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The development and evaluation of a μ-capture ELISA detecting chlamydia-specific IgM

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SUMMARY

A μ-capture enzyme-linked immunosorbent assay (ELISA) for detecting chlamydia-specific IgM was developed by use of the heat stable, lipopolysaccharide group-specific antigen and an alkaline phosphatase-labelled anti-chlamydia group-specific monoclonal antibody conjugate. The test was used to study the serological response in chlamydial respiratory tract infection among patients with acute respiratory tract symptoms in Cambridgeshire during the past 7 years. Results were compared with those of the complement fixation test (CFT) in routine use as well as those of a whole inclusion indirect immunofluorescence (WIF) test for IgM. Correlation between results of the μ-capture ELISA and those of the WIF test was 87·5%.

The percentage of patients in whom specific IgM was found fell with increasing age. This may be due to lack of recall of IgM as a response to reinfection. Chlamydia-specific IgM was more likely to be detected when the CFT titre was $\geqslant 64$ and was rarely detected more than 6 months after the onset of symptoms. However, several patients < 20 years of age were found to have specific IgM with CF antibody titres < 64. We have found the μ-capture ELISA a useful test for the diagnosis of respiratory tract chlamydial infections, particularly in younger patients.

INTRODUCTION

The genus Chlamydia consists of at least two species, *Chlamydia trachomatis* and *Chlamydia psittaci*. *C. trachomatis* is a major cause of both non-gonococcal urethritis (NGU) and post-gonococcal urethritis (PGU) in men. In women cervical infection may be asymptomatic but the organism can ascend to the fallopian tubes

and can give rise to severe systemic infections such as pelvic inflammatory disease which may lead to infertility (Conway *et al.* 1984).

Infection with *C. psittaci* may lead to respiratory symptoms of varying severity (MacFarlane & Macrae, 1983) that include a dry or productive cough and pneumonia in some patients. Headache, arthralgia, myalgia and malaise are common. Systemic complications reported include endocarditis, renal failure and a toxic confusional state (Byrom, Walls & Mair, 1979). *C. psittaci* infection is frequently associated with avian contact. Ovine strains of *C. psittaci* cause abortion and other complications of pregnancy in ewes and have been associated with spontaneous abortion in women (McGivern *et al.* 1988).

Recently, there have been reports of a new chlamydia strain (TWAR) isolated from students in Washington with acute upper respiratory tract symptoms (Grayston *et al.* 1986). Further work on TWAR isolates has shown that they are morphologically, antigenically and epidemiologically different from *C. trachomatis* and more closely related to, but probably distinct from *C. psittaci* (Kuo *et al.* 1986; Campbell, Kuo & Grayston, 1987).

Respiratory tract chlamydial infection is usually diagnosed by means of serological tests. The complement fixation test (CFT) (Bradstreet & Taylor, 1962) is most commonly used although it is unable to distinguish between *C. psittaci*, *C. trachomatis* or TWAR infections. Other serological tests for measuring chlamydial antibody include indirect ELISA (Lewis, Thacker & Mitchell, 1977), micro-immunofluorescence (MIF) (Wang *et al.* 1977) or whole inclusion immuno-fluorescence (WIF) (Richmond & Caul, 1982), haemagglutination inhibition (Meyer, Eddie and Schachter, 1969), and radio-immunoassay (Terho & Meurman, 1981). Lewis, Thacker & Mitchell (1977) dismissed the agglutination test as requiring antigen in unavailable quantity and quality, the radioisotope precipitation test as requiring equipment not readily available, the immuno-fluorescence test as requiring specially trained personnel as well as being prone to the variability of human optical reading, the HA inhibition test as lacking specificity and the CFT as being hampered by anti-complementary serum samples and the occasional presence of anti-chlamydial antibodies in some batches of complement derived from supposedly healthy guinea-pigs. For the CFT the heat-stable group-specific antigen is used. This however, does not allow discrimination to be made between *C. psittaci* and systemic *C. trachomatis* infections.

This report describes the development and application of a μ-capture ELISA for detecting a specific IgM response to chlamydia group-specific antigen and the comparison with the whole inclusion indirect immunofluorescence (WIF) test.

MATERIALS AND METHODS

Serum samples

Between 1980 and 1987, 1249 serum samples from 862 patients with respiratory tract symptoms were tested for antibodies to respiratory viruses, chlamydiae and *Mycoplasma pneumoniae* in the Clinical Microbiology and Public Health Laboratory at Addenbrooke's Hospital, Cambridge. All samples were stored at -20 °C before further testing. All those with a chlamydia CF antibody titre $\geqslant 16$ (including follow-up samples from some patients) were included in the study.

Samples from persons who had been in contact with birds or with known cases of human chlamydial respiratory tract infection and from patients in the acute stage of suspected or subsequently proved chlamydial respiratory tract infection were also included.

A sample of serum with a chlamydia CF antibody titre of 512 was selected as the reference μ-capture ELISA IgM positive control serum and was assigned an arbitrary value of 100 units of chlamydia-specific IgM. This positive control serum was diluted in chlamydia-specific IgM negative control serum with CF titre of < 8 to give dilutions containing 33, 10, 3·3, 1, 0·33 and 0·1 arbitrary units of chlamydia-specific IgM.

Preparations of C. psittaci *antigen*

Various preparations of antigen were tried initially in the test. These included preparations containing the elementary and reticulate bodies of duck and parrot strains of *C. psittaci* grown in McCoy cells, heat-inactivated at 56 °C and purified on a urografin gradient (kindly made available by Dr Stuart Chalmers, Animal Health Trust, Newmarket). *C. psittaci* CFT antigen provided in freeze-dried form by the Central Public Health Laboratory Division of Microbial Reagents and Quality Control (DMRQC), Colindale, London, NW9, was also used.

μ-capture ELISA

Falcon flexible plates (Becton Dickinson) were coated overnight at 4 °C with 100 μl rabbit anti-human IgM (DAKO, High Wycombe, Bucks) diluted 1 in 2000 in carbonate/bicarbonate buffer, pH 9·6. After the coating antibody had been aspirated, all wells were washed three times with IDEIA chlamydial washing buffer (Boots Celltech, Slough, UK) allowing 5 min between the second wash and aspiration. 100 μl human test or control serum diluted 1 in 100 in phosphate-buffered saline (PBS) with 0.08% Tween 20 (PBST) were then added to duplicate wells. Reference control sera diluted 1 in 100 in PBST and containing 100, 33, 10, 3·3, 1, 0·33, 0·1 and 0 arbitrary units of chlamydia-specific IgM were included in each assay. The plates were then incubated in a moist chamber for 3 h at 37 °C.

After the plates had been washed as before, 100 μl PHLS, DMRQC CFT chlamydia antigen diluted 1 in 10 in PBST with 1% fetal calf serum were added to each well and the plates stored overnight at 4 °C. The plates were again washed as described previously before 100 μl monoclonal anti-chlamydia LPS Fab'2 alkaline phosphatase conjugate were then added to each well. The conjugate was prepared according to the method of Ishikawa *et al.* (1983) and diluted 1 in 10 in Tris-buffered saline pH 7·5 with 1% bovine serum albumin (BSA) (kindly provided by Novo BioLabs Ltd, Cambridge, UK). Plates were incubated in a moist chamber at 37 °C for 3 h. The optimal dilutions of antigen and conjugate were previously determined by chequerboard titrations.

Plates were then washed four times as described previously, and 50 μl freshly constituted NADP substrate (Novo BioLabs Ltd, Cambridge, UK) were added to each well and the plates incubated in a moist chamber for 30 min at room temperature. Following this, 100 μl freshly reconstituted amplifier (Novo BioLabs Ltd, Cambridge, UK) were added to each well and after 5 min the colour development was stopped by the addition of 50 μl of 0·1 M sulphuric acid.

Absorbances were read by means of a Titertek plate reader at 492 nm, blanking on air. The ratio of test to negative absorbance (T:N) was calculated for each serum, the negative value being taken from the wells containing the serum with 0 units of chlamydia-specific IgM in the dilution series.

CFT

The CFT was performed as described by Nagington (1984). The chlamydial group-specific CFT antigen was supplied by DMRQC, Central PHL, London, NW9 5HT.

Whole-inclusion indirect immunofluorescence (WIF) test

The WIF test was performed as described by Richmond & Caul (1982). The antigen was that prepared from a strain of *C. psittaci* (enzootic abortion agent of ewes (EAE)), which had been grown in McCoy cells. Serum samples with titres $\geqslant 8$ were regarded as positive for chlamydia-specific IgM.

RESULTS

The μ-capture ELISA clearly distinguished chlamydia-specific IgM-positive samples from those which were negative. The best discrimination between absorbance readings of reference positive and negative control sera was found when the monoclonal anti-chlamydia LPS Fab'2 alkaline phosphatase-conjugated antibody was used in conjunction with DMRQC *C. psittaci* CFT antigen.

The chlamydial CF antibody titre and μ-capture ELISA T:N ratios were compared for each serum. A large proportion of serum samples had μ-capture ELISA T:N ratios < 2. There was a positive correlation between the *C. psittaci* CF antibody titre and the μ-capture ELISA T:N ratios (Fig. 1). Of those samples with CF antibody titres $\leqslant 32$, 80% gave μ-capture ELISA T:N ratios < 2 (Fig. 2). Those CF antibody titres $\leqslant 32$ which gave μ-capture T:N ratios $\geqslant 2$ tended to have been taken in the acute stage of infection or from patients < 20 years of age.

Patients whose serum samples were positive for chlamydial CF antibody (titre $\geqslant 64$) and/or IgM by μ-capture ELISA (T:N ratio $\geqslant 2$) were analysed by age. The proportion of CF antibody-positive patients which were also IgM positive fell with increasing age (Fig. 3). The proportion of samples which gave μ-capture ELISA T:N ratios $\geqslant 2$ but had low CF antibody titres ($\leqslant 32$) was particularly high for the age group 11–20 years. The maximum chlamydia-specific IgM response obtained for each patient was analysed by age, and it was found that the value of the μ-capture ELISA T:N ratio declined with age. Chlamydial respiratory tract infection was not particularly associated with either sex (males 168, females 165).

In patients who had μ-capture ELISA T:N ratios $\geqslant 2$, the IgM appeared mostly between 1 and 2 weeks after the onset of symptoms. Persistence of IgM was examined in 14 patients for whom serial serum samples were available (Fig. 4). Most were found to revert to μ-capture T:N ratios < 2 between 1 and 6 months after the onset of symptoms. The time taken for patients to become μ-capture ELISA negative depended on the initial IgM response for each patient (Fig. 4).

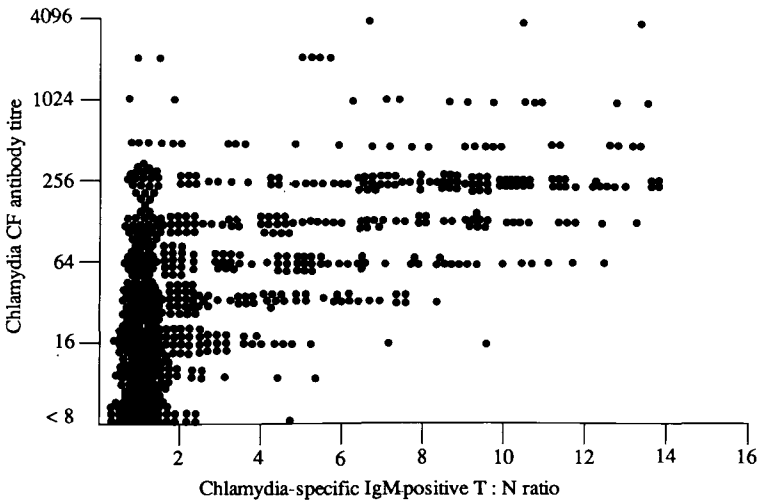

Fig. 1. Correlation between chlamydia CF antibody titre and μ-capture ELISA chlamydia-specific IgM T:N ratio.

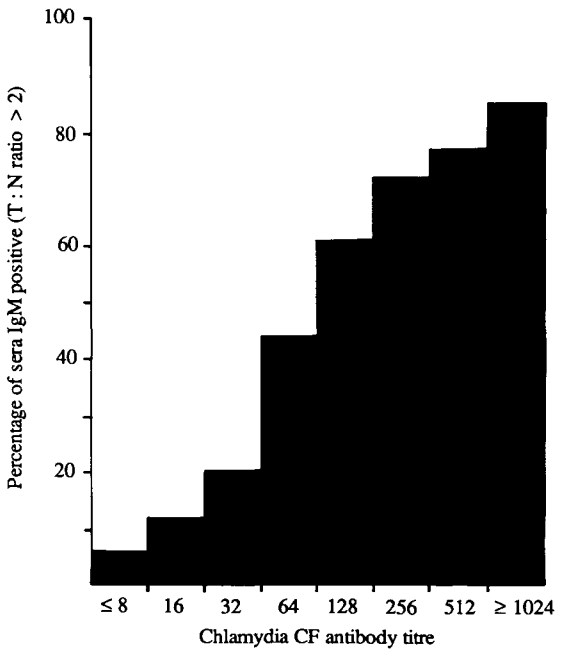

Fig. 2. Correlation between chlamydia CF antibody titre and percentage of serum samples with chlamydia-specific IgM by μ-capture ELISA.

Altogether, 73 coded serum samples with a range of chlamydial CF antibody titres were tested 'blind' by μ-capture ELISA and by the WIF test. They were classed as chlamydia-specific IgM-negative or positive by each test. An 87·5% agreement was obtained between the two tests (Table 1).

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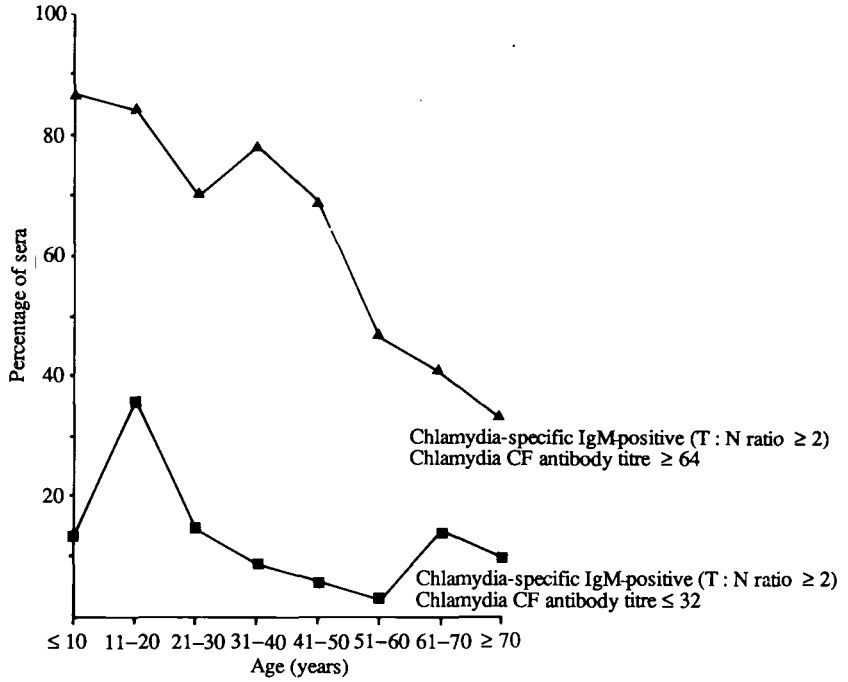

Fig. 3. Relationship between age, chlamydial CF antibody and μ-capture ELISA chlamydia-specific IgM in patients with respiratory tract symptoms.

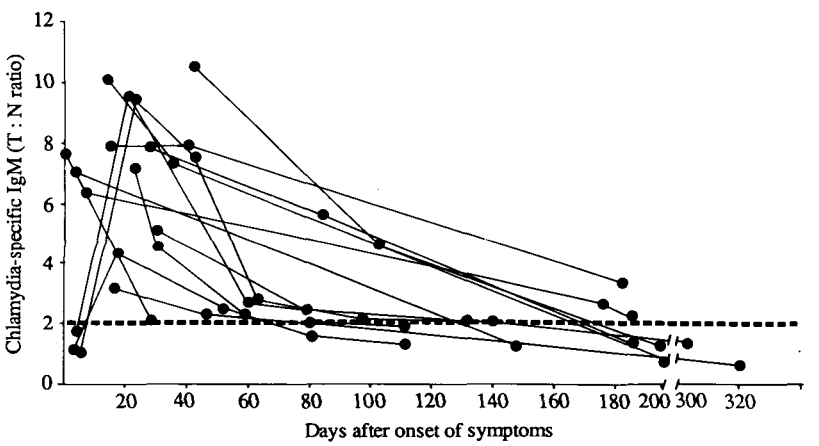

Fig. 4. The decline of chlamydia-specific IgM in 14 patients for whom multiple samples were available.

DISCUSSION

A particular advantage of the μ-capture ELISA over the indirect ELISA, is that use of an anti-human IgM capture antibody avoids false positive results attributable to rheumatoid factor if present in the test serum. In addition, competition between specific IgM and IgG for the antigen coated on the solid

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Table 1. *Correlation betweeen results of μ-capture ELISA and IFA tests for detecting chlamydia-specific IgM*

Whole inclusion indirect immunofluorescence test	μ-capture ELISA	
	Positive	Negative
Positive	21	5*
Negative	4	42

* Three samples from one patient with CFT titres ⩾ 256.

phase does not occur. It has been shown that indirect ELISAs may be unsuitable for detecting specific IgM (Wreghitt & Sillis, 1985).

The μ-capture ELISA for chlamydia-specific IgM measures antibody to the chlamydial group antigen, and is therefore unlikely to be able to distinguish between antibodies formed as a result of *C. psittaci*, *C. trachomatis* or TWAR infections. Serum samples investigated were from patients with respiratory tract symptoms. Those whose serum reacted in our chlamydial antibody assays were most probably suffering from infection with *C. psittaci* or TWAR strains rather than *C. trachomatis*, which is rarely a pathogen of the respiratory tract, except in neonates (Schachter *et al.* 1975). However, further work with serum samples from patients with TWAR infections will be necessary to establish the ability of our μ-capture ELISA to detect IgM produced as a result of TWAR infection. Further studies are also indicated to establish the prevalence of TWAR strains in the UK.

On the basis of our results, we chose a T:N ratio ⩾ 2 as indicating the presence of chlamydia-specific IgM in the μ-capture ELISA, since most low T:N ratios were clustered between 0·8 and 2. Agreement between the μ-capture ELISA and the WIF test for detecting chlamydia specific IgM was 87·5%. The WIF test uses the *C. psittaci* enzootic abortion agent of ewes (EAE) as antigen. The discrepancies may be due to the fact that the tests use different antigens.

When specific IgM was present, the μ-capture ELISA T:N ratio generally correlated with the CF antibody titre. When serial samples were available chlamydia-specific IgM became undetectable within 6 months of the onset of symptoms. Strauss (1967) showed that CF antibody peaked at 3 weeks after onset of symptoms and that titres remained ⩾ 16 for 2–3 years. This highlights the need for an alternative assay such as the μ-capture ELISA for detecting recent infection.

The percentage of patients whose serum samples were μ-capture ELISA positive and which had CF titres ⩾ 64 fell with age. This phenomenon has been noted with *M. pneumoniae* (Wreghitt & Sillis, 1987; Moule, Caul & Wreghitt, 1987). Conversely, a high proportion of patients < 20 years of age gave μ-capture ELISA positive results but had CF titres between 8 and 32. This is further evidence of usefulness of μ-capture ELISA in this age group. Wang & Grayston (1971) pointed out that IgM is not recalled in trachoma when reinfection is caused by the same serotype. It has been shown that the CF antibody response may be minimal after respiratory tract chlamydial infection (Schachter & Dawson, 1978; Grayston *et al.* 1986). In a study of an outbreak, 8 of 15 cases had CF antibody

titres < 32 5–9 weeks after exposure, which the authors attributed to either a poor response or a low degree of sensitivity of the CF test (Palmer, Andrews & Major, 1981). It has also been observed that children may fail to produce CF antibody in chlamydial respiratory tract infection (Berman *et al.* 1955) and that tetracycline, the preferred antibiotic for treating human respiratory tract infection, may suppress the antibody response (Meyer & Eddie, 1956). Again, a sensitive detection system for chlamydial IgM may be a valuable diagnostic tool for these groups of patients.

Although respiratory chlamydial infection are rarely fatal (Byrom, Walls & Mair, 1979) unless treatment is late, five patients died in Cambridgeshire between 1980 and 1987. The μ-capture ELISA for detecting chlamydia-specific IgM may well prove a useful adjunct to the WIF test in assisting the early diagnosis of chlamydial respiratory tract infection, and lead to more rapid and appropriate treatment with a reduction in morbidity and mortality.

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