Comparison of three serological methods for diagnosing *Mycoplasma pneumoniae* infection

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Abstract

Aims—To compare the novel Serofast latex agglutination test (International Mycoplasma, Toulon-Cedex, France) with the complement fixation test and enzyme immunoassay (EIA) for diagnosing acute *Mycoplasma pneumoniae* infection.

Methods—Paired sera from 60 patients with respiratory infection who had tested positive for *M pneumoniae* by complement fixation test were analysed with Serofast and indirect EIA for specific IgG and IgM antibodies.

Results—Serofast was less sensitive than the two other tests. Only 30 (50%) out of 60 paired sera which showed a diagnostic seroconversion or had high positive, unchanged antibody titres by complement fixation test or EIA, or both, tested positive with Serofast. Positive test results with Serofast were associated with the presence of a complement fixation test titre of ≥ 512 and high positive IgM antibody titres measurable by EIA; virtually all patients with a complement fixation test titre of <256 or those responding primarily in the IgG class tested negative with Serofast. Based on analysis of sera taken at the acute phase of infection, 10 (17%) of the 60 patients tested positive by complement fixation test, 10 (17%) by EIA, and only four (7%) by Serofast.

Conclusions—Serofast was less sensitive than complement fixation test and EIA and it cannot be recommended as a replacement for either test in routine diagnostic use. It might prove useful in laboratories where non-specific tests, such as the determination of cold agglutinins, are still used for the diagnosis of *M pneumoniae* infection. Testing paired sera is, however, a prerequisite for obtaining acceptable sensitivity by Serofast as well as other serological methods currently available.

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Mycoplasma pneumoniae is a common respiratory pathogen the symptoms of which range from mild respiratory infection to pneumonia. The infection is endemic but epidemics occur every few years.^{1,2} The infection spreads especially easily in institutions, where people are in close contact with each other. This fact

explains, at least partially, why school children and their parents are those most at risk and children under three years of age and adults over 40 are less frequently affected.^{1,3,4}

M pneumoniae grows slowly and its culture is difficult and time consuming. The diagnosis of *M pneumoniae* infection, therefore, is usually based on serology. Even today, the complement fixation test is the most widely used method for the diagnosis of *M pneumoniae* infection, though its limitations in terms of specificity and sensitivity are well recognised.⁵⁻⁸ Thus there is a need for serological tests which could be used in the early diagnosis of *M pneumoniae* infection. In fact, several test applications are currently being offered for this purpose—indirect enzyme immunoassay (EIA), μ -capture enzyme immunoassay, and gelatin particle agglutination methods.^{8,9}

We evaluated the novel Serofast latex agglutination test (International Mycoplasma, Toulon-Cedex, France) by comparing it with an indirect enzyme immunoassay (EIA) for immunoglobulin G (IgG) and IgM antibodies, and conventional complement fixation test in the serological diagnosis of *M pneumoniae* infection. We studied the test performance of the three serological methods by analysing acute and convalescent phase sera from patients with probable *M pneumoniae* infection.

Methods

The study population comprised 55 men and five women with a mean age of 21 years, who presented with an illness compatible with acute mycoplasmal respiratory disease and tested positive for *M pneumoniae* by complement fixation test. Most of the patients were military conscripts. Both acute and convalescent phase sera were available, taken at a mean interval of 17 days (range five to 73).

Complement fixation test was performed as described before.¹⁰ A fourfold or more seroconversion in paired sera or a stable high positive titre of ≥ 128 was considered indicative of acute *M pneumoniae* infection.

M pneumoniae strain 133A was grown in Hayflick's medium¹¹ until a change in the indicator colour was apparent. The broth culture was centrifuged at $7700 \times g$ for 20 minutes at $+4^\circ\text{C}$. The pelleted *M pneumoniae* cells were suspended into phosphate buffered saline (PBS), pH 7.2, and centrifuged as above. This washing procedure was repeated three times and, finally, the cells were disrupted by sonication. The resulting antigen

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Distribution of positive Serofast test results in relation to results of complement fixation test and EIA in paired sera from 60 patients

	Acute phase sera		Convalescent phase sera	
	Positive* (n = 10)	Negative (n = 50)	Positive (n = 49)	Negative (n = 11)
No of positive Serofast tests in:				
EIA positive† sera	4/8‡	0/2	29/48	0/4
EIA negative sera	0/2	0/48	0/1	0/7

*Complement fixation test titres of ≥ 128 were considered positive.

†IgG or IgM antibody titres exceeding the mean of normal population by ≥ 2 SD were considered positive in the EIA.

‡The nominator shows the number of positive Serofast tests in each group. The denominator shows the total number of sera in each group.

was divided into small aliquots and stored at -20°C until used.

Microtitre plates (Maxisorp, Nunc, Denmark) were sensitised with $150\text{ }\mu\text{l}$ (per well) of the antigen diluted to a protein concentration of $1.4\text{ }\mu\text{g/ml}$ in carbonate-bicarbonate buffer, pH 9.6, incubated overnight at $+37^{\circ}\text{C}$ and washed three times with PBS containing 0.5% v/v Tween 20. The serum samples were diluted 1 in 100 in PBS containing 0.05% v/v Tween 20, 5% v/v horse serum, and NaCl (final concentration, 0.5M). They were then added in duplicate ($100\text{ }\mu\text{l}$ per well) to separate plates for determination of IgG and IgM and incubated for 90 minutes at $+37^{\circ}\text{C}$. After three washings rabbit anti-human IgG and IgM (Dako, dilutions 1 in 4000 and 1 in 2000, respectively) were added to the appropriate plates, $100\text{ }\mu\text{l}$ per well, and incubated 45 minutes at $+37^{\circ}\text{C}$. Finally, after three washings alkaline phosphatase conjugated anti-rabbit Ig (Orion Diagnostica, Espoo, Finland), diluted 1 in

100, $100\text{ }\mu\text{l}$ per well, was incubated two hours at $+37^{\circ}\text{C}$. After three additional washings $100\text{ }\mu\text{l}$ of p-nitro-phenylphosphate- MgCl_2 -buffer, pH 10.0, was incubated for 30 minutes at $+37^{\circ}\text{C}$. Reaction was stopped with $100\text{ }\mu\text{l}$ 0.3M NaOH per well. Optical densities were recorded with a spectrophotometer (Titertek Multiscan, Eflab, Helsinki, Finland) at a wavelength of 405 nm. Antibody titres were expressed in arbitrary EIA units (EIU), relative to negative and positive control sera, to give 0 EIU and 100 EIU, respectively.

The cutoff values for high positive antibody titres in the EIA had been predetermined in our routine laboratory as ≥ 100 EIU for the IgM assay and ≥ 75 EIU for the IgG assay, corresponding to the mean $+2$ SD of apparently healthy blood donors' sera. Based on monitoring the intra- and interassay variations in EIA, an increase of ≥ 20 EIU in the IgM or IgG antibody titres between paired sera was also considered indicative of recent infection.

The Serofast test (Lot SB2351) was performed according to the manufacturer's instructions. In brief $25\text{ }\mu\text{l}$ of the antigen suspension (latex particles coated with *M pneumoniae* antigen) was mixed on the plate with $25\text{ }\mu\text{l}$ of serum diluted 1 in 10, 1 in 20, and 1 in 40 and rotated gently. Reaction was read after four minutes. A positive reaction appeared as agglutination. A titre of $\geq 1/20$ was regarded as positive.

Results

A fourfold or higher seroconversion in complement fixation test occurred in 55 (92%) of the 60 patients. In five the peak titre remained at ≤ 64 . Five (8%) of the 60 patients already had a stable high (≥ 128) complement fixation test titre in the acute phase serum.

When all the serum samples were considered, altogether 56 (95%) of the 59 acute or convalescent phase sera with a high positive complement fixation test titre of ≥ 128 tested positive in the EIA for IgM or IgG. Of the 61 sera with a complement fixation test titre of <128 , 55 (90%) tested negative in the EIA for IgM and IgG (table). Thus in discriminating between high positive and negative sera, the overall consistency between EIA and complement fixation test was 93%. The correlation between EIA for IgM antibodies and complement fixation test titre was stronger ($r = 0.824$) than that between EIA for IgG antibodies and complement fixation test titre ($r = 0.341$) (figure).

Thirty (50%) out of 60 cases, all of which were positive by complement fixation test or EIA, tested positive with Serofast. When only acute phase sera were considered, 10 (17%) out of 60 cases tested positive for *M pneumoniae* in EIA, 10 (17%) in complement fixation test, and four (7%) in Serofast. If a titre of 1/10 was considered positive with Serofast, two additional positive cases were detected.

Of the 33 sera testing positive by Serofast,

Correlation of serum antibodies to *M pneumoniae* in paired sera from 60 patients with mycoplasmal respiratory infection as determined by enzyme immunoassay (EIA) for specific IgM and IgG antibodies, complement fixation (CF) test, and Serofast latex agglutination test.

28 (85%) were associated with a complement fixation test titre of ≥ 512 . Twenty nine (88%) of them were associated with positive IgM in EIA, two (6%) with positive IgG, and two (6%) with both positive IgM and IgG (figure). The 55 seroconversions with complement fixation test were associated with positive IgG, IgM, or both by EIA in 12, 24, and 18 cases, respectively, with one pair remaining totally negative in EIA (data not shown). Of the former, one (8%), 14 (58%), and 12 (67%), respectively, tested positive by Serofast.

Discussion

The early diagnosis of *M pneumoniae* respiratory tract infections is difficult. Culture of the organism is time consuming and requires a specialised laboratory. Moreover, colonisation of the upper respiratory tract by *M pneumoniae* in asymptomatic subjects has been reported,^{3,12} making the clinical value of a positive culture result uncertain. The sensitivity of Mycoplasma culture has been questioned, emphasising the importance of serological methods.^{13,14} Novel methods available for the direct detection of *M pneumoniae* include a species specific cDNA probe against *M pneumoniae* RNA¹⁵ and a polymerase chain reaction test.¹⁶ The difficulty of obtaining adequate samples from the lower respiratory tract, however, will limit the routine use of these sophisticated methods.¹⁵

Complement fixation test is still the most widely used serological method in the diagnosis of *M pneumoniae* infection. The need for paired sera and the recognition of false positive reactions in complement fixation test among patients with some extrapulmonary manifestations, such as meningitis and pancreatitis,⁵⁻⁷ has prompted the search for more advanced serological methods. The advantage of EIA based¹⁷ and indirect immunofluorescence methods⁹ is the possibility of detecting specific immunoglobulin classes separately: the demonstration of specific IgM antibodies allows earlier diagnosis of acute infections. The IgM antibodies reach diagnostic titres seven to 10 days from the onset of symptoms, while it usually takes longer for specific IgG to reach diagnostic titres.¹⁸⁻²⁰

Considering the good agreement between test results using CF and EIA in all except two pairs of sera, it is reasonable to assume that most, if not all, of the 60 patients really had *M pneumoniae* infection. The good correlation between results for IgM by EIA and complement fixation test was expected.²¹ In contrast to an earlier report²² we did not, however, notice any significant correlation between EIA for IgG and complement fixation test.

The sensitivity of Serofast was much less than that of the other tests. Of 60 paired sera showing diagnostic seroconversion or stable high positive complement fixation test titres of ≥ 128 , only 30 (50%) cases tested positive in Serofast. In only four (7%) patients did the first serum sample taken at the acute phase of

the infection test positive by Serofast. No significant increase in sensitivity was achieved by decreasing the cutoff value of Serofast. The specificity of Serofast was not assessed in this study.

The association between positive Serofast test results and the presence of high IgM antibody titres measurable by EIA was expected because IgM is far more efficient than IgG in agglutination reactions. The inability of Serofast to detect IgG class antibodies is probably a weakness inherent in many other agglutination tests.²³

Among the relatively young patients in the present study the determination of IgG antibodies by EIA proved of limited diagnostic value. The IgG:IgM ratio is lower among this age group than older patients,²¹ and the superior test performance of the IgM assay to that of the IgG assay was anticipated. By using a μ -capture EIA, a better sensitivity yet might have been obtained.⁹ The need to determine IgG antibodies in routine testing, on the other hand, is based on the fact that the IgG:IgM ratio increases in older age groups,^{21,24} and in reinfected patients the IgM response may be totally absent.^{15,19}

In conclusion, the Serofast latex agglutination test is no better for the rapid diagnosis of *M pneumoniae* infection than currently available methods. It is important to examine both acute and convalescent phase sera while using any of the serological methods currently available.

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