

## Laboratory diagnosis of *Mycoplasma pneumoniae* infection

### 1. Direct detection of antigen in respiratory exudates by enzyme immunoassay

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#### SUMMARY

Direct and indirect antigen capture enzyme immunoassays (Ag-EIA) have been developed for the detection of *Mycoplasma pneumoniae* in nasopharyngeal aspirates or sputum from respiratory infection. The sensitivity of the two Ag-EIA were similar, but the indirect method using polyclonal rabbit and guinea-pig antisera was more convenient. The Ag-EIA had a detection limit of  $10^{4-4.5}$  colony-forming units/ml of sample. It was specific for *M. pneumoniae* and gave a low level response with *M. genitalium*. There were no cross-reactions with 10 other species of mycoplasmas. Tests with a wide range of bacteria and chlamydia group antigen, representing agents sometimes found in the respiratory tract, were also negative. At the current level of development, the Ag-EIA detected about 90% of specimens that were also positive for culture; 43% of specimens from culture-negative – seropositive patients gave a positive result. The overall pattern of results indicated that while antigen detection is a quick and effective substitute for the slow culture method, serological examination for specific IgM antibody is also necessary to give a complete diagnostic coverage.

#### INTRODUCTION

*Mycoplasma pneumoniae*, first isolated from patients with atypical pneumonia by Eaton, Meiklejohn & van Herick (1944), has remained a consistent and significant cause of acute respiratory disease. For example, infection rates ranging from 15–25% are found in children with lower respiratory tract disease (Chanock *et al.* 1960; Clyde 1985); our recent experience in Adelaide mirrors this – in 1983,

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85 (20%) of 423 children, 1–15 years, with respiratory tract illness, were positive serologically or by culture. Higher infection rates – 25–68% of pneumonias – may be observed in institutional or family groups (Chanock *et al.* 1961; Hers, Masurel & Gans, 1969; Foy *et al.* 1979).

Despite these conclusions from planned surveys, in day-to-day practice the significance of the mycoplasma as a pathogen tends to be obscured by the suboptimal performance of commonly used methods for its laboratory diagnosis.

*M. pneumoniae* grows slowly in cell-free media. It may be necessary to incubate diphasic media for 2–3 weeks with periodic subculture on to serum agar plates before an isolate is obtained. Furthermore, it appears that the widely-used Hayflick medium may not be optimal for the culture of all strains of *M. pneumoniae* (Tully *et al.* 1979).

The complement fixation (CF) test for antibody – on which many laboratories rely exclusively – suffers from the limitations (a) that not all culture-positive patients develop CF antibody or a diagnostic increase in CF antibody level (Grayston, Foy & Kenny, 1969; Kok, Varkanis & Marmion (unpublished observations), and (b) that the CF test is less sensitive than the measurement of antibody by immunofluorescence (IF) on infected CE lung sections, by indirect haemagglutination or by metabolic inhibition (Taylor-Robinson *et al.* 1966). In particular, there are difficulties in interpreting the diagnostic significance of high, unchanging CF antibody levels in culture-negative patients with an illness clinically compatible with *M. pneumoniae* infection.

Recent experience in Adelaide indicates that some two-thirds of patients with suspected *M. pneumoniae* infection present in mid, or late disease when CF antibody has already reached a high, unchanging level (titre  $\geq 160$ ); as 90% of these patients also have specific IgM antibody to the mycoplasma, it is probable that in fact they represent current infection with the organism (see Discussion).

Because of these limitations of standard methods and of the need for a rapid test to detect *M. pneumoniae* infection to facilitate decisions about appropriate chemotherapy, attention has turned to direct detection of *M. pneumoniae* antigen or specific nucleotide sequences in respiratory secretions. An early direct method utilized immunofluorescence detection of *M. pneumoniae* antigen on cells from sputum or respiratory exudates (Hers, 1963; Hers & Masurel, 1967), but this technically exacting approach has not been widely adopted. Antigen has also been detected by enzyme immunoassay (Ag-EIA) (Helbig & Witzleb, 1984) although the method was not applied to clinical samples. Ag-EIA has also been used after short term culture of samples in mycoplasma broth (Weiner *et al.* 1985). Antigen may be detected in clinical samples by counter immunoelectrophoresis (Wiernik, Jarstraud & Tunevall, 1978). Most recently, Madsen *et al.* (1988) have described detection of antigen in clinical specimens by immunoblot with a monoclonal antibody to a 43 kilodalton (kDa) membrane associated protein of *M. pneumoniae*. Detection of genomic or specific ribosomal sequences in respiratory secretions is discussed by Harris *et al.* (1988).

We now report our experience since 1983 with a direct and an indirect Ag-EIA for *M. pneumoniae* based on polyclonal antibody, together with the validation of these assays with specimens from natural infections or with simulated positive samples.

An accompanying paper compares the results of antigen detection with the detection of the organism by a probe for its specific ribosomal RNA sequences (Harris *et al.* 1988).

## MATERIALS AND METHODS

*Mycoplasma* strains; culture and quantitation

*Mycoplasma orale* (strain CH19299), *M. buccale* (strain CH20247), *M. faucium* (strain DC-333), *M. hominis* (strain PG 21), *A. laidlawii* A (strain PG 8), *A. laidlawii* B (strain PG 9), *M. pulmonis* (strain ASH PG 34) and *M. hyorhinis* (strain BTS-7) were obtained from the Reference Reagents, National Institute of Allergy and Infectious Diseases Research. *M. salivarium* (strain PG 20) and *M. fermentans* (strain PG 18) were gifts from Dr Wallace Clyde (Chapel Hill, NC, USA). *M. genitalium* (NCTC 10195) was from the National Collection of Type Cultures (Colindale, London).

Standard strains and recent isolates were grown either in SP-4 liquid culture (Tully *et al.* 1977) or on cycloheximide-inhibited, mycoplasma-free HeLa 229 cells in SP-4 medium. For determination of colony-forming units (CFU), growth was on mycoplasma agar (Cruickshank *et al.* 1975) for 7 days (or longer for *M. genitalium*) followed by microscopic counting of colonies. Diphasic medium consisted of SP-4 liquid medium overlaid on a mycoplasma agar slant.

*Preparation of antisera in rabbits and guinea-pigs*

Rabbits and guinea-pigs were immunized with *M. pneumoniae* whole cell antigen (Wellcome Laboratories, Beckenham, Kent; 1 vial resuspended in 1 ml PBS). The initial dose was 0.5 ml of a 1:1 v/v mixture of incomplete Freund's adjuvant and the suspension of *M. pneumoniae* organisms. It was injected in both hind thigh muscles (IM). This was repeated after 2 and 2½ months. One week after the last IM injection, 0.5 ml of *M. pneumoniae* organisms in normal saline was given intravenously to the rabbit or intramuscularly in the guinea-pig. Five days later the animals were bled from the heart and the serum separated. Antibody titres of > 5120 were obtained by indirect haemagglutination (Dowdle & Robinson, 1964) with tanned sheep erythrocytes sensitized with sonicates of *M. pneumoniae* (Wellcome). A range of tests indicated a broad antibody response to both glycolipid and protein antigenic determinants of the organism. The globulin fraction of the sera was fractionated with ammonium sulphate, and dialysed against PBS pH 7.3 at 4 °C.

Sera were absorbed serially with normal human lung homogenate, HeLa 229 cells and glutaraldehyde-insolubilized (Avrameas & Ternynck, 1969) horse serum (because preliminary experiments used *M. pneumoniae* cultures grown in horse serum containing media). When sera from dissimilar animal species were used in the different 'layers' of the indirect Ag-EIA, sera were also absorbed with glutaraldehyde-insolubilized normal serum from the relevant animal species.

The rabbit *M. pneumoniae* antiserum was conjugated with horseradish peroxidase (HRP) by the methods of Avrameas & Ternynck (1971), and O'Sullivan & Marks (1981). Rabbit anti-guinea-pig antibody conjugated with horseradish peroxidase (DAKOPATTS, Denmark) was used in the indirect Ag-EIA.

*Enzyme immunoassay (EIA) procedure for detection of M. pneumoniae antigen*

Duplicate flat-bottom microtitre wells (NUNC, certificated, Cat. No. 439454) were coated at 37 °C for 1.5 h with either the fractionated absorbed anti-*M. pneumoniae* antibody (100 µl/well) (capture antibody), or with a similarly absorbed and fractionated serum from an unimmunized rabbit (control antibody). Sera were diluted in 0.1 M NaHCO<sub>3</sub>, pH 9.6 for coating; the particular dilution used had been determined by previous titration in 'chessboard' fashion with a suspension of *M. pneumoniae* (Wellcome; 1 vial resuspended in 2 ml PBS and sonicated 10 min, 4 °C with cooling). The dilution used was usually *ca.* 25 µg/ml of protein and was usually a 1 in 50 dilution. This sonicate had an antigen titre of 80–160 in the haemagglutination inhibition assay (using four antibody units).

The coated plates were washed with 0.05% v/v Tween 20 in PBS pH 7.3 (PBS/T) then 100 µl of the positive control antigen or of a simulated or clinical specimen was added to the wells. Before addition, the specimens were mixed with an equal volume of 5% w/v non-fat skim milk-PBS/T, and sonicated for 30 sec at 4 °C (Branson B15 sonifier).

Overnight incubation of plates at room temperature gave a more sensitive test than 2 h at 37 °C or overnight at 4 °C. After incubation, unbound antigen was removed by washing with PBS/T.

The detection system in the direct Ag-EIA comprised 100 µl of *M. pneumoniae* rabbit antiserum (8 antibody units) conjugated with horseradish peroxidase (HRP). This was added and incubated for 1 h at 37 °C. In the indirect Ag-EIA, 8 antibody units of the unconjugated guinea-pig *M. pneumoniae* antiserum was added and incubated for 1 h at 37 °C. The plates were washed with PBS/T and finally a rabbit anti-guinea-pig (HRP) conjugate was added and incubated for 1 h at 37 °C. After washing with PBS/T, 100 µl of freshly prepared substrate (1 mg/ml of *o*-phenylenediamine . 2HCl in 0.1 M citrate-phosphate buffer, pH 5.0 containing 0.015% H<sub>2</sub>O<sub>2</sub>) was added and incubated for 15 min/RT. Reactions were terminated with 150 µl 1 M H<sub>2</sub>SO<sub>4</sub>, and the absorbances at 492 nm were determined (Dynatech MR 600 spectrophotometer).

The specific antigen binding percentage was calculated as follows:

$$\text{Antigen binding \%} = \frac{100(\text{At} - \text{Ac})}{2},$$

where At = absorbance for specimen in antibody coated well;

Ac = absorbance for specimen in well coated with normal rabbit serum (control);

2 = maximum absorbance at 492 nm.

*Preparation of mycoplasmas for calibration of the Ag-EIA*

*M. pneumoniae* strains FH-Liu and Ca were grown on mycoplasma-free HeLa 229 cells in SP-4 medium. Portions of the fluid phase and cells from the culture were used to determine the optimal dilutions of coating antibody in the plates, and also as positive antigen controls. In order to determine the dose-response curve of the Ag-EIA assay in terms of colony-forming units (CFU) of the organism, half log

dilutions were made in 5% skim milk-PBS/T and assayed. Colony-forming units were determined for each dilution (see 2 above). Other mycoplasma species were similarly grown and tested at  $> 10^6$  c.f.u./ml.

Other organisms tested for cross-reactivity in Ag-EIA were *Streptococcus MG* and its L-phase derivative, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (including a strain forming protein A: Oxford staphylococcus NCTC 6571), *Candida albicans* and *Nocardia brasiliensis*. The organisms were grown on nutrient agar plates, or in nutrient broth, as appropriate, and the confluent growth, or centrifuged deposit was suspended in 5% w/v skim milk-PBS/T for test. In view of the report of Allen & Prescott (1978), *Strep. pneumoniae* serotypes 3, 4, 6, 14, 19 and 23 – common pathogenic pneumococci (Dr James Paton, Adelaide Children's Hospital) – were tested as heavy suspensions. Cross-reactivity with pathogenic pneumococci was also checked with Pneumovax 23 (Merck, Sharp & Dohme (Australia) Pty Ltd), a mixture of capsular polysaccharides from 23 most prevalent or invasive pneumococci (5 µg/0.1 ml for each polysaccharide). In the light of the report by Grady & Gilfillan (1979), *Legionella pneumophila* serotypes 1–12 and *Legionella longbeachae* 1 and 2 (grown on yeast charcoal agar) were tested. In addition, all antisera used were tested by IF on slide preparations of *L. pneumophila* serotypes 1–10, *L. longbeachae* 1 and 2, *L. bozemani*, *L. mcdadei*, *L. dumoffi*, *L. gormanii*, *L. sainthelensi* and *L. anisa*, all with negative results (data not shown). Cross-reaction with the group antigen of chlamydias was excluded by test of a known chlamydia control antigen and by titration of the rabbit and guinea-pig antisera for chlamydia antibody.

#### *Patients sampled*

The majority of the nasopharyngeal aspirates (NPA) examined were collected from children (1–15 years) at the Adelaide Children's Hospital for routine respiratory diagnosis. Sputum, NPA samples, or *M. pneumoniae* isolates from adults with pneumonia or other illness were kindly routed to us from the Royal Adelaide Hospital (Dr I. Lim and Dr D. Shaw), Flinders Medical Centre (Dr J. Turnidge), Modbury Hospital (Dr E. R. Smith), Royal Children's Hospital, Melbourne (Dr L. Gilbert) and Royal Hobart Hospital, Tasmania (Ms K. Dahlenburg).

#### *Preparation of clinical and artificially infected specimens (simulated positive)*

Mucus (NPA) aspirated from the posterior nasopharyngeal space was washed out of the aspirating catheter with transport medium (CMRL 1969, 1% v/v foetal calf serum, 250 µg/ml ampicillin or 50 µl/ml of cefotaxime). Simulated positive samples consisted of *M. pneumoniae* cultures diluted in a pool of antigen negative (by Ag-EIA) NPA. The inflammatory cells and desquamated respiratory epithelium cells of a natural infection were mimicked by the addition of  $10^5$  HeLa 229 cells/ml.

#### *Culture of clinical specimens for viruses and M. pneumoniae*

Cells deposited from these specimens were examined for viral antigens by immunofluorescence. The supernatants were examined for viruses by standard methods and for *M. pneumoniae* by inoculation of diphasic media with agar

subculture or a HeLa 229 cell monolayer culture-agar subculture system (Kok, Varkanis & Marmion, unpublished observations). Subcultures from the HeLa cell monolayers or other media were made on Hayflick and other mycoplasma agar and suspect colonies of *M. pneumoniae* were identified initially by colony-haemadsorption and then by IF staining of colonies transferred to slides by the method of Clark, Fowler & Brown (1961). Identification was also made by Ag-EIA, as detailed above.

#### *Serological studies*

Acute and convalescent phase sera, or single convalescent samples were tested for CF and specific IgM antibodies to *M. pneumoniae*. CF antibody was measured with a lipid solvent extracted antigen (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia) or an inactivated whole cell antigen (Wellcome).

Specific IgM antibody to *M. pneumoniae* was measured by a solid phase enzyme immunoassay with sonicated whole cell *M. pneumoniae* antigen on the cup and a urease conjugated anti-human IgM or by a  $\mu$  chain capture modification (M-IHA) of the indirect haemagglutination antibody test of Dowdle & Robinson (1964). Total antibody was estimated using the test of Dowdle & Robinson (1964). A survey of 649 respiratory infections showed that the M-IHA was positive in at least 88% of cases found positive by CF test but that it detected an extra 35% of cases with CF responses negative by conventional criteria. The levels of specific IgM antibody as measured by M-IHA decline within 1 year to levels below those taken as significant in the present study (Kok, Varkanis & Marmion, unpublished observations).

## RESULTS

#### *Calibration of direct and indirect Ag-EIA*

Figure 1 shows dose-response curves of specific antigen binding (%) versus dilutions of the Wellcome whole cell antigen in both the direct and the indirect Ag-EIA. The log-log plots give a satisfactory straight line relationship and correlation coefficients of 0.97 and 0.99 respectively. At the 'cut-off' point of 5% (see below) the antigen titre was 5000 in the direct Ag-EIA which used the polyclonal rabbit antiserum to *M. pneumoniae*. This antigen titre corresponded to 25 ng/ml of mycoplasma protein; the CF antigen titre of the whole cell preparation was 128 against 8 units of antibody.

The indirect Ag-EIA with the polyclonal guinea-pig antiserum to *M. pneumoniae*, gave a greater sensitivity than the direct method with a titre of 9500 at the 5% cut-off point. A 'cut-off' value of 5% specific antigen binding was chosen as 'positive' for routine diagnostic purposes. This was well in excess of three standard deviations above the mean of the binding values with NPA from 100 patients without evidence of *M. pneumoniae* infection (mean 1%  $\pm$  0.1%).

Figure 2 shows dose-response curves with the direct and indirect Ag-EIA and falling dilutions of a culture of the FH-Liu strain of *M. pneumoniae* (see Materials and Methods simulated positives). In the titrations the indirect Ag-EIA with guinea-pig antiserum gave slightly more sensitive results than the direct Ag-EIA. Similar curves to those in Fig. 2 were also obtained with two local isolates of *M. pneumoniae* (data not shown).

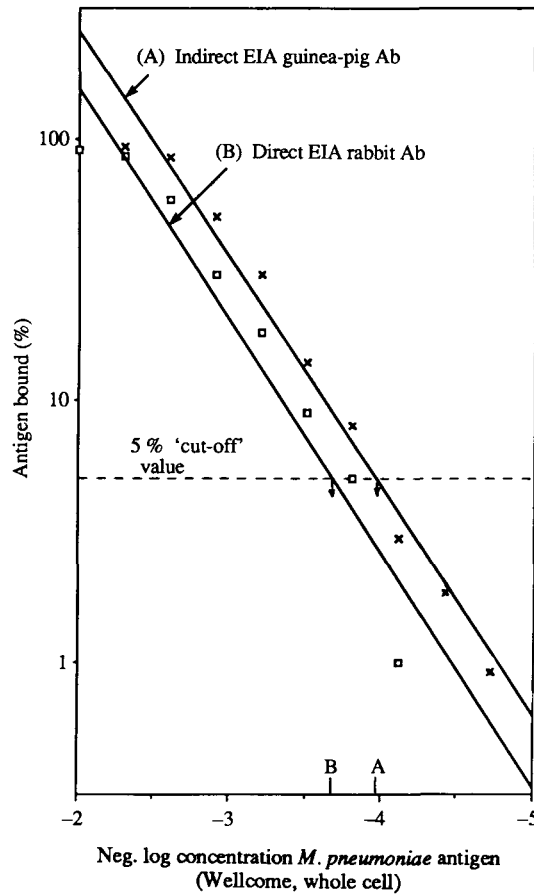


Fig. 1. Log-log plots of the specific binding values (%) obtained in the direct enzyme immunoassay (using peroxidase-conjugated antibodies to *M. pneumoniae*) and the indirect enzyme immunoassay with guinea-pig antibodies to *M. pneumoniae* against falling dilutions of the *M. pneumoniae* complement fixing antigen (Wellcome). Line equations: (A)  $Y = 14260 \times X^{0.8708}$  ( $R = 0.99$ )  
 (B)  $Y = 9332 \times X^{0.889}$  ( $R = 0.97$ )

The 'cut-off' level of 5% specific antigen binding was reached with samples with a final concentration between  $10^4$  and  $10^{4.5}$  CFU of *M. pneumoniae*/ml, in the direct or indirect Ag-EIA tests. In all of these comparative experiments with simulated positive specimens, it was found that culture would detect suspensions containing  $10^2$  c.f.u./ml; 100-300 fold less than either of the enzyme immunoassays.

*Specificity of the direct and indirect Ag-EIA for M. pneumoniae*

The specificity of the direct and indirect (guinea-pig antiserum) Ag-EIA was validated in two ways, (a) by tests with other species of mycoplasma and with bacteria (including the chlamydia group antigen) which might be present in respiratory tract specimens, and (b) by tests with a large number of NPA from patients without evidence of infection with *M. pneumoniae* and with evidence of infection with one of a range of respiratory viruses.

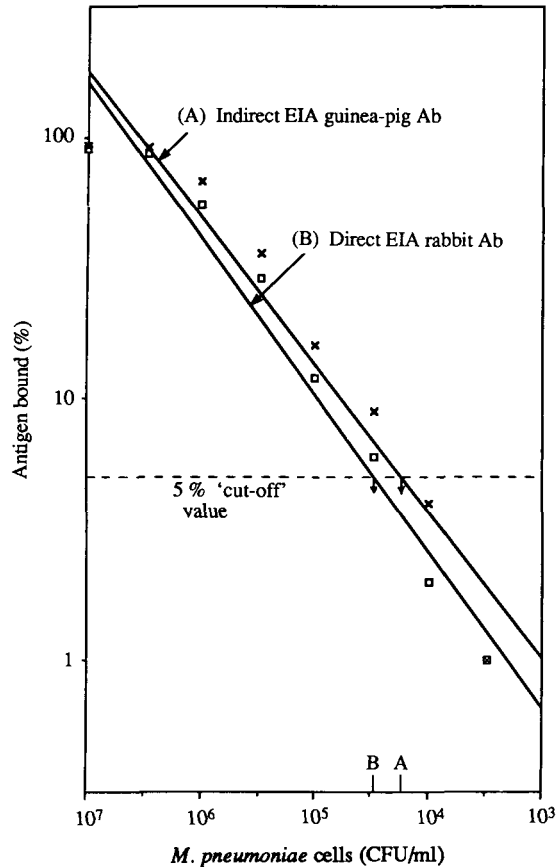


Fig. 2. Log-log plots of the specific binding values (%) obtained in the direct enzyme immunoassay (using peroxidase-conjugated antibodies to *M. pneumoniae*) and indirect enzyme immunoassay with guinea pig antibodies to *M. pneumoniae* against simulated positive clinical samples prepared by inoculation of falling dilutions of a culture of *M. pneumoniae* (CFU/ml) added to a pool of negative nasopharyngeal aspirates. Line equations:

$$(A) Y = 0.0212 \times X^{0.56} \quad (R = 0.97)$$

$$(B) Y = 0.0107 \times X^{0.5969} \quad (R = 0.98)$$

Table 1 shows the specific binding values (%) obtained with *M. pneumoniae* and 11 other species of mycoplasma in the direct and indirect Ag-EIA. High binding values were obtained with *M. pneumoniae* (60–69%); all the remainder were well under the 5% cut-off level except for *M. genitalium* (9%, indirect Ag-EIA). Additionally, specific binding values of less than 1% were obtained with all other organisms listed in Materials and Methods.

Table 2 shows the results with the direct Ag-EIA and 1864 NPA samples from patients with no evidence of *M. pneumoniae* infection (groups A & B) either on culture or by serological tests, or ones taken during a period when *M. pneumoniae* was not prevalent in the community (group C). Approximately one-half of the latter NPA were culture-positive for viruses such as respiratory syncytial virus, parainfluenza, adenovirus, herpes simplex, rhinovirus, cytomegalovirus or poliovirus. Some of the NPA were also positive for bacteria or fungi (namely many



Table 1. Specific binding values obtained in the direct and indirect enzyme immunoassay with polyclonal antisera to *M. pneumoniae* tested with the homologous organism, *M. pneumoniae*, and with other mycoplasma species and bacteria diluted in negative nasopharyngeal aspirates and tested at a final concentration of  $> 10^6$  c.f.u./ml

Mycoplasma and bacterial species added to nasopharyngeal aspirate	Binding values (%) in	
	Direct EIA	Indirect EIA
<i>M. pneumoniae</i>	60	69
<i>M. orale</i>	2	1
<i>M. buccale</i>	2	0
<i>M. faucium</i>	2	1
<i>M. hominis</i>	0	0
<i>M. pulmonis</i>	1	1
<i>M. hyorhinae</i>	1	0
<i>A. laidlawii</i> A	2	0
<i>A. laidlawii</i> B	3	1
<i>M. salivarium</i>	1	2
<i>M. fermentans</i>	0	2
<i>M. genitalium</i>	N/T	9
Legionella, <i>Str. pneumoniae</i> , <i>Str. MG</i> and other bacterial species (see Methods)	< 1	< 1
Uninoculated cell culture medium (CMRL 1969 with 1% foetal calf serum)	0	0

N/T, not tested.

of those indicated in Materials and Methods and additionally *Streptococcus viridans* and *S. epidermidis*. All binding values with these specimens were below the cut-off level of 5%. The binding of antibody globulin by protein A of *Staphylococcus aureus* (Gosting *et al.* 1984) was not a significant problem in these assays in the sense that many samples containing *Staphylococcus aureus* were negative. However, direct tests with Oxford staphylococcus NCTC 6571, a known producer of protein A, gave binding in both the test serum and control serum wells and would be scored as non-specific and negative.

On the other hand, 6 (86%) of 7 NPA from patients in the 1983/84 prevalence with a positive culture for *M. pneumoniae* (groups D, E and F), and 3 (27%) of 11 NPA from patients with negative cultures but reliable serological evidence of infection (see footnote to Table 2), were positive in the Ag-EIA.

#### Results with the indirect Ag-EIA with specimens from patients with and without evidence of *M. pneumoniae* infection

A total of 207 nasopharyngeal aspirates and sputa from patients with respiratory tract infections in the 1986-7 prevalence of infection were tested in the indirect Ag-EIA; the results are tabulated in groups A-F (Table 3). As with the direct Ag-EIA, all 104 specimens from patients negative for *M. pneumoniae* (by culture and serological tests, group A, Table 3), were negative in the indirect assay. Sixty-nine specimens (group B) were tested by culture only; these were also

Table 2. *Specific antigen binding values (%) obtained in the direct enzyme immunoassay with a polyclonal rabbit antiserum to M. pneumoniae on respiratory secretions from patients with respiratory illness due to M. pneumoniae or to other agents*

Group	Category of patient/ evidence of infection	No. of patients	Binding (%) values
A	Culture negative* Serum tests negative†	81	0-2
B	Culture negative Sera not tested	132	0-3
C	Routine specimens taken in non-epidemic period; not cultured for <i>M. pneumoniae</i>	1633	0-2
D	Culture positive‡ Serum tests positive§	2	<b>45, 9</b>
E	Culture positive‡ Serum tests negative	3	<b>4, 9, 13</b>
F	Culture positive‡ No sera available	2	<b>13, 8</b>
G	Culture negative Serum tests positive§	11	0, 1, 0, 1, 4, 0, 4, 0, <b>5, 10, 27</b>
	Total	1864	

\* Culture negative; *M. pneumoniae* not isolated on HeLa 229 cell monolayers and/or on agar subculture.

† Convalescent phase sera CF < 5; specific IgM Ab ≤ 80.

‡ *M. pneumoniae* isolated on HeLa 229 cell monolayers and/or agar subculture.

§ Specific IgM antibody titre ≥ 320, and/or fourfold increase in CF or specific IgM Ab.

negative for antigen. Approximately one half of the specimens in groups A and B were subsequently shown to be culture-positive for viruses or bacteria.

Of the 34 specimens in groups C-F of Table 3, 31 were from patients who had either culture or serological evidence of *M. pneumoniae* infection; of the latter, 21 (67%) were positive (values in bold type) for *M. pneumoniae* in the indirect Ag-EIA. The remaining three patients - one of whom had the highest recorded value (63%) for antigen in any sample - did not have culture or clear serological evidence of infection (group F, Table 3). The patient with the 63% antigen binding value, a male aged 52 with pneumonia involving multiple lobes, previously treated with cytotoxic drugs for carcinoma of the lung, showed a titre of 160 in the IHA on serial samples, but no specific IgM antibody. Repeated attempts to culture *M. pneumoniae* on HeLa 229 cells, in diphasic medium and in chick embryo amnion, were unsuccessful; large doses of broad spectrum antibiotics had been given to the patient. The remaining two patients were children with pneumonia who could not be bled until more than 100 days after onset of illness and at that time showed no or only low titres (< 80) of antibody.

If the results with antigen detection by direct and indirect Ag-EIA, serological testing and culture are combined from Tables 2 and 3, it is found that eight (89%) of respiratory samples from nine patients who were culture-positive for *M. pneumoniae* were also antigen-positive (seven of the nine had been tested serologically and were positive).

Table 3. Specific antigen binding values (%) obtained in the indirect enzyme immunoassay (with rabbit antiserum as capture antibodies and guinea-pig antiserum as the detecting antibody) with respiratory secretions from patients with respiratory illness caused by *M. pneumoniae* or other agents

Group	Category of patient/ evidence of infection	No. of patients	Range of antigen binding (%) values or individual values
A	Culture negative* Serum tests negative†	104	0-2
B	Culture negative Sera not tested	96	0-3
C	Culture positive‡ Serum tests positive§	2	<b>17, 15</b>
D	Culture negative Serum tests positive§	12	1, 0, 3, 0, 2, <b>9,</b> <b>16, 26, 5,</b> <b>18, 5, 15</b>
E	Culture not done Serum tests positive§	17	2, 2, 3, 4, 2 <b>8, 25, 13,</b> <b>11, 21, 16,</b> <b>16, 9, 6, 7,</b> <b>26, 17</b>
F	Culture negative or not done Serum tests equivocal	3	<b>63, 13, 10</b>
	Total	207	

\* Culture negative; *M. pneumoniae* not isolated on HeLa 229 cell monolayer or agar subculture.

† Convalescent phase sera CF < 5; specific IgM Ab ≤ 80.

‡ *M. pneumoniae* isolated on HeLa 229 cell monolayers and/or agar subculture.

§ Specific IgM antibody titre ≥ 320, and/or fourfold increase in CF or specific IgM Ab.

On the other hand, 26 (59%) of samples from 44 seropositive patients were antigen-positive, substantially fewer than among the culture-positive ( $P = 0.024$ ; Fisher's exact test).

#### *Antigen and other diagnostic markers for M. pneumoniae in relation to the stage of illness*

Table 4 relates the results of antigen detection, serodiagnosis and culture to the stage of the disease at which the patient was first seen and sampled. It will be seen that in both the 1983 and 1986-7 prevalences of the infection, a substantial proportion of the patients were not seen and sampled until 8 or more days after onset of illness. This timing limits the usefulness of the serological convention that the diagnosis of a current infection requires the demonstration of a fourfold or greater increase in antibody during the course of the illness. Thus, in the subgroup of patients in Group A seen between 15 and 60 days after onset, only one (3%) met this criterion, the remaining 28 had raised, unchanging antibody titres ≥ 320 by M-IHA or CF test, in the absence of evidence of current infection with a range of

Table 4. Serological responses, antigen detection by direct or indirect techniques and culture as diagnostic markers for infection with *M. pneumoniae* at various stages of illness

Diagnostic category	Duration of illness when patient first seen and sampled (Number/total in subgroup: proportion)			Total
	1-7	8-14	15-60 days	
Group A 1985-7				
Numbers and proportions	19 (25%)	28 (37%)	29 (38%)	76*
with $\geq$ fourfold increase in CF or specific IgM antibody	11/19 (58%)	5/28 (18%)	1/29 (3%)	17/76*
Numbers and proportions with <i>M. pneumoniae</i> antigen in respiratory exudate	12/16 (75%)	7/16 (44%)	5/9 (56%)	24/41*
Group B 1983-4				
Numbers and proportions culture-positive for <i>M. pneumoniae</i>	9/26 (34%)	10/24 (42%)	11/21 (52%)	30/71*

\* All patients had serological evidence of infection with *M. pneumoniae* either in the form of a fourfold or greater increase of CF or specific IgM antibody (as designated) or had raised, unchanging titres ( $\geq 320$ ) of specific IgM antibody and were negative for evidence of current infection with respiratory viruses.

respiratory viruses. It is concluded that they were in fact currently infected with *M. pneumoniae*.

On the other hand, in Group A, antigen was detected by Ag-EIA in each of the three subgroups presenting at different stages of the disease although the rate was a little higher (12/16 or 75%) in patients seen in the first 7 days of the illness. The longest period between onset and presentation was 60 days and of two patients presenting at that time one was Ag-EIA positive. The mean duration in the subgroup 15-60 days was 29 days.

The prolonged persistence of the antigen (and by inference the organism) in the respiratory tract may seem surprising though there are similar indications from other studies (see Discussion). To substantiate the point an analysis has also been made of the results of culture in relation to time of sampling in the 1983-4 prevalence of infection (Group B, Table 4). This group of 71 patients is different from that (N = 76) seen in 1985-7, although the serological criteria for infection are the same. Again it will be seen that the organism can be isolated late in the disease, 11/21 (52%) being culture-positive 15-60 days after onset.

#### DISCUSSION

The work described in this paper establishes that an Ag-EIA, with a direct or indirect detection system, is capable of demonstrating *M. pneumoniae* antigen in respiratory exudates from a substantial percentage of naturally infected patients.

The dose-response curves in the Ag-EIA show a satisfactorily linear log-log relationship between the binding values and the numbers of organisms detected.

and at the cut-off point (5% antigen binding) about  $10^4$  c.f.u./ml of *M. pneumoniae* are detected. In titrations with the Wellcome whole cell *M. pneumoniae* antigen the 5% cut-off level was reached at an antigen titre of 5000 and a protein concentration of 25 ng/ml. Madsen *et al.* (1988), using an immunoblot assay and a monoclonal antibody to a 43 Kd membrane associated protein of the mycoplasma, detected the 43 kDa protein at a limit concentration equivalent to 2.5  $\mu$ g of whole organism; in turn this corresponded to a concentration of 100 ng of mycoplasma protein per ml of specimen. They further calculated that this end-point was equivalent to  $10^{3.5}$  colony forming units.

There is, therefore, reasonable agreement between these two methods of detecting the organism in respiratory secretions. Allowance has to be made for the differences in the methods of protein estimation and for the difficulties of translating values for antigen and protein concentrations into CFU with an organism that grows in variable-sized clumps rather than as a single cell suspension.

The Ag-EIA appears to be specific for *M. pneumoniae* with no cross-reactions with a range of saprophytic mycoplasmas or bacteria found periodically in the human respiratory tract. Only *M. genitalium* showed a cross-reaction in the Ag-EIA; it is known to share antigens with *M. pneumoniae* (Lind *et al.* 1984) but has not, so far as we are aware, been reported in samples from the respiratory tract.

From time to time attention is drawn to the theoretical pitfalls of Ag-EIA based on a polyclonal antiserum to the whole antigenic mosaic of *M. pneumoniae*. Certainly, it is well established that the carbohydrate chains of the mycoplasma glycolipids may cross-react with similar groupings on bacteria such as *Strep. MG* (Marmion, Plackett & Lemcke, 1967), *Strep. pneumoniae* (Allen & Prescott, 1978) or with those of cell surface markers such as cytolipin H (Plackett *et al.* 1969) or the I antigen of human erythrocytes (Costea, Yakulis & Heller, 1971). However, in our experience with the Ag-EIA we have not observed such reactions with the bacteria listed in Materials and Methods or with a suspension of I antigen positive erythrocyte ghosts. We conclude that haptenic cross-reactions demonstrated with concentrated solvent or other extracts of mycoplasmas or bacteria may not necessarily predict effects with intact organisms in which the spatial presentation, density, accessibility and binding affinities of haptenic groups may be different.

The use of a monoclonal antibody to a defined protein of the mycoplasma (Madsen *et al.* 1988) could offer a further safeguard against the perceived difficulties of haptenic cross-reactions related to similarities in carbohydrate chains. In our experience with the Ag-EIA the substitution of a monoclonal antibody to the P1 protein of the mycoplasma for the polyclonal 'capture' antibody gave less sensitive results (data not shown).

In the present series the Ag-EIA detected about 90% of the samples from natural infections that (ultimately) gave a positive culture; overall, it also detected antigen in about 60% of samples taken from patients with good serological evidence of current infection irrespective of culture result. Collier & Clyde (1974) examined sputa from 10 patients with *M. pneumoniae* infection and found that the number of organisms present ranged from  $10^2$  to  $10^6$  c.f.u./ml of sputum, with a geometric mean value of  $10^{4.1}$ . The sensitivity of our Ag-EIA is

compatible with their findings and also with the view that for the most part the number of organisms in the nasopharynx of an infected patient will be above the threshold sensitivity of the Ag-EIA.

As stated earlier, with NPA artificially seeded with *M. pneumoniae* adapted to growth in artificial media, culture was 100–300 times more sensitive than antigen detection. Despite this, in patients with natural infection and reliable serological evidence of infection the Ag-EIA was more often positive than culture. It is presumed that either antigen persists when viable mycoplasma cells have declined in the respiratory tract as the result of the immune response or antibiotic treatment or that with wild strains the number of viable organisms declines below that required to initiate growth in the insensitive artificial media employed.

The dichotomy between culture results and antigen detection highlights a central difficulty in assessing the efficacy of direct diagnostic techniques with *M. pneumoniae*. If culture-positive is taken as the gold standard then the Ag-EIA – and the immunoblot technique of Madsen *et al.* (1988) – give a 90–100% efficiency of detection of samples from infected patients. However, it is clear from our own results and those of Grayston, Foy & Kenny (1969) that only about 50–64% of samples from the upper respiratory tract of patients with *M. pneumoniae* infection are culture-positive; i.e. there is a significant group with good serological evidence of current infection who are culture-negative. It is our view that the direct detection methods should also be assessed against the standard of serological response as well as against culture of the organism.

The data presented in this paper illustrate the complexities of the diagnostic problems in practice. Samples taken in the first 7 days of illness are frequently (75%) Ag-EIA positive, and a good proportion of patients (58%) show a rising titre of antibody to substantiate the Ag-EIA results. However, with patients presenting later in the disease, the Ag-EIA is less often positive and a higher proportion of patients have raised, unchanging antibody titres; assays of specific IgM antibody are required to establish that the latter infections are current, and that the antibody is not due to a past infection.

It will also be noted that a significant proportion (44–56%) of patients were Ag-EIA positive later in the disease (> 8 days from onset). Similar findings with culture were obtained in the 1983–4 prevalence of infection. Prolonged carriage of the organism in the nasopharynx has been noted previously. Thus, Couch, Cate & Chanock (1964) recovered the organism from experimentally inoculated volunteers up to 27 days after its instillation into the respiratory tract. Even an agar adapted, low virulence strain persisted for a similar period.

In summary, we conclude that practical diagnostic terms, while Ag-EIA appears to be a quick and sensitive substitute for culture of the organism, and when positive is of immediate value in assisting decisions on appropriate antibiotic therapy or clinical management, the existence of a substantial group of serologically positive, Ag-EIA negative cases means that it cannot be the sole diagnostic marker used for diagnosis of *M. pneumoniae* infection. For complete diagnostic coverage antigen detection must be coupled with serological examination, in particular for high titres of specific IgM antibody which will distinguish between antibody from a current infection and that from a past infection.

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