et al. Chlamydia serology: inter-laboratory variation in microimmunofluorescence results. In: Stephens RS, Byrne GI, Christiansen G, et al, eds. *Chlamydial infections. Proceedings of the Ninth International Symposium on Human Chlamydial Infection*. San Francisco: International Chlamydia Symposium, 1998:159–62.
- 12 Ekman MR, Leinonen M, Syrjala H, et al. Evaluation of serological methods in the diagnosis of Chlamydia pneumoniae pneumonia during an epidemic in Finland. *Eur J Clin Microbiol Infect Dis* 1993;12:756–60.
- 13 Ladany S, Black CM, Farshy CE, et al. Enzyme-immunoassay to determine exposure to Chlamydia pneumoniae (strain TWAR). *J Clin Microbiol* 1989;27:2778–83.



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