Detection of IgM antibodies against *Chlamydia trachomatis* by enzyme linked fluorescence immunoassay

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SUMMARY A simple, sensitive enzyme linked fluorescence immunoassay has been developed to detect IgM antibodies against *Chlamydia trachomatis*. Reticulate bodies and elementary bodies from *C trachomatis* L₂/434 Bu strain were isolated and used as antigens in the assay. Of 113 serum samples obtained from infants with pneumonia, 27 (23.9%) had IgM antibodies to *C trachomatis* L₂ reticulate bodies and nine (8.0%) had IgM antibodies to *C trachomatis* L₂ elementary bodies (titre $\geq 1/500$). Specific IgM antibodies were not detected in 20 control serum samples obtained from healthy adults and children. The possible use of enzyme linked fluorescence assay to determine IgM antibodies in the serodiagnosis of *C trachomatis* infection is discussed.

Chlamydia trachomatis causes a wide range of infections in adults and children, including trachoma, infantile pneumonia, neonatal inclusion conjunctivitis, non-gonococcal urethritis and other diseases of the eye and genital tract. *C trachomatis* infection may be shown either by isolating the organisms in tissue culture or less often by serological means; in infantile pneumonia it is usual to make the diagnosis serologically.¹ In a previous study we isolated *C trachomatis* from infants with pneumonia and detected IgM antibodies to *C trachomatis* by the indirect immunofluorescence test.^{2,3}

Up to now the most reliable technique for the serodiagnosis of *C trachomatis* infection has been the microimmunofluorescence test of Wang and Grayston,⁴ which uses chlamydial elementary bodies as the test antigen. As this test requires specific antigens as well as trained personnel, it has not been readily available to small laboratories. Similar immunofluorescence tests using purified reticulate bodies or infected cells as the test antigen have also been described.⁵⁻⁷ More recently, chlamydial antibodies have been detected by means of an enzyme linked immunosorbent assay (ELISA). Lewis *et al*⁸ described an ELISA for measuring antibodies to *C psittaci*, and Evans and Taylor-Robinson⁹ used an ELISA to determine IgG and IgM antibodies to *C trachomatis* based on passively absorbed extracted

chlamydial group antigen. Mahony *et al*¹⁰ also described an ELISA for measuring IgG and IgM antibodies to *C trachomatis* using Renografin purified *C trachomatis* L₂ elementary bodies grown in cycloheximide treated McCoy cells.

We have developed a simple, sensitive enzyme linked fluorescence immunoassay (ELFA) for measuring of IgG antibodies to *C trachomatis* and have compared the results with those obtained with the ELISA and a microimmunofluorescence test for detecting serum IgG antibodies to *C trachomatis*.¹¹ This report describes the use of the ELFA to measure antichlamydial IgM antibodies in infants with pneumonia and in patients with a variety of diseases of the eye.

Material and methods

SERUM SAMPLES

Serum samples were obtained from 113 infants with pneumonia who were admitted to Sapporo Medical College Hospital and Hokkaido Children's Hospital during December 1981 to February 1984. The infants' ages ranged from 4 days to 2 years. Samples were also obtained from 37 patients with trachoma (stage VI), 21 patients with Behçet's disease, and 15 patients with acute follicular conjunctivitis attending eye clinics in Sapporo City. Patients were aged 20 to 80 years. In addition, samples were obtained from three sets of parents and a mother (aged 20 to 30

years) attending eye clinics, in whose babies *C trachomatis* had been found in conjunctival specimens. Serum samples were obtained from twenty age matched adults and children aged 0 to 80 years without infectious diseases to act as controls. All samples were frozen at -20°C before being tested.

ANTIGEN PREPARATION

C trachomatis L₂/434/Bu was kindly provided by Dr Julius Schachter, University of California, San Francisco. The elementary body antigen used in the microimmunofluorescence test was prepared from HeLa 229 cells infected with the above strain as described elsewhere.¹² Reticulate bodies were harvested from HeLa 229 cells after inoculation of the same strain of *C trachomatis* by a modification of the procedure of Yong *et al.*⁶ After the cells had been disrupted by sonication, reticulate bodies were separated by differential centrifugation (500 g for 10 min and 30 000 g for 30 min) and purified through a 30% Renografin cushion at 22 000 g for 40 min. The cells were then fixed in 6% formalin in phosphate buffered saline (PBS). For use in the ELFA, elementary bodies from *C trachomatis* L₂/434/Bu were harvested from HeLa 229 cells after inoculation and subjected to differential centrifugation as described above. The elementary body pellet was treated with RNase and DNase (Miles Laboratories, Illinois) at 37°C for 60 min before final separation on Renografin gradients. The elementary body pellet was then washed in PBS and suspended in PBS containing 0.1% formalin. The reticulate body antigen used in the ELFA was prepared in the same way as for the microimmunofluorescence test. Antigen concentrations of 10 $\mu\text{g/ml}$ for elementary bodies and 2.5 $\mu\text{g/ml}$ for reticulate bodies were used in subsequent experiments.

ELFA PROCEDURE

Wells of a polyvinyl microtitration plate (B plate; Dynatech Laboratories) were coated with reticulate body and elementary body antigens, which had been diluted to the appropriate concentration in 0.05M carbonate and bicarbonate buffer (pH 9.6). After incubation overnight at 4°C , the plate was washed once with PBS (pH 7.3) and 0.05% (vol/vol) Tween 20 (Sigma Chemical Company, St Louis, Missouri) (PBST). The wells were then filled with 100 μl of 1.0% bovine serum albumin in PBS and left at room temperature for at least 4 h to block the remaining sites of the well. After this the wells were washed once with PBST and 100 μl of diluted serum was added to each well. The plate was then incubated for 2 h at 37°C and washed five times with PBST. Next, 100 μl of a 1/40 000 dilution in PBST of β -galactosidase labelled goat antihuman IgM

(Zymed Laboratories, South San Francisco, California) was added to each well. After further incubation for 2 h at 37°C the conjugate was removed by aspiration, and each well was washed five times with PBST. Substrate containing 0.0025% 4-methylumbelliferyl- β -D-galactopyranoside (Koch-Light Laboratories, Colnbrook, Bucks) in 0.01M sodium phosphate buffer (pH 7.0) was added to a volume of 100 μl /well. The enzyme was allowed to act for 20 min and then stopped with 100 μl of 0.1 M glycine and NaOH buffer. The fluorescence unit (FU) value of each well was determined with a Dynatech MicroFLUOR reader (1 fluorescence unit = 0.5×10^{-12} M methylumbelliferone).

MEASUREMENT OF SERUM IgG AND IgM BY ELISA

One hundred microlitres of commercial antihuman IgG and IgM antibodies (obtained from Dako Immunoglobulin, Copenhagen, Denmark), diluted to the required concentration in carbonate and bicarbonate buffer (pH 9.6), was added to each well of a Nuncleon plate (Nunc, Denmark). The plate was incubated overnight at 4°C for passive absorption and washed once with PBST. The wells were then filled with 100 μl of 1.0% bovine serum albumin in PBS and left at room temperature for 4 h. Each well was washed once with PBST. Test samples were diluted in PBST and 100 μl was added to appropriate wells. The plate was incubated for 2 h at 37°C and washed three times with PBST. Next, 100 μl of a 1/2000 dilution of alkaline phosphatase conjugated goat antihuman IgG or IgM (Cappel Laboratories, West Chester, Pennsylvania) was added to each well. After further incubation for 2 h at 37°C the conjugate was removed by aspiration, and each well was washed three times with PBST. Substrate, consisting of p-nitrophenyl-phosphate (1 mg/ml), 50 mM Na_2CO_3 , and 1mM MgCl (pH 9.6), was then added to a volume of 100 μl /well. The plate was left at room temperature for 30 min after which time the reaction was stopped with 50 μl of 1 N NaOH. The absorbance at 405 nm was determined in a spectrophotometer.

SUCROSE DENSITY GRADIENT CENTRIFUGATION

Half a millilitre of the test serum diluted 1/2 was layered over 10 ml of 12 to 36% (vol/vol) linear sucrose gradient and centrifuged at 100 000 g for 20 h. Twenty fractions were collected with a density gradient fractionator (Hitachi Co).

MIF TEST

The microimmunofluorescence test was performed with reticulate bodies and elementary bodies of *C trachomatis* L₂ as single antigens according to the methods described previously.^{4 11}

Fig. 1 Detection of serum IgG and IgM from *Chlamydia trachomatis* antibody positive (a) and antibody negative (b) serum by ELISA.

Results

DETECTION OF ANTICHLAMYDIAL IgM ANTIBODIES BY ELFA AND COMPARISON WITH ELISA TITRES AFTER SUCROSE DENSITY GRADIENT CENTRIFUGATION

ELISA titres of serum IgG and IgM were determined in three IgM positive (titre, $\geq 1/36$) and three IgM negative samples against L_2 reticulate bodies and elementary bodies by means of the microimmunofluorescence test after sucrose density gradient centrifugation (Fig. 1). The main peak of IgG appeared in fractions 5 to 7, and those of IgM

appeared in fractions 11 to 13. IgM antibodies to *C. trachomatis* L_2 elementary bodies and reticulate bodies were determined in microimmunofluorescence test positive and negative samples by ELFA at the dilution of 1/100. The peaks that appeared in ELFA were roughly matched with the peak of IgM by ELISA. In microimmunofluorescence test negative sera no peak appeared by ELFA (Figs. 2 and 3). To investigate whether rheumatoid factor caused any false positive reactions with the IgM assay, three microimmunofluorescence test positive and three microimmunofluorescence test negative samples were tested. One of the microimmunofluorescence

Fig. 2 Detection of IgM antibodies to *C. trachomatis* L_2 reticulate bodies from microimmunofluorescence test positive (a) and microimmunofluorescence test negative (b) serum by enzyme linked fluorescence immunoassay.

Fig. 3 Detection of IgM antibodies to *C. trachomatis* L₂ elementary bodies from microimmunofluorescence test positive (a) and microimmunofluorescence test negative (b) serum by enzyme linked fluorescence immunoassay.

Fig. 4 Enzyme linked fluorescence immunoassay titration curve for serum IgM antibodies to *Chlamydia trachomatis* L₂ reticulate bodies.

Fig. 5 Enzyme linked fluorescence immunoassay titration curve for serum IgM antibodies to *Chlamydia trachomatis* L₂ elementary bodies.

Seropositivity of IgM antibodies against *Chlamydia trachomatis* from different populations at the dilution of 1/500

Population	No tested	Reticulate body positive (%)	Elementary body positive (%)
Pneumonia	113	27 (23.9%)	9 (8.0%)
Trachoma	37	1 (2.7%)	0 (0.0%)
Behçet's disease	21	1 (4.8%)	1 (4.8%)
Acute follicular conjunctivitis	15	2 (13.3%)	2 (13.3%)
Parents	7	3 (42.9%)	2 (28.6%)
Controls	20	0 (0.0%)	0 (0.0%)

test negative samples gave a positive reaction with the slide latex agglutination test for rheumatoid factor.

POSITIVE AND NEGATIVE CONTROL CURVE TO ASSIGN ELFA TITRES

To establish cut off values of fluorescence units, which distinguished positive from negative samples, we determined the fluorescence unit value of each serum by subtracting the value shown on the control coated plates. Positive samples had values over three times the standard deviation for these negative samples. Based on these findings, we set the standard cut off value at 100 FU for ELFA (Figs. 4 and 5). Subsequent tests showed that this arbitrary cut off value did not compromise the sensitivity of the assay.

TITRATION OF HUMAN SERUM SAMPLES BY ELFA
Of the 113 infants with pneumonia, 27 (23.9%) had

IgM antibodies to *C trachomatis* reticulate bodies (titre $\geq 1/500$), and nine (8.0%) had IgM antibodies to elementary bodies by ELFA (Table). Of the 37 patients with trachoma (stage VI), one (2.7%) had IgM antibodies to reticulate bodies and none of them had IgM antibodies to elementary bodies. Of 21 patients with Behçet's disease, one (4.8%) had antibodies to reticulate bodies and elementary bodies. Of the 15 patients with acute follicular conjunctivitis, two (13.3%) had IgM antibodies to reticulate bodies and elementary bodies. Of the three parents and a mother of infants from whom *C trachomatis* was isolated (from their conjunctival swabs), three had IgM antibodies to reticulate bodies and two had IgM antibodies to elementary bodies. On the other hand, none of the 20 age matched adults and children in the control group had antibodies either to reticulate bodies or to elementary bodies. The results for reticulate body

Fig. 6 Fluorescence unit (FU) in *Chlamydia trachomatis* L₂ reticulate bodies (a) and elementary bodies (b) enzyme linked immunofluorescence assays with serum from different populations at the dilution of 1/500.

and elementary body ELFA showed that the infants with pneumonia had higher fluorescence unit values than the other groups examined (Fig. 6).

Discussion

ELISA, originally described by Engvall and Perlmann¹³ and Van Weeman and Shuurs,¹⁴ has been used to detect antibodies to a number of infectious agents, including chlamydia.¹⁵⁻¹⁷ More recently, ELFA, using a fluorogenic substrate, has proved more sensitive than standard ELISA for detecting antibodies to some infectious agents.¹⁸ Yolken and Stopa¹⁹ found that ELFA was about 100 times more sensitive than the corresponding ELISA or radioimmunoassay for the detection of human rotavirus in a standard stool suspension. ELFA has proved to be a simple, reliable, and sensitive method for the rapid detection of some infectious agents.²⁰ There have been no reports, however, on the use of ELFA for the detection of IgM antibodies to *C trachomatis*.

We have previously found that ELFA is a useful method for detecting IgG antibodies to *C trachomatis*,¹¹ and the present results suggest that specific IgM antibodies to *C trachomatis* reticulate bodies and elementary bodies can be determined by ELFA. Although elementary body antigens from some strains have common antigenicity with other strains by ELISA, no elementary body antigen from a single immunotype can detect antibodies against all immunotypes of *C trachomatis*. In contrast, reticulate bodies of *C trachomatis* have group specific antigens shared by all immunotypes of *C trachomatis* and by some strains of *C psittaci* by ELISA.⁹ The same result was found in ELFA.

Serological tests are not particularly useful in the diagnosis of *C trachomatis* infections except in patients experiencing their first episode of the disease. The chronic nature of *C trachomatis* infections makes rising antibody titres a very unlikely finding.^{1,22}

IgM antibodies to *C trachomatis* are most likely to be detected in cases of systemic chlamydial infection, especially infantile pneumonia. The presence of high concentrations of IgM antibodies is not completely specific for the diagnosis of pneumonia in infants, as some infants who do not have pneumonia may develop high concentrations of these antibodies. Such cases occur in 0-1% of the population.¹

In a previous study we isolated *C trachomatis* from Japanese infants with pneumonia and detected IgM antibodies by the indirect immunofluorescence test.^{2,3} Subsequent tests showed good correlation between the isolation of *C trachomatis* and the detection of IgM antibodies.

Of 109 infants with pneumonia, 32 (29.3%) were positive for IgM antibodies (titre, $\geq 1/16$) by the indirect immunofluorescence test. *C trachomatis* was isolated from 21 (65.6%) of 32 IgM antibody positive infants compared with five (6.5%) of 77 IgM antibody negative infants. This study showed that about 24% of the infants with pneumonia had IgM antibodies to *C trachomatis* L₂ reticulate bodies (titre $\geq 1/500$).

Among patients with eye diseases (trachoma, stage VI, and Behçet's disease) and healthy controls concentrations of IgM antibodies against *C trachomatis* were significantly lower. The meaning of the seropositivity of the patients with acute follicular conjunctivitis is unclear. The seropositivity of parents may be influenced by the presence of sexually transmitted diseases caused by *C trachomatis*. The positive fluorescence unit values of infants with pneumonia were higher than in other groups tested. From these results, it would appear that the ELFA is group reactive or species reactive rather than type reactive.

The present results have shown the ELFA to be sensitive and able to determine accurately IgM antibodies against *C trachomatis* from infants with pneumonia.

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