Enzyme-Linked Immunosorbent Assay for Immunoglobulin G and M Antibodies to *Chlamydia trachomatis* in Human Sera

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Microimmunofluorescence methods used for detection of immunoglobulin G and M antibodies to *Chlamydia trachomatis* are not available to many clinical laboratories. We evaluated a simple enzyme-linked immunosorbent assay in which serotype L2 elementary bodies are used as antigen. The enzyme-linked immunosorbent assay proved satisfactory for the detection of serum IgG. A total of 160 human sera were tested, and the results correlated well with those obtained by microimmunofluorescence. Results for IgM antibody detection were not as successful, and correlation with current methods was poor.

*Chlamydia trachomatis* is a major cause of sexually transmitted disease in adults and a significant cause of respiratory tract infection in infants (9, 10).

Serological methods may be an adjunct to cell culture techniques in the diagnosis of certain chlamydial infections and are particularly useful for epidemiological studies. Recent studies of infants with chlamydial pneumonia suggest that detection of immunoglobulin M (IgM) chlamydial antibody may be the method of choice for diagnosis of this disease (10a). At present, the most widely used methods for detection of chlamydial antibodies in human serum are the complement fixation test and various modifications of the microimmunofluorescence (micro-IF) method of Wang and Grayston (12). Unfortunately, both of these tests have limitations. The complement fixation test is useful for the diagnosis of lymphogranuloma venereum and psittacosis but is not useful in other genital-tract or eye infections caused by *C. trachomatis*. The micro-IF test, although useful, requires highly skilled personnel, special reagents, and does not lend itself to automation.

A simple, reproducible, and sensitive serological method for determination of specific IgG and IgM levels would provide an important diagnostic tool for chlamydial pneumonia in infants and for screening adults for certain genital chlamydial diseases. It would also be useful for the accumulation of data for use in epidemiological studies on this group of organisms.

Enzyme-linked immunosorbent assays (ELISAs) have been applied to determination of specific antibody levels in a number of infectious diseases since their introduction by Engvall and Perlmann (4). The ELISA method appears to offer advantages as a routine method for determination of chlamydial antibodies in human sera.

We examined the use of an ELISA suitable for the determination of specific IgG and IgM antibodies to *C. trachomatis* in which a single, broadly reactive L2 serotype is used as antigen. We compared the results obtained with this assay with those of the micro-IF test and also examined the value of the assay for the early diagnosis of chlamydial pneumonia.

**MATERIALS AND METHODS**

**Antigen.** Elementary bodies (EB) of *C. trachomatis* strain L2/434/Bu were purified from L929 suspension cultures by the method of Caldwell et al., with minor modifications (1). Briefly, L2 EB were harvested from cell cultures 36 h postinoculation by differential centrifugation. Crude preparations of EB were treated with RNase and DNase (Miles Laboratories, Inc., Elkhart, Ind.) at 37°C for 60 min before final separation on Renographin (E. R. Squibb & Sons, Princeton, N.J.) gradients. The purified EB were killed with 0.1% Formalin, washed, and suspended in phosphate-buffered saline (PBS), pH 7.4. A sample of the preparation of EB was solubilized in 2% sodium dodecyl sulfate-1.5 mM EDTA-PBS (pH 8.0), and the protein content was estimated by the method of Lowry et al. (8).

Control antigen was prepared in the same way from uninfected L929 cells.

**Antisera.** Horseradish peroxidase-labeled rabbit anti-human IgG (γ chain specific) and anti-human IgM (μ chain specific) were obtained from DAKO-immunoglobulins, Copenhagen, Denmark. The human serum specimens were obtained from 42 infants and 144 adults and were selected to provide a wide range of micro-IF titers.
Micro-IF. All sera were tested for chlamydial antibodies of the IgG and IgM classes by the micro-IF method as described elsewhere (11). Each serum was tested against all 15 C. trachomatis serotypes.

Preparation of antigen-coated plates. Polyvinyl U-bottom microtiter trays (model 1-220-24; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with antigen by passive adsorption overnight at 4°C. The optimal dilution of the purified preparation of EB for plate coating was determined by titration against known positive and negative sera. This dilution was 1:1,000 in 0.05 M carbonate-bicarbonate buffer (pH 9.6), giving a final protein concentration of approximately 2.5 μg/ml.

ELISA procedure. The technique used for the ELISA was adapted from that of Voller et al. (11). Coated plates were washed once with PBS containing 0.05% (vol/vol) Tween 20 (Sigma Chemical Co., St. Louis, Mo.) (PBST), and then the wells were filled with 5.0% (vol/vol) normal horse serum (HS) in PBS (PBS-HS) and left at room temperature for at least 4 h to block remaining sites on the plastic. Plates were then washed once with PBST, rinsed with distilled water, and used immediately or dried and stored at 4°C. Serial twofold dilutions of human sera in PBST-HS were made in the plate to give a final volume of 100 μl per well. Each serum was diluted in test and control antigen-coated wells. The plate was then incubated for 2 h at 37°C and washed three times with PBST and twice with distilled water. The PBST wash fluids were left in the wells for 5 min each, and the distilled water wash was used to flood the entire plate surface to ensure the removal of any material that may have splashed onto the lids of the wells. Conjugate diluted in PBST-HS was then added in a volume of 100 μl per well. The optimal conjugate dilutions were determined to be 1:2,000 for rabbit anti-human IgG and 1:1,000 for anti-human IgM. The plate was then incubated for 2 h at 37°C and washed again as described above. Substrate consisting of 50 ml of 50 mM citrate buffer (pH 5.1) containing 20 mg of o-phenylenediamine (Sigma) and 20 μl of 30% hydrogen peroxide (Mallinckrodt Inc., St. Louis, Mo.) was then added to a volume of 100 μl per well. The enzyme was allowed to act for 15 min and then stopped with 50 μl of 4 N sulfuric acid. Color was read without delay at 492 nm with Multiskan plate reader (Flow Laboratories, Inc., Rockville, Md.), and serum antibody titers were determined by using a cutoff absorbance based on the absorbance of known negative controls. All readings were made against a blank row of wells which received all of the above treatments, except human serum was replaced by PBST.

Rheumatoid factor. Sera with positive IgM titers by ELISA were tested for the presence of rheumatoid factor with a latex slide agglutination test (RAPI/TEX-RF kit; Calbiochem-Behring Corp., La Jolla, Calif.). The 47 sera tested were diluted 1:20 in test buffer, and 1 drop was mixed with 1 drop of latex reagent on a slide. The slide was rocked for 2 min and examined visually for agglutination.

RESULTS

Determination of optimal test conditions. Results of experiments to determine optimal coating conditions showed that variation of the pH and the ionic strengths of coating buffers had only marginal effects on coating efficacy. Alkaline pH buffers with higher ionic strengths gave slightly better results, and 50 mM carbonate-bicarbonate buffer (pH 9.6) was chosen for all subsequent coating. Coating with antigen concentrations of ≥2.5 μg/ml gave equally good results in the assay, as determined by the ratio of absorbance with positive and negative sera (Fig. 1), and this concentration was used for plate coating. Polystyrene and polyvinyl plates from various manufacturers were compared for use as the solid phase in this assay. The polyvinyl plates chosen gave equal or better results than polystyrene plates, including some which are marketed especially for use in the ELISA. We also found polyvinyl preferable to work with because it showed less light reflection, making it easier to see which wells had been filled and which were empty. Incubation times and temperatures were chosen to combine optimal results with completion of the assay in a normal work day. Experiments were also performed to determine dilution buffers and blocking agents which would allow maximum binding of serum antibodies while reducing nonspecific background readings. It was found that antigen-coated plates needed to be blocked with a protein solution before the addition of human sera, even though 0.05% Tween was incorporated into the dilution buffers. HS (5.0%) in PBS was used for blocking, and PBST-HS was used for dilution of human sera and conjugates. Bovine se-

![FIG. 1. Effect of the concentration of antigen solution used to coat a polyvinyl chloride plate. A total of 100 μl of various dilutions of LGV/434 EB was allowed to adsorb in wells overnight at room temperature. An ELISA was performed with a 1:64 dilution of a known positive (●) and a known negative (○) serum. Optimal reactivity with the lowest concentration of antigen was seen at a concentration of 2.5 μg/ml.](http://jcm.asm.org)
rum albumin at a concentration of 1.0% in PBS could also be used to block the plates. With many sera, the absorbance with control or test antigens was reduced from 0.2 to 0.4 to readings of <0.1 by incorporating HS (or, to a lesser extent, bovine serum albumin) into blocking steps.

**Determination of titration endpoint in ELISA.**

To establish a cutoff absorbance reading which would distinguish a positive from a negative reading, two methods were examined. Titration of a specimen on both L2 and control antigen-coated wells should distinguish binding due to chlamydial antibody from nonspecific reactivity. This method proved unsatisfactory, as the absorbance readings of negative sera on L2 antigen-coated wells were not equivalent to those on control antigen-coated wells.

The alternative was to establish a cutoff absorbance value based on the standard deviation of absorbance readings of micro-IF-negative sera in separate test runs. We ran a series of known negative and positive sera on L2 and control antigen-coated plates. We then determined the net absorbance of each serum by subtracting the absorbance readings on control antigen-coated plates from that detected on L2 antigen-coated plates. We found that positive sera always had net absorbance values exceeding the absorbance plus twice the standard deviation of those of negative sera. For example, a series of nine negative sera was tested and the highest mean absorbance was 0.076; one of these sera was tested in eight separate tests (in quadruplicate), with a mean absorbance of 0.043 ± 0.029. On the basis of these findings, we set standard cutoff values of 0.100 for the IgG assay and 0.200 for the IgM assay. In the IgG assay, for example, sera whose net absorbance at a given dilution exceeded 0.100 were considered positive. Subsequent tests showed that this choice of an arbitrary cutoff value did not compromise the sensitivity of the assay. Often, 2- to 10-fold increases over micro-IF endpoints were seen by ELISA.

**Titration of human sera by ELISA.**

Titers of IgG and IgM class antibodies to *C. trachomatis* were determined for 160 human sera. The lowest serum dilutions tested in the ELISA were chosen to be 1:64 for both IgG and IgM. Because of the high sensitivity of this assay, dilutions below this level were considered to give results of doubtful significance and to lead to problems with nonspecific reactions. Representative titration curves for positive and negative sera are shown in Fig. 2 and 3 for IgG and IgM determinations, respectively. Comparison of the titration results for all sera tested with the ELISA and with the multiple-antigen micro-IF test is shown in Fig. 4 and 5.

*Fig. 2.* ELISA titration curves for serum chlamydial IgG antibody by ELISA. Results are shown for micro-IF-negative serum (○) and for serum dilutions of 1:256 (●), 1:2,048 (∆), and 1:8,192 (□).

The correlation coefficient for IgG results with the two methods was 0.79, indicating good agreement of ELISA results with those of the currently accepted micro-IF procedure. Correlation of results for the IgM assay was poor, although a number of sera were positive in both tests. Over half of the sera gave negative results in one or the other test. None of the IgM-
positive sera were positive for rheumatoid factor.

Results of experiments done to test the stability of the antigen coat on the plates and the reproducibility of the method are shown in Table 1. Antigen-coated plates could be stored for at least 4 months at room temperature without causing any loss of titer.

**DISCUSSION**

We have developed an ELISA in which the EB of a single *C. trachomatis* serotype are used as antigen for the detection of chlamydial antibody in human serum. For the detection of IgG class antibody, the ELISA proved to be more sensitive than the micro-IF test presently used. Even though the ELISA described used only a single serotype of *C. trachomatis*, cross-reactivity was sufficient to give antibody titers two- to fourfold higher than those seen by micro-IF in more than 80% of cases. ELISA titers were lower than those of micro-IF when the serum reactivity was monospecific for serotypes A, C1, or 1. In light of previous experience with the micro-IF test, some of these serum reactions are of doubtful significance (10a). A means to overcome this problem may be to include more serotypes in the ELISA or to use reticulate bodies as antigen instead of the EB used here. Reticulate bodies have previously been reported to give equal cross-reactivity for all serotypes when used in the micro-IF test.

There have been two previous reports on the use of ELISA for detection of *Chlamydia* anti-bodies in which EB attached are to plastic by Formalin treatment (2, 7). A more recent report compared the use of an ELISA to a single-antigen micro-IF test for detection of *C. trachomatis* antibody (6). Like us, these authors found the ELISA to be useful for chlamydial serology. However, the optimal conditions they found for their assay differed from ours, and higher concentrations of antigen were used to coat the plastic microtiter trays. Our assay also required more rigorous washing procedures than were thought to be necessary in other studies.

We found that, to ensure reproducible results, it was important to adhere rigidly to technical detail with the ELISA, especially during the washing steps of the procedure. In previous reports on the use of ELISA for detection of antibody to a variety of infectious agents, investigators have used a number of means to distinguish a positive from a negative reading and to determine titration endpoints. After trying several methods, we found best results with the use of a cutoff absorbance value greater than readings obtained with negative control sera. We used sera from 1- to 2-year-old infants who showed no evidence of chlamydial infection by other tests.

There have been no reports on the use of ELISA for detection of IgM class antibody to *C. trachomatis*. When we used ELISA for the detection of IgM class antibody, results were not as good as those found with the IgG assay. In the IgM assay, many sera showed high levels of nonspecific reactivity with control antigen. Serum titers could still be determined, but owing to
TABLE 1. Results of ELISA for chlamydial IgG antibody on 10 sera

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<th>Serum no.</th>
<th>Titer on day:</th>
<th>Titer from plate for 4 mo at:</th>
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</table>

* Assays were done on different days to test the reproducibility of the method. Plates which had been stored for 4 months at room temperature and at 4°C were also used to assess the stability of the antigen coat.

the need for high cutoff absorbance values, sensitivity of the assay was, at best, equivalent to that of the micro-IF test. There was also a sensitivity problem, as some micro-IF-reactive sera were negative in the ELISA assay. High absorbance readings on the control antigen also made this assay more difficult to read visually. We have tried using a capture system for this assay, as reported by Duermeyer and van der Veen (3), but were unable to obtain satisfactory results with this method, again owing to nonspecific reactivity of the sera. Work is continuing to try to improve the IgM assay. One possibility for use of the assay as a screening test is to use polystyrene plastic balls in place of the microtiter tray to reduce nonspecific reactions, as reported by Kalimo et al. (5). Despite its current limitations, the IgM ELISA may ultimately prove to be a useful screening test for chlamydial pneumonia in infants, as these patients have been consistently found to have high levels of IgM antibody to *C. trachomatis* by micro-IF assay and the infecting serotypes are usually those cross-react strongly with the L2 serotype used in the ELISA reported here.

In conclusion, the results of this study confirm the usefulness of the ELISA as a screening test for detection of specific IgG antibody to *C. trachomatis*. A similar assay for IgM antibody was inadequate, being less specific and less sensitive. The ELISA offers some advantages over the micro-IF test, which is difficult to read and requires specialized equipment and an ob-server trained to distinguish specific from nonspecific staining.

The ELISA may be performed with minimal technical training and can be read either visually or with a colorimeter. It uses only small quantities of reagents, most of which are available commercially, and all reagents, including antigen-coated plates, are stable for long periods of time.

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LITERATURE CITED