Enzyme-Linked Immunosorbent Assay for Chlamydial Antibodies

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An enzyme-linked immunosorbent assay (ELISA) detected chlamydial antibodies in human sera. The assay antigen produced in cell cultures infected with *Chlamydia psittaci* was Formalin-fixed to microplates. Single convalescent-phase sera positive for chlamydial antibodies by a complement-fixation test were positive at even higher dilutions by ELISA. Paired sera with diagnostic rises in complement-fixing antibody showed seroconversion by ELISA also. Control sera from persons with no history of chlamydial infection were negative by both tests. Sera from patients with psittacosis or lymphogranuloma venereum were ELISA positive, indicating that the assay with the antigen used in this study is genus specific rather than species specific.

The complement fixation (CF) test is the most widely used serological technique for diagnosing chlamydial infections (18). The numerous alternative tests proposed, however, attest to the need for a practical and sensitive technique to circumvent problems caused by anti-complementary sera and the presence of chlamydial antibodies in many lots of supposedly normal guinea pig complement. Unfortunately, most of the proposed alternatives also have shortcomings. The agglutination test requires antigen in quantity and quality not readily available (24). A radioisotope precipitation technique (6) requires equipment unavailable to many diagnostic laboratories. The hemagglutination inhibition test for chlamydial antibody is plagued by nonspecificity (13) and, like the indirect hemagglutination test (1, 12), is not generally used. The indirect fluorescent-antibody (IFA) technique, although employed to great advantage by specialized laboratories (8, 10, 17, 24), requires highly skilled personnel and specialized immunofluorescence equipment. A fluorescent cell-counting neutralization test (7) appears to be complex, and other neutralization tests lack sensitivity (18).

Engvall and Perlmann (5) recently described a novel assay for detecting antibodies in which they allowed antigen to adsorb to sensitized polystyrene tubes and then added dilutions of test serum. After washing away those serum immunoglobulins that did not combine with the antigen, they added anti-immunoglobulin conjugated to an enzyme. They next washed away excess uncombined conjugate and added substrate for the enzyme. Color development from the enzymatic reaction indicated that antibody

was present in the serum.

Adaptation of this enzyme-linked immunosorbent assay (ELISA) to disposable microplates (23) makes it more practical for a diagnostic laboratory, and the highly sensitive yet simple assay is being used more often for serological diagnosis of bacterial (2, 9, 16), viral (21, 22), and parasitic (15, 19) infections. This report describes an ELISA technique for diagnosing chlamydial infections that avoids difficulties encountered with other serological procedures.

MATERIALS AND METHODS

Antigen production. Trypsinized McCoy cells were suspended at a concentration of 10^5 per ml in Eagle medium (4) containing 10% fetal bovine serum (GM) and 25 μ g of 5-iodo-2-deoxyuridine per ml, and 50 ml of the suspension was added to each of nine 32-ounce (ca. 96 ml) prescription bottles. After it was incubated at 36°C for 3 days, each culture was inoculated with 10^6 mouse intracerebral mean lethal doses (11) of a *Chlamydia psittaci* strain isolated from a parrot. Incubation was continued for 5 days in iodo-deoxyuridine-free GM. The cells were then frozen and thawed three times, and the cellular debris was pelleted from the medium by centrifugation at $800 \times g$ for 10 min and discarded. The antigen was collected from the supernatant fluid by centrifugation at $12,000 \times g$ for 90 min, suspended in 10 ml of 0.85% saline buffered at pH 7.2 with 0.01 M phosphate (PBS), and stored at -65°C. The optimal dilution of the final antigen suspension was 16 for the CF test (3).

Microplate sensitization. A 0.05-ml drop of antigen suspension was added to each well of a microplate and dried at 40°C. The microplates were then stored at -65°C in plastic bags. When they were used, the plates were removed from the bags and dried at 24°C for 15 min; then 0.05 ml of 10% Formalin was dropped into each well. The plates were kept at 4°C for 15

min and were then washed three times with PBS.

Sera. Sera tested had been sent to the Center for Disease Control (CDC), Atlanta, Ga., from persons throughout the United States with clinically diagnosed psittacosis, lymphogranuloma venereum, or infections unrelated to chlamydiae. The specimens included convalescent-phase sera from 13 persons with pneumonia caused by microbes other than chlamydiae.

ELISA reagents. Horseradish peroxidase type VI was conjugated to sheep anti-human immunoglobulin G as described by Nakane and Kawaoi (14). Sera known to contain chlamydial antibodies and sera without these antibodies were assayed by ELISA with conjugate dilutions in PBS containing 1% bovine albumin fraction V (PBS-alb) to determine the optimal dilution of conjugate. A dilution of 600 was chosen because it detected antibody as well as lower dilutions and did not react with normal sera. Undiluted conjugate was stored at -65°C in 0.1-ml aliquots.

To detect the peroxidase by color change, a solution containing H_2O_2 and 5-amino-2-hydroxybenzoic acid was prepared as described by Ruitenberg et al. (15).

Performance of ELISA. PBS-alb in 0.1-ml volumes was added to antigen-sensitized microplate wells, and the plates were placed on a test tube rack in a water bath at 37°C so that their bottom surfaces touched the water. After 30 min, the wells were washed three times with PBS, and 0.05 ml of serial twofold dilutions of serum in PBS, prepared in final volumes of 0.2 ml in test tubes with the aid of an automatic pipette (BB460422, Baltimore Biological Laboratory, Division of Bioquest, Cockeysville, Md.), were added beginning at a dilution of 8. Incubation at 37°C was repeated, and the plates were washed three times with distilled water. A 0.05-ml volume of distilled water containing 1% bovine serum albumin was then added to each well. The plates were incubated as before and again washed three times with distilled water, and 0.05 ml of a 600 dilution of conjugate was added to each well. Incubation and the three washings with distilled water were repeated a final time; 0.05 ml of the enzyme substrate solution was added to each well; and the plates were then placed at 24°C . Plates were examined 1 h later, and the greatest serum dilution which by that time had resulted in an unmistakable darkening of the substrate solution in a well was considered the end-point dilution. Positive reactions were unmistakable, and spectrophotometric measurements were unnecessary. However, we found that the absorbance levels of substrate recorded as positive invariably exceeded 0.21 when read at 450 nm in a spectrophotometer (Spectronic 20, Bausch & Lomb, Rochester, N.Y.).

CF test. Chlamydial genus-specific antigen for the CF test was supplied by the Biological Products Branch of the CDC. CF tests were done by the Laboratory Branch CF microtiter method (3).

RESULTS

Although microplates are usually sensitized for ELISA by passive adsorption of antigen, this method produced inconsistent results with our chlamydial antigen. We obtained reproducible sensitization when the antigen-coated wells were

treated with Formalin as described above.

To determine an optimal antigen dilution for sensitization with Formalin treatment, we sensitized microplates with various dilutions of antigen in PBS and tested positive sera. The serum end-point dilutions were decreased only when the antigen dilution exceeded 160. Antigen from a single McCoy cell monolayer at this optimal dilution sensitized approximately 3,000 wells.

The Formalin-treated antigen presented little risk of laboratory infection. Infectivity was not demonstrated when antigen was scraped from 10 wells into 0.5 ml of PBS immediately after the Formalin treatment and then inoculated intracerebrally into weanling mice. The diluted-antigen solution contained $10^{1.8}$ mouse intracerebral lethal doses per 0.03 ml (11) before it was added to the wells.

Single convalescent-phase sera from six persons with clinical lymphogranuloma venereum, 25 persons with clinical psittacosis, and 21 persons with no history of chlamydial infection were tested for chlamydial antibodies by ELISA and by CF tests. The laboratory worker testing sera by one method did not usually know the results by another method or the associated clinical histories. All sera positive by one method were positive also by the other (Table 1). End-point dilutions were always higher by ELISA than by the CF test. The geometric mean titer of the positive sera was 419 by ELISA and 94 by the CF test. Results by the ELISA technique were clear-cut inasmuch as sera either appeared completely nonreactive or showed definite positive end points (Fig. 1).

Acute- and convalescent-phase sera from 14 employees of a Nebraska turkey-processing plant who had clinically diagnosed psittacosis during an outbreak in 1976 (20) became available for testing. As shown in Table 2, diagnostic rises in antibody titer were demonstrated in all 14 cases, by both ELISA and the CF test. Rises in titer were always greater by ELISA.

Reproducibility of ELISA appeared acceptable. When two positive sera were assayed weekly for 6 weeks, end-point dilutions for the same serum differed only once, and then by a single twofold dilution.

Microplates used in ELISA studies are usually made of polystyrene, and we initially used microplates (type IS-MRC 96, Linbro Chemical Co., New Haven, Conn.) of this type. However, polyvinyl chloride, in contrast to polystyrene, is easily cut with scissors. If polyvinyl chloride microplates could be sensitized, the exact number of wells required at one time could be cut from stored plates and waste thus reduced. The same positive end-point dilutions were obtained in tests with three positive sera in parallel on

polystyrene and polyvinyl chloride ("U" Plate 220-24, Cooke Engineering Co., Alexandria, Va.) microplates.

Undiluted stock antigen suspension, sensitized microplates, and enzyme-conjugated immunoglobulin preparations did not noticeably deteriorate during storage at -65°C for more than a year. When samples of the same ELISA-

positive sera were tested with these materials at the beginning and end of this period, results were identical.

DISCUSSION

Laboratories now depend on the CF test or IFA technique for serological diagnosis of chlamydial infections. The results of this study indicate that ELISA offers advantages over both of these methods. It is more sensitive and rapid than the CF test. Simple to perform, it requires neither the highly trained personnel nor the specialized equipment demanded by the IFA technique.

Even laboratories that only occasionally test sera for chlamydial antibodies can easily keep the highly stable ELISA reagents on hand for immediate use. Excellent conjugates are available commercially, eliminating the necessity for laboratories of limited resources to prepare them. A single anti-immunoglobulin conjugate can be used to test human serum, not only for chlamydial antibodies, but also for antibodies to any other microbe for which a suitable sensitizing antigen is obtainable.

We elected to determine the ELISA test results by visual inspection of the microtiter plates, as is usually done in the CF test, and positive reactions were readily recognizable. Spectrophotometric measurements would, however, provide numerical definition of the product of the enzymatic reaction. The automation possible spectrophotometrically and, indeed, for the entire ELISA, could allow large numbers of sera to be tested with high efficiency. Even when all steps

TABLE 1. *Chlamydial antibody titer of convalescent-phase sera by ELISA and the CF test*

Case	Clinical diagnosis	Titer ^a	
		ELISA	CF test
1	Psittacosis	2,048	256
2	Psittacosis	2,048	64
3	Psittacosis	1,024	512
4-5	Psittacosis	1,024	256
6	Psittacosis	1,024	128
7-8	Psittacosis	512	128
9-11	Psittacosis	512	64
12-13	Psittacosis	256	128
14-17	Psittacosis	256	64
18-23	Psittacosis	128	64
24	Psittacosis	64	8
25	Psittacosis	16	8
26	Lymphogranuloma venereum	2,048	512
27-28	Lymphogranuloma venereum	2,048	128
29	Lymphogranuloma venereum	1,024	256
30-31	Lymphogranuloma venereum	1,024	128
32-65	Nonchlamydial infection	<8	<8

^a Titers are expressed as end-point dilution factors of sera dilutions.

FIG. 1. Typical ELISA test results. Twofold serial dilutions of sera were tested in duplicate beginning at a dilution of 8 in vertical row 1. Positive end-point dilutions were 64 for the serum of rows A and B, 256 for the serum of rows C and D, 16 for the serum of rows E and F (known positive control serum), and <8 for the serum of rows G and H (known negative control serum).

TABLE 2. *Chlamydial antibody titers of paired sera from psittacosis cases by ELISA and the CF test*

Case	Day after onset (serum collected)	Titer ^a	
		ELISA	CF test
1	1	<8	8
	22	512	64
2	1	<8	8
	26	128	32
3	2	<8	8
	20	512	64
4	2	<8	8
	24	1,024	128
5	2	<8	<8
	22	256	64
6	2	<8	8
	22	512	64
7	3	<8	8
	20	512	64
8	3	<8	<8
	21	512	256
9	3	<8	<8
	25	1,024	128
10	3	<8	<8
	25	512	128
11	4	<8	<8
	26	2,048	128
12	4	<8	8
	26	512	64
13	5	<8	8
	26	512	64
14	7	<8	8
	28	1,024	256

^a Titers are expressed as end-point dilution factors of sera dilutions.

were done manually, however, we consistently recorded test results 3 h after serum dilutions were prepared.

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