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The serological diagnosis of *Mycoplasma pneumoniae* infection: a comparison of complement fixation, haemagglutination and immunofluorescence

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SUMMARY

A total of 193 sera were examined for antibody to *Mycoplasma pneumoniae* by three techniques – complement fixation (CF), haemagglutination (HA) and immunofluorescence (IF), the last method being used to assess IgM, IgG and IgA antibodies. The most reliable single test for diagnosis was HA, and the most useful combination of tests was HA with IF (IgM and IgG). The IgA IF was not found to be diagnostically helpful.

INTRODUCTION

The serological methods which have been described for the diagnosis of *Mycoplasma pneumoniae* (MP) infection include complement fixation (CF) (Taylor-Robinson *et al.* 1966; Fernald, Clyde & Denny, 1967), immunofluorescence (IF) (Taylor-Robinson *et al.* 1966; Fernald, Clyde & Denny, 1967; Skaug *et al.* 1976; Sillis & Andrews, 1978; Carter & Carter, 1983), haemagglutination (HA) (Taylor-Robinson *et al.* 1966; Taylor, 1979), tetrazolium reduction (Taylor-Robinson *et al.* 1966), enzyme-linked immunosorbent assay (ELISA) (Raisanen, Suni & Leinikki, 1980; Busolo, Tonin & Meloni, 1983; Van Griethuysen *et al.* 1984; Wreghitt & Sillis, 1985), and radioimmunoassay (RIA) (Hu *et al.* 1983). In the UK, CF is probably still the most commonly used diagnostic test for MP infection, despite a lack of specificity (Raisanen, Suni & Leinikki, 1980), and the need often for two specimens to demonstrate a rise in titre. In recent years an HA kit (Serodia Myco) has become available and has been assessed by Taylor (1979). Using this kit, it is possible to make a strong presumptive diagnosis of MP infection on a single serum sample, and at an earlier stage of infection than with CF (Taylor, 1983).

The detection of MP-specific IgM also allows a serological diagnosis to be made on a single specimen, and methods described include CF following fractionation (Chamberlain & Saeed, 1983), IF (Skaug *et al.* 1976; Sillis & Andrews, 1978), ELISA (Van Griethuysen *et al.* 1984; Wreghitt & Sillis, 1985), and RIA (Hu

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et al. 1983). CF following fractionation is time consuming, μ-capture ELISA requires conjugates that are not readily available at present, and specialized equipment is needed for RIA. On the other hand the reagents required for IF are readily available or easily prepared. Of tests for the detection of MP-specific IgM, we feel that only IF lends itself readily to use in a routine diagnostic laboratory. We therefore have compared CF, HA and IF to see which single test or combination of these tests provides the most clinically useful information in a diagnostic laboratory.

MATERIALS AND METHODS

Patients/samples

A total of 193 serum samples from 111 patients were included in the study. They were all routine samples and representative of those received in a diagnostic laboratory.

Within the total population there were two main groups.

Group A. This included 145 specimens from 87 patients under investigation for febrile illnesses, most of which had a pneumonic component. A patient qualified for this group if one of three tests on at least one specimen was positive at a minimal titre – 8 for CFT, 40 for HA and 4 for IF IgM – and the clinical history warranted further investigation. This group was subdivided as follows.

Subgroup A1. 65 patients presenting with a clinical picture which we judged to be consistent with, but not necessarily diagnostic of, MP infection.

Subgroup A2. 22 patients who presented with illnesses in which the subsequent clinical course, or other laboratory findings, made MP infection unlikely.

Group B (control group). A total of 48 specimens were selected from 24 patients in whom a respiratory illness caused by another pathogen was supported by laboratory findings – usually a diagnostic CF titre. These comprised: influenza A, 6 patients; influenza B, 2 patients; adenovirus infection, 2 patients; respiratory syncytial virus infection (RSV), 3 patients; Q fever, 11 patients.

LABORATORY METHODS

The study samples were assayed in batches over a period of months. For each test system, every batch run included a standard positive control sample. Where more than one sample was received from a single patient, the complete patient set was always tested within a single batch.

Haemagglutination. The Serodia Myco test (Fujirebio, Tokyo, Japan) was used. This was carried out and interpreted according to the manufacturer's instructions. Briefly, one volume of a 1/10 dilution of the test serum was allowed to react with three volumes of chicken cells which were either sensitized with *Mycoplasma pneumoniae* (MAC strain) antigen or were unsensitized (control) cells. The tests were carried out in microtitre trays, and examined for a smooth mat of haemagglutination after 1 h at RT in quiet conditions.

The cells were lyophilized, and had to be reconstituted with diluent before the test was carried out. Each serum could be doubly diluted further to obtain a titre, but for this study they were tested at three dilutions only – 1/10, 1/20 and 1/40,

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and we describe the final dilutions as suggested by the manufacturers, i.e. 1/40, 1/80 and 1/160.

Complement fixation. This was carried out using a method standard in most laboratories (Bradstreet & Taylor, 1962).

Immunofluorescence. A 'sandwich' IF method for detecting antibodies to MP, as developed by the PHLS Mycoplasma Reference Laboratory, Norwich (Sillis & Andrews, 1978; Wreghitt & Sillis, 1985) was used. In essence doubling dilutions of test serum from 1/4 to 1/128 in PBS (pH 7·4) were incubated for 30 min at 37 °C with the antigen air-fixed to the wells of PTFE-coated slides (Hendley Ltd, Loughton, UK). This antigen was a washed granular broth culture of MP (FH strain – NCTC 10119).

After incubation the slides were washed and bound antibody was detected with FITC-labelled anti-human IgM, IgA and IgG (sheep anti-IgG and IgM, Wellcome Diagnostics, Dartford, UK; goat anti-IgA, Miles-Yeda, Miles Laboratories (UK) Ltd, Stoke Poges, UK). The slides were mounted in buffered glycerol and examined under epi-illumination with a mercury vapour lamp and an excitation filter passing 450–490 nm wavelengths. Fluorescence was graded on an arbitrary scale ($-, \pm, +, ++, +++, ++++$), and any sample showing 1+ or greater was designated positive.

RESULTS

Because single specimens only were received from about one-third of the patients in the series and high stable titres were found in many of the rest, we have attempted to define a level or a set of criteria by which a particular result could be designated as clinically significant or positive. To achieve this, the set of results for each patient in subgroup A1 were assessed and are summarized in Table 1.

We therefore deduce that the following constitute positive results.

(1) CF: any titre of $\geqslant 128$ or where there is a $\geqslant$ 4-fold rise, to at least 32.

(2) HA: any titre $\geqslant 160$.

(3) IF IgM: any titre of $\geqslant 16$ (a titre of 8 being considered equivocal).

(4) IF IgG: any $\geqslant$4-fold rise.

Among the 65 patients in subgroup A1 (clinically MP infection), we identified 53 patients in whom at least two of the four tests (CF, HA, IF IgM, IF IgG) were positive by the above criteria. These we have redesignated the 'core' group of definite MP infection.

The remaining 12 patients in subgroup A1 either showed one positive result unsupported by another test (7 patients: 4 by CF, 2 by HA, 1 by rising IgG IF), or gave equivocal results (5 patients: 2 with an HA titre of 80, 3 with an IF IgM level of 8).

Of the 22 patients in subgroup A2 (MP infection unlikely), 6 were positive by CF, 1 by HA, and 3 with an IF IgM level of 8. None of these positive results was supported by the results of other tests.

Of the 24 control patients (Group B), none gave positive results by our criteria in any of the four test systems. A single patient aged 3 with an RSV infection was, however, found to have an IF IgM titre of 8.

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Table 1. *Results, using each test system, for total patients and subgroup A1*

Test	Results	Patients: Total	Patients: No. in sub-group A1
CF	Patients showing ⩽ 64 and no rise in titre	27	8 (30%)
	Patients showing ⩾ 4-fold rise to at least 32 or any sample ⩾ 128	59	51 (86%)
HA	Patients with any result equal to but not higher than 40	13	3 (23%)
	Patients with any result equal to but not higher than 80	10	5 (50%)
	Patients with any result ⩾ 160	48	47 (98%)
IF IgM	Patients with any result equal to but not higher than 4	7	2 (29%)
	Patients with any result equal to but not higher than 8	17	13 (76%)
	Patients with any result ⩾ 16	37	37 (100%)
IF IgG	Patients showing ⩾ 4-fold rise in titre	19	19 (100%)

Table 2. *Positive results within and without 'core' group*

Test	CF	HA	IF IgM ⩾ 16	IF IgM ⩾ 8	IF IgG ⩾ 4-fold rise
Total no. of patients with positive result	59	48	37	54	19
No. of patients with positive result and in 'core' group	49 (83%)	45 (94%)	37 (100%)	47 (87%)	18 (95%)
No. of patients with positive result and not in 'core' group	10 (17%)	3 (6%)	0	7 (13%)	1 (5%)

Sera from 70% of the patients in the 'core' group were reactive in the IF IgA tests, but about half of these were at low titre (4 or 8). The results showed no particular pattern of correlation with those from other tests. Sera from six patients in subgroup A2 (27%) had low titres by IF IgA, while all the sera from group B patients were negative. Titres ⩾16 of IF IgA were specific for MP infection, all occurring in the 'core' group, but none of these results would have influenced the final serological diagnosis.

The data are analysed further to assess the reliability of each separate test system in Tables 2–4. Table 2 shows the percentages of positives that are (*a*) supported both clinically and by one other test, or (*b*) unsupported by another test, while Table 3 gives the percentages of patients from the 'core' group with positive results for each test system.

Table 4 summarizes the percentages of the patients from the 'core' group whose diagnosis can be confirmed serologically from the results of either of two techniques.

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Table 3. *Positive results in the 'core' group of patients with each test system*

Test	No. of patients from 'core' group (53) with positive result No. (%)
CF	49 (92)
HA	45 (85)
IF IgM ($\geqslant$ 16)	37 (70)
IF IgM ($\geqslant$ 8)	47 (89)
IF IgG ($\geqslant$ 4-fold rise)	18 (34)

Table 4. *Diagnostic results when a combination of tests was used*

Test combination	No. of patients from core group (53) 'diagnosed' as MP infection by a combination of tests No. (%)
HA *or* IF IgM ($\geqslant$ 16)	50 (94)
HA *or* IF IgM ($\geqslant$ 16) *or* IF IgG ($\geqslant$ 4-fold rise)	53 (100)
HA *or* CFT	52 (98)
CF *or* IF IgM ($\geqslant$ 16)	53 (100)

We found evidence of IgM by IF (a titre of $\geqslant$16) in patients of any age, and among the 53 cases of MP infection, only 6 were negative (a titre of $<$ 4). In five their ages were known and were 2, 15, 36, 41 and 55 years.

DISCUSSION

This study was designed to assess these tests in the environment of a routine diagnostic service. Accurate timing of the onset of infection can probably only be achieved by volunteer studies, or by the most careful prospective epidemiological survey. We did not have control over the timing of our specimens since these reflected unpredictable clinical demands, but we felt strongly that it was precisely this type of demand to which these tests should be subjected. Our patients were grouped primarily by broad clinical criteria, and the test results had to be compared against each other because there is no recognized reference serological test, and culture for MP is impracticable for us.

It is clear that if only one of the tests we tried is to be used for diagnosis, then the highest percentage of positives will be found by CF. However, this will mean that a number of positives will be unsupported by clinical findings, or by a second laboratory test. Seventeen per cent of our positive CF results were thus unsupported in this way, and a similar percentage has been reported by other workers (Sillis & Andrews, 1978). Some of these may be residual high titres from past infection, and some were possible re-infections. The HA test may not be helpful, as this is thought to detect mainly IgM (Biberfeld, 1968). It is interesting to note that Van Griethuysen *et al.* (1984) found that their ELISA for IgM

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correlated better with CF than did their ELISA for IgG, but this may be related to the methodology of the ELISA (an indirect method for measuring IgM). The influence of variations in the methodology has been commented upon by other workers (Wreghitt & Sillis, 1985).

We found that the HA test was extremely useful, with a low rate of positives unsupported by other tests (3/48). All three of these patients, however, presented with respiratory illnesses compatible with MP infection. We share Taylor's (1980) caution over low positive titres, which my be non-specific. Among the 13 patients with a titre of 40, only three had illnesses compatible with MP infection and, among the other 10, there was laboratory support from the CF in only one patient. Ten patients had titres of 80, of which five were originally considered to have clinical histories compatible with MP infection (subgroup A1). In only two of these five was supportive evidence obtained by another technique. We found it unnecessary to find the end-point titre in all sera provided each was tested to at least 160. This makes a considerable financial saving.

With regard to the IF test for IgM, a positive result at a dilution of 1/16 was probably the definitive test of current or recent infection in our hands, although this level of reliability is at the expense of sensitivity; 30% of our 'core' group of patients were negative at this titre. Carter & Carter (1983) were cautious about titres of 8, and we agree with their advice to pursue such cases. We had 17 patients with titres of 8. Thirteen of these had an illness compatible with MP infection, of which 11 had at least one other 'positive' test. We did not find any clear evidence that a lack of specific IgM antibody was associated with disease in adulthood, and even the equivocal IgM titres of 8 occurred at all ages from 2 to 44. We are still uncertain whether the cut-off for IF IgM should be 8 or 16, but we note that Wreghitt & Sillis (1985) consider 8 to be significant.

Rising titres of IF IgG had a high association with MP infection (Table 2), although only a minority of our 'core' group showed rising titres (Table 3). This is partly because only single specimens were received from a third of the patients, and high static titres were found in many of the rest.

Although IF IgA titres of $\geqslant 16$ occurred only in 'core' group patients, only 38% of 'core' group patients gave such a reaction. Another 32% of 'core' group patients gave low IgA titres of 4 or 8. Such low titres were also found in 27% of subgroup A2 patients. We conclude that low titres of IF IgA are not reliable indicators of MP infection, and that titres of $\geqslant 16$ do not provide any information that cannot be gained from other tests.

No single test system will confirm a diagnosis of MP infection in 100% of cases if it is assumed that the 'core' group are definite MP infections. However, if a combination of two tests to investigate cases of suspected MP infection can be used, then serological confirmation would be likely in all genuine cases by using both CF and IF IgM, provided that a positive result in either test system is assumed to confirm the diagnosis. Nevertheless by relying on CF when the IF IgM is negative, there will be an unacceptably large number of false positives. A combination of HA and IF IgM will fail to provide serological confirmation in about 6% of cases, but if a test for rising titres of IgG is included as well, serological confirmation will be made in 100%. Since it is technically convenient to test for IgG as well as IgM when performing IF tests, the use of HA and IF (both IgM

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and IgG) would be a suitable combination of tests for the routine diagnostic laboratory, because this would give only a small number of false positives.

We propose that single samples should be screened by HA at 80 and 160 only. Titres of 160 can be considered almost certainly diagnostic and an indication for appropriate chemotherapy. Sera should be checked by IF for specific IgM antibody, which may help to clarify HA titres of 80. Paired samples failing to reach a titre of 160 by HA should be tested by IF for IgM and for rising titres of IgG.

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