The role of IgA determination by ELISA in the early serodiagnosis of *Mycoplasma pneumoniae* infection, in relation to IgG and μ-capture IgM methods

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Summary. Enzyme-linked immunosorbent assay (ELISA) for IgA, IgG and IgM was evaluated with sera from 50 adult patients with pneumonia, selected on the basis of a positive complement fixation (CF) test for diagnosis of *Mycoplasma pneumoniae* infection and with sera from 105 healthy blood donors. The ELISA antigen for IgG and IgA was a sonicated suspension of *M. pneumoniae* solubilised by deoxycholate. For the IgM assay, the same antigen was directly conjugated to alkaline phosphatase and used in a μ-capture format. ELISA gave positive results with high or rising titres for one or several antibody classes in 47 (94%) patients. In two of the three ELISA-negative cases, the diagnosis of *M. pneumoniae* infection indicated by the CF test seemed unlikely on clinical grounds. Specific IgA antibodies was developed more regularly and more rapidly than IgM. IgA titres also started to decrease earlier than IgM or the late-peaking IgG response. Thus, the determination of IgA antibodies was found to be valuable for the early diagnosis of *M. pneumoniae* infection. The study also demonstrated that the determination of all three antibody classes is necessary to obtain an optimal level of serodiagnosis.

Introduction

*Mycoplasma pneumoniae* is one of the commonest causes of bacterial respiratory disease, particularly amongst older children and young adults. A study of community-acquired pneumonia in the UK, over a period that included a *M. pneumoniae* epidemic peak year, showed that *M. pneumoniae* was responsible for 18% of cases in adults, being the most common aetiological agent after *Streptococcus pneumoniae*, which was associated with 34% of cases. *M. pneumoniae* has been predicted to remain an important cause of community-acquired pneumonia. Cough and tracheobronchitis are the commonest features of *M. pneumoniae* infection but do not distinguish *M. pneumoniae* disease from that due to other common respiratory pathogens, which may require different forms of treatment. Therefore, availability of rapid laboratory diagnosis of *M. pneumoniae* infection is important, in particular so that appropriate antibiotic treatment can be initiated.

As culture of *M. pneumoniae* from clinical samples is difficult and time-consuming, routine laboratory diagnosis is currently based on serology. The complement fixation (CF) test is used by many laboratories, even though non-specific reactions may occur. In conclusive results, especially moderately raised titres, are common with this method and may be the result of infection several years previously. Presence of *M. pneumoniae*-specific IgM is a reliable indicator of recent primary infection, but since this antibody is produced less frequently during re-infection, a negative result does not exclude current infection, especially in patients over the age of 45 years. The IgA response to *M. pneumoniae* has been little studied but it has been suggested that IgA antibodies could be reliable indicators of recent infection.

The aim of the present study was to develop and evaluate a *M. pneumoniae* ELISA, including tests for IgA as well as for IgM and IgG, in order to compare the kinetics of the antibody responses in the different immunoglobulin classes for adult patients with community-acquired pneumonia, previously diagnosed as due to *M. pneumoniae* by the CF test.

Materials and methods

Preparation of antigens and conjugate

*M. pneumoniae* strain FH (ATCC 15531) was grown as attached cell sheets in polystyrene flasks in a medium containing (L) PPLO broth (Difco) 14 g, yeast extract (Difco) 25 g, 20 mmol HEPES-buffer, glucose 4 g,

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5 mmol KOH, horse serum 200 ml and penicillin 4 x 10^4 IU. After incubation for 48–72 h at 37°C, the medium was removed and the adhering mycoplasmas were washed and harvested as described previously. The bacteria were then suspended in 0.1 M glycine, pH 9.8, with the addition of 10 mM sodium deoxycholate (Sigma), sonicated four times for 30 s and centrifuged to remove debris. The supernate was dialysed against phosphate-buffered saline (PBS), pH 7.2. Visible precipitates were removed by centrifugation. The antigen in the supernate was used in the IgG and IgA assays at appropriate dilutions (see below). For the IgM assay, the antigen was conjugated to alkaline phosphatase by the method described by Engvall and Perlmann in all essentials. The use of a μ-capture assay for IgM was mainly to eliminate false positive reactions due to rheumatoid factor (RF), which is not an important problem for IgG and IgA assays. The antigen conjugate used in the IgM assay was found to be stable for at least 6 months at 4°C. The protein content of the antigen and conjugate preparations was determined by the method of Lowry et al.

**ELISA procedures**

Cobalt-irradiated polystyrene 96-well microtitration plates (Immunolon II; Dynatech, Chantilly, VA, USA) were coated overnight at room temperature (22–25°C) with, per well, 100 μl of antigen (protein 10 μg/ml) in PBS, pH 7.2, for IgG and IgA determination. Coating for IgM determination was done under the same conditions with 100 μl of goat anti-μ-chain IgM (Sigma). Serum and conjugate dilutions were added in 50-μl volumes and incubated on a shaker. The substrate was added in 100-μl volumes with stationary incubation and all washings were done in four cycles with 300 μl of wash solution (PBS containing Tween 20 0.05%). A 1-in-1000 dilution of patient’s serum, identified as optimal by previous titrations, was used for screening. When necessary, sera were diluted further by two-fold dilution steps to obtain an end-point titre. The serum dilutions were incubated for 1 h at 37°C with shaking. Incubation with alkaline phosphatase-conjugated anti-human IgG and IgA (Sigma) and with the in-house conjugated antigen at a concentration of 2.5 μg/ml for IgM was done for 2 h at 37°C with shaking. The reaction time for the substrate, p-nitrophenyl phosphate, was 20–30 min, the time being adjusted to obtain predetermined values for the positive controls.

A serum dilution on the linear part of the titration curve was used to calculate the ELISA titre, defined as the mean absorbance value at 405 nm (A405) of duplicate determinations multiplied by the serum dilution factor, e.g., an A405 value of 0.7 at a serum dilution of 2000 represents an ELISA titre of 1400. A significant rise in titre was defined as at least a two-fold increase between paired samples, based on an intra-assay variation of < 20%. High titres in single sera, representing positive results, were defined as values above the upper limit for 98–100% of healthy individuals, as determined in sera from 105 blood donors (see Results). The blood donor sera were not tested or selected by CF testing.

**Patients and blood samples**

Adult patients admitted with community-acquired pneumonia had been investigated for aetiological diagnosis in two separate studies. From these two studies, paired sera were available from a total of 50 patients who were diagnosed as having *M. pneumoniae* infection on the basis of at least four-fold rises in titre by the CF test, performed by a routine procedure in a microtitration plate assay. The diagnosis was confirmed by culture from sputum samples in 27 (57%) of 47 cases. The mean age of the patients was 41-9 years (range 17–92 years). A total of 118 serum samples was tested in the present study. The first samples from the 50 patients were drawn on mean day 8.1 (range 1–20 days) after onset of disease. The second to fourth blood samples were drawn on mean day 31 (median 23, range 4–177 days) after onset of clinical symptoms as stated by the patient.

**Results**

To establish the background antibody levels against *M. pneumoniae*, sera from 105 healthy blood donors were tested for IgG, IgM and IgA antibodies. The distribution of the ELISA titres is shown in fig. 1. IgA and IgM titres were below 200 in the vast majority of the samples and none was above 400. An arbitrary upper limit for normal values, i.e., the cut-off limit for a positive result, was set at 400 for IgM and IgA, thus yielding a specificity of 100%. The IgG antibodies were scattered over a wider range and a cut-off limit of 1000 was selected, with 103 (98%) of 105 healthy donors having titres below that level.

Determination of IgG, IgM and IgA antibodies in the 118 samples from the 50 patients with *M. pneumoniae* infection (defined as giving a positive CF test) showed that 47 (94%) of the patients gave positive ELISA results by either 2-fold titre rises or by high titres for one or more antibody classes. Of the 27 culture-confirmed cases, 26 gave positive results by ELISA by the same criteria. The most regular response was seen in IgG with 44 (94%) of the 47 patients giving positive results by titre rise or by high titres, i.e., titres above the cut-off level. The second most common response was in IgA with 39 (83%) patients giving positive results, followed by IgM with 32 (68%) patients giving positive results.

Samples from 25 (53%) patients gave positive results in all three antibody classes. Twelve patients had positive results in IgA but not in IgM and samples from six patients were IgM-positive but IgA-negative. Samples from three patients gave positive results only for IgG, from
Fig. 1. Distribution of IgG (■), IgM (□) and IgA (■) antibody titres against *M. pneumoniae* in serum samples from 105 healthy blood donors. Numbers on the X-axis indicate intervals in titre levels and on the Y-axis the percentage of samples within each titre range.

Fig. 2. Percentage rate of positive IgG (■), IgM (□) and IgA (■) titres, i.e., high titres above cut-off level, in serum samples from 47 ELISA-positive patients in relation to time after onset of disease; n, number of available serum samples for the time period indicated.

two patients only for IgA and from one patient only for IgM.

Sera from three patients gave negative results by ELISA for all three antibody classes. Two of these patients had the lowest titre rises in the CF test of all patients included in the study, from < 2 to 8 and to 16, respectively. Both patients also had negative culture results for *M. pneumoniae*. The clinical pictures, additional bacteriological findings and a prompt response to penicillin treatment in one of these cases
were suggestive of a non-mycoplasmal aetiology in these two patients. The third patient, however, had a culture-confirmed infection, a greater than four-fold rise in CF titre and blood samples that were well-spaced, being drawn on days 7, 9 and 21 after the onset of symptoms.

The kinetics of the antibody responses in the 110 sera from the 47 ELISA-positive patients are shown in fig. 2. There were only a few positive samples during the first week of disease and IgA was the most commonly detected antibody. The role of IgA as the most regular early antibody response was even clearer during the second, third and fourth weeks of disease. Peak IgA response was observed during the third week after onset of disease, when all 17 samples drawn during this period gave positive results. The kinetics of IgM and IgA antibody responses were similar, both starting to decrease during the fifth week after onset of symptoms. However, in this respect, the IgA response was slightly different from that of IgM, as the number of positive results decreased more rapidly for IgA than for IgM. The maximal response for IgG was reached later than for IgA or for IgM, i.e., during the fifth week after onset of disease; all 21 samples drawn during this period were IgG-positive.

Titres in samples considered to be positive by the criterion of high titre varied between the three antibody classes. The highest mean titre of 6090 (median 4000, range 1040–57600) was obtained for the 61 positive samples and a mean of 2000 (median 910, range 410–8320) in the 49 IgM-positive samples. The kinetics of the antibody responses in the 110 sera from the 47 ELISA-positive patients are shown in fig. 2. There were only a few positive samples during the first week of disease and IgA was the most commonly detected antibody. The role of IgA as the most regular early antibody response was even clearer during the second, third and fourth weeks of disease. Peak IgA response was observed during the third week after onset of disease, when all 17 samples drawn during this period gave positive results. The kinetics of IgM and IgA antibody responses were similar, both starting to decrease during the fifth week after onset of symptoms. However, in this respect, the IgA response was slightly different from that of IgM, as the number of positive results decreased more rapidly for IgA than for IgM. The maximal response for IgG was reached later than for IgA or for IgM, i.e., during the fifth week after onset of disease; all 21 samples drawn during this period were IgG-positive.

The value of specific IgM antibody determination by μ-capture ELISA for the early diagnosis of M. pneumoniae infection has been documented in several studies in recent years.23-26 The μ-capture technique was not directly compared with an indirect IgM assay but was considered preferable, mainly due to the potential problem of false positive reactions due to RF. Direct conjugation of the antigen to the enzyme, as in our present and previous studies,11-13 decreases the number of steps in the μ-capture procedure, compared to the method in which a further antibody and a conjugated anti-antibody are used.10,23 The conjugation of the M. pneumoniae antigen to alkaline phosphatase, as used in this study, resulted in a more stable antigen-conjugate complex than the horseradish peroxidase previously used. The present results confirm earlier studies on the value of μ-capture IgM determination, although the diagnostic sensitivity of this antibody class was lower than for IgA. Therefore, the determination of both IgM and IgA is recommended to obtain maximal sensitivity in the determination of the early antibody response.

The onset of a M. pneumoniae infection is often gradual and only slowly progressive. As illustrated by the present study, a duration of illness of more than a week before the patient seeks medical assistance is not uncommon. On the basis only of the presenting clinical features and chest radiography, it can be difficult on hospital admission to distinguish between a mycoplasmal and a pneumococcal pneumonia.20,21 Failure to recognise M. pneumoniae infection is also a common cause of unsuccessful empirical β-lactam antibiotic treatment for clinical pneumonia.22

The CF test, used as the reference method in the present study, has been used widely for the serological diagnosis of M. pneumoniae infection. However, this test has several drawbacks, including technical complexity and difficulty of adaptation to fully automated procedures. The issue of the sensitivity of the CF test was not addressed in the present investigation, but another recent study has indicated that it would be lower than that for an estimate based upon a combination of IgM and IgA antibody results.14 The differentiation of high titres due to a current infection from those due to residual antibodies from earlier infections can be difficult in the absence of a significant titre rise. Reports on the kinetics of CF antibodies are conflicting; they have been reported to rise slowly, although in some recent studies significant levels of CF antibodies were detected in the second week.11,13,24,25 Furthermore, problems of specificity due to antiglycolipid antibodies cross-reacting with membranes of host tissues and some other bacterial agents have been described.5 This could have been the case in our two patients whose sera gave positive results by low CF-titre rises but negative results by ELISA, with clinical support for a non-mycoplasmal aetiology.

The main finding of the present study was the value of the ELISA-determined IgA antibody for the early serodiagnosis of M. pneumoniae infection in adult patients. The rapid rise of IgA during the first 3 weeks and the rapid decrease during the second month after onset of disease allows for specific diagnosis of current infection. The role of IgA as an acute reactant has earlier been investigated only by Sillius14 with an immunofluorescence technique. The author suggested that IgA antibodies could be valuable in confirming a current infection in the absence of an IgM response. Our results confirm and expand this observation, showing that an IgA response is more regular than an IgM response in adults with pneumonia caused by M. pneumoniae.

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classes, 26 of 27 patients with culture-confirmed disease were also positive by serology.

In conclusion, the present investigation has shown that the combined determination of IgA and IgM antibody responses allows for a serological diagnosis during the second or third week of disease in the majority of adult patients with *M. pneumoniae* pneumonia. The determination of IgG antibodies was necessary in a few cases and served as a confirmatory test in others. The rapid disappearance of IgA and IgM antibody titres is of value for the differentiation of current and past infections, since the CF test as well as IgG ELISA may remain positive for extended periods after *M. pneumoniae* infections.

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### References


