Evaluation of an indirect haemagglutination kit for the rapid serological diagnosis of *Mycoplasma pneumoniae* infections

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SUMMARY A recently introduced indirect haemagglutination kit for the detection of *Mycoplasma pneumoniae* antibodies is compared with the standard method of complement fixation. It is concluded that the kit provides a rapid, simple, and moderately sensitive means of detecting antibodies and, in some cases, enables these to be detected in the early, acute stage of infection.

A complement fixation test (CF) is adopted by most laboratories for the routine serological diagnosis of *Mycoplasma pneumoniae* infections. The particular advantage of this test is that it may be combined with other CF tests for evidence of infection with respiratory viruses but, because an overnight complement fixation is normally used, the results cannot be assessed for 18 hours or more. More sensitive serological techniques include indirect immunofluorescence (Skaug *et al.*, 1976), radio-immunoprecipitation, and complement-mediated killing (Brunner *et al.*, 1973). These latter methods are technically demanding in both time and equipment and hence are restricted to specialised laboratories.

An indirect haemagglutination (IHa) test for *M. pneumoniae* antibody using tanned erythrocytes was first described by Dowdle and Robinson (1964) and has been modified by Lind (1968) and Ghyka *et al.* (1977). The IHa is both sensitive and specific, takes one hour to perform, and may detect antibody before other techniques will do so. It seems to have distinct advantages over other tests for rapid serological diagnosis but the preparation and standardisation of tanned erythrocytes is technically laborious, thus limiting its routine use. Diamed Diagnostics have recently introduced a *M. pneumoniae* indirect haemagglutination kit (Fujizoki, Serodia-Myco) which utilises freeze-dried sensitised erythrocytes. This paper compares the new Fujizoki IHa kit with CF for the serological diagnosis of *M. pneumoniae* infections.

Material and methods

TEST SERA

Sera were received in the Brompton Hospital virus laboratory for routine serological examination between August 1971 and March 1978; the majority were from patients diagnosed as 'pneumonia'. The patients were grouped as follows:

Category I

335 patients whose serum did not show a raised CF titre to *M. pneumoniae* in a single sample taken during the acute stage of pneumonia, although raised levels of antibody (1/160 or greater) were detected in some sera to one of the following agents: influenza A and B, respiratory syncytial virus, adenovirus, *Chlamydia psittaci*, and *Coxiella burnetii*.

Category II

108 patients whose serum showed a raised CF titre to *M. pneumoniae* (1/160 or greater) in a single sample taken during the acute stage of pneumonia, suggesting recent infection with *M. pneumoniae*.

Category III

23 patients whose paired sera (acute and convalescent) showed a fourfold or greater rise in CF titre to *M. pneumoniae*, thus indicating recent infection.

TEST METHODS

The CF test employed throughout was a microtitre adaptation of Bradstreet and Taylor's (1962) method using antigens supplied by the Standards Laboratory, Colindale. Microtitre plates with U-shaped wells together with microdiluters and micro-

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droppers (Gibco-Biocult Ltd, Paisley) were used for both the CF and IHa tests. The Fujizoki kit contains a phosphate buffered saline diluent which was used for reconstitution of both the lyophilised chicken erythrocytes (sensitised and unsensitised) and dilution of the test sera. Reagents and sera were allowed to reach ambient temperature before the test was started. Sera were not heat inactivated as with the CF test. All sera were initially examined at a dilution of 1/10 as follows: 0.1 ml of diluent was added to well A and 0.025 ml to each of two adjacent wells B and C. A microdiluter was used to transfer 0.025 ml of serum to well A, which was rotated, and then 0.025 ml of the mixture was transferred to well B and rotated before being blotted dry and washed. A further 1/10 dilution was similarly prepared in well C; 0.075 ml of sensitised cells were added to well B and 0.075 ml of unsensitised cells to well C. A positive control serum supplied with the kit was incorporated into each series of tests performed. Serial dilutions were performed on some sera by double diluting 0.025 ml from well A in 0.025 ml of diluent contained in further wells D, E, F, etc. The plate was 'tapped' by hand to mix the cells and sera and covered to prevent evaporation; the cells were then allowed to settle at ambient temperature for between 50 and 60 minutes.

Agglutination patterns were considered reactive when a smooth lattice of cells, sometimes with its edges slightly folded, covered the base of the well. The manufacturers of this kit recommend that the final dilution of serum should be 1/40 since 3 volumes of cells have been added to the 1/10 dilution of serum. In this study a dilution of 1/10 (the initial dilution of serum) has been used in order that a direct comparison can be made with the CF test which does not compensate for the addition of reagents. When agglutination was observed with unsensitised cells attempts were made to absorb the non-specific agglutination with packed adult chicken cells (Tissue Culture Services, Slough).

Results

A comparison of the CF and IHa titres on sera from patients in categories I and II is shown in Table 1. Of the 335 sera with CF titres below 1/160, two with CF titres of 1/20 and 1/40 had IHa titres of 1/320 and 1/20 respectively. No significant CF titres to other respiratory pathogens were detected in these sera. The time of onset of symptoms was not known in these two patients. Sera with CF titres of 1/160, 1/320, and 1/640 or more were positive by IHa in 48%, 67%, and 86% of cases respectively. Non-specific agglutination occurred in 3.2% (14/443) of sera in these groups. Such agglutination could not be absorbed by treatment with fresh chicken erythrocytes.

Table 1 Comparison of CF and indirect haemagglutination tests on 443 single sera from patients with pneumonia

No. of sera tested	CF titre	Indirect haemagglutination		
		Negative (<1/10)	Positive (≥1/10)	Non-specific agglutination
Category I				
174	<1/20	169	0	5
64	1/20	63	1	0
79	1/40	76	1	2
18	1/80	17	0	1
Category II				
27	1/160	14	13	0
18	1/320	5	12	1
63	≥1/640	4	54	5
Total 443		348	81	14

In order to determine whether the IHa test will detect antibody at an earlier stage than CF the results of both tests on sera from patients within category II have been tabulated in relation to the time between onset of symptoms and collection of serum sample (Table 2). Of the 23 sera received up to two weeks after onset, 17% were negative by IHa whereas no negatives were found in the 10 sera from patients with illnesses of three weeks' duration. The

Table 2 Complement fixation and indirect haemagglutination titres produced at varying times after infection with *M. pneumoniae* (category II sera)

Weeks after onset	No. of sera tested	Complement fixation titre			Indirect haemagglutination		
		1/160	1/320	≥1/640	Negative (<10)	Positive (≥10)	Non-specific agglutination
≤2	23	8	3	12	4	18	1
3	10	0	1	9	0	8	2
4	11	0	3	8	2	7	2
≥5	18	3	6	9	4	13	1
Unknown	46	16	5	25	13	33	0

number of sera negative by IHa increased four and five weeks after onset, the most sera negative by this test being observed when no information was available concerning the onset of symptoms. The majority of sera (90%) tested three weeks after the onset of symptoms showed CF titres of 1/640 or above.

Figure Comparison of CF and indirect haemagglutination titres on acute and convalescent sera from patients with *M. pneumoniae* infections (category III sera).

The results of 21 of the paired sera in category III are presented in the Figure. Sera from two patients exhibited non-specific agglutination and were therefore excluded. Sera from three patients failed to react in the IHa test. In two patients in whom the CF rise was minimal the sera had been collected several weeks after infection (4 and 6 weeks and 7 and 9 weeks respectively). The actual titres overall were much higher by CF than by the IHa test. In nine cases the IHa test showed fourfold rising titres: in three twofold rises were obtained, in three there were declining IHa levels, and in three other cases there were no changes in IHa titres. These results, in part, demonstrate that the two tests do not measure identical proportions of IgM and IgG antibody. Five of the 'acute' sera had detectable IHa titres when CF titres were not raised beyond 1/80, suggesting that IHa may be more sensitive than CF in the early detection of infection.

Discussion

The Fujizoki IHa test is a simple one requiring only a small volume of serum which does not have to be

inactivated. It is rapid, the results being read after incubation for one hour. For these reasons the test is an improvement over CF. The only other specific serological test that compares with IHa, with respect to simplicity and speed, is counterimmunoelectrophoresis (CIEOP) (Menonna *et al.*, 1977). Preliminary experiments (Taylor, unpublished results) indicate that CIEOP, unlike IHa, is able to detect only cases in which CF titres are 1/160 or greater even where the 'acute' serum shows CF and IHa titres of 1/80. CIEOP is therefore considered to have little advantage in the examination of specimens collected shortly after the onset of illness. One of the minor disadvantages of IHa is the presence of non-specific agglutinins in certain sera. In the present study these could not be removed with chicken erythrocytes; other methods of treatment, including differential titration of sera with unsensitised and sensitised cells together with prior treatment of sera with 2-mercaptoethanol (Roberts and Hobbs, 1977), are currently being investigated. However, this problem is not common since only 3.7% of all the sera tested contained non-specific agglutinins. The incidence was much lower in fresh sera or in those that had been stored at -30°C for only a few weeks.

A titre of 1/10 or greater in the IHa test has, in this study, been taken as evidence of recent exposure. Higher baseline titres have been used by Lind (1968), who regarded titres of 1/160 or more as significant, and Ghyka *et al.* (1977) used 1/128 as their borderline for significance. The low positive titre with the Fujizoki kit is, however, beneficial since it enables a qualitative screening test at a dilution which requires minimal titration.

The sera of two patients within category I produced positive IHa titres in the absence of raised CF levels which may indicate early infection with *M. pneumoniae*. This suggestion is supported by titres of 'acute' sera from five patients in category III which gave similar results. In some cases, therefore, the IHa test has been shown to be more sensitive than CF in confirming 'acute' infections and single sera may suffice for diagnosis.

The results suggest that IHa is specific for *M. pneumoniae* infections, firstly by the absence of IHa titres in those sera in category I which had raised CF titres to respiratory viruses, *C. psittaci* and *C. burneti*, and secondly by the general correlation between CF and IHa in sera from category II and III patients. The higher the CF titre with the single sera in category II, the greater were the number of positive IHa titres. A possible explanation is that CF titres may in some instances remain at a level of 1/160 long after recovery from infection. Consequently some of the CF titres of 1/160 may

represent past rather than current infection. The IHa like the CF test seems to be most sensitive between two and three weeks after the onset of symptoms. This finding is in agreement with the results of Clyde and Denny (1967). Taylor-Robinson *et al.* (1966) suggested that IHa may detect antibody to *M. pneumoniae* at a much earlier stage than other methods which they tested including CF.

Towards the latter part of the study sera from patients with 'atypical pneumonia' were examined by IHa testing on the same day that they were received in the laboratory. This resulted in 10 cases being reported immediately as containing antibodies to *M. pneumoniae* detected by IHa, allowing chemotherapy to begin promptly. All of these 10 cases were subsequently confirmed by raised CF levels but two had low initial CF levels and required convalescent sera to show fourfold rises in titre.

Since a few sera with raised or fourfold rising CF titres were negative by IHa it is suggested that all sera giving negative IHa results should be retested by CF before being reported.

In conclusion, the Fujizoki indirect haemagglutination kit is a simple, rapid, and relatively sensitive test for the detection of antibodies to *M. pneumoniae*. It is a valuable aid to early diagnosis and can be recommended for adoption routinely in combination with CF tests in a diagnostic clinical service.

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