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Laboratory diagnosis of *Mycoplasma pneumoniae* infection

3. Detection of IgM antibodies to *M. pneumoniae* by a modified indirect haemagglutination test

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SUMMARY

The indirect haemagglutination (IHA) test was compared with the complement-fixation (CF) test for the measurement of antibodies to *Mycoplasma pneumoniae*. A modification of the IHA was used to measure *M. pneumoniae* IgM antibodies. Sera were obtained from various groups of patients who were either culture or antigen positive for *M. pneumoniae* in nasopharyngeal aspirates or who had fourfold or greater increase in CF antibody or a titre $\geqslant 320$.

The results of these comparisons showed that the modified IHA test was specific and more sensitive (89% as opposed to 64%) than the CF test. The modified IHA test for the detection of IgM antigody was highly effective in the recognition of recent or current infection with the mycoplasma. It was also of equal sensitivity to an indirect enzyme immunoassay for the detection of IgM antibodies to *M. pneumoniae*.

INTRODUCTION

For many years, after an initial enthusiasm for the culture of *Mycoplasma pneumoniae* in cell-free solid or liquid media as a diagnostic measure, the complement-fixation (CF) test has been the most commonly used method for the serodiagnosis of *M. pneumoniae* infection. It is conveniently incorporated into a battery of CF tests and since some patients with *M. pneumoniae* infection present to the doctor in mid disease (1), it may give an answer more rapidly than the slow and insensitive culture method.

Nevertheless, the CF test has a number of disadvantages. For example, it is less sensitive for antibody measurement than immunofluorescence (IF) on infected chick embryo lung section or metabolic inhibiton tests, and detects fewer diagnostic increases of antibody titre than IF when paired sera from patients with *M. pneumoniae* infection are assayed (2).

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Further, Grayston and colleagues (3) found that only 58% of patients from whom *M. pneumoniae* was cultured showed a diagnostic increase of CF antibody: similarly, Clyde (4) states that only about 60–65% of culture-positive pneumonia patients have a significant increase of CF antibody.

Finally, the CF test, as commonly used, measures total antibody and predominantly that in the IgG class and detection of antibody of other immunoglobulin classes by this method involves time-consuming and expensive separation of the IgM, IgG and IgA fractions.

The measurement of *M. pneumoniae* IgM antibodies is important because not infrequently a serum designated as acute phase is taken when pneumonia is apparent rather than at the onset of respiratory infection. Consequently, it and subsequent sera from the patient may show high unchanging CF antibody titres to *M. pneumoniae*. It may then be difficult to tell whether the antibody relates to the current illness or an infection in the past.

For the above reasons, and also because experience with the CF test during two prevalences (1978 and 1983) of *M. pneumoniae* infection in Adelaide showed inadequacies similar to those described by Grayston and colleagues (3) and Foy and colleagues (5), we now report the development of an indirect haemagglutination (IHA) assay and its modification to measure the *M. pneumoniae* IgM antibody responses in various groups of patients with respiratory infections.

MATERIALS AND METHODS

Fixation, tanning and sensitisation of sheep red blood cells

These procedures were modified from those of Dowdle & Robinson (6). Fresh sheep red blood cells (SRBC) were fixed in 1% v/v glutaraldehyde and then treated with an equal volume of freshly prepared tannic acid (final concentration 1 in 80000 w/v).

M. pneumoniae whole organisms (freeze-dried) obtained from Wellcome Diagnostics (Beckenham, Kent, England). The organisms were resuspended in 2·0 ml of 0·01 M phosphate buffered saline (PBS) pH 7.2 and sonicated at 4 °C in a Branson Sonifier for 10 min with two 1 min cooling intervals. This sonicate, without centrifugation, was then used for the sensitization of the tanned red cells.

Equal volumes of *M. pneumoniae* sonicate and of 2% v/v tanned cells were mixed and incubated in a roller drum at 37 °C for 1 h. The sensitized SRBC were washed three times with PBS. A 4% v/v cell suspension was prepared in 2·5% v/v fetal calf serum in PBS (the diluent buffer in this system). After 2 days, the cells were resuspended in a fresh volume of diluent buffer and then used in the IHA test. Such sensitized cells could be stored up to 2 months at 4 °C without appreciable loss of activity. Before each test run, the cells were sedimented and resuspended in fresh diluent buffer.

Indirect haemagglutination (IHA) assay for measurement of total antibody to M. pneumoniae

Twofold dilutions of the test sera were made in microtitre (U-bottom) trays (25 μl/well) (Cooke, Linbro or Nunc) from a starting dilution of 1 in 10. An equal volume of 0·4% v/v *M. pneumoniae* sensitized SRBC (SRBC-Mp) was added to

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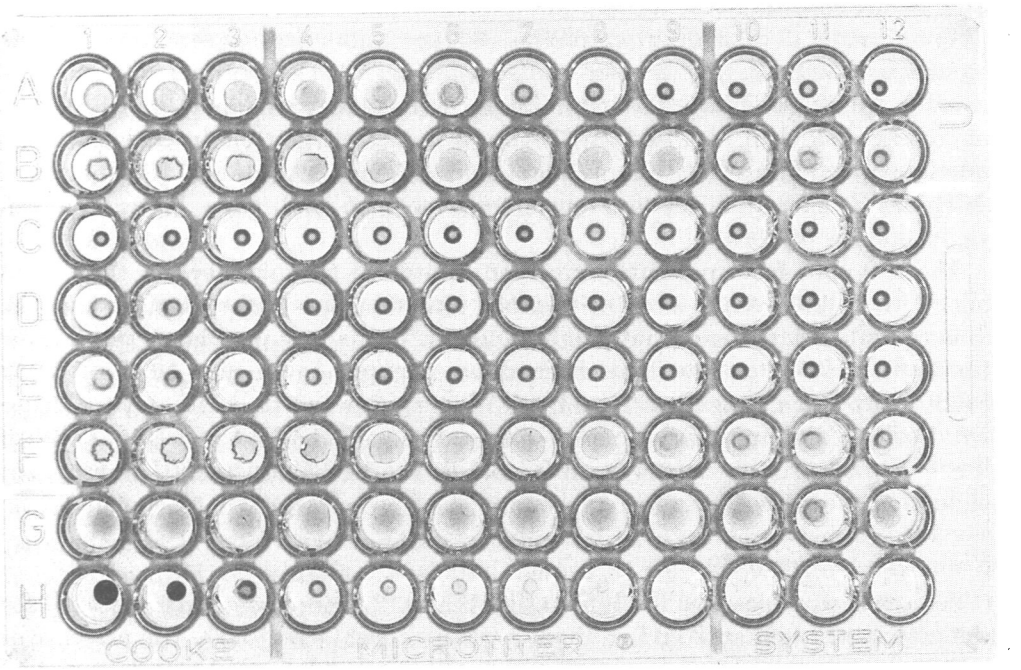

Fig. 1. Indirect haemaggluttination test using glutaraldehyde-fixed, tanned sheep erythrocytes sensitized with sonicated *M. pneumoniae* organisms. Serum dilution in row 1 is 1 in 20. Row A, Rabbit anti-*M. pneumoniae* serum (titre 640); row B, Human serum positive for *M. pneumoniae* antibody (titre 5120); row C, Normal rabbit serum (titre < 20); row D, Human serum negative for *M. pneumoniae* antibody (titre < 20); row E, Duplicate of row D; row F, Duplicate of Row B; row G, Mouse monoclonal antibody to *M. pneumoniae* (titre 10240); row H, Twofold falling dilution of 1·6% sensitized cells made in 50 μl diluent buffer, starting in Well 1. Thus *Well 4* had 0·2% sensitized cells – similar to the other wells (rows A–G) in the plate.

each well. Hence, the final concentration of cells in each well was 0·2% v/v and the first dilution of the serum was 1 in 20. The plate was read after 2–3 h at room temperature (RT), or after overnight incubation at 4 °C. Incubation at the latter temperature generally increased the end point titre by one doubling dilution, compared with incubation at RT. An agglutinated mat of cells covering the bottom of the well indicated the presence of specific antibodies; a button of cells at the bottom of the well indicated a negative result (Fig. 1). In routine testing of human samples, sera with titres of $\geqslant 320$ were considered positive for *M. pneumoniae* antibodies. Titres of 160 were scored as equivocal, and titres of $\leqslant 80$ were taken as negative for *M. pneumoniae* antibodies.

Modified IHA test for IgM antibodies to M. pneumoniae

Rabbit anti-human μ-chain immunoglobulins (Dakopatts, Denmark) were added to each well (100 μl/well) of a polystyrene microtitre tray (U-bottom) (Cooke, Linbro or Nunc). The optimal dilution of antiserum for coating the wells was determined by chessboard titration of increasing dilutions of the anti-μ chain or other antiserum, against twofold dilutions of (*a*) a convalescent phase serum

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from a currently infected patient, previously shown to be positive by CF test and (*b*) as a control, twofold dilutions of pre- and post-immunization sera from a rabbit immunized with *M. pneumoniae* (data not shown). The optimal dilution was usually a 1 in 50 dilution of the anti-μ chain antiserum (made in PBS). The microtray was then air-dried at 37 °C and immersed for 10 s in cold methanol previously dehydrated with molecular sieves (Sigma, 3 A) at −20 °C. The use of dehydrated methanol for fixation was important and gave better red cell agglutination patterns.

The test sera from patients were then diluted in twofold steps in the coated microtitre plate, using a multi-channel pipette (Flow Laboratories) and the diluent buffer. After overnight incubation at 4 °C, the tray was then washed three times with PBS, excess fluid drained on a paper towel and 50 μl of 0·2% SRBC−Mp was then added to each well. The plate was left at RT and read after 1 h. Positive and negative patterns were read as with the IHA test (Fig. 1). With this modified IHA test, it was preferable to use SRBC-Mp cells that were not older than 2 weeks.

Rheumatoid factor (RF) test

Sera that were positive for IgM antibodies to *M. pneumoniae* were checked for the presence of IgM anti-IgG antibodies (rheumatoid factor; RF). The presence of RF was determined by slide agglutination of latex beads coated with human IgG (Commonwealth Serum Laboratories (CSL), Parkville, Victoria, Australia). Such sera were then absorbed with washed, glutaraldehyde cross-linked human serum (7) and then re-tested for *M. pneumoniae* IgM antibodies.

Complement-fixation tests

These used a chloroform–methanol extracted antigen from CSL.

Enzyme immunoassay for IgM antibody to M. pneumoniae

This technique, devised in our laboratories, was a solid phase indirect enzyme immunoassay (IgM-EIA) for the detection of *M. pneumoniae* IgM antibody and resembled that of Busolo and colleagues (8). The soluble extract of the sonicated CF antigen (Wellcome Diagnostics) was used to coat the microwells. Test sera were diluted 1 in 400, added to the wells and incubated for 1 h at 37 °C. After washing three times with PBS (containing 0·1% Tween-20), an optimal dilution of a peroxidase-labelled anti-human IgM serum was added and incubated for 30 min at 37 °C. After a further wash (3×), *O*-phenylenediamine.2HCl (containing 0·02% hydrogen peroxide) was added to each well and the enzyme reaction allowed to proceed for 30 min. The colour reaction was read spectrophotometrically at 490 nm. Absorbance values greater than 3 × S.D. plus the mean of 3 negative sera were taken as positive.

Patients used for validation of the assay in Table 1

Group A. Patients with respiratory illness and either a ⩾ 4-fold rise in CF antibody titre or with a high unchanging titre of ⩾ 160 to *M. pneumoniae*. The CF antibody titres to chlamydiae, Q fever and respiratory viral antigens were all < 5. Note that the acute phase, as well as the convalescent phase sera are included in this group.

Group B. Patients with respiratory illness from whom evidence of virus infection was obtained by showing CF antibody titre $\geqslant 160$ to adenovirus, or parainfluenza or influenza. *M. pneumoniae* CF antibody titres were all < 5.

Group C. Patients with respiratory infection but with no CF antibody response to *M. pneumoniae*, Chlamydiae, Q fever or viral antigens.

Group D. There were 32 patients in this group who showed CF antibody titre $\geqslant 160$ to adenovirus, parainfluenza or influenza viruses. They were different from those in Group B.

Patients used to determine performance of the tests in routine practice

Group E. The group consisted of 649 patients, with the majority in the 1–4-year age group. Acute and convalescent phase sera were collected from these patients during the normal course of our diagnostic virological service. The acute blood sample was collected usually within 2 days of taking the NPA and the convalescent blood was obtained between 10–14 days after onset of illness. Antibodies to the respiratory viruses (namely adenovirus, influenza A and B, parainfluenza 1, 2 and 3 and respiratory syncytial virus), Chlamydiae and Q fever, in addition to *M. pneumoniae*, were also assayed by the CF test. The majority of the patients also provided an NPA which was tested for the presence of respiratory viruses by a fluorescent antibody test on cells (9) and by virus culture, in addition to culture for *M. pneumoniae*.

RESULTS

Comparison of the measurement of complement fixing antibody to M. pneumoniae *with the total antibody levels detected by indirect haemagglutination*

The specificity of the IHA test for total antibodies to *M. pneumoniae* was compared with the CF test using 214 sera from three different groups of patients (Table 1). The results show that there is a good correlation in the distribution of antibody between CF and IHA in group A. The sensitivity of the IHA test is greater than the CF test as judged by the larger number of responders with titres $\geqslant 2560$ and the higher mean titre.

The specificity of the IHA test is shown by the predominant responders with low IHA titres ($\leqslant 40$) in Group B (88%, 37/42) and Group C (98%, 46/47) (Table 1).

Detection of IgM antibody to M. pneumoniae *by modified IHA*

The specificity of the assay both for the measurement of total antibody and for IgM antibody is illustrated by the results of test on sera from patients with a fourfold rise in CF antibody titre (Table 2).

Titres of IgM antibody *M. pneumoniae* were similar to the total antibody titres in the IHA test. In those acute phase sera with low ($\leqslant 80$) *M. pneumoniae* CF titres (patients A–D), the corresponding IgM antibody titres were significantly higher, an indication of the greater sensitivity of the modified IHA test for the early IgM response compared to the CF test.

Treatment of sera with 0·01 M 2-mercaptoethanol removed or reduced the IgM antibody to *M. pneumoniae*, again showing the specificity of the assay (Table 2). (Similar levels of IgM antibody to *M. pneumoniae*, again sensitive to 2-ME, were present in convalescent-phase sera from eight patients who had been shown to be culture positive for the organism on cell–free media (data not included).)

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Table 1. *Assay of* M. pneumoniae *antibody by indirect haemagglutination and complement fixation in groups of patients with and without evidence of* M. pneumoniae *or virus infection*

Category	Number of patients with *M. pneumoniae* antibody at stated titre determined by CF or IHA								Mean titre	Total number
	⩽ 20	40	80	160	320	640	1280	⩾ 2560		
Group A										
IHA	16	1	7	9	12	26	13	41	1156	125
CFT	14	3	4	34	27	23	14	6	502	125
Group B										
IHA	29	8	5	0	0	0	0	0	31	42
CFT	42	0	0	0	0	0	0	0	20	42
Group C										
IHA	35	11	1	0	0	0	0	0	26	47
CFT	47	0	0	0	0	0	0	0	20	47

Table 2. *Analysis of IgM antibody response to* M. pneumoniae *measured by indirect haemagglutination (IHA) in seven patients who were originally diagnosed by a ⩾ 4-fold rise of CF antibody*

Patient	Serum	Total IHA titre	CF titre	IgM-IHA titre	2-ME†	RF‡
A	1	1280	80	1280	20	Neg
	2	1280	320	2560	20	Neg
B	1	320	80	320	20	Neg
	2	320	320	640	20	Neg
C	1	NT	40	640	40	Neg
	2	640	320	640	20	Neg
D	1	320	5	640	160	Neg
	2	640	160	640	160	Neg
E	1	40	320	20	NT	Neg
	2	640	1280	1280	20	Neg
F	1	20	5	20*	NT	Pos
	2	1280	80	2560*	20	Pos
G	1	20	10	20	NT	Pos
	2	320	320	640*	20	Pos

* The IgM titres remained unchanged after absorption with glutaraldehyde-linked human serum.

† IgM titres after treatment with 2-mercaptoethanol.

‡ Rheumatoid factor, screened at 1/20 dilution of the serum.

NT, not tested.

The specificity of the IgM-IHA test was also shown with the results of sera from patients in Group D. No IgM antibodies to *M. pneumoniae* were detected in the latter group of sera (data not shown).

The presence of rheumatoid factor in the paired sera from patients F and G (Table 2) did not affect the specific IgM antibody titre; absorption of sera from patients F and G with glutaraldehyde-fixed human sera removed the RF. but the IgM antibody to *M. pneumoniae* titres remained unchanged. Nevertheless, in

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Table 3. *Relation between the CF antibody responses and those measured by the IgM antibody test for* M. pneumoniae (*IgM-IHA*) *in 649 patients with respiratory infection*

No. patients with antibody responses as measured by CF test	Number of patients with antibody titre or designated increase in antibody titre as measured by IgM-IHA		
	4-fold rise or ⩾ 320	⩽ 160	Total
4-fold rise or ⩾ 160	72*	9‡	81
⩽ 80	40†	528§	568
Total	112	537	649

* *M. pneumoniae* isolated from 25/47 (53%) patients tested.
† *M. pneumoniae* isolated from 5/24 (21%) patients tested.
‡ *M. pneumoniae* isolated from 1/3 (33%) patients tested.
§ M. pneumoniae isolated from 16/308 (5%) patients tested.

subsequent routine tests for *M. pneumoniae* IgM antibodies, sera were also screened for the presence of rheumatoid factor by the latex agglutination test and adsorbed if positive.

Persistence of IgM antibody to M. pneumoniae *1–3 years post infection*

The IgM-IHA was used to measure antibody in sera from 55 patients taken 1, 2 and 3 years after *M. pneumoniae* infection. These patients (age 3–10 years) had initially shown high (⩾ 320) or fourfold rising CF antibody titres, and included some that were also culture-positive for *M. pneumoniae*. Only one of the 55 patients showed an IgM antibody titre to *M. pneumoniae* > 320 one year after the infection; this patient was < 40 at 2 years.

Experience with the routine assay of IgM antibody in patients with suspected M. pneumoniae *infection*

Sera from 649 patients (Group E) were tested for antibody to *M. pneumoniae* by IHA and CF (Table 3). Of the group, 17% (112/649) showed a ⩾ 4 fold rise in IgM antibody or a titre ⩾ 320 in the IgM-IHA test (Table 3). In contrast, significantly fewer – 12% (81/649)had a fourfold Ab rise in, or antibody titre ⩾ 160 in the CF test ($\chi_1^2 = 348$, $P < 0{\cdot}00001$).

The sensitivity of the IgM-IHA for antibody to *M. pneumoniae*, when compared with the CF test as the standard is 89% (72/81) and its specificity is 93% (528/568). When the IgM antibody assay is used as the standard, the sensitivity of the CF test is significantly lower at 64% (72/112) ($\chi_1^2 = 18{\cdot}4$, $P < 0{\cdot}001$).

Table 4 shows the relationship between the IgM antibody responses to *M. pneumoniae* and the results of culture for the organism from nasopharyngeal aspirates (NPA) taken from 423 patients. The culture method used was a cell sheet culture/immunofluorescence method with subculture on mycoplasma agar (unpublished data). Among 47 culture positive patients, 33 (70%) showed IgM antibody titres ⩾ 160 or with a fourfold rise in antibody titre – an efficiency of

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Table 4. *Relation between serological responses in the IgM-IHA test and cultures of nasopharyngeal aspirates (NPA) for* M. pneumoniae *by the cell culture/immunofluorescence method*

M. pneumoniae culture results on NPA	Number of patients with stated IgM-IHA result: 4-fold or ⩾ 320	160	⩽ 80	Total
Positive	30 (64)*	3 (6)	14 (30)	47 (100)
Negative	41 (11)	11 (3)	324 (86)	376 (100)
Total	71	14	338	423

* Figures in parentheses = percent of total (100).

Table 5. *Relation between assay results in the modified IHA test (IgM-IHA) and a solid-phase indirect enzyme immunoassay (EIA) for the detection of* M. pneumoniae *IgM antibodies. Comparison made under code with paired sera from 29 patients who showed either a fourfold or greater rise of antibody or a CF titre of ⩾ 320 to* M. pneumoniae

Antibody response in IgM-IHA	Reactions in IgM-EIA: Positive*	Negative	Total
4-fold rise in antibody or titre ⩾ 320	23	2	25
⩽ 160	2	2	4
Total	25	4	29

* See Materials and Methods for definition of colourometric values taken as positive.

70% with culture positive patients ($\chi^2_2 = 88$, $P < 0{\cdot}001$). Nevertheless, there were discrepancies in both directions, with 30% of culture-positive patients failing to give a positive IgM-IHA and 18% of IgM-IHA positives failing to yield a positive culture.

A positive culture on an NPA, and a seropositive in the CF test, may reflect the sampling of a subgroup with a heavy or prolonged infection. It was therefore of interest to compare the performance of the IgM-IHA and the CF test with sera from patients in which infection with *M. pneumoniae* had been identified by the antigen capture method (1) rather than by culture. In a group of 15 patients who were positive for *M. pneumoniae* by antigen capture, ten had either a fourfold or greater rise in IgM antibodies or a titre of ⩾ 320 by the IgM-IHA test. In the latter group, there were two who showed a greater than fourfold rise of CF antibody titre; sera from the remaining 13 patients were CF negative. Thus, in these more stringent conditions the difference in the effectiveness of the IgM-IHA and the CF test was even more marked.

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Comparison of the IgM-IHA test with an enzyme immunoassay for detection of M. pneumoniae *IgM antibodies*

The IgM-IHA test was also compared with a solid phase indirect enzyme immunoassay (IgM-EIA) for the detection of *M. pneumoniae* IgM antibodies. Acute and convalescent phase sera from 29 patients with either a fourfold or greater increase in CF antibody, or an unchanging titre of $\geqslant 320$, were examined under code in the IgM-IHA and in the IgM-EIA.

Twenty-three of the 29 paired sera were positive for *M. pneumoniae* IgM antibodies by both tests (Table 5), with each test showing two discordant results. Two pairs of sera were negative by both tests (some reinfections with *M. pneumoniae* are not accompanied by formation of IgM antibody (10)); overall, the IgM-IHA appears to have a similar sensitivity to the IgM-EIA, but the period of persistence of IgM in the latter test has not yet been determined.

DISCUSSION

The serological results of the preliminary tests and the trial with routine clinical specimens illustate the greater sensitivity of the IHA and IgM-IHA tests when compared to the CF test; a high specificity is retained. This is probably due to the greater efficiency of the haemagglutination assay in measuring IgM class antibodies. Moreover, the *M. pneumoniae* CF test with chloroform-methanol extracted antigen used in these trials measures antibodies to a glycolipid antigen, whereas, the IHA measures antibodies to both protein and glycolipid antigens.

The efficiency of the IgM−IHA test for antibody was 70% in culture-positive patients with respiratory disease – a higher rate than that obtained with the CF test by Grayston and colleagues (3), who found that 58% of culture-positive patients with pneumonia had rises in CF titres.

The decline of IgM antibody to *M. pneumoniae*, as measured by IgM-IHA was also satisfactory. Only 1 of 55 (2%) was still positive 1 year after illness. This is also similar to the study reported by Coombs and colleagues (11) using the reverse passive haemagglutination test. Thus, the IgM-IHA appears to be a more useful marker of current infection than the CF test, and it is not complicated by the persistence of detectable specific IgM antibody. By contrast, Biberfeld (12) reported the persistence of IgM specific antibodies 2–4 years after the infection using an IF test.

There have been various reports of the successful use of enzyme immunoassays (8) or radioimmunoassays (10, 13–15) for IgM antibody measurement. It has been shown that they are more sensitive than the CF, metabolic inhibition or mycoplasmacidal tests. Again, care must be taken in the interpretation of a positive result because of the high sensitivity of the test, and the possibility of the persistence of specific IgM antibody. This aspect had not been systematically investigated with some of the assays described.

Subsequent to the completion of the work described here utilizing the *M. pneumoniae* whole cell preparation from Wellcome Diagnostics, the product has been withdrawn. However, the IgM-IHA can be performed with *M. pneumoniae*, available from the Central Public Health Laboratories, Division of Microbiological

Reagents & Quality Control, 61 Colindale Avenue, London, NW9 5HT, or grown in the laboratory from prototype strains (1, 16, 17).

A direct comparison (data not shown) of the whole cell antigen from Wellcome Diagnostics, and that from PHL Colindale, using 75 acute and convalescent phase sera from *M. pneumoniae* infections, showed a close correlation (Spearman Rank Order Correlation = 0·84778, $P < 0·00001$).

Once the sensitized cells are prepared, the IHA assay is easy and economical to do on small or large numbers of sera, without the necessity of expensive equipment and a result may be obtained within 2 h of doing the assay. Antibody in other immunoglobulin classes (e.g. IgA, IgG) can also be detected as easily by this method using the appropriate anti-human immunoglobulin as the capture antibody (data not shown).

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