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Evaluation of an Indirect Hemagglutination Test for *Legionella pneumophila* Serogroups 1 to 4

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Parallel testing of 895 sera by indirect hemagglutination and indirect fluorescent-antibody techniques showed 97.3% agreement. Although the indirect hemagglutination technique usually showed more cross-reactivity among serogroups than the indirect fluorescent-antibody technique with Formalin-fixed antigens and a conjugate which detected primarily immunoglobulin G antibodies, heterologous serogroup reactions were significantly lower than homologous serogroup titers and the etiological serogroup could be easily defined. The indirect hemagglutination technique showed no cross-reactivity with a crude extract of *Escherichia coli* O13:K92:H4. Since the indirect hemagglutination technique was shown to detect both immunoglobulin M and immunoglobulin G antibodies and was found to be rapid, simple, and inexpensive, it appears to be an excellent alternative to the indirect fluorescent-antibody test for serodiagnosis of legionellosis.

The indirect fluorescent-antibody (IFA) test for legionellosis (10, 15) has become an important diagnostic tool in the 3 years since its introduction. Performing the test on a routine basis, however, is becoming an increasingly complex task; with the recent addition of the agents TATLOCK (13), WIGA (1), and NY 23 (4) to the genus *Legionella* (proposed designations *L. micdadei* [9], *L. bozemanii*, and *L. dumoffii* [2], respectively), proper serodiagnosis of Legionnaires disease involves the use of nine separate antigens. The use of polyvalent antigens (7, 15) can simplify testing somewhat, but even this does not discount some inherent disadvantages of the technique, i.e., it is technically difficult and expensive to perform. As a practical alternative, Edson and associates (5) have introduced an indirect hemagglutination (IHA) technique for legionellosis. The test is rapid, inexpensive, and simple to perform and shows a 94.9% agreement with IFA serogroup 1 results. The IHA test has recently been expanded to include *L. pneumophila* serogroups 2, 3, and 4, and this study was made to evaluate the sensitivity and specificity of the method.

MATERIALS AND METHODS

Sera for parallel testing of IHA and IFA techniques. Sera were received at the Michigan Department of Public Health (Lansing, Mich.) for routine IFA legionellosis testing between March and October 1979. All sera received during this period were included in the study except for those in which the quantity of serum was insufficient for testing by both techniques. The sample tested included a total of 895 sera. There were single serum specimens from 483 patients and paired or serial sera from 188 patients.

Special sera selected for comparison of IHA and IFA techniques. In addition to the above-mentioned, unselected sera, sera from 55 patients with IFA-confirmed (fourfold or greater rise in titer) or presumptive (titer of ≥1:128) legionellosis were selected for comparative studies. Single serum specimens were available from 8 patients, and paired or serial sera were available from 47 patients. These cases represent 41% of the 134 cases of legionellosis diagnosed by serology between October 1977 and August 1980. The only additional criterion for inclusion in the study was sufficient serum volume for testing.

Sera for cross absorption and blocking fluid studies. The 13 sera with serogroup 1 titers used for these studies (see Table 6) were from IFA-confirmed or presumptive cases. Of the 12 sera with *Escherichia coli* titers, 4 were from Legionnaires disease patients, and 8 were from patients without legionellosis.

Sera for sucrose density gradient fractionation. Twelve sera selected from the 55 patients described above were used. An additional three sera which showed fourfold or greater rises or titers of ≥1:128 by IHA and were IFA negative were fractionated for analysis.

Bacterial strains. Bacterial strains were grown in Roux bottles on enriched chocolate agar (GC medium base with heated sheep blood and vitamin supplements). The *L. pneumophila* Flint 1, Flint 2, and Detroit 1 strains were maintained as stock cultures at the Michigan Department of Public Health. The *L.*

pneumophila Knoxville 1, Togus 1, Bloomington 2, and Los Angeles 1 strains and *E. coli* O13:K92:H4 were obtained from the Centers for Disease Control (Atlanta, Ga.).

IFA antigen preparation. Bacterial growth was washed from the surface of the Roux bottle after 72 h of incubation with 0.4% formalinized phosphate-buffered saline, filtered through a fine-mesh wire screen to remove agar debris, and then refrigerated at 4°C for 1 week. The suspension was then washed three times in formalinized phosphate-buffered saline, and the opacity was standardized to 300 international opacity units per ml by comparison with World Health Organization opacity standards. Suspensions of formalinized whole organisms, adjusted to 2 international opacity units per ml, served as the antigen.

IHA antigen preparation. All IHA antigens were supplied as experimental antigens by Difco Laboratories (Detroit, Mich.). The method of preparation was similar to that of Edson et al. (5) for other IHA antigens. The antigens consisted of turkey erythrocytes stabilized by glutaraldehyde treatment and sensitized with boiled, sonicated antigen from agar-grown isolates by the bis-diazotized benzidine technique. Antigens were prepared from the following strains: serogroup 1 antigen as a mixture of Flint 1, Flint 2, and Detroit 1; serogroup 2 from Togus 1; serogroup 3 from Bloomington 2; and serogroup 4 from Los Angeles 1. The polyvalent antigen was made from a mixture of Knoxville 1, Togus 1, Bloomington 2, and Los Angeles 1.

IFA technique. A standard microtiter technique with 0.005-ml amounts of antigen on 12-well, acetone-resistant glass microscope slides was employed. Conjugates were diluted in phosphate-buffered saline with 2% Tween 80. Rabbit anti-human globulin conjugate was purchased from Beckman Instruments, Inc. (Fullerton, Calif.). Anti-rabbit globulin conjugate (Cappel Laboratories, Downington, Pa.) was used to determine titers on hyperimmune rabbit antisera. Anti-human immunoglobulin M (IgM) conjugate was supplied by Burroughs Wellcome Co. (Research Triangle Park, N.C.).

IHA technique. The IHA technique was similar to that of Edson et al. (5). Unless otherwise noted, sera were screened at an initial 1:16 dilution against polyvalent sensitized cells and unsensitized control cells. Any serum showing a reaction with the test cells but not with the control cells was subsequently screened with the four monovalent antigens (serogroups 1 to 4) and corresponding control cells. Sera were titrated to endpoint in serial, twofold dilutions with appropriate monovalent antigens.

Any serum showing agglutination of both antigen-sensitized and unsensitized control cells was treated in the following manner. A 0.02-ml amount of packed, unsensitized (control) cells was added to 0.12 ml of serum. After overnight incubation at 4°C, the cell suspension was centrifuged at 3,000 rpm for 15 min, the absorbed serum was removed, and the IHA test was repeated. Sera with high titers of anti-cell antibody required two or three successive absorptions for complete removal of these agglutinins.

Cross absorption. For absorption, 0.1 ml of serum was added to approximately 3.7×10^{10} organisms which had been washed three times in phosphate-buffered saline to remove Formalin. The absorption mixture was incubated overnight at 4°C and centrifuged at 3,000 rpm for 15 min, and the absorbed serum was removed.

Preparation of hyperimmune sera. Rabbits weighing >2 kg each were immunized with an initial intramuscular injection of 1 ml of antigen mixed with an equal amount of Freund complete adjuvant, followed by five intravenous boosters of 1 ml of antigen at days 22, 24, 26, 28, and 30. Rabbits were exsanguinated 9 days after the last booster.

Comparison of immune sera prepared against IFA and IHA antigens. Rabbits were divided into four groups of two rabbits each and immunized as described above. Groups 1 and 3 received injections of formalinized IFA antigens at 16 international opacity units per ml. Groups 2 and 4 received injections of IHA antigen (boiled and sonicated) at 16 international opacity units per ml. Groups 1 and 2 were inoculated with the Flint 1 strain of *L. pneumophila*. Groups 3 and 4 were immunized with the Detroit 1 strain of serogroup 1. Sera from two rabbits in each immunization group were pooled, and IFA and IHA titers were then determined on each of the pools.

Blocking fluid preparation. Blocking fluids of the Flint 1 strain and *E. coli* O13:K92:H4 were prepared in the manner described by Wilkinson et al. (14). Sera were initially diluted in IHA diluent or phosphate-buffered saline as a control and in both blocking fluids. Subsequent dilutions were made in diluent or phosphate-buffered saline, and IHA or IFA titers were determined.

Sucrose density gradients. Sera were fractionated on 10 to 50% sucrose density gradients (12) by centrifugation at 33,500 rpm for 17 h in a Spinco ultracentrifuge with an SW50.1 rotor. Ten 0.5-ml fractions were collected for analysis.

Immunodiffusion. The presence of IgM or IgG or both in each of the samples was confirmed with radial immunodiffusion kits (Meloy Laboratories, Springfield, Va.). These kits employ the Fahey and McKelvey (6) technique to measure immunoglobulins.

RESULTS

Parallel testing of 895 sera by the IHA and IFA techniques. Results of parallel testing of 895 sera by the IHA and IFA techniques are shown in Table 1. There were 796 nonreactive

TABLE 1. *Results of parallel testing by the IHA and IFA techniques on 895 sera*

Results of testing	No. of sera in serogroup:			
	1	2	3	4
IHA−[a] and IFA−	796	879	885	858
IHA+[b] and IFA+	46	1	5	15
IHA− and IFA+	14	0	2	2
IHA+ and IFA−	39	15	3	20

[a] −, Titer of <1:16.
[b] +, Titer of ≥1:16.

sera and 46 sera which reacted in both techniques in serogroup 1. There were 14 sera reactive only in the IFA technique which were nonreactive in the IHA technique at a 1:16 dilution. There were 39 sera reactive only in the IHA technique that failed to react in the IFA technique at a 1:16 dilution. Discrepancies between IFA and IHA reactivities with serogroups 2, 3, and 4 were due largely to the cross-reactive nature of the serogroup 1 antibody and not to sera with specific serogroup 2, 3, or 4 antibody. Agreement (percentage of sera positive by both techniques plus percentage of sera negative by both techniques) was 94.1, 98.3, 99.4, and 97.5% for serogroups 1, 2, 3, and 4, respectively. There was an overall agreement for serogroups 1 to 4 of 97.3%.

Among the 188 paired or serial sera, there were nine instances of fourfold or greater increases in titer and four discrepancies by both the IHA and IFA techniques. One patient with a fourfold rise in IFA titer showed a twofold rise by IHA, another patient showed the reverse (i.e., a fourfold rise by IHA and a twofold rise by the IFA technique). Another patient with a greater than fourfold increase by the IFA technique had a high stable titer (1:2,048) by IHA. Finally, serum from a patient with successive IFA titers of 1:256 and 1:128 in serogroup 1 was IHA negative.

Some of the diverse cross-reactivity among serogroups of the human antibody response noted by Wilkinson et al. (15) in the IFA technique was observed with the IHA technique. However, with Formalin-fixed antigens, the IFA technique did not appear to be so cross-reactive in this study (Table 2).

When cross-reactivity was seen, it was found that the etiological serogroup could best be defined by cross absorption. Absorption of a serum with a serogroup-specific antigen removed the cross-reacting antibody as well as the homologous antibody. Absorption with a heterologous serogroup antigen removed only the cross-reacting antibody but did not decrease the serogroup-specific titer. Ten cases of serogroup 1 and one case of serogroup 4 were identified by cross absorption. Two examples are shown in Table 3. Patient 1 appears to have serogroup 1 legionellosis, and patient 2 appears to have Legionnaires disease of serogroup 4 etiology.

Comparison of IHA and IFA techniques on selected sera from 55 patients. Of the 55 patients with IFA-confirmed or presumptive legionellosis, 54 were identified as serogroup 1, and 1 was identified as serogroup 3. The IFA technique detected fourfold or greater rises in titer in 42 patients, 41 of whom were in serogroup 1 and 1 of whom was in serogroup 3. Of these patients, 37 (36 in serogroup 1 and 1 in serogroup 3) also showed significant rises in titer by the IHA technique (Table 4). In three of the remaining five patients, the IHA technique showed a rise from <1:16 to 1:16, and in two cases the IHA technique was nonreactive. Of 13 patients with presumptive legionellosis, 7 showed IHA titers of ≥1:128, 1 showed a rise from <1:16 to 1:16, 2 showed titers between 1:16 and 1:128, and 3 were nonreactive in the IHA technique (Table 4).

TABLE 2. *Diverse serogroup reactivity of the human antibody response*

Serogroup antigen	Convalescent serum titer					
	Serum 1		Serum 2		Serum 3	
	IFA	IHA	IFA	IHA	IFA	IHA
1	64[a]	512	64	16	128	2,048
2	—[b]	—[c]	—	256	—	32
3	—	—	512	512	—	—
4	—	32	16	128	8	128

[a] Reciprocal of highest dilution showing reactivity.
[b] IFA titer of <1:8.
[c] IHA titer of <1:16.

TABLE 3. *Cross absorption to define etiology*

Patient	Serum	Serum IHA titer of serogroup:			
		1	2	3	4
1	Unabsorbed	256[a]	—	—	64
	Absorbed with Flint 1 (serogroup 1)	—[b]	—	—	—
	Absorbed with Los Angeles 1 (serogroup 4)	256	—	—	—
2	Unabsorbed	256	—	—	2,048
	Absorbed with Flint 1 (serogroup 1)	—	—	—	2,048
	Absorbed with Los Angeles 1 (serogroup 4)	—	—	—	—

[a] Reciprocal of highest dilution showing reactivity.
[b] Titer of <1:16.

TABLE 4. *Comparison of IFA and IHA results from 55 patients with IFA-confirmed or presumptive legionellosis*

IFA	IHA (no. of patients)				
	Confirmed[a]	Presumptive[b]	Change in titer from negative[c] to 16[d]	Stationary titers[e]	Negative[c]
Confirmed	37	0	3	0	2
Presumptive	0	7	1	2	3

[a] Fourfold or greater rise in titer.
[b] Titer of ≥1:128.
[c] Titer of <1:16.
[d] Reciprocal of highest dilution showing reactivity.
[e] Between 1:16 and 1:128.

It was considered that differences between the results from the IHA and IFA techniques might be explained by: (i) measurement of two different antigen-antibody systems, (ii) reactions with a cross-reacting antigen(s), or (iii) differences in immunoglobulin specificity. To explore each of these possibilities, the studies discussed below were conducted.

Comparison of immune sera prepared against IFA and IHA antigens. Results of IFA and IHA testing of hyperimmune rabbit sera that were prepared with IFA and IHA antigens are shown in Table 5. If two different antigen-antibody systems are involved, titers with homologous antigens might be expected to be higher than those with heterologous antigens. However, there were no significant differences in titers with IFA and IHA antigens, regardless of the antigen used to prepare the antisera.

Blocking fluid and cross absorption studies. Wilkinson and associates reported that a crude extract of *E. coli* O13:K92:H4 can block 97% of IFA-positive reactions with a variety of gram-negative bacterial species and block 6% of *L. pneumophila* titers (14). This strain of *E. coli* was obtained from H. W. Wilkinson, and blocking fluids of it and the Flint 1 strain were prepared.

Twelve sera with anti-serogroup 1 IHA titers were tested by comparing titrations done with blocking fluid as diluent with titrations done with regular test diluent (Table 6). Dilution in *E. coli* blocking fluid had no significant effect on any of the 12 sera tested, whereas Flint 1 blocking fluid caused a fourfold or greater decrease in all of the sera tested.

Blocking fluid studies by the IFA technique showed similar results. *E. coli* blocking fluid had no effect on the 13 sera tested. Dilution in Flint 1 blocking fluid reduced serogroup 1 titers by at least fourfold in 100% of the cases tested. Twelve sera with initial IFA *E. coli* titers were similarly tested (Table 6). Flint 1 blocking fluid caused no significant decreases in titer in any of the 12 sera, whereas *E. coli* blocking fluid significantly decreased titers in all of the sera. There was one patient who showed a concurrent fourfold rise in titer to both *E. coli* and the Flint 1 strain in which blocking was specific, i.e., Flint 1 blocking fluid reduced only the Flint 1 titer, and *E. coli* blocking fluid reduced only the *E. coli* titer. Titers in another patient who showed a fourfold rise in serogroup 1 titer to 1:128 by the IFA technique and a maximum IHA titer of 1:16 were blocked by Flint 1 blocking fluid but not by *E. coli* blocking fluid.

Cross absorption was felt to be a more conclusive test for common antigen than was the use of crude blocking fluid extract. Absorption of 11 sera that had serogroup 1 IHA titers with whole-cell *E. coli* antigen caused no significant decrease in any serogroup 1 titers, whereas absorption with the whole-cell Flint 1 strain caused a fourfold or greater decrease in all titers (Table 6). Similarly, in the IFA technique, absorption with whole-cell *E. coli* did not change Flint 1 titers in nine sera, whereas absorption with the Flint 1 strain significantly decreased all of the titers. Cross absorption studies of eight sera with *E. coli* IFA titers proved specific for all but one serum. This serum had an initial *E. coli* titer of 1:32, and absorption with *E. coli* decreased the titer to <1:8. Absorption with the whole-cell Flint 1 strain decreased the titer fourfold to 1:8.

Fractionation of sera on sucrose gradients to determine the immunoglobulin class reactive in the IHA and IFA tests. A total of 15 sera were fractionated by the sucrose density gradient technique, and the fractions were analyzed by both the IFA and IHA methods (Table 7). Three sera from cases diagnosed

TABLE 5. *Comparison of antisera prepared against IFA and IHA antigens*

Immunization group	Immunizing antigen	Titer[a] by:	
		IHA	IFA
1	Flint 1, IFA	4,096[b]	2,048[c]
2	Flint 1, IHA	4,096	2,048[c]
3	Detroit 1, IFA	4,096	2,048[d]
4	Detroit 1, IHA	8,192	4,096[d]

[a] Results shown are the average values of triplicate determinations.
[b] Reciprocal of highest dilution showing reactivity.
[c] Flint 1 served as the test antigen.
[d] Detroit 1 served as the test antigen.

TABLE 6. *Results of blocking fluid and cross-absorption studies*

Test	Initial serum titer to:	BF[a] or Abs.[b] used	Decrease in titer[c]
IHA	Serogroup 1	Flint 1 BF	12/12
IHA	Serogroup 1	*E. coli* BF	0/12
IHA	Serogroup 1	Flint 1 Abs.	11/11
IHA	Serogroup 1	*E. coli* Abs.	0/11
IFA	Flint 1	Flint 1 BF	13/13
IFA	Flint 1	*E. coli* BF	0/13
IFA	Flint 1	Flint 1 Abs.	9/9
IFA	Flint 1	*E. coli* Abs.	0/9
IFA	*E. coli*	Flint 1 BF	0/12
IFA	*E. coli*	*E. coli* BF	12/12
IFA	*E. coli*	Flint 1 Abs.	1/8
IFA	*E. coli*	*E. coli* Abs.	8/8

[a] BF, Blocking fluid used for initial dilution of serum.
[b] Abs., Whole-cell antigen used to absorb sera.
[c] Fourfold or greater decrease in titer.

TABLE 7. *Immunoglobulin reactivity of 15 sera fractionated on sucrose density gradients*

Technique	No. of sera with reactivity in:	
	IgM fractions	IgG fractions
IFA	0	12
IHA	13	11

TABLE 8. *Comparison of IHA and IFA results on 10 sucrose density gradient fractions*

Fraction no.	IHA	IFA[a]	IFA-M[b]	Immunodiffusion[c]	
				IgM	IgG
1	8[d]	—[e]	—	−	−
2	32	—	2	+	−
3	512	—	32	+	−
4	128	—	8	+	−
5	32	4	—	−	+
6	32	16	—	−	+
7	4	32	—	−	+
8	—	2	—	−	+
9	—	—	—	−	−
10	—	—	—	−	−

[a] Anti-human globulin conjugate.
[b] Anti-human IgM conjugate.
[c] Presence (+) or absence (−) of IgM and IgG in each fraction.
[d] Reciprocal of highest dilution showing reactivity.
[e] —, No reaction with undiluted sample.

by the IHA technique but not by the IFA technique showed reactivity in only the IgM fractions. Ten sera showed reactivity in the IgM and IgG fractions by the IHA test but showed reactivity in only the IgG fractions by the IFA test. Another serum showed reactivity in the IgG fractions by both techniques. The remaining serum showed reactivity in the IgG fractions by the IFA test but was nonreactive in all fractions by the IHA test. This serum had an initial IFA titer of 1:128 (serogroup 1) and was nonreactive by the IHA test. It appeared that the standard antiglobulin conjugate used in the IFA technique did not adequately detect IgM. To test this hypothesis, the fractions of 10 sera which showed the presence of IgM by the IHA test but not by the IFA test were reexamined by the IFA test with an IgM-specific conjugate. All sera then showed reactivity in the IgM fractions (Table 8).

DISCUSSION

Parallel testing of 895 sera showed that the IHA and IFA tests yielded essentially the same results for serogroups 1 to 4. There was approximately 97% overall agreement between the two techniques, and discrepancies were observed in both directions. IHA-positive and IFA-negative discrepancies were associated with the inability to detect IgM with the antiglobulin conjugate used in this study. Nagington and associates (11) reported that 3 of 22 Legionnaires disease patients showed a rise in IgM antibody without a concurrent rise in IgG antibody. Such cases would go undetected with a conjugate of this type. Polyvalent immunoglobulin conjugates should be used for maximum sensitivity (11, 14). This requirement poses no problem in the IHA technique, as it was conclusively shown to detect IgM and IgG antibodies.

The problem of IFA-positive and IHA-negative sera is yet to be resolved. One such serum showed reactivity in only the IgG fractions. Continued fractionation of sera of this nature may provide an explanation for the differences between the two techniques.

Correlation of clinical data with laboratory data provides further insight into the sensitivity and specificity of the two techniques. Two cases of Legionnaires disease defined by the IHA which were undiagnosed by the IFA technique had clinical symptoms consistent with legionellosis and responded promptly to erythromycin therapy. Among six patients diagnosed by the IFA test but not by IHA, three had clinical histories consistent with Legionnaires disease and responded to treatment with erythromycin; one patient had symptoms consistent with the disease but responded well to treatment with gentamicin and cephalothin, two antibiotics which are of low efficacy in the treatment of legionellosis. Clinical histories of the two remaining patients were not consistent with the diagnosis made by IFA serology. Clinical data were not available on the remaining discrepancies described in this study.

Even when cross absorption was used in addition to the cruder blocking technique, the IHA test showed no significant cross-reactivity with *E. coli*. The IFA test showed evidence of cross-reactivity with *E. coli* in only one instance, but it should be noted that this serum had a low initial titer. Studies with 12 common gram-negative bacterial antigens, including *Haemophilus*, showed no cross-reactivity in the IFA test with *Legionella* antigens (B. Wentworth, personal communication). Cherry and associates (3) found that of 400 bacterial strains tested, one strain of *Pseudomonas fluorescens* is antigenically related to *L. pneumophila* serogroup 1.

Although the IHA test showed more cross-reactivity among serogroups than did the IFA test with Formalin-fixed antigens, heterologous reactivity is usually significantly lower than homologous serogroup reactivity. The IHA test adequately defined the etiological serogroup in

most cases. If there was any question as to the etiological serogroup, cross absorption could be used to define it.

Microagglutination and micro-enzyme-linked immunosorbent assays have also been described for the detection of *Legionella* antibodies (8). However, microagglutination is less sensitive than the IFA test, and the micro-enzyme-linked immunosorbent assay technique, although sensitive, is more expensive and difficult to perform than the IHA technique.

The IHA technique is rapid, simple, inexpensive, and well suited to large-scale studies and routine use. It provides a suitable alternative to the use of the more complicated and expensive IFA technique.

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