Detection of Antibodies to Legionnaires Disease Organism by Microagglutination and Micro-Enzyme-Linked Immunosorbent Assay Tests

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Microagglutination and micro-enzyme-linked immunosorbent assay (ELISA) tests with easily prepared, safe, heat-killed antigens for detecting antibodies to the legionnaires disease organism have been developed. A safranin-stained whole-cell antigen is used in the microagglutination test, and a simply prepared soluble antigen is used in the micro-ELISA tests. The microagglutination test detected elevated titers in 97.2% of the sera from patients with legionnaires disease. Three variations of the micro-ELISA test with anti-human immunoglobulin G, immunoglobulin M, and Fab peroxidase-labeled conjugates revealed elevated titers with 74.3, 82.9, and 88.6% of the sera, respectively. The microagglutination and the micro-ELISA tests used in combination detected 100% of the elevated titers.

American Legion members attending a convention in Philadelphia in July and August, 1976, were stricken with a pneumonia of unknown etiology. The death rate in this outbreak was 16% (2). It was not until early 1977 that an unidentified gram-negative bacterium presumed to be the etiological agent was isolated and partially characterized (6). Much remains to be learned about the causative agent of the Philadelphia outbreak, an organism which has now been implicated in several other outbreaks of pneumonic disease in the U.S.A. and abroad, as well as in a growing number of sporadic cases (2). To date, the only published method for serodiagnosis of the disease is a technically difficult indirect fluorescent-antibody procedure (6). The present study was undertaken in response to the urgent need for relatively simple and, above all, safe serological tests that could be used by any competent laboratory personnel to detect antibodies to the legionnaires disease organism. Because of intense interest in this disease and in the diagnostic procedures used, the procedures are described in detailed, step-wise fashion.

MATERIALS AND METHODS

Stock antigen preparations. Center for Disease Control (CDC) strain 1 of the legionnaires organism was grown for 72 h at 37°C in a candle jar on Mueller-Hinton agar (BBL) plates supplemented with 1% IsoVitaleX (BBL) and 1% hemoglobin. Each standard-size petri dish was harvested under a microbiological safety hood by adding 3 ml of sterile phosphate-buffered saline (PBS), pH 7.2. Growth was dislodged by gentle rubbing with a bent sterile capillary pipette.

The harvested growth was pooled in a flask and steamed in an Arnold sterilizer for 1 h at 101°C. The temperature was verified at 15-min intervals. An aliquot of the steamed preparation was streaked on petri dishes with the Mueller-Hinton agar medium described above and incubated to ensure that the organisms were nonviable.

The killed-cell suspension was centrifuged in Falcon (Becton, Dickinson, and Co.) plastic 15-ml graduated conical centrifuge tubes for 30 min at $1,600 \times g$ in a table-top International centrifuge. The supernatant fluid was removed, and 0.5 ml of packed cells was resuspended in 10 ml of PBS, pH 7.2. This preparation (designated first wash) was left in the refrigerator at 4°C for 10 days to allow for extraction of the soluble antigen from the cells and then was centrifuged at $1,600 \times g$. The supernatant fluid was decanted and designated the stock solution of soluble antigen. The total protein content of this stock solution was determined to be 0.155 $\mu\text{g}/\text{ml}$ by the method of Lowry et al. (5). The stock solution of soluble antigen has remained stable for more than a month at 4°C. The 0.5 ml of packed cells was resuspended in 10 ml of PBS, pH 7.2. This stock suspension was further diluted and used as the antigen in the microagglutination test.

Sera. A total of 304 sera were tested. Fifty-two sera from 35 patients with suspected legionnaires disease were kindly provided by the Bureau of Epidemiology, CDC. We obtained one acute- and one convalescent-phase serum specimen from 13 patients; from 2 patients we obtained one acute- and two convalescent-phase sera; and from each of 20 patients we obtained a single serum specimen. One hundred and thirty-five sera had been obtained from apparently healthy "normal" individuals for routine physicals, premarital serological tests, etc. Thirty-nine sera were from patients clinically diagnosed as having bacterial, parasitic, or viral diseases, with 6 of the 39 having high titers for *Brucella abortus*. Seventy-eight sera were from the

Department of Health, Education and Welfare (HEW) employees in a Federal office building where one case of legionnaires disease was confirmed. All sera were diluted 1:10 with sterile PBS, pH 7.2, and used in both of the following tests.

Microagglutination test. Our microagglutination test is an adaptation of a test for brucellosis described by Gaultney et al. (3). We use 0.025-ml volumes of serial serum dilutions and 0.025-ml volumes of antigen dilution. Plates are incubated overnight (16 h) at room temperature (22°C) and then refrigerated for 2 h at 4°C. The optimal antigen dilution is determined by block titration of various dilutions of the stock suspension of cells in PBS, pH 6.4, against a known positive serum. The optimal dilution of this particular lot of antigen proved to be 1:50 of the stock cell suspension. It was determined that PBS, pH 6.4, was the best diluent for the microagglutination test. A 1:50 dilution of the antigen suspension without safranin gave an optical density reading of 0.155 on the Coleman Junior spectrophotometer at 420 nm. We recommend that an antigen suspension giving an optical density of 0.155 to 0.160 be used for this test.

The working suspension of the stained antigen is prepared by adding 2 ml of the stock cell suspension to 98 ml of PBS, pH 6.4, containing 0.005% safranin (from a 2% stock solution in alcohol).

Rigid polystyrene U plates (Linbro Scientific Co.) are labeled so that each specimen to be tested is assigned a row of eight wells, leaving seven wells of one row per run for a positive reference (control) serum of known titer and the remaining well for a negative (control) serum. An antigen control consisting of PBS and antigen is not included because this may show a false agglutination pattern unless a trace of serum is present.

PBS, pH 6.4, diluent in 0.025-ml volumes is added to the second through eighth wells for each specimen and the second through seventh wells for the positive reference serum. Sera being tested are diluted 1:10, and 0.05-ml volumes of each are added to the first well of each row. Each row of serum is diluted by the microdilution technique with 0.025-ml microdiluters (Cooke Engineering Co.). This results in serum dilutions of 1:10 through 1:1,280. Safranin-stained antigen in 0.025-ml volumes is added to every well, and the plates are placed on a vertical vibrator (Arthur H. Thomas Co.) and mixed for 20 s. The plates are removed and covered with an empty plate or with plastic microtiter plate sealers, incubated at room temperature (22°C) overnight (16 h), and refrigerated for 2 h at 4°C to sharpen the end points. The plates are read with a test reading mirror (Cooke Engineering Co.) and a fluorescent lamp. A piece of thin white translucent paper, such as onionskin, is placed over the top of the plate to facilitate reading. A positive reaction consists of a matte of agglutinated cells with no definite button. A negative reaction consists of a definite button of stained cells. The end point is the highest total dilution of serum (after addition of antigen) resulting in a matte of cells with no button or with only a very slight trace of a button. Titers are recorded as the dilution factor of the total end-point dilution when the first well is considered as a 1:20 dilution.

Micro-ELISA immunoglobulin G (IgG) test. Our micro-ELISA test for legionnaires disease antibodies is a modification of the original enzyme-linked immunosorbent assay (1) adapted to the Microtiter (Cooke Engineering Co.) system (7). The test is carried out in Cooke micro-ELISA substrate (protein-binding polystyrene no. 1-223-29) plates and requires 0.1-ml volumes of antigen, serum, and peroxidase-labeled conjugate dilutions as well as 0.1 ml of substrate. The substrate used is *o*-phenylenediamine (OPD; Eastman Chemical Co.) in methanol with hydrogen peroxide (Mallinckrodt).

The optimal working dilution of antigen is determined by block titration of the stock solution of soluble antigen against a serum of known titer. The optimal dilution of antigen turned out to be 1:40 for the lot of soluble antigen used. A 1:40 working dilution of the soluble antigen is made in PBS, pH 7.2, rather than in carbonate buffer.

Diluted soluble antigen is dispensed in 0.1-ml volumes into every well of several Cooke micro-ELISA plates. Plates are sealed with Cooke microtiter plate sealers, incubated at 37°C for 3 h, and stored at 4°C until used. Plates may be stored for 3 weeks and possibly longer.

Excess antigen is removed from the wells by aspiration, and each well is washed three times with PBS, pH 7.2, containing 0.05% Tween 20. Each wash should last 4 min. If a large number of sera are to be tested, they may be screened at a 1:10 dilution, which requires only one well per specimen. The end point of sera positive at 1:10 may be determined by further dilution. Cooke micro-ELISA plates are labeled so that each specimen is assigned a row of eight wells, leaving one row per plate for a negative reference (control) serum. One row per run is also included for a positive reference (control) serum of known titer and one row for PBS control. PBS, pH 7.2, in 0.1-ml volumes is added to the second through the eighth wells for all sera with a 100- μ l MLA micropipette. One-tenth milliliter of each specimen (diluted 1:10) is added to the first and second wells of each row. Dilutions are made in the wells by mixing the contents of the second well eight times with a 100- μ l MLA micropipette and transferring 0.1 ml to the third well and repeating as above through the eighth well. This results in serum dilutions of 1:10 through 1:1,280. Plates are sealed and incubated at 37°C for 1 h. Serum dilutions are removed by aspiration, and each well is washed three times with PBS containing Tween 20 as before, after which 0.1 ml of appropriate conjugate dilution is added to every well, including all control wells. The appropriate conjugate dilution is determined by performing a block titration against known positive and known negative sera which conjugate diluted in PBS, pH 7.2, plus Tween 20. For initial studies, Miles Laboratories peroxidase-labeled anti-human IgG was used. After the conjugate is added, plates are sealed and incubated at 37°C for 1 h. Conjugate is removed by aspiration, and the plates are washed as before. One-tenth milliliter of working dilution of OPD is added to each well. The substrate is light sensitive and should be prepared just before it is used. Stock substrate contains: 1% (wt/vol) OPD in methanol (100 mg of OPD/10 ml of methanol). Working substrate contains: 1 ml of stock plus 99 ml of

distilled water; add 0.1 ml of 3% hydrogen peroxide. After the substrate is added, plates are sealed and incubated at 37°C in the dark for 1 h, after which plates are removed from the incubator; then 1 drop of 8 N sulfuric acid is added to each well to terminate the reaction and to intensify the color. Plates are placed on a white background and read by comparing the specimens against the negative control serum. The end point is the highest serum dilution showing a distinct color difference from the first dilution of the negative control. The titer is recorded as the dilution factor of the end-point serum dilution.

Micro-ELISA IgM and Fab tests. Additional studies were carried out with anti-human IgM and Fab peroxidase-labeled conjugates. The IgM conjugate was obtained from Cappel Laboratories, and the Fab conjugate was from Immunological Products Branch, Biological Products Division, CDC. Tests with these two conjugates were performed according to the micro-ELISA procedure for IgG conjugate. The sera were those from the 35 patients with suspected legionnaires disease.

RESULTS

A rise in titer between an acute-phase serum specimen and a convalescent-phase specimen is the ideal serological criterion for detecting an infection with the legionnaires disease organism. However, it is not always possible to obtain specimens during both the acute and convalescent stages, e.g., if the patient is not seen until the acute stage has passed. When paired specimens are not available, an "upper limit of normal" titer can be used as a base line for determining the significance of the titers of single specimens. The upper limit of normal value is usually defined as a titer that is not exceeded by 85% of an apparently healthy or normal population (4).

Microagglutination test. The serum titers obtained with the microagglutination test are listed in Table 1 according to groups of sera tested. The titers of sera from 135 apparently healthy normal adults, consisting of approxi-

mately equal numbers of black females, white females, black males, and white males, were determined with the microagglutination test. One hundred and thirty-four (99.3%) had titers of 40 or less, and 128 (94.8%) had titers of 20 or less. Thus the upper limit of normal titer for this group is actually 20; however, to allow for a possible equivocal area, a titer of ≥ 80 is considered to be elevated and indicative of infection.

Thirty-nine sera with elevated titers for various diseases such as bacterial, viral, and parasitic infections as well as rheumatoid arthritis and lupus erythematosus were tested with the microagglutination test to determine whether they would cross-react with the legionnaires antigen. Thirty-six (92.3%) of the sera gave titers of 20 or less, which is in the range of the sera from the normal group. Two sera, one from a patient with leprosy and the other a hyperimmune herpes-rubella serum, gave a titer of 40, which is within normal limits. One serum from a patient with trichinosis had a titer of 80, which is above the upper limits of normal, but we do not know whether the patient had had contact with the legionnaires organism. Sera from 78 HEW employees who worked in the same building with a person with a known case of legionnaires disease were tested to determine whether any of them had elevated titers. Seventy-six (97.4%) of these sera had titers of 20 or less, which is below the upper limits of normal. However, 55.1% of these sera had titers of 20 or 40, whereas only 37.7% of the sera from the normal group and 17.9% from the group with other diseases had titers of 20 or 40. We do not know whether these somewhat higher normal titers were due to exposure to the legionnaires organism.

Fifty-two sera from 35 patients with suspected legionnaires disease (about one-third of the sera being paired) were tested. Only one of the paired sera failed to show a rise in titer between the acute- and convalescent-phase specimens, and

TABLE 1. *Distribution of microagglutination test titers*

Titer	Normal		Other diseases		HEW employees		Legionnaires disease	
	No.	%	No.	%	No.	%	No.	%
<20	83	61.5	31	79.5	35	44.9		
20	45	33.3	5	12.8	41	52.6	1	2.9
40	6	4.4	2	5.1	2	2.5		
80	1	0.7	1	2.6			6	17.1
160							9	25.7
320							8	22.9
640							6	17.1
$\geq 1,280$							5	14.3
Total	135	99.9	39	100.0	78	100.0	35 ^a	100.0

^a 35 patients with 52 sera (see Materials and Methods).

the titer for this pair was 20 for each of the specimens. All of the single serum specimens and sera from 34 of the 35 (97.1%) patients with legionnaires disease had elevated titers (≥ 80).

Micro-ELISA. Table 2 shows the range of titers obtained with the micro-ELISA test for the four groups of sera. One hundred and thirty-one (97.0%) of the 135 sera from the normal group had titers of 10 or less. An elevated titer for the micro-ELISA test is considered to be ≥ 40 . Thirty-eight (97.4%) of the 39 sera from patients with other diseases had titers of less than 10, and the remaining specimen had a titer of 10. Seventy-seven (98.7%) of the 78 sera from HEW employees had titers of 10 or less. The remaining specimen had a titer of 40 with both the micro-ELISA and microagglutination tests. Twenty-six (74.3%) of the sera from the 35 patients with legionnaires disease showed elevated titers with the micro-ELISA test. Nine (25.7%) of the cases had micro-ELISA titers of 10 or less.

Comparison of tests. Table 3 shows the distribution of micro-ELISA titers obtained on

sera from 35 patients with legionnaires disease when three different peroxidase-labeled anti-human conjugates were used (IgG, IgM, and immunopurified Fab). Twenty-six (74.3%) of the 35 patients had elevated titers (>40) with the IgG conjugate, 29 (82.9%) with the IgM conjugate, and 31 (88.6%) with the Fab conjugate. The micro-ELISA test with the Fab conjugate detected the greatest number of elevated titers; it detected five (14.3%) cases that were missed by the IgG conjugate.

DISCUSSION

Results from our comparison of the microagglutination test and three variations of the micro-ELISA test indicate that the microagglutination test is the best of the four for detecting antibodies to the legionnaires disease organism for the limited number of sera tested. Not only did the microagglutination test detect more elevated titers than the micro-ELISA test, but it is a simpler test to perform and requires fewer man-hours per specimen tested. The micro-

TABLE 2. *Distribution of micro-ELISA test titers*

Titer	Normal		Other diseases		HEW employees		Legionnaires disease	
	No.	%	No.	%	No.	%	No.	%
<10	129	95.5	38	97.4	72	92.3	7	20.0
10	2	1.5	1	2.6	5	6.4	2	5.7
20								
40	4	3.0			1	1.3	4	11.4
80							3	8.6
160							8	22.8
320							5	14.3
640							3	8.6
$\geq 1,280$							3	8.6
Total	135	100.0	39	100.0	78	100.0	35 ^a	100.0

^a 35 patients with 52 sera (see Materials and Methods).

TABLE 3. *Distribution of titers of sera from 35 cases of legionnaires disease: comparison of IgG, IgM, and Fab micro-ELISA test results with results of microagglutination test*

Titer	ELISA						Microagglutination	
	IgG		IgM		Fab		No.	%
	No.	%	No.	%	No.	%		
<10	7	20.0			1	2.9		
10	2	5.7	1	2.9	1	2.9		
20			5	14.3	2	5.7	1	2.9
40	4	11.4	3	8.6	5	14.3		
80	3	8.6	4	11.4	3	8.6	6	17.1
160	8	22.8	10	28.6	6	17.1	9	25.7
320	5	14.3	5	14.3	4	11.4	8	22.9
640	3	8.6	2	5.7	6	17.1	6	17.1
$\geq 1,280$	3	8.6	5	14.3	7	20.0	5	14.3
Total	35	100.0	35	100.1	35	100.0	35	100.0

ELISA test with Fab conjugate proved to be the most sensitive of the three micro-ELISA tests compared; it detected 14.3% more elevated titers than were detected with the IgG conjugate and 5.7% more than with the IgM conjugate. Therefore, it is the test of choice if the micro-ELISA test is to be used to detect legionnaires disease antibodies. The combined use of both the microagglutination and the micro-ELISA tests appears to be better than the use of either test alone. With combination of the two tests, elevated titers were detected in all 35 (100%) patients, whereas with the microagglutination test alone, elevated titers were detected in 34 (97.2%) of the 35 patients.

As with nearly all infectious diseases, a rise in titer between an acute- and a convalescent-stage specimen is the most convincing serological evidence of infection with the legionnaires organism. However, in the absence of paired specimens, a single serum specimen can be of value if an upper limit of normal base line is established for apparently healthy subjects. The upper limit of normal value should be established by each laboratory performing serological tests for legionnaires disease because it may vary geographically or otherwise, although these parameters have not yet been adequately studied. The interpretation of the serological results is dependent upon the clinical findings.

One of the most important features of the tests we have described is the preparation of the antigen used in the tests. We use legionnaires disease organisms grown on modified Mueller-Hinton agar plates instead of infected yolk sacs (6). We kill the organism by steaming it at 101°C for 1 h rather than by using Formalin, which in CDC experience has not always been effective in killing the legionnaires organism. A brief study conducted in this laboratory has indicated that agglutinating antigen containing organisms killed by 101°C heat was as sensitive as antigen killed by 0.5% phenol, 0.3% Formalin, or 56°C heat in detecting antibody by agglutination tests (J. C. Feeley, unpublished data). The soluble antigen used in the micro-ELISA test is merely the supernatant of a suspension of the whole

cells; therefore it is easy to prepare and does not require apparatus for disrupting the cells. This simple and safe antigen preparation can be easily prepared for use in the microagglutination and/or micro-ELISA test by any competent laboratory personnel.

It should be emphasized that further study of agglutination and ELISA procedures may result in improvements. The many experimental variables that can be manipulated in such serological test systems need to be studied more extensively. However, the need for more widespread availability of serological test procedures for the legionnaires disease organism dictates that these methods be described at this time while further studies are being conducted.

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