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Serology of Legionnaires Disease: Comparison of Indirect Fluorescent Antibody, Immune Adherence Hemagglutination, and Indirect Hemagglutination Tests

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An immune adherence hemagglutination (IAHA) test for the measurement of antibodies to *Legionella pneumophila* was developed and evaluated for the diagnosis of Legionnaires disease. Its sensitivity was compared to that of the indirect fluorescent antibody (IFA) test and a recently developed indirect hemagglutination (IHA) test. The sensitivity of the three tests appeared to be similar, with the IFA test giving slightly higher titers. Both the IHA and IAHA tests appear useful for the serodiagnosis of Legionnaires disease; the IAHA test has the advantage that it can be used with many other serological antigens.

Infection with *Legionella pneumophila* may result in a disease characterized by fever, diarrhea, and confusion, followed by pneumonia (11). Known as Legionnaires disease, after the 1976 outbreak among those attending an American Legion convention in Philadelphia, Pa., the illness occurs both in outbreaks and as sporadic cases (2). The sporadic cases are not readily recognized, as they resemble and have been misdiagnosed as other respiratory infections, including psittacosis, influenza, mycoplasmal pneumonia, and Q-fever (11). This problem in diagnosis arises partly from the difficulty in culturing *L. pneumophila*, so that few cases are confirmed by recovery of the organism. Although there is interest in the direct detection of the organism in clinical specimens by means of immunofluorescence (3) and other techniques, it seems likely that the principal means of laboratory diagnosis will continue to be serology. Since the indirect fluorescent-antibody (IFA) test currently used (13) is both technically difficult and prone to some error, there is a need for a more convenient serological test of good sensitivity and specificity. The recent development of a serological test based on microagglutination appears to be a first step in this direction, as the method is technically simple, and the antigen required is easily prepared (6). We recently extended earlier work (8, 9) on immune adherence hemagglutination (IAHA) to develop a serological test for *L. pneumophila* and then compared it to the widely used IFA test and to a recently developed indirect hemagglutination (IHA) test (4).

MATERIALS AND METHODS

Study design. Since the significance of single serum titers of antibody to *L. pneumophila* is uncertain, the sensitivity of the IAHA test was evaluated by examining paired sera for "significant" (defined as fourfold or greater) rises in antibody titer. The IFA test was selected as the reference test, as it is the only test that is in widespread use for serology of *L. pneumophila*. To obtain enough paired sera to evaluate the IAHA test, panels of well-tested paired sera were solicited from several state reference laboratories which were running the IFA test on a routine basis. The sera were tested at each originating laboratory as "panels" to minimize variations. The sera were shipped and stored frozen and tested under code. The IAHA tests were run in two laboratories, and all sera were tested at least twice in each laboratory. Some serum samples were retested by IFA or IHA techniques after completion of the IAHA tests.

Sera. Human sera, previously tested for antibodies to *L. pneumophila* by IFA test, were obtained from the Allentown-Sacred Heart Hospital, Allentown, Pa., the New Jersey Department of Public Health, Trenton, N.J., and the Michigan Department of Public Health, Lansing, Mich. The paired sera available were all from patients who had been described as having had an illness compatible with Legionnaires disease. Additional paired sera, previously tested for antibodies to other agents, were obtained from the diagnostic virology laboratory at Hahnemann Hospital, Philadelphia, Pa. Detailed clinical information about patients whose sera were studied was not available.

Antigens. Antigen for the IAHA tests was prepared as described for the microagglutination tests (6), by using the Philadelphia 1 strain of *L. pneumophila*. Later lots of antigen prepared from bacteria grown on FG agar (7) were not aged at 4°C, but were made up as 5% suspensions in phosphate-buffered saline (pH 7.4) and stored at −80 or −20°C.

L. pneumophila. The Philadelphia 1 strain of *L. pneumophila* was obtained from R. Weaver, Special Bacteriology Branch, Bureau of Laboratories, Center for Disease Control, Atlanta, Ga. The organism was maintained by passage at 6-week intervals on FG agar slants.

IAHA test. The IAHA test procedure was performed as described previously (9). Briefly, diluted sera (1:4) were inactivated at 56°C, then serially diluted (in duplicate) on V-bottom microtest plates; antigen was added to one set of dilutions, and diluent was added to the other set, which served as serum control dilutions. The titer of the antigen was previously determined by block titration. After incubation at 37°C for 1 h, diluted guinea pig complement was added to the test plates, which were incubated 40 min further at 37°C. The reaction was stopped by the addition of ethylenediaminetetraacetate and dithiothreitol, and then a 0.8% suspension of type O human erythrocytes was added to each well of the test, and the plates were examined after the cells settled, usually about 1 h at room temperature. Positive reactions were indicated by agglutination produced by immune complexes cross-linking the erythrocytes through attachment to their C3b receptors. Serum titer endpoints were read as the last serum dilution producing complete agglutination with the test antigen, in the absence of agglutination in the serum control dilution.

IFA test. The IFA tests were carried out in the different laboratories providing sera for this study, by methods described by Wilkinson et al. (13). The IFA titers of a few sera were confirmed from tests performed at the Center for Disease Control, Atlanta, Ga.

IHA test. The hemagglutination technique used was similar to the microtiter method for detection of treponemal antibodies described by Wentworth et al. (12). Antigen was supplied by Difco Laboratories, Detroit, Mich., as turkey erythrocytes sensitized by the bis-diazotized benzidine technique with a mixture of three agar-grown strains of *L. pneumophila* (Flint 1 and 2 and Detroit 1 isolates). Sera were heated at 56°C for 30 min before testing and then examined at an initial dilution of 1:16. Each serum was tested against an unsensitized cell control as well as against antigen-sensitized cells. Sera that agglutinated sensitized cells on initial screening were titrated in twofold dilutions to an endpoint titer. Reactive and nonreactive control sera were tested in parallel with all test sera. All IHA tests were performed at the Michigan Department of Public Health, Lansing, Mich., simultaneously with the IFA tests.

RESULTS

Antigen reactivity. Initially, antigen for the IAHA test was prepared as described by Farshy et al. for the microagglutination test (6) (supplied by M. V. French) and found to be satisfactory at dilutions of 1:50 or 1:100 by block titration. Later antigen preparations differed only in that the organism was grown on FG agar rather than the original MHIH agar (7) and that the antigen was not stored at 4°C for 10 days before use, but was immediately stored at −80°C. Sonication of some lots of antigen led to smoother (less granular) hemagglutination patterns, but no differences in serum titers were seen between untreated and sonicated antigen. One lot of antigen prepared from the Philadelphia 2 strain of *L. pneumophila* (supplied by G. Lattimer) was tested and found to give the same results as other antigen lots.

Test sensitivity. The criterion of a fourfold or greater increase in antibody titer was selected to evaluate the sensitivity of the various tests in detecting infection by *L. pneumophila*. The paired acute and convalescent sera had previously been tested by the IFA method at the Michigan and New Jersey State Laboratories; some of these sera had also been tested by the IHA procedure (Michigan panel). As shown in Table 1, all but one of the 26 sets of sera that were positive by the IFA test were also positive by the IAHA test. The single exception was a pair of sera (MI-1 in Table 1) that was positive by IFA test, but negative in both the IAHA and IHA tests. An additional 10 pairs of sera from the Michigan panel (not shown in Table 1), which were negative for antibody to *L. pneumophila* by the IFA test, were tested by the IAHA procedure and were all negative by that test also.

Test specificity. Previous reports (10, 11) had indicated that the IFA test for *L. pneumophila* antibodies may exhibit some non-specificity; sera from patients known to have been infected with agents other than *L. pneumophila* have shown diagnostic increases in antibody titer with the IFA test. Therefore, the IAHA test was examined for evidence of any obvious non-specificity. Over 200 sera were selected from those submitted for serological testing unrelated to respiratory illness and Legionnaires disease. All of these sera were negative for antibodies to *L. pneumophila* by IAHA test. Tested next were a small number of paired sera from patients previously found to have been infected with agents other than *L. pneumophila*. The respiratory infections included psittacosis, influenza (type A), *Mycoplasma pneumoniae*, respiratory syncytial virus, and cytomegalovirus. None of the paired sera for these infections, nor for toxoplasmosis or infectious mononucleosis, were positive for antibodies to *L. pneumophila* by the IAHA test. The latter two diseases were included because a small number of sera were available from patients with toxoplasmosis or infectious mononucleosis that had previously

TABLE 1. *Comparison of serological tests on paired sera*[a]

Serum pair number	Days between paired sera	*L. pneumophila* titer			Serum pair number	Days between paired sera	*L. pneumophila* titer		
		IAHA	IFA	IHA			IAHA	IFA	IHA
MI-01	23	Neg.	60	16	MI-14	25	256	64	512
		Neg.	512	16			2,048	8,192	4,096
MI-02	24	Neg.	Neg.	Neg.	MI-15	8	512	256	256
		128	512	512			2,048	1,024	256
MI-03	14	Neg.	Neg.	Neg.	MI-16	19	Neg.	Neg.	Neg.
		16	128	64			128	128	128
MI-04	13	Neg.	Neg.	NS	MI-17	12	Neg.	Neg.	Neg.
		128	128	NS			1,024	4,096	2,048
MI-05	6	Neg.	Neg.	Neg.	MI-18	13	Neg.	Neg.	Neg.
		128	256	128			64	128	256
MI-06	11	Neg.	Neg.	Neg.	MI-19	40	Neg.	Neg.	ND
		128	256	128			256	128	ND
MI-07	26	Neg.	16	64	MI-20	14	Neg.	Neg.	Neg.
		32	256	256			512	2,048	2048
MI-08	?	Neg.	Neg.	Neg.	NJ-01	19	Neg.	Neg.	ND
		32	128	64			40	512	ND
MI-09	25	Neg.	Neg.	Neg.	NJ-02	16	10	Neg.	ND
		128	256	256			80	2,048	ND
MI-10	64	Neg.	Neg.	Neg.	NJ-03	10	40	Neg.	ND
		256	256	128			640	1,024	ND
MI-11	38	Neg.	Neg.	Neg.	NJ-04	13	Neg.	Neg.	ND
		128	256	256			40	1,024	ND
MI-12	14	32	16	128	NJ-05	91	Neg.	Neg.	ND
		128	128	512			640	4,096	ND
MI-13	20	Neg.	Neg.	Neg.	NJ-06	?	10	128	ND
		256	256	128			40	1,024	ND

[a] Antibody titers to *L. pneumophila* in 26 pairs of sera were measured by IAHA, IFA, and IHA and compared. NS, Test results were non-specifically reactive; ND, test was not done. A titer of <1:8 is considered negative. Neg., Negative.

been found to be reactive in the IFA test at titers as high as 1,024 (G. Lattimer, unpublished data). These limited data indicated no obvious problem of non-specificity with the IAHA test, but do not permit an evaluation of the relative specificities of the various tests for *L. pneumophila* antibodies.

DISCUSSION

Symptoms of Legionnaires disease are similar to those of many other respiratory infections with agents that are difficult to cultivate; thus there is a need for laboratory tests to distinguish infection with *L. pneumophila* from other similar infections. Cultivation of *L. pneumophila* is difficult and slow, as it will not grow on ordinary bacteriological media. Although improved media for the recovery of this organism from clinical specimens are being developed (7), its recovery on artificial media has been infrequent to the present (2). An alternative approach is the use of direct immunofluorescence to detect *L. pneumophila* in tissue or sputum (1, 3). Although this method is rapid, it is unlikely to be used routinely, and suitable specimens may not be available from most patients. Furthermore, when applied to sputum this approach may not have adequate sensitivity. In one study (1), only 6 of 63 (9%) of the sputum smears, or 24% of patients shown to have Legionnaires disease by other

criteria, were positive by this test. The test was, however, specific (1). Most infections with *L. pneumophila* have been diagnosed by serology (2), and it is likely that this will remain the principal means of laboratory diagnosis due to its effectiveness and availability. The presently used IFA test, however, is difficult to perform and is poorly suited to the examination of large numbers of sera. Also, it may give false-positive results; on the basis of fourfold or greater increases in titer found by the IFA test, patients with plague, tularemia, and leptospirosis have been misdiagnosed as having Legionnaires disease (11). Sera from patients with toxoplasmosis and infectious mononucleosis have also been found to give positive results with the IFA test (G. Lattimer, unpublished data). In addition, serological cross-reactions have been found between *Chlamydia psittaci* and *L. pneumophila* with human sera in the IFA test, but were not seen in a complement fixation test (10). The basis for these cross-reactions has not yet been explained.

Since the IAHA test has been shown to be sensitive and specific with a number of other antigens (9), it seemed likely to be suitable for the serodiagnosis of *L. pneumophila* infections. We evaluated the IAHA test primarily with paired sera which had previously been tested for *L. pneumophila* antibodies by the IFA test. Our results showed excellent agreement between the IAHA and IFA methods. Of 26 pairs of sera that were positive and 10 that were negative by IFA test, only one disagreement was observed with the IAHA test (MI-1). Antibody titers were also measured by IHA for 20 of these pairs; the IAHA and IHA tests were positive for 19 of these 20 pairs and were negative for the serum pair MI-1 which was positive only by IFA test. The reason for the discrepancy between the IFA and other tests for this pair is unknown.

Paired sera from a number of patients with various infections other than *L. pneumophila* were tested by the IAHA method with *L. pneumophila* antigen to see whether the IAHA test was likely to give false-positive results (fourfold or greater increase in titer with paired sera). The results showed that none of the tested sera reacted with *L. pneumophila* antigen.

Apart from the IAHA test, two other alternatives to the IFA test have recently been used to detect antibodies to *L. pneumophila*, the microagglutination and IHA tests. The latter, when compared to the IAHA test, showed equal sensitivity and specificity in the detection of antibodies to *L. pneumophila*. However, the IHA test may be somewhat less versatile, as the antigen-sensitized cells must be specially prepared. The microagglutination test is the simplest to perform, but we do not have data to compare it to the other tests. Also, the microagglutination test has been reported (5) to depend upon the presence of immunoglobulin M antibodies. Both the IHA and IAHA tests appear to be satisfactory alternatives to the IFA test for Legionnaires disease. In addition, the IAHA test is widely applicable to the serological diagnosis of other infections. This single procedure can be used to differentiate Legionnaires disease, mycoplasmal pneumonia, influenza, and other viral respiratory diseases. Both the IAHA and IHA procedures are well suited to the testing of large numbers of sera and are easily performed.

LITERATURE CITED

1. **Broome, C. V., W. B. Cherry, W. C. Winn, and B. R. MacPerson.** 1979. Rapid diagnosis of Legionnaires' Disease by direct immunofluorescent staining. Ann. Intern. Med. **90**:1-4.
2. **Center for Disease Control.** 1978. Legionnaires' disease: United States. Morbid. Mortal. Weekly Rep. **27**:439-441.
3. **Cherry, W. B., B. Pittman, P. P. Harris, G. A. Hebert, B. M. Thomason, L. Thacker, and R. E. Weaver.** 1978. Detection of Legionnaires disease bacteria by direct immunofluorescent staining. J. Clin. Microbiol. **8**:329-338.
4. **Edson, D. C., H. E. Stiefel, B. B. Wentworth, and D. L. Wilson.** 1979. Prevalence of antibody to Legionnaires' Disease. Ann. Intern. Med. **90**:691-693.
5. **Farshy, C. E., D. D. Cruse, G. C. Klein, H. W. Wilkinson, and J. C. Feeley.** 1979. Immunoglobulin specificity of the microagglutination test for the Legionnaires' Disease bacterium. Ann. Intern. Med. **90**:690.
6. **Farshy, C. E., G. C. Klein, and J. C. Feeley.** 1978. Detection of antibodies to Legionnaires disease organism by microagglutination and micro-enzyme-linked immunosorbent assay tests. J. Clin. Microbiol. **7**:327-331.
7. **Feeley, J. C., G. W. Gorman, R. E. Weaver, D. C. Mackel, and H. W. Smith.** 1978. Primary isolation media for Legionnaires disease bacterium. J. Clin. Microbiol. **8**:320-325.
8. **Lennette, E. T., G. Henle, W. Henle, and C. A. Horwitz.** 1978. Heterophil antigen in bovine sera detectable by immune adherence hemagglutination with infectious mononucleosis sera. Infect. Immun. **19**:923-927.
9. **Lennette, E. T., and D. A. Lennette.** 1978. Immune adherence hemagglutination: alternative to complement-fixation serology. J. Clin. Microbiol. **7**:282-285.
10. **Ormsbee, R. A., M. G. Peacock, G. L. Lattimer, L. A. Page, and P. Fiset.** 1978. Legionnaires' Disease: antigenic peculiarities, strain differences, and antibiotic sensitivities of the agent. J. Infect. Dis. **138**:260-264.
11. **Tsai, T. F., and D. W. Fraser.** 1978. The diagnosis of Legionnaires' Disease. Ann. Intern. Med. **89**:413-414.
12. **Wentworth, B. B., M. A. Thompson, C. R. Peter, R. E. Bowdon, and D. L. Wilson.** 1978. Comparison of a hemagglutination treponemal test for syphilis (HATTS) with other serologic methods for the diagnosis for syphilis. Sex. Transm. Dis. **5**:103-111.
13. **Wilkinson, H. W., B. J. Fikes, and D. D. Cruce.** 1979. Indirect immunofluorescence test for serodiagnosis of Legionnaires disease: evidence for serogroup diversity of Legionnaires disease bacterial antigens and for multiple specificity of human antibodies. J. Clin. Microbiol. **9**:379-383.