A μ-capture ELISA for detecting *Mycoplasma pneumoniae* IgM: comparison with indirect immunofluorescence and indirect ELISA

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SUMMARY

A μ-capture ELISA was developed for detecting *Mycoplasma pneumoniae*-specific IgM, and compared with an indirect immunofluorescent antibody (IFA) technique and an indirect ELISA. μ-capture ELISA and IFA compared well and were found to be the most sensitive assays. The IFA test can be completed in 2 h whilst the results of the μ-capture ELISA can be available in 24 h. Both tests are amenable to routine diagnostic use and have similar sensitivity. Indirect ELISA was found to be less sensitive and less specific, giving high assay values with several sera having undetectable *M. pneumoniae* CF antibody or CF antibody in low titre. Serum samples obtained from 11 patients at various times after *M. pneumoniae* infection showed maximum antibody levels within the first month by all assays, with a gradual fall in amount of IgM with time when assayed by μ-capture ELISA, a more gradual decline by IFA and hardly any decline with indirect ELISA. It was concluded that the indirect ELISA is unsuitable for the investigation of possible *M. pneumoniae* infection because the sustained high assay values with serum samples taken many months after infection, make interpretation of the test results very difficult.

INTRODUCTION

Mycoplasma pneumoniae may cause serious respiratory infection in man. There is a wide spectrum of disease ranging from mild upper respiratory tract infection to pneumonia, which is the most serious manifestation. Myringitis (Rifkind *et al.* 1962), haemolytic anaemia (Finland *et al.* 1945), erythema multiforme (Swift & Stevens, 1978), and neurological symptoms (Mardh, Ursing & Lund, 1975) are seen less often associated with *M. pneumoniae* infection.

M. pneumoniae is difficult to culture, requiring specialized media, and diagnosis is most often made either by demonstrating a high titre of antibody in a single sample of serum or a significant rise in antibody titre in paired serum samples obtained during the acute and convalescent stages of disease. The complement fixation test (CFT) is most often used in these determinations, but CF antibody

may not be detectable until 7–10 days after the onset of respiratory symptoms. In practice, it is often very difficult to interpret CFT results obtained with single samples of serum taken in the early convalescent phase of the disease, unless very high titres are found.

As in most other microbial infections, the initial antibody response after *M. pneumoniae* infection is of the IgM type, although IgG is produced in quick succession (Skaug *et al.* 1976). IgM antibody disappears first (Skaug *et al.* 1976) and it is for this reason that several investigators have designed assays to detect *M. pneumoniae*-specific IgM as an indication of recent infection with this agent. These tests include indirect staphylococcal radioimmunoassay (Brunner *et al.* 1978) indirect immunofluorescence (Biberfield & Sterner, 1971; Skaug *et al.* 1976) complement fixation (Chamberlain & Saeed, 1983) and indirect ELISA (Raisanen, Suni & Leinikki, 1980; Dussaix, Slim & Tournier, 1983).

While being very sensitive, indirect ELISA for detecting IgM is prone to false positive results due to the presence of rheumatoid factor in the serum (Dussaix, Slim & Tournier, 1983). An assay which has been developed to diagnose infection with various viruses (Duermeyer, Wielaard & van der Veen, 1979), is widely believed not to detect non-specific rheumatoid factor in serum. This assay involves coating the solid phase in the test with anti-human-IgM and is known as μ-capture assay. A radioimmunoassay based on this principle was developed for detecting *M. pneumoniae* IgM by Price (1980). Although a very sensitive and specific assay, it is not applicable to general use because of the need for expensive counting equipment as well as the lability and hazard of the radiolabelled conjugate.

We have adapted this technique to ELISA for detecting *M. pneumoniae* IgM and report here on the development and standardization of this assay, comparing it with an indirect immunofluorescence technique (Sillis & Andrews, 1978) and with indirect ELISA.

MATERIALS AND METHODS

Sera

Twenty-three samples of serum with *M. pneumoniae* complement fixing (CF) antibody titres of < 16 were regarded as negative; one of these was chosen as a reference negative serum with zero units of *M. pneumoniae*-specific IgM. A serum with an *M. pneumoniae* CF titre of > 256 and with the highest *M. pneumoniae*-specific IgM activity was chosen as the reference positive control serum. This was assigned an arbitrary value of 100 units of *M. pneumoniae*-specific IgM. This positive control serum was diluted in negative control serum, so as to give dilutions containing 33, 10, 3·3, 1, 0·33, and 0·1 units.

Twenty samples of serum from 17 patients with clinical features of severe lower respiratory tract infection and for which the indirect immunofluorescence *M. pneumoniae* IgM titres were known, were tested in the three assays. Thirty-three sera taken from 11 patients at various times during and following their *M. pneumoniae* infection were used to determine how long *M. pneumoniae*-specific IgM is detectable after infection. All of these patients had clinical features which were consistent with *M. pneumoniae* infection and *M. pneumoniae* was cultured from sputum or throat swab specimens from each of these patients.

Twenty samples of serum positive for rheumatoid factor (RF), 20 with high anti-streptolysin-O (ASO) titres, 20 with CF titres of $\geqslant 64$ to influenza A virus, influenza B virus, adenovirus and *Chlamydia psittaci*, as well as 20 positive in the Paul Bunnell test were also tested in the three assays in order to evaluate their specificity. None of these sera had significant amounts of *M. pneumoniae* CF antibody.

Preparation of M. pneumoniae *antigen*

Antigen for all three assays was prepared as follows: the FH strain of *M. pneumoniae* was initially grown in 8ml of modified Hayflick's broth with 1% glucose and phenol red as indicator (Leach, 1973). The culture was grown to form an adherent monolayer in a 4 oz 'medical flat' on its side during incubation at 37 °C until an acid colour developed. The culture was further maintained by replacing the spent broth with fresh broth and reincubating the bottle until the monolayer was confluent.

The broth was decanted and the monolayer gently washed with three changes of phosphate buffered saline, pH 7·6 (PBS). The antigen was then scraped from the glass with the aid of a cell scraper and suspended in PBS to produce a suspension equivalent to Browns opacity tube no. 9.

In order to produce *M. pneumoniae* slides for IFA, it is important to minimize homogenization of the suspension so as to retain the antigen in a clumped suspension. Ten microlitres of antigen were applied to wells of a Teflon-coated microscope slide and allowed to dry in air. The slides could then be stored, unfixed, at 4 °C and retained their activity for many months.

Antigen for use in ELISA tests was frozen and thawed once, sonicated at maximum setting for 10 min, dispensed in 1 ml volumes and stored at −20 °C until required.

Indirect immunofluorescence (IFA)

Dilutions of sera from 1 in 4 to 1 in 128 were made in PBS and one drop of each applied in duplicate in parallel rows to antigen-coated wells on a microscope slide. A positive and negative control serum was included in each assay. After incubating in a moist chamber for 30 min at 37 °C, the slides were washed in three changes of PBS with periodic agitation and then gently blotted. Ten microlitres of anti-human IgM-FITC (Wellcome Reagents) was applied to the wells in one row and anti-human IgG-FITC (Wellcome Reagents) to wells in the second row. After further incubation at 37 °C for 30 min, the slide was washed as previously described, blotted dry and mounted in glycerol buffered to pH 8–9 with carbonate/bicarbonate buffer.

Slides were examined at × 80 magnification, the endpoint chosen being the highest serum dilution giving strong fluorescence. Sera with IFA titres $\geqslant 4$ were considered positive, the cut-off value being determined by the background fluorescence observed in sera with no detectable *M. pneumoniae* CF antibody at dilution of < 4.

Indirect ELISA

The tests were performed in Falcon flexible assay plates (Becton Dickinson). A unit volume of $100 \mu l$ *M. pneumoniae* antigen in carbonate/bicarbonate buffer, pH 9·6, at a dilution previously determined in a chequerboard assay (1 in 25) was adsorbed overnight at 4 °C on to wells in the plate. The plates were then washed three times (with 0·85 % sodium chloride containing 0·08 % Tween 20). One hundred microlitres of serum diluted 1 in 100 in PBS with 0·08 % Tween 20 (PBS/Tween) was then placed in the wells of the plate, which was incubated at room temperature for 2 h and then washed. Positive and negative controls were included in each assay. Conjugate (horseradish peroxidase labelled anti-human IgM; DAKO, Mercia Brocades Ltd; 1 in 1000 in PBS/Tween) was added and the plates incubated for 3 h at room temperature and then washed. Substrate solution ($100 \mu l$ of *o*-phenylenediamine 1 mg/ml and hydrogen peroxide 0·4 μl/ml in citric acid/phosphate buffer pH 5) was added and the plates incubated for approximately 30 min until a strong colour change was observed in the positive control wells, when the reaction was stopped by adding 25 μl 3 M sulphuric acid. Absorbance was read at 492 nm in a Titertek multiscan (Flow Laboratories Ltd), and the test results were compared with those of reference positive and negative sera.

μ-capture ELISA

Falcon flexible and Dynatech 129A plates were compared for use as the solid phase in the assay.

The horseradish peroxidase conjugate was prepared from mule anti-*M. pneumoniae* (FH) serum (NIH Research Reference Reagent) as previously described (Smith & Tedder, 1981).

The method that was adopted for routine use employed Falcon flexible plates, the wells of which were coated overnight at 4 °C with 100 μl of DAKO rabbit anti-human IgM (diluted 1 in 2000 in carbonate/bicarbonate buffer, pH 9·6). Plates were washed three times as previously described and 100 μl of human serum (diluted 1 in 100 in PBS/Tween) was then added, and the plates incubated at 37 °C for 3 h. Serum dilutions were not placed in the top row of each plate. Reference control sera containing 100, 33, 10, 3·3, 1, 0·33, 0·1 and 0 units of *M. pneumoniae*-specific IgM were included in each assay.

· The plates were washed and 100 μl of *M. pneumoniae* antigen (1 in 10 in PBS/Tween with 10 % fetal calf serum) were added to each well which had contained serum. The plates were stored at 4 °C overnight and then washed three times, before 100 μl of peroxidase-conjugated mule anti-*M. pneumoniae* antibody was added to all wells, except those in the top row. The plates were incubated at 37 °C for 3 h. The dilutions of antigen and conjugate used had been previously determined by chequerboard titrations. The plates were washed three times and 100 μl of substrate solution (*o*-phenylenediamine, 1 mg/ml and hydrogen peroxide, 0·4 μl/ml in citric acid/phosphate buffer pH 5) was added to all wells and the plates incubated for 30 min at room temperature, when the reaction was stopped with 25 μl 3 M sulphuric acid. Absorbance was read at 492 nm. The top row of wells, coated with anti-human IgM and containing substrate and stop solution, were used to blank the plate.
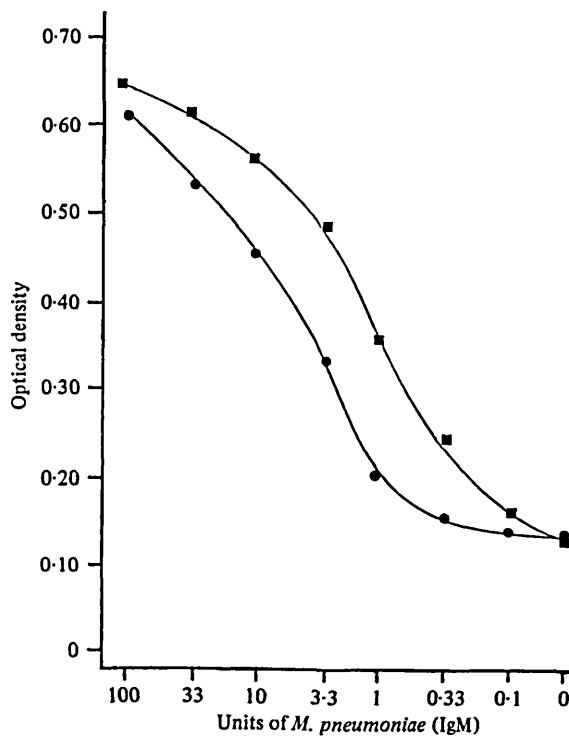
Fig. 1. Optical density readings against units of *M. pneumoniae* IgM for the positive control serum diluted in the negative control serum, assayed by *μ*-capture ELISA (■) and indirect ELISA (●).

RESULTS

Reference positive control serum diluted in negative control serum, to give samples containing between 100 and 0·1 arbitrary units of *M. pneumoniae* IgM were assayed in the indirect and *μ*-capture ELISA assays (Fig. 1). This shows the *μ*-capture assay to be more sensitive with a positive cut-off of 0·33 units of *M. pneumoniae* IgM. On the other hand, the indirect ELISA was found to be less sensitive, with a positive cut-off between 1 and 3·3 units of *M. pneumoniae* IgM. The cut-off values for the ELISA assays were derived from Fig. 1 and represent the lowest arbitrary units of IgM in the positive control serum dilution which gave O.D. values which were significantly greater than O.D. values for the negative control serum. The *μ*-capture ELISA was also found to be more robust and reproducible than indirect ELISA. The amount of *M. pneumoniae* IgM in the 53 sera from patients with confirmed *M. pneumoniae* infection was estimated in the indirect and *μ*-capture ELISA tests in units of *M. pneumoniae* IgM and expressed as a titre in the IFA. The correlation between the results of these tests is shown in Fig. 2. There was a good correlation between IFA and *μ*-capture ELISA, but the correlation between *μ*-capture and indirect ELISA and IFA and indirect ELISA tests was not as good. Several sera contained high levels of IgM in the indirect ELISA test but low levels of IgM when tested by IFA or *μ*-capture ELISA. Most of these sera were taken many months after recorded *M. pneumoniae* infection.
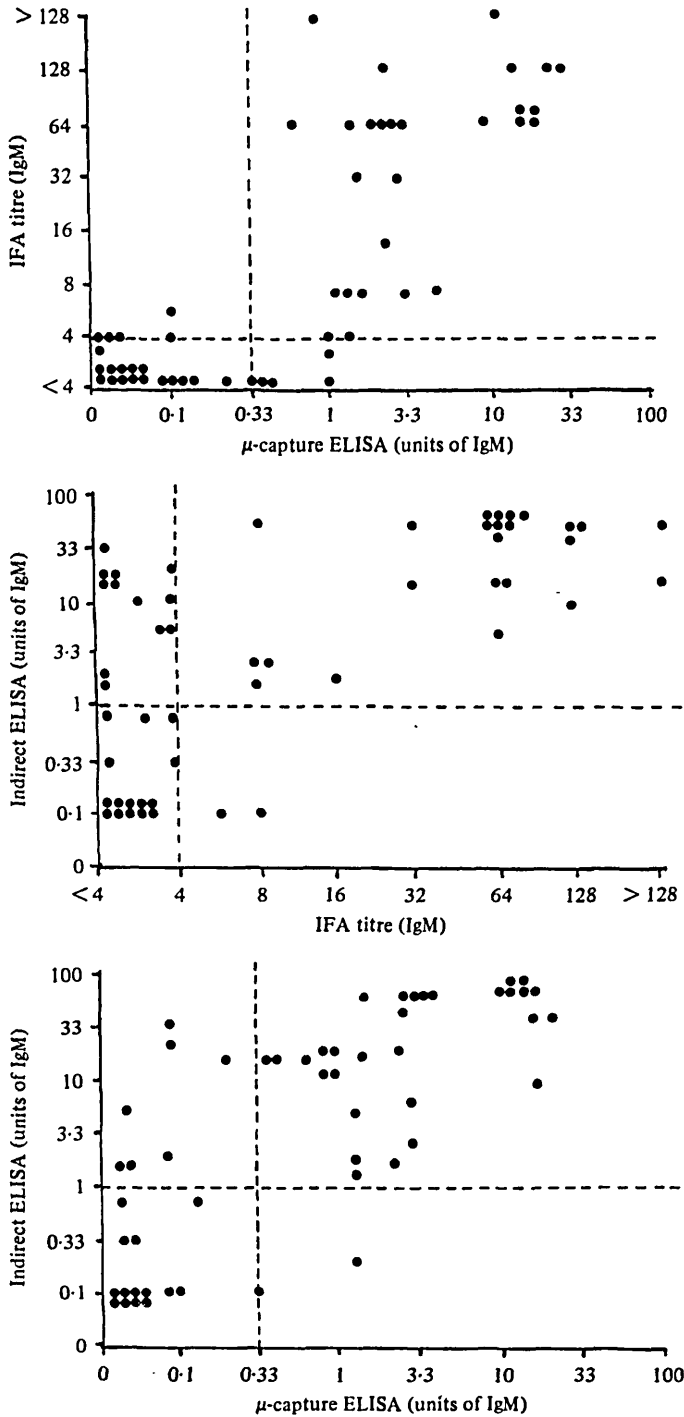
Fig. 2. Correlation of results obtained in the three assays with sera from patients with proven *M. pneumoniae* infection. Dotted lines indicate positive cut-off values.
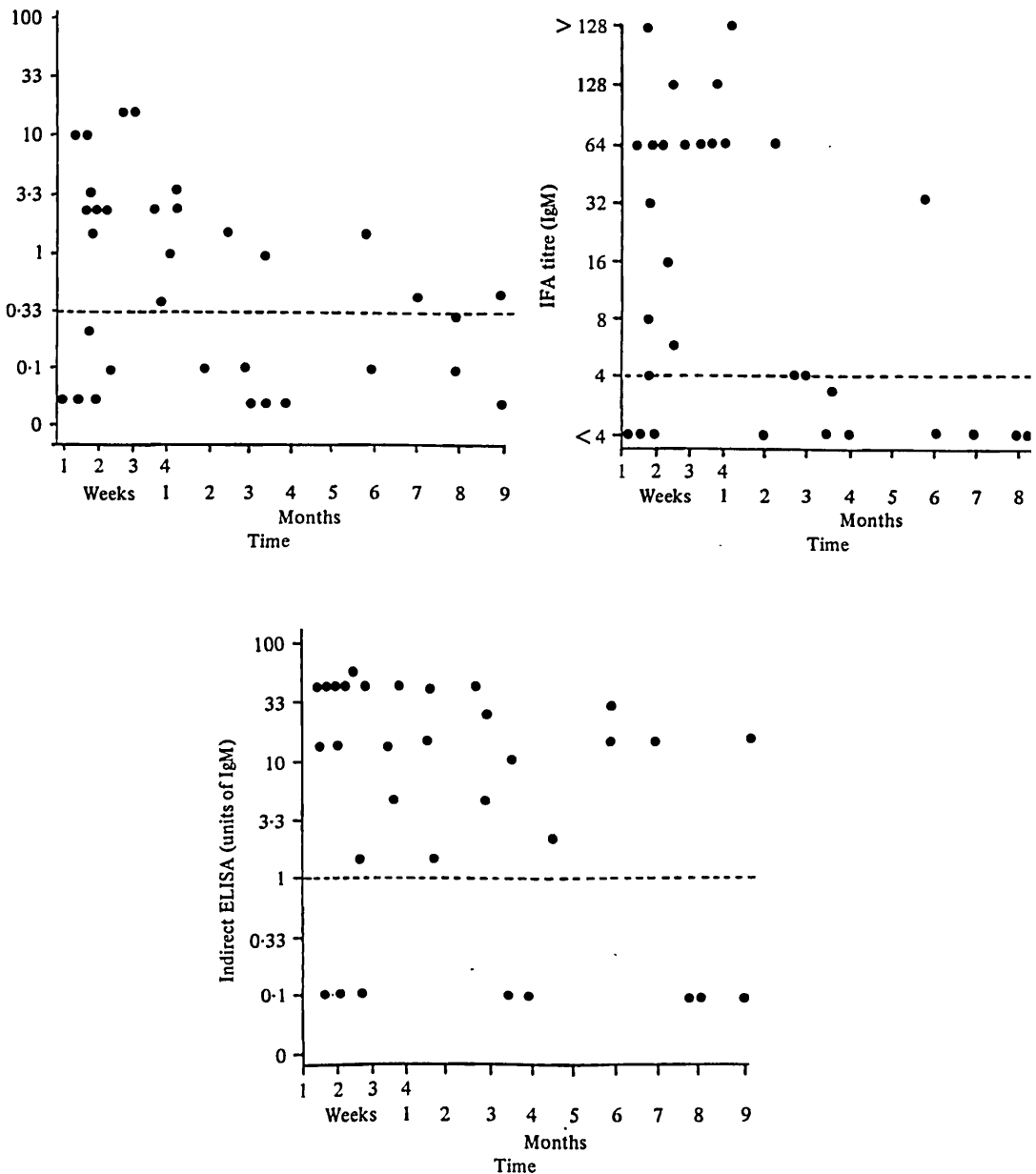
Fig. 3. Levels of *M. pneumoniae*-specific IgM detected by the three assays in sera taken at various times after proven *M. pneumoniae* infection. Dotted lines indicate positive cut-off values.

The amount of anti-*M. pneumoniae* antibody measured by μ-capture ELISA, indirect ELISA and IFA in the 33 sera taken from 11 patients at various times after their illness, is shown in Fig. 3. Maximum antibody levels were detected by all assays in the first month, with a gradual fall off with time; faster when sera were assayed by μ-capture ELISA, more gradual by IFA and hardly any decline at all with indirect ELISA.
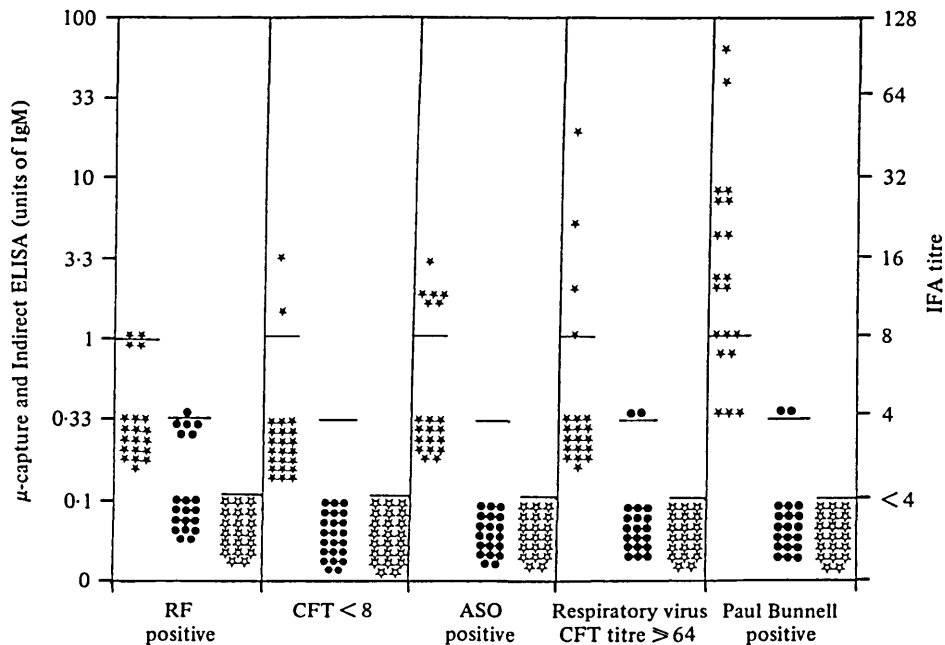
Fig. 4. Results of indirect ELISA (★), μ-capture ELISA(●) and IFA (☆) tests on sera likely to give non-specific results. Bar marks indicate the positive cut-off values for each assay.

Test results on the sera used to investigate the specificity of the three assays are shown in Fig. 4. The 20 rheumatoid factor positive sera did not react significantly in the three assays, but two sera gave borderline positive results in the indirect ELISA and one serum gave a very low positive result in the μ-capture ELISA.

Of the 20 sera with relatively high ASO antibody titres, six had moderate to low levels of *M. pneumoniae* IgM by indirect ELISA, but none was positive by IFA or μ-capture ELISA. Similar findings were noted with 23 sera which had *M. pneumoniae* CF antibody titres of < 8 (Fig. 4).

When sera containing high titres of CF antibody to influenza A virus, influenza B virus, adenovirus and *Chlamydia psittaci* but low or undetectable titres of *M. pneumoniae* CF antibody, were tested in the three assays, three sera with high titres of influenza A virus antibody had moderate to high levels of *M. pneumoniae* IgM by indirect ELISA. None of these sera was positive in the IFA and μ-capture ELISA tests (Fig. 4).

Twelve of the 20 sera with Paul Bunnell titres of 40 to 5120 gave medium to high levels of *M. pneumoniae* IgM in the indirect ELISA test, with two containing more than 33 units of *M. pneumoniae* IgM (Fig. 4). Five sera gave borderline results and three were negative. In marked contrast, only two sera gave borderline results in the μ-capture ELISA and none was positive by IFA.

DISCUSSION

The previous reports of *M. pneumoniae* IgM detection by ELISA have involved the indirect method. Either ether/tween treated (protein) antigen (Raisanen, Suni & Leinikki, 1980; Dussaix, Suni & Tournier, 1983), or whole sonicated antigen (van Griethuysen *et al.* 1984) was employed. Protein antigen was used in the earlier studies because *M. pneumoniae* lipid antigen shares glycosyl diglyceride groups with streptococcus MG (Raisanen, Suni & Leinikki, 1980) and similarities can be found with cellular components of some host tissues (Lemcke, Marmion & Plackett, 1967). Commercial *M. pneumoniae* protein antigen was unavailable at the time of this study and protein antigen produced by the method of Raisanen, Suni & Leinikki (1980), gave very poor results in our ELISA tests, as did antigen used in the complement fixation test. Therefore, we used whole sonicated antigen in our ELISA systems.

As in previous studies (Wreghitt & Nagington, 1983; Wreghitt *et al.* 1984), we found that Falcon flexible plates were very reliable and their use resulted in assays with greater sensitivity than when Dynatech 129A plates were employed. Comparison of the results obtained with the three assays using the 53 sera from patients with proven *M. pneumoniae* infection showed that μ-capture ELISA and IFA gave the best correlation, with values in the two assays showing a predominantly linear relationship.

Comparison of results obtained in these two assays with those obtained by indirect ELISA showed poorer correlation. In particular, there were several sera which gave high assay values in the indirect ELISA but low levels in the other two assays. Most of those serum samples were taken many months after *M. pneumoniae* infection had taken place.

The *M. pneumoniae* IgM results on sera taken at various times after proven *M. pneumoniae* infection show that IFA and μ-capture ELISA antibody reached a peak at about 2 weeks after infection and then steadily declined, reaching very low or negative levels by 6 months. Indirect ELISA antibody also reached a peak 2 weeks after infection, but declined at a much slower rate, several patients having relatively high levels of IgM 6–9 months after known infection. These higher levels in the indirect ELISA cannot be due to this test being more sensitive than μ-capture ELISA because the opposite was shown in the titration.

Van Griethuysen *et al.* (1984), who used sonicated lysed antigen, reported that *M. pneumoniae*-specific IgM detected by indirect ELISA persisted for up to a year after proven *M. pneumoniae* infection, which agrees with our results. A test which gives relatively high levels of IgM for up to a year after infection is not useful in the investigation of recent *M. pneumoniae* infection, being liable to misinterpretation. Much more confidence can be placed in the results of the μ-capture ELISA and IFA tests in which the level of IgM detected corresponds with the length of time after infection.

To investigate the possibility of non-specific results in the three assays, we examined several groups of sera previously shown to give problems with similar assays. Kurtz & Malic (1981) showed that sera containing high levels of rheumatoid factor gave non-specific positive results in μ-capture ELISA tests for detecting rubella-specific IgM, but Mortimer *et al.* (1981) did not detect non-specific IgM to

rubella by μ-antibody capture radioimmunoassay (MACRIA). In our tests with 20 rheumatoid factor positive sera we did not encounter false positive results in the three assays. We did however note some low level non-specific IgM in the indirect ELISA with sera having *M. pneumoniae* CF antibody titres of < 8 and in sera with high ASO titres. None of these sera were positive by IFA or μ-capture ELISA.

When investigating sera with high CF antibody titres to other respiratory pathogens, we similarly found some, particularly those with high titre influenza A virus antibody to have significant levels of *M. pneumoniae* IgM in the indirect ELISA, but none was positive by IFA or μ-capture ELISA.

The substantial proportion (12/20) of sera with significant Paul Bunnell positive titres giving high levels of *M. pneumoniae* IgM by indirect ELISA was in contrast to the results by IFA and μ-capture ELISA. Morgan-Capner, Tedder & Mace (1983) noted that eight sera from 125 cases of infectious mononucleosis were reactive for rubella-specific IgM by MACRIA.

In general, the lower sensitivity and lack of specificity of the indirect ELISA using sonicated *M. pneumoniae* antigen promote serious doubts about its usefulness for the routine detection of *M. pneumoniae* IgM, particularly since it gives positive values for sera taken many months after infection. We found the μ-capture ELISA and IFA the most robust sensitive and specific techniques for detecting *M. pneumoniae*-specific IgM. Both are suitable for routine diagnostic use, and the choice of test depends on what facilities are available. The IFA takes only 2 h to perform and provided the antigen used is in a suitably clumpy suspension, the assay may be read with relative ease. The μ-capture ELISA takes a little longer to perform, requiring an overnight incubation step, but results may be obtained in 24 h. The use of flexible assay plates, enables tests to be done on a few sera; plates coated with anti-human IgM may be stored at 4 °C for up to a week and strips of wells cut with scissors on demand. The test can be read by eye provided that suitable controls are included but it is better read by machine because the level of *M. pneumoniae* IgM taken into consideration with date of onset of symptoms has diagnostic significance.

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