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Enzyme Immunoassay To Determine Exposure to *Chlamydia pneumoniae* (Strain TWAR)

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Recent studies suggest that a group of *Chlamydia* strains known as TWAR, which are now proposed to be a new species called *Chlamydia pneumoniae*, may be a frequent cause of respiratory disease in the United States and many other countries. Current serotesting methods do not allow rapid screening of large numbers of samples to distinguish *C. trachomatis* exposure from *C. pneumoniae* exposure. We developed an enzyme immunoassay to decrease cross-reactivity between immunoglobulin G antibodies reactive with *C. trachomatis* and *C. pneumoniae*. Elementary bodies of *C. trachomatis* or *C. pneumoniae* were treated with a detergent-chelating solution to decrease the reactivity of the common lipopolysaccharide antigens. Sera from four groups of patients, totaling 143 persons, were tested by this assay. The prevalences of titers of ≥128 to *C. trachomatis* and *C. pneumoniae*, respectively, were as follows: (i) for 23 women seropositive for *C. trachomatis* by the microimmunofluorescence test, 21 (91%) and 18 (78%); (ii) for 50 adult blood donors, 13 (26%) and 39 (78%); (iii) for 40 sexually transmitted disease clinic patients, 20 (50%) and 32 (80%); (iv) for 30 healthy children 5 to 7 years old, 0 (0%) and 8 (27%). Western blots (immunoblots) of each antigen corroborated the differential reactivity of *C. trachomatis*-positive, *C. pneumoniae*-negative and *C. trachomatis*-negative, *C. pneumoniae*-positive serum samples. Western blots of serum samples from rabbits immunized with either *C. trachomatis* or *C. pneumoniae* elementary bodies revealed at least two protein bands (30 and 80 kilodaltons) which appeared to represent unique *C. pneumoniae* antigens.

Chlamydia trachomatis is the most frequent cause of sexually transmitted disease (STD) in industrialized nations (4, 17, 25, 36). Recent studies suggest that a group of atypical *Chlamydia* strains known as TWAR (15, 21), which have been proposed to represent a new species called *C. pneumoniae* (10), may be a frequent cause of acute respiratory disease (11, 14, 22, 26). Serologic studies using the microimmunofluorescence test (MIF) have demonstrated a high frequency of antibodies to *C. pneumoniae* strains in humans (5, 8, 9, 34). Because of the high prevalence of antibodies to both *C. trachomatis* and *C. pneumoniae* in healthy adults (8, 34), screening tests for chlamydial seroprevalence must distinguish between *C. trachomatis* and *C. pneumoniae* exposure. The MIF test differentiates between antibodies to *C. pneumoniae* and antibodies to *C. trachomatis* strains (9, 34). This test is tedious and time-consuming and is therefore not suitable for processing large numbers of samples. Enzyme immunoassays are simple, inexpensive, and applicable to large-scale testing. In this report, we describe the development and evaluation of an enzyme-linked immunosorbent assay (ELISA) that differentiates immunoglobulin G (IgG) antibodies reactive to *C. trachomatis* from those reactive to *C. pneumoniae*. Substantial cross-reactivity between *C. trachomatis* and *C. pneumoniae* was eliminated by extraction of common lipopolysaccharide (LPS) antigens with a detergent-chelating solution.

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MATERIALS AND METHODS

Antigen preparation. *C. trachomatis* L_2/434/Bu was grown in HeLa 229 cell monolayers as described by Kuo et al. (16). Elementary bodies (EBs) were then obtained as described by Kuo et al. (16), with minor modifications. Infected monolayers were removed from culture flasks by agitation with 4-mm-diameter glass beads, suspended in an aqueous solution of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–0.2 M sucrose–15 mM $CaCl_2$–1 mM $MgCl_2$–90 mM NaCl (HSC), and sonicated for 30 s at 20% output (W-375 Sonifier Cell Distributor; Heat Systems-Ultrasonics Inc., Plainview, N.Y.). HeLa cell debris was sedimented by centrifugation at 500 × *g* for 15 min at 4°C, and EBs from the supernatant were pelleted by centrifugation at 31,000 × *g* for 60 min at 4°C. The pellet was suspended in HSC and stored at −70°C.

EBs of *C. pneumoniae* TW-183 (Washington Research Foundation, Seattle) were obtained by the same procedure, except that the HeLa monolayers were treated with DEAE-dextran (30 μg/ml) in HSC for 30 min at room temperature before inoculation, preincubated with the inoculum for 2 h, and incubated for 72 h.

For ELISA and MIF, a sample of the EB preparation or (for ELISA only) the control cell preparation was formalinized for use as the antigen as described by Wang et al. (35). The protein content of the preparation was determined by the method of Lowry et al. (19) as modified by Markwell et al. (21), with bovine serum albumin as the standard.

Human sera. Sera were obtained from the following four populations: group 1, 23 pregnant women with *C. trachomatis* IgG antibody titers of >1:16 by MIF (13); group 2, 50 adult blood donors; group 3, 30 healthy children 5 to 7 years old; group 4, 40 patients of STD clinics in Atlanta, Ga. Serum specimens were also obtained from a 50-year-old

woman who contracted pneumonia after contact with a cockatoo.

Hyperimmune rabbit antisera to L_2/434/Bu and TW-183. For preparation of the inoculum, pelleted EBs were suspended in 10 ml of HSC, sonicated, and centrifuged at 20,000 × *g* for 60 min through a 10-ml cushion layer of 30% Renografin-76 (E. R. Squibb & Sons, Princeton, N.J.). The Renografin was removed from the resulting pellet by suspension in HSC, followed by centrifugation at 30,000 × *g* for 60 min. The pellet was suspended in HSC and inactivated by being heated in a 56°C water bath for 30 min. Immunization and bleeding of rabbits were performed as described by Caldwell et al. (2).

ELISA. Formalinized antigen or control antigen was diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6, to a protein concentration of 20 μg/ml, and 0.05 ml was dispensed into each well of a microtiter plate (Immulon-2 flat-bottom plates; Dynatech Laboratories, Inc., Alexandria, Va.). After incubation overnight at 4°C, plates were washed once with phosphate-buffered saline (PBS), and 0.05 ml of PBS containing 1% sodium deoxycholate and 5 mM disodium EDTA salt were placed in each well. Following 1 h of incubation at 37°C, the plates were washed three times with 1% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) in PBS (PBST), once briefly and twice for 4 min, and 0.05 ml of diluted serum per well was added. Sera were diluted in PBS containing 0.3 M NaCl, 0.001 M $MgCl_2$, 0.1% bovine serum albumin (fraction V; Sigma), and 0.5% gelatin (Difco Laboratories, Detroit, Mich.) (20). The plates were then incubated at 37°C for 2 h. After the plates were washed with PBST as described above, they were incubated at 37°C for 2 h with peroxidase-conjugated rabbit immunoglobulins to human IgG (gamma chains; DAKO-Immunoglobulins a/s, Copenhagen, Denmark) diluted 1:400 or peroxidase-conjugated goat anti-rabbit IgG (heavy and light chains; ICN Immunobiologicals, Lisle, Ill.) diluted 1:2,000 at 0.05 ml per well. Conjugates were diluted in the same buffer as the serum samples. After 2 h of incubation at 37°C and washing with PBST as described above, 0.05 ml of substrate solution (99 ml of distilled water, 1 ml of a freshly prepared 1% solution of *o*-phenylenediamine in methanol, 0.1 ml of 3% hydrogen peroxide) was placed in each well. The plates were left at room temperature in the dark for 12 min. The enzyme reaction was stopped by addition of 0.025 ml of 8 N H_2SO_4 to each well, and the A_{492} was read in a Dynatech Micro ELISA MR 580 reader. Each serum dilution was tested in duplicate against both antigen and control antigen, and the net absorbance was obtained by subtracting the mean absorbance of the control antigen from that of the test antigen. A positive reference serum, positive by MIF and ELISA, and a negative reference serum, negative by MIF and ELISA, were included in each test series.

Definition of positive ELISA readings. A net absorbance of ≥0.100 was considered positive. This value was at least 5 standard deviations above the mean net absorbance of four serum samples (from 5- to 7-year-old children) nonreactive in the MIF assay for IgG antibodies to either *C. trachomatis* or *C. pneumoniae*, each tested twice, on separate occasions, over a period of 2 months.

Construction of standard curves for assigning ELISA titers. Serum samples from patients of STD clinics were used to construct standard curves. Nine serum samples positive by MIF for anti-*C. trachomatis* and anti-*C. pneumoniae* IgG antibodies were assayed by ELISA with serial twofold dilutions, beginning at 1:32. The endpoint titer was defined as the reciprocal of the highest serum dilution yielding a net absorbance of ≥0.100. For each antigen (L_2/434/Bu and TW-183) and dilution of serum, a linear regression line was fitted to the net absorbance as a function of the endpoint titer. The coefficients of linear correlation for the 1:128 dilution were 0.947 for L_2/434/Bu and 0.935 for TW-183; correlations at other dilutions were similar. In subsequent experiments, serum samples were tested at one dilution only, and antibody titers were read off the respective standard curves.

Net absorbances of test samples were corrected to a reference serum which was positive by both MIF and ELISA by the following formula: corrected net absorbance of sample = measured net absorbance of sample × (net absorbance of positive reference serum obtained when the standard curve was constructed/net absorbance of positive reference serum).

MIF. MIF was performed by the method of Wang and Grayston (33). Serial twofold dilutions of serum were tested, beginning at 1:16.

SDS-polyacrylamide gel electrophoresis. Antigens for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis consisted of EBs purified by centrifugation through a cushion of 30% Renografin as described above. Purified, pelleted EBs were divided into aliquots and stored at −70°C. Protein concentrations of EBs solubilized in 0.1% SDS were determined by the method of Smith et al. (29) (BCA Protein Assay Kit; Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as the standard. For electrophoresis, purified EBs were solubilized by being boiled for 5 min in SDS sample buffer. Samples containing approximately 70 μg of EB protein were loaded into trough wells and resolved on 12.5% polyacrylamide slab gels (140 by 1.5 by 120 mm) with the discontinuous-buffer system of Laemmli (18). Protein profiles were visualized by silver staining with the method of Morrissey (23). Apparent molecular weights were estimated by comparison with a plot of migration distance versus log molecular weight with phosphorylase *b* (97.4K protein), bovine serum albumin (66.2K protein), ovalbumin (42.7K protein), carbonic anhydrase (31K protein), soybean trypsin inhibitor (21.5K protein), and lysozyme (14.4K protein) as standards (Bio-Rad Laboratories, Richmond, Calif.). Gels were run at 20 mA per gel.

Immunoblotting. Proteins resolved by SDS-polyacrylamide gel electrophoresis were transferred to 0.45-μm-pore-size nitrocellulose membranes by electrophoresis at 30 V for 16 to 18 h at 14°C as previously described by Towbin et al. (32). After transfer, each nitrocellulose membrane was incubated in 5% nonfat dry milk (Carnation Co., Los Angeles, Calif.)–PBS for 4 h at 4°C with rocking to block excess protein-binding sites. Each wide lane of transferred protein on the nitrocellulose membrane was cut into five identical antigen strips and incubated overnight at 4°C with rocking in a 1:100 dilution of human serum (from one of the groups described) or 1:500 dilutions of hyperimmune rabbit serum samples (prepared as described above) in 5% nonfat dry milk. Following this incubation, the strips were washed three times with PBST and then incubated for 2 h in a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains) (Protoblot System; Promega Biotec, Madison, Wis.) or a 1:1,000 dilution of goat anti-rabbit IgG (heavy and light chains) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). The strips were washed twice in PBST and once in PBS and then developed by incubation in a solution of Nitro Blue Tetrazolium (0.33 mg/ml)–5-bromo-4-chloro-3-indolylphosphate (0.165 mg/ml) in buffer (100 mM Tris hydrochloride [pH 9.5], 100 mM

TABLE 1. Effect of sodium deoxycholate extraction of ELISA antigens on cross-reactivity of *C. trachomatis* and *C. pneumoniae*

Serum	Antibody titer			
	L_2/434/Bu antigen		TW-183 antigen	
	PBS[a]	Sodium deoxycholate[b]	PBS[a]	Sodium deoxycholate[b]
Rabbit anti-L_2	4,096	4,096	256	<64
Rabbit anti-TW	<128	<128	1,024	1,024
Pneumonia, acute	256	<32	1,024	512
Pneumonia, convalescent phase	256	64	1,024	1,024

[a] Coated plates were treated with PBS alone.
[b] Coated plates were treated with sodium deoxycholate in PBS.

NaCl, 5 mM $MgCl_2$ [all from Sigma]). To visualize proteins, the nitrocellulose membrane was stained by incubation overnight in 1.0 μl of India ink (Fount India; Pelikan AG, Hannover, Federal Republic of Germany) per ml of PBST (12). As a control against nonspecific binding reactions, serum samples that were representative of the observed range of banding patterns were selected and blotted against HeLa cell antigens as described above.

RESULTS

Effect of antigen extraction. To assess the effect of sodium deoxycholate extraction of ELISA antigens, hyperimmune rabbit antiserum to L_2/434/Bu, hyperimmune rabbit antiserum to TW-183, and acute and convalescent serum samples from a person who contracted pneumonia after contact with a cockatoo were tested against both extracted and unextracted L_2/434/Bu and TW-183 antigens by ELISA. The results (Table 1) demonstrated that the cross-reactivity between TW-183 and L_2 decreased considerably after sodium deoxycholate extraction of the antigens, whereas the titers obtained with the homologous antigen remained practically unchanged.

Use of EAELISA to detect IgG antibodies to *C. trachomatis* and *C. pneumoniae* in various populations. Four groups (pregnant women seropositive for *C. trachomatis* by MIF, STD clinic patients, adult blood donors, and healthy children 5 to 7 years old) totaling 143 subjects were tested by extracted-antigen ELISA (EAELISA). Table 2 summarizes the prevalences and the geometric means of titers of ≥128. The prevalences of antibodies to *C. pneumoniae* were similar among all three adult groups (about 80%) but considerably lower (only 27%) in the children. In contrast, the prevalence of antibodies to *C. trachomatis* was variable: 91% in pregnant, MIF-positive women, 50% in STD clinic patients, 26% in blood donors, and 0% in children. The geometric mean titers for *C. pneumoniae* and *C. trachomatis* showed similar trends. Of 113 adults tested, 54 were seropositive for *C. trachomatis*, 89 were seropositive for *C. pneumoniae*, and 43 were seropositive for both antigens.

TABLE 2. Seropositivity of different populations for IgG antibodies to *C. trachomatis* and TWAR in the sodium deoxycholate-extracted ELISA at a serum dilution of 1:128

Population	Total no. tested	No. (%) and geometric mean of titers ≥ 128	
		L_2-positive samples	TWAR-positive samples
Pregnant, MIF-positive[a] women	23	21 (91), 333	18 (78), 621
STD clinic patients	40	20 (50), 284	32 (80), 546
Blood donors	50	13 (26), 218	39 (78), 668
Children, 5–7 yr old	30	0 (0)	8 (27), 304

[a] Reactive by MIF for anti-*C. trachomatis* IgG antibodies.

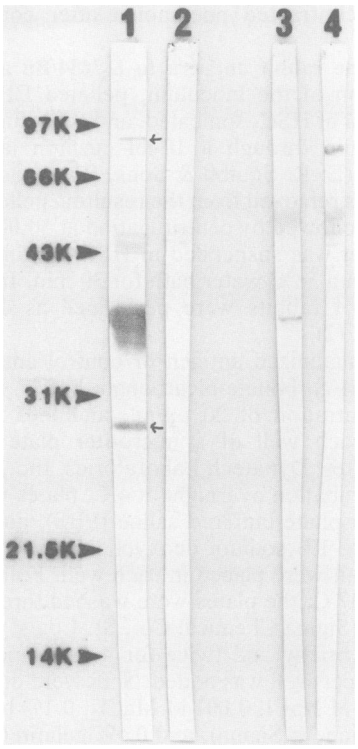

FIG. 1. Immunoblots of hyperimmune serum samples from rabbits immunized with either L_2/434/Bu or TW-183 and reacted with autologous or heterologous antigen. Lanes: 1, TW-183 antigen with autologous serum; 2, L_2 antigen with anti-TW-183 serum; 3, TW-183 antigen with anti-L_2 serum; 4, L_2 antigen with autologous serum. The arrows beside lane 1 indicate *C. pneumoniae*-specific protein bands. The positions of the molecular weight markers described in Materials and Methods are shown on the left.

Western blotting of *C. trachomatis* and *C. pneumoniae* antigens. To compare the protein antigen profiles of the two *Chlamydia* species, hyperimmune sera from rabbits immunized subcutaneously with purified L_2 and TW-183 EBs were blotted against autologous and heterologous protein antigens (Fig. 1). Cross-reacting antigens were present as shown by the appearance of common protein bands at molecular masses corresponding to 86 to 90 and 45 to 50 kilodaltons (kDa). Anti-L_2 serum also reacted with a protein band of approximately 35 kDa present in both *C. trachomatis* and *C. pneumoniae* antigen preparations. The cross-reactivity seen in these blots indicates that not all genus reactivity resides on the LPS, as has been noted by other investigators (31). Two *C. pneumoniae*-specific protein bands were noted at molecular masses of approximately 30 and 80 kDa.

Human serum samples that were characterized for *C. trachomatis* and *C. pneumoniae* reactivity in the EAELISA were also immunoblotted (Fig. 2). Sera from patients that were reactive in the EAELISA with L_2 antigens and not TW-183 antigens showed a greater degree of cross-reactivity than sera from patients that were reactive with TW-183 and not L_2 EAELISA antigens. The cross-reacting protein bands

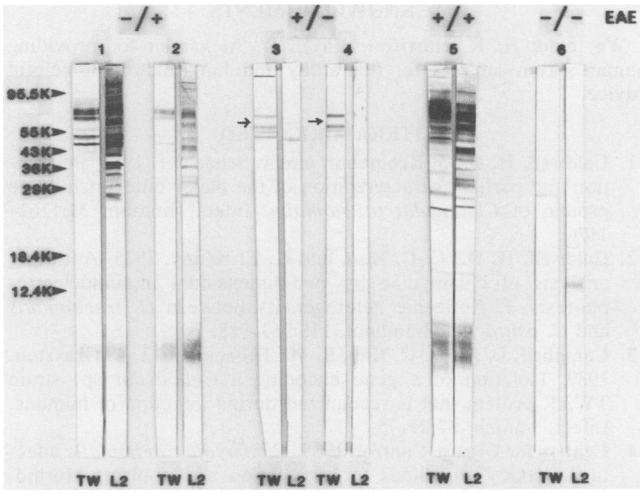

FIG. 2. Immunoblots of human serum samples tested by EAELISA (EAE) and reacted with L_2/434/Bu (L2) or TW-183 (TW) antigen. Lanes 1 to 6 show EAELISA results (TW-183 reactivity/L_2 reactivity is marked above the appropriate lanes) obtained with sera from patients. The arrows indicate the *C. pneumoniae*-specific bands.

in the former case were commonly observed at molecular masses of approximately 60 and 45 kDa. Protein bands that appeared to be *C. pneumoniae* antigen specific were observed at a molecular mass range of 50 to 60 kDa. No bands were observed on chlamydial antigen strips blotted against HeLa cell antigens; thus, the trace amounts of HeLa antigens which may contaminate chlamydial antigen grown in HeLa cells were not responsible for the reactivity we observed.

Correlation between EAELISA and MIF. Human serum samples were tested for chlamydial antibody by MIF. The correlation between antibody titers for MIF and EAELISA was high for both *C. trachomatis* and *C. pneumoniae* antigen reactivity (Fig. 3). With antibodies to *C. trachomatis*, results from assays of sera from at least three of the patients tested were represented in datum points that did not correlate well between MIF and EAELISA, i.e., MIF^+ $EAELISA^-$.

DISCUSSION

An ELISA in which the antigen is from a broadly reactive organism, such as *C. trachomatis* serotype L_2, has the potential of detecting antibodies to most serotypes of *C. trachomatis*. However, because of the presence of genus-specific antigens in chlamydial EBs, cross-reaction with antibodies to other *Chlamydia* species is not unexpected. This is particularly significant in view of recent reports of the high prevalence of antibodies to *C. pneumoniae* (5, 8, 9, 34). When assays that detect antibodies to genus-specific antigens are used (9), detection of *C. pneumoniae*-specific antibodies could lead to overestimation of the prevalence of *C. trachomatis* infections. Since chlamydial LPS is a major genus-specific antigen (6), it is logical to expect that removal of LPS from EBs will decrease cross-reactivity between *C. trachomatis* and *C. pneumoniae*. Chlamydial LPS can be extracted from EBs (24). Obviously, removal of LPS has to be done under mild conditions which minimize loss of species-specific antigens. Evans and Taylor-Robinson (7) and Schmeer and Krauss (28) used sodium deoxycholate to extract a genus-specific antigen from chlamydiae. Caldwell et al. (1), who studied the effects of different detergents on

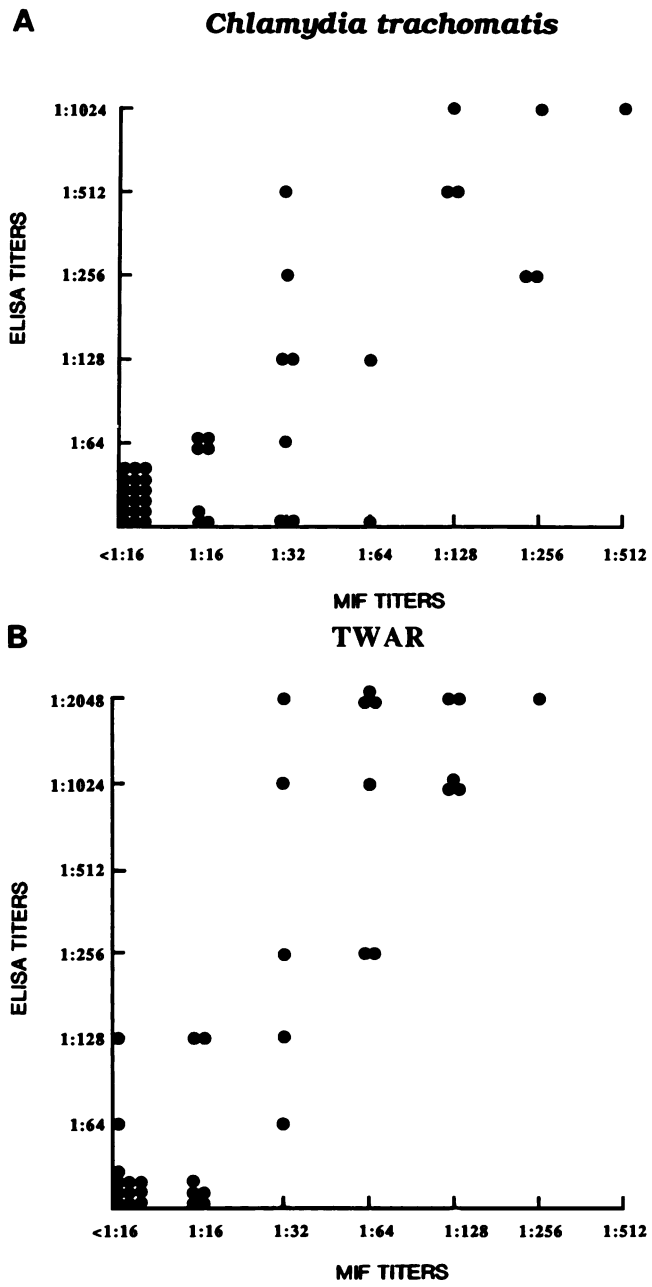

FIG. 3. Correlation between the EAELISA and MIF titers of IgG antibodies to *C. trachomatis* (A) and *C. pneumoniae* (TWAR) (B).

extraction of proteins from EBs, found that a solution of sodium deoxycholate and disodium EDTA extracted very little protein from EBs. Thus, we assumed that a sodium deoxycholate-disodium EDTA solution had the potential of removing genus-specific antigens from EBs without impairing species-specific antigenicity. Our data suggest that extraction on ELISA plates of LPS from our EB antigen preparations of either *C. trachomatis* L_2 or *C. pneumoniae* TW-183 with a solution of sodium deoxycholate and disodium EDTA effectively reduced cross-reactivity between the two species without decreasing reactivity with antibodies of the homologous species.

The specificity of EAELISA is indicated by the fact that

antibodies to *C. trachomatis* were not detected in the sera of 5- to 7-year-old children, although 27% of them were seropositive for *C. pneumoniae*. Of 23 pregnant women seropositive for *C. trachomatis* by MIF, 21 (91%) were positive by EAELISA. These figures could indicate that EAELISA is less sensitive than MIF; however, one of the two women who showed discrepant results had an MIF titer of only 1:32. Some of the results positive for *C. trachomatis* obtained by MIF might have been due to cross-reacting antibodies to *C. pneumoniae* (5, 27), especially since *C. pneumoniae* antigen was not included in the MIF during testing of these two women (14). In addition, discrepancies between the MIF and EAELISA in *C. trachomatis* titers may be due to MIF titers that are based on reactivity with the HI serotype pool, which does not cross-react with L_2 antigen (33) and thus would fail to react in the EAELISA. This finding applies to at least three of the patients we tested, who are represented in Fig. 3A by datum points that do not correlate well between the axes.

It is difficult to compare our EAELISA results with results of previous studies done by MIF, since prevalence of antibodies to *C. pneumoniae*, as well as that of antibodies to *C. trachomatis*, varies according to geographic areas, socioeconomic status, and other factors. Thus, the prevalence of IgG antibodies to *C. pneumoniae* in mixed populations in 14 countries in Africa, Asia, the Middle East, and Europe varied from 2.8% in Zimbabwe to 67% in Iran (8). We detected antibodies to *C. pneumoniae* in 78 to 80% of the three groups of adults tested; two of them were from Atlanta, Ga., and one was from Tucson, Ariz. A similar prevalence of *C. pneumoniae* antibodies (79%) was observed by Wang and Grayston (34) in aborigines in Taiwan. In agreement with previous reports (8, 34), we found a much lower prevalence of antibodies to *C. pneumoniae* in children than in adults. The rate of *C. trachomatis* seropositivity we observed in a group of blood donors in Atlanta (26%) was much higher than the 3% reported by Darougar et al. (5) for male blood donors in London, England. However, the blood donors in our study were young (20 to 35 years old), 80% were male, and all donated blood at a commercial rather than a volunteer blood bank—characteristics of a population with relatively high exposure to STD. In the current study, antibodies to *C. trachomatis* were detected in 50% of STD clinic patients, similar to the 53% prevalence found by Stamm et al. (30) in a Seattle STD clinic and higher than those detected by Forsey et al. (8) in a London genitourinary medicine department (26% in males and 40% in females).

The EAELISA is a practical and simple technique. Determination of endpoints is objective and does not require special expertise. The technique lends itself to automation and is thus suitable for central laboratories where large numbers of samples are tested. The technique is also useful for smaller laboratories with relatively modest equipment. A technician working manually can test 60 to 80 serum samples in a working day.

In conclusion, our data suggest that EAELISA distinguishes IgG antibodies reactive with *C. trachomatis* from those reactive with *C. pneumoniae* and may be a useful tool for detection of *C. trachomatis* and *C. pneumoniae* exposure. We have found preliminary evidence of *C. pneumoniae*-specific protein antigens that appear to be different from the *C. pneumoniae* protein antigen reported by Campbell et al. to react with serum against *C. psittaci* or *C. trachomatis* (3). Our findings warrant further study of these species-specific protein antigens for development of additional diagnostic tests and potential chlamydial vaccines.

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