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Detection of Antichlamydial Immunoglobulin G and M Antibodies by Enzyme-Linked Immunosorbent Assay

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Chlamydia trachomatis causes a wide range of infections in adults and conjunctivitis and pneumonia in neonates. The complement fixation test for chlamydial antibody is broadly reactive, but possesses low sensitivity, whereas the microimmunofluorescence test is highly sensitive, but technically difficult to perform. A simple, rapid enzyme-linked immunosorbent assay (ELISA) has been developed for the measurement of immunoglobulin G (IgG) and IgM antibodies to *C. trachomatis*. Wells of microtiter plates were coated with Renografin-purified elementary bodies (serotype L2) grown in cycloheximide-treated McCoy cells, and serum antibody was detected with peroxidase-labeled goat antihuman IgG and IgM antibody. Of 41 sera tested from patients with lymphogranuloma venereum, pelvic inflammatory disease, cervicitis, or urethritis there was a 90 and 63% correlation of positive results for IgG and IgM, respectively, by microimmunofluorescence and ELISA. Of the positive correlates, ELISA titers were up to 128 times higher than microimmunofluorescence titers for IgG and IgM. The ELISA detected no false-positive results, but missed two positive results for IgG. Both of these sera were reactive against serotypes C and J, suggesting that the ELISA with LGV L2 antigen may not measure antibodies to serotypes within the C serogroup. The IgM ELISA detected 7 negative and 4 positive results not detected by the microimmunofluorescence test. Of four paired sera examined by ELISA, three showed a fourfold rise in IgG antibody titer, and one showed a twofold rise. Further evaluation of this ELISA will be required to determine how useful it will be in seroepidemiological studies and as a diagnostic tool.

Chlamydial infections in humans include hyperendemic blinding trachoma, lymphogranuloma venereum (LGV), psittacosis, urethritis, proctitis, epididymitis, cervicitis, salpingitis, conjunctivitis, and pneumonia (13, 16–19, 24). Existing serological methods for diagnosing human chlamydial infections are not satisfactory. The complement fixation (CF) test is useful in diagnosing psittacosis and LGV, but much less useful in diagnosing oculogenital infections and virtually useless in diagnosing trachoma. A major disadvantage of the CF test is its lack of sensitivity and low antibody titers associated with certain infections. The microimmunofluorescence (MIF) test is useful in diagnosing oculogenital infections and trachoma and is more sensitive than the CF test, detecting high antibody prevalences in certain groups. A commercial antigen is available for the CF test; however, none is available for the MIF test. Each laboratory performing the MIF test must therefore prepare chlamydial antigen grown in yolk sacs. This is a major drawback of this test and precludes small laboratories from performing the test.

A number of alternative serological tests have been proposed, but they are not without drawbacks. The haemagglutination inhibition test for chlamydial antibody is plagued by nonspecificity (15) and, together with the indirect haemagglutination test (1, 10), is generally not used. The radioisotope precipitation technique (7) requires a scintillation counter unavailable to many laboratories. A fluorescent cell-counting neutralization test (9) and other neutralization tests (22) are either too complex or lack sufficient sensitivity. Lewis et al. described an enzyme-linked immunosorbent assay (ELISA) for the measurement of antibodies in patients with psittacosis and LGV (11), and Cevenini et al. recently reported on an ELISA for the measurement of antibodies in patients with nongonococcal urethritis (4).

This report describes an ELISA for the measurement of chlamydial immunoglobulin M (IgM) and IgG antibodies of several serotypes in

patients with a variety of genital tract infections, including LGV, pelvic inflammatory disease, cervicitis, and nongonococcal urethritis.

MATERIALS AND METHODS

Cells and media. Mycoplasma-free heteroploid mouse McCoy cells were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.), 2 mM glutamine, and 50 μg of streptomycin per ml (growth medium).

Serology. Sera were collected from persons attending sexually transmitted disease clinics and gynecological practices in San Francisco and Hamilton and from patients with LGV, pelvic inflammatory disease, cervicitis, and nongonococcal urethritis.

CF test. A standard microtiter CF test was performed as previously described (14). Sheep erythrocytes were obtained from Woodlyn Laboratories (Guelph, Ontario) and washed in Veronal buffer. Hemolysin and guinea pig complement were obtained from Flow Laboratories (Mississauga, Ontario) and pretitrated before use. Chlamydia LGV antigen was obtained from Behring, Germany.

MIF test. The MIF test was performed with prototype antigens grown in yolk sac culture by the method of Wang et al. (23). Each serum was tested at an initial dilution of 1:8 with nine twofold dilutions. The chlamydial serotype specificity of serum antibody in reactive serum specimens was characterized according to the pattern of reactivity with prototype antigens.

ELISA. (i) Preparation of antigen. *Chlamydia trachomatis* strain L2 434/Bu was grown in cycloheximide (2 μg/ml)-treated McCoy cells to 95% infectivity as assessed by iodine staining (8). Monolayers in 25-cm^2 Falcon flasks were inoculated with 5 ml of chlamydia (10^6 inclusion-forming units per ml) then centrifuged at 2,800 rpm (2,400 × *g*) for 30 min at 35°C in an International centrifuge (model PR-J). Flasks were incubated for 2 h at 37°C, and then the inoculum was replaced with 5 ml of growth medium supplemented with 2 μg of cycloheximide per ml. Chlamydial elementary bodies were harvested at 72 h postinfection by disruption of cell monolayers with 5-mm glass beads. Cell debris was pelleted by centrifugation at 500 rpm for 10 min, and chlamydiae were collected by centrifugation at 18,500 rpm (30,000 × *g*) for 20 min. Elementary bodies from six flasks were suspended in 3 ml of phosphate-buffered saline, pH 7.4 (PBS), layered over 2 ml of 30% (vol/vol) Renografin-76 (E. R. Squibb & Sons Ltd., Montreal, Canada), and centrifuged in an SW 50L rotor at 15,000 rpm (20,000 × *g*) for 40 min in a Beckman model L3-50 ultracentrifuge. The final pellet was suspended in 1 ml of PBS (for six flasks). Chlamydiae prepared by this method yielded 400 μg of protein per ml (determined by the Lowry et al. method [12] with bovine serum albumin as the standard) and 2 × 10^8 inclusion-forming units per ml by titration in cycloheximide-treated McCoy cells.

(ii) Assay. Ninety-six-well polystyrene microhaemagglutination plates (no. 1-220-24X) and Immulon 1 "U" plates (no. 011-010-3650) were obtained from Dynatech Laboratories Inc. (Alexandria, Va.) and used without prewashing since washing with either PBS or carbonate buffer greatly reduced the binding capacity for antigen. Chlamydial antigen prepared as described above was diluted 1:100 in 0.05 M carbonate buffer (pH 9.6), and 0.1 ml was dispensed into the wells of microtiter plates. In some experiments antigen was treated with 0.1% (wt/vol) sodium dodecyl sulfate (SDS) in PBS for 1 h at 37°C and then diluted 1:800 in carbonate buffer for coating plates. Various times and temperatures were used for adsorbing antigen to the solid phase (see below). Plates were washed three times with PBS containing 1% Tween 20. Serial twofold dilutions of serum (0.1 ml) in buffer G (0.01 M sodium phosphate [pH 7.2], 0.3 M NaCl, 0.001 M $MgCl_2$, 0.1% bovine serum albumin [fraction V; Sigma Chemical Co., St. Louis, Mo.], 0.5% gelatin) were incubated for 1 h for IgG or 3 h for IgM. The plates were washed, and 0.1 ml of horseradish peroxidase-labeled goat antihuman IgG or anti IgM (Cappell Laboratories, West Chester, Pa.) diluted 1:4,000 in buffer G was added, and the plates were incubated for 1 h at 37°C. After washing, 0.1 ml of ortho-phenylenediamine (Sigma) (0.1 mg/ml in phosphate citrate buffer, pH 5.0) was added, and plates were left for 30 min at room temperature in the dark. Fifty microliters of 1 N NaOH was added to each well, and the plates were read with a Dynatech automated micro ELISA reader (model MR 580) at a wavelength of 492 nm. In some experiments, HCl was used to intensify the color, but in a small comparison of the two stop solutions there were no differences in antibody titers.

RESULTS

Comparison of antigen adsorption in microtiter plates at different temperatures in PBS (pH 7.4) and carbonate buffer (pH 9.6) revealed no difference in binding at 4, 22, and 37°C for either buffer. Titration of antigen with human or mouse antisera to LGV L2 434/Bu demonstrated an inability to saturate the solid phase with antigen in either PBS or carbonate buffer. At high concentrations of antigen (1:160) binding with carbonate buffer exceeded that in PBS (Fig. 1). In an attempt to saturate plates with antigen in carbonate buffer, antigen was treated with 0.1% SDS by heating for 1 h at 37°C. Figure 2 shows that saturation was obtained with SDS-treated antigen, as binding was maximum at an antigen dilution of 1:400, decreased with further dilution to 1:6,400, and then fell quickly. Identical antibody titers were obtained when sera were tested with untreated antigen at 1:100 or SDS-treated antigen at 1:800. Detergent-treated antigen at 1:800 was therefore used in subsequent experiments.

Formalin has been used to fix antigen to polystyrene plates to prevent desorption of antigen during washing steps (2). We found no difference between antigen-coated plates treated with 0.1% Formalin and antigen-coated plates not treated with Formalin. Plates coated with antigen and not treated with Formalin yielded good reproducibility. Fifteen sera tested on successive days all gave identical antibody titers.

To determine whether chlamydial IgG and IgM antibodies could be measured quantitative-

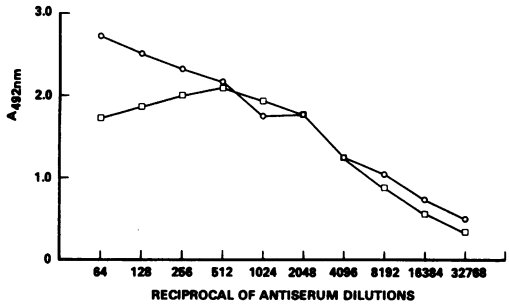

FIG. 1. Comparison of coating plates with antigen in carbonate buffer and PBS. LGV antigen (L2 434/Bu) diluted 1:160 in either (○) carbonate buffer (pH 9.6) or (□) PBS (pH 7.4) was incubated overnight at room temperature (22°C). After a wash (PBS plus 1% Tween 20), serial dilutions of human serum (CF titer, 1:128; MIF IgG titer, 1:16,384) were added and incubated for 1 h at 37°C. Peroxidase-labeled goat anti-human IgG antibody diluted 1:500 was added and incubated for 1 h at 37°C. After the addition of substrate, the optical density at 492 nm ($A_{492\ nm}$) was determined by transferring the contents of the well to a test tube and reading in a spectrophotometer.

ly, 102 negative specimens with CF titers <1:4 were assayed to determine the negative cutoff value. Of these 102 sera, 26 were nonreactors by ELISA. These were tested for chlamydial antibody and for nonspecific reactivity with chlamydial antigen- and control antigen-coated wells, respectively. The value for the control antigen was subtracted from the value for chlamydial antigen at each serum dilution. A plot of the optical density versus serum dilutions for high-positive sera versus the mean of the negative sera revealed that the greatest difference in optical density between positive and negative sera occurred at a serum dilution of 1:32. The normal distribution of the negative sera was obtained by plotting the number of sera versus their optical density at a dilution of 1:32 (data not shown). The mean optical density of the negative sera was calculated from the normal distribution, and a value of 2 times the negative mean was chosen as the cutoff value for determining antibody titer. To compensate for day-to-day variation in optical densities, 20 representative negative sera were selected and tested on the same day with unknown sera to establish an accurate cutoff value for determining the antibody titer. The last dilution of serum giving an optical density greater than 2 times the mean optical density of the 20 negative sera at the same dilution was taken as the antibody titer. The results with representative negative (<1:32), low-titer (1:1024), and high-titer (>1:16,384) IgG sera are illustrated in Fig. 3.

To evaluate the ELISA for chlamydial IgG and IgM antibody, sera were tested by CF, MIF, and ELISA. Sera from adult patients attending general gynecological practice or a sexually transmitted disease clinic and having a variety of chlamydial infections, including LGV, pelvic inflammatory disease, cervicitis and urethritis were used in the evaluation. Fifteen of 41 sera were tested by CF, MIF, and ELISA, and the remainder were tested by MIF and ELISA only. Of 41 sera tested from patients infected with different serotypes, there was a 90 and 63% correlation of positive results for IgG and IgM, respectively, by MIF and ELISA. As expected,

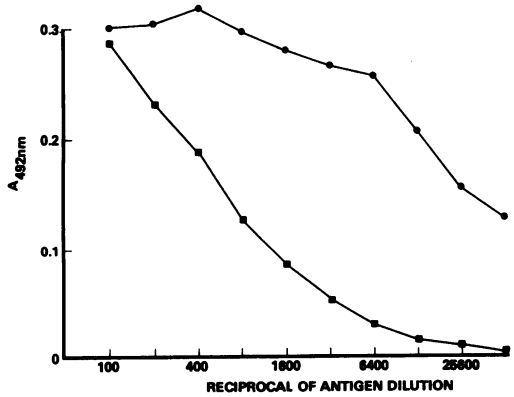

FIG. 2. Optimal concentration of untreated and detergent-treated antigen for the ELISA. Serial dilutions of (■) untreated or (●) SDS-treated (0.1% SDS at 37°C for 1 h) antigen in carbonate buffer were adsorbed overnight at room temperature (22°C). Rabbit antichlamydia antiserum (diluted 1:800) and peroxidase-labeled goat anti-rabbit IgG (diluted 1:4,000) were used to measure the binding of antigen to the solid phase. Plates were read on a Dynatech automated plate reader.

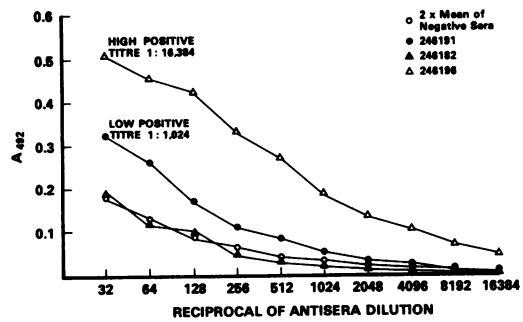

FIG. 3. ELISA IgG titers for negative and low- and high-titer human sera. Twenty negative sera (CF titer, <1:4; ELISA titer, <1:32) were run to determine antibody titers as described in the text. Peroxidase-labeled goat antihuman IgG was diluted 1:4,000 in buffer G.

there was little correlation between CF and MIF titers, since the former measures genus-specific antibodies and the latter measures serotype-specific antibodies. Of the positive correlates, ELISA titers were up to 128 times higher than MIF titers for IgG and IgM (Table 1). The geometric mean titer for IgG was 7,086 by ELISA and 1,602 by MIF, and that for IgM was 540 by ELISA and 48 by MIF. The ELISA gave no false-positive results, but missed two positive results for IgG. The 2 positive sera not detected by the ELISA were both reactive with serotypes C and J. The use of an L-2 antigen therefore failed to detect IgG antibodies to some serotypes within the C serogroup. The IgM ELISA detected four positive results not seen with the MIF test, but seven sera positive by MIF were negative by ELISA.

Paired sera from patients attending a sexually transmitted disease clinic were tested by CF, MIF, and ELISA to evaluate the diagnostic potential of the ELISA. Acute and convalescent sera from four *C. trachomatis* culture-positive women with cervicitis were tested (Table 2). Three of the four serum pairs showed a fourfold rise in IgG by ELISA, whereas only one pair showed a rise by MIF, and none showed a rise by CF. Paired sera from the first patient which showed a fourfold rise in IgG titer also showed a rise in IgM titer from 1:128 to 1:512. The second patient's sera demonstrated a fall in titer in the ELISA IgM (1:64 to <1:8), whereas the third and fourth patients had no detectable IgM by ELISA.

TABLE 1. Comparison of antibody titers by CF, MIF, and ELISA

Serum	Reciprocal of antibody titer				
	CF	MIF		ELISA	
		IgG	IgM	IgG	IgM
246 034	512	16,384	256	>16,384[a]	512
246 173	16	1,024	64	4,096	<32
246 182	16	<8	<8	32	<32
246 183	64	256	16	>16,384	2,048
246 191	32	128	16	1,024	1,024
246 195	<8	256	8	1,024	<32
246 196	256	4,096	128	16,384	256
246 200	<8	1,024	64	16,384	<32
246 205	16	128	32	1,024	512
246 206	8	128	32	128	256
246 208	<8	64	32	512	2,048
246 213	16	256	16	>16,384	<32
246 230	<8	128	16	16,384	512
246 231	<8	128	32	128	256
246 302	16	32	8	32	512

[a] ELISA antibody titers were taken as the last serum dilution having an optical density greater than twice the mean of 20 negative sera at the same dilution.

DISCUSSION

Serological diagnosis of chlamydial infections in the past has relied heavily on the CF and MIF tests. The lack of sensitivity of the CF test and the difficulty in performing the MIF test have fostered the search for new serological tests for the measurement of chlamydial antibodies. We have described an ELISA which measures IgM and IgG antibodies to *C. trachomatis* by using the broadly reactive antigen L2 434/Bu. The test is rapid, easy to perform, and much more sensitive than the CF test. It employs LGV antigen grown in cycloheximide-treated McCoy cells, thus eliminating the difficulties associated with growing chlamydia in yolk sacs for use in the MIF test. Antigen-coated plates and other reagents can be stored at 4°C ready for immediate use. Commercial antiglobulin conjugates are readily available, eliminating the necessity for laboratories with limited resources to prepare them. Results can be read visually or with an automated ELISA reader for efficient testing of a larger number of sera.

Lewis et al. used a parrot strain of *Chlamydia psittaci* in an ELISA and showed a diagnostic rise in 14 sera from patients with clinically diagnosed psittacosis (11). They used antigen at a dilution of 1:160 and employed an albumin-blocking step to minimize nonspecific absorption of antibody. We have employed an SDS-treated LGV antigen at a dilution of 1:800 and found a blocking step unnecessary when high dilutions (1:4,000) of conjugate are used. Treatment of antigen with SDS likely solubilizes the antigen and facilitates adsorption to the plastic, thus decreasing the amount of antigen necessary to measure antibody accurately. Lewis et al. reported geometric mean titers for positive sera of 419 by ELISA and 94 by CF (11). Cevenini et al., using the L2 434/Bu antigen, reported geometric mean titers of 229 by ELISA and 29 by immunofluorescence for 53 positive sera from patients with nongonococcal urethritis (4). Our results using the same antigen indicate geometric mean titers for IgG of 7,086 by ELISA, 1,602 by MIF, and 66 by CF, demonstrating that our ELISA is more sensitive than the MIF and much more sensitive than that reported by Cevenini et al. These authors reported three false-negative results for IgG by ELISA out of 53 positive sera, whereas we observed two false-negative results out of 41 sera, both of which reacted with C or J serotypes. Our two false-negative results may be explained by the fact that serotypes C and J possess little antigenic relatedness with serotype L2; therefore, antibodies to C and J would not be expected to react with the L2 serotype.

TABLE 2. Comparison of CF, MIF, and ELISA antibody titers of paired sera from four patients

Patient	Clinical signs	Date serum taken (1982)	Antibody titer			
			CF	MIF IgG	ELISA	
					IgG	IgM
1	Cervicitis	2 February	1:16	1:128	1:2,048	1:128
		4 March	1:16	1:128	1:8,192	1:512
2	Cervicitis	9 February	<1:4	1:16	1:1,024	1:64
		2 March	<1:4	1:16	1:4,096	<1:8
3	Cervicitis, dysuria, discharge	28 January	1:8	1:16	1:256	<1:8
		25 February	1:8	1:64	1:512	<1:8
4	Cervicitis	25 February	1:8	1:64	1:4,096	<1:8
		9 March	1:8	1:64	1:16,384	<1:8

Cevenini et al. presented no data on antibody serotypes measured by their ELISA. Since our ELISA appears to measure antibodies to the B complex (strains B, D, E, F, G, K, and L) and not the C complex (strains C, J, H, I, and A), we are currently developing an assay employing a mixture of either serotype C or J and L2 antigen in an attempt to measure antibodies to both the B and C complexes of *C. trachomatis*. Detection of rising or falling antibody titers in two cases of clinically diagnosed ornithosis (data not shown) suggested that ELISA was capable of measuring genus-specific antibodies in addition to serotype-specific antibodies.

Further study is required to determine whether the present ELISA can be used successfully to diagnose infections. We have tested paired sera from four patients with proven chlamydial infections and have found significant rises to IgG in all four. Chlamydia-specific IgM antibody was detected in two of four patients by ELISA, with titers ranging from 1:64 to 1:512. Where dates of onset of symptoms were recorded (three of four patients tested) IgM titers became negative 21 days after chlamydia was recovered from the cervix of one patient, were not detected in the acute serum collected 23 days after the onset of symptoms in one patient, and remained positive as long as day 49 in a third patient. Serial serology on a greater number of patients with documented onset of symptoms is underway.

Although measurement of chlamydia-specific IgM and a fourfold rise or fall in IgG would indicate that the ELISA may prove useful for the diagnosis of certain types of chlamydial infections, serology has not been a particularly useful approach to diagnosis. A better application of serology has been its use in seroepidemiological studies for the determination of serotype prevalences and transmission patterns. If the ELISA measures the same types of antibodies as detected by the MIF test, then it is not likely to be useful in diagnosing chlamydial infections. If, however, the ELISA measures different antibodies, then it may provide some diagnostic assistance in certain clinical situations. This question will only be resolved by further experimentation.

The basis for the limited sensitivity of IgM detection by ELISA is not understood at this time. Attempts to accurately measure IgM by ELISA using outer membrane proteins prepared by the method of Caldwell et al. (3) have also been nonproductive. We have attempted to develop a capture IgM assay using goat antihuman IgM antibody attached to a solid phase and measuring specific IgM by adding chlamydial antigen followed by a detector antibody, but have met with little success. This type of assay has proven useful for the measurement of IgM antibody to *Toxoplasma* (6), cytomegalovirus (21), Epstein-Barr virus (20), parainfluenza virus (5), influenza A virus (H. Schmitz, B. Flehmig, and A. Vallbracht, Abstr. 5th Int. Congr. Virol., Strasbourg, France, abstr. W13108, p. 165, 1981) and herpes simplex virus (A. M. vanLoon, A. M. Heesen, J. van der Logt, and J. van der Veen, Abstr. 5th Int. Congr. Virol., Strasbourg, France, abstr., P13120, p. 171, 1981). We have demonstrated the capture of serum IgM, but failed to demonstrate the capture of chlamydial antigen by specific IgM. The failure of the capture IgM and indirect ELISAs to accurately measure IgM may be the result of an inability of antigen and pentameric IgM to react due to altered conformational changes of either antigen or antibody or to stearic hindrance resulting from the binding of antigen to a solid phase.

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