Validation of *Legionella pneumophila* Indirect Immunofluorescence Assay with Epidemic Sera

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Sera from six outbreaks of legionellosis and four outbreaks of pneumonia of other etiologies were tested with the indirect immunofluorescence assay (IFA) as currently performed. The current IFA is at least as sensitive as the original test in detecting cases of Legionnaires disease (78 to 91%). By using Center for Disease Control criteria for a positive (fourfold increase in titer during convalescence to ≥128) or presumptive (single titer ≥256) serological test, the specificity exceeded 99%. No cross-reactions against *Legionella pneumophila* antigens were observed among sera from epidemic cases of Q fever, tularemia, and psittacosis; the only positive *L. pneumophila* IFA titer among the epidemic *Mycoplasma* pneumonia sera was reduced to a negative titer with an immunosorbent extracted from *Escherichia coli* strain O13:K92:H4. The slight increase in specificity (to 100%), however, was offset by a slight decrease in sensitivity. The sensitivity of the IFA was maximal when a conjugate that detected immunoglobulins G, M, and A was used. IFA titers were not significantly altered by replacing the monovalent serogroup 1 antigen with a polyvalent antigen (serogroups 1 through 4) nor by the presence of rheumatoid factor or heat-labile serum factors.

Since the discovery that Legionnaires disease (LD) is caused by a bacterium (16), the indirect immunofluorescence assay (FA) has been used to demonstrate antibody responses to *Legionella pneumophila* infections and provide a retrospective serodiagnosis. Validating serological tests for legionellosis has been difficult, however, because of the lack of sufficient numbers of known positive sera from patients with a definite diagnosis of legionellosis. Confirmation by isolation of *L. pneumophila* has not been possible in the majority of cases because of the absence of appropriate media. The original IFA was validated because cases could be defined by the clinical and epidemiological characteristics, i.e., onset of fever and cough in persons attending the 1976 Philadelphia American Legion Convention (12).

Since 1976, knowledge has increased about the bacteria and about the immune response in humans to legionellosis, and the IFA has been modified accordingly. For example, the in vitro cultivation of *Legionella* on several generations of laboratory media (10) made possible the change from an IFA antigen prepared from infected hen yolk sacs to one harvested from the surface of agar. Discovery of multiple serogroups of *L. pneumophila* (17) necessitated the change from an ether-killed to a heat-killed antigen to avoid extracting serogroup 2 antigenic determinants with ether (24). Cross-reactive antibodies against a variety of gram-negative bacteria could be blocked by including in the test an immunosorbent prepared from an *Escherichia coli* O13:K92:H4 strain (23). Testing sera from sporadic cases of legionellosis demonstrated that an antihuman immunoglobulin conjugate that binds immunoglobulin G (IgG), IgM, and IgA is necessary to detect the maximum number of cases (23).

We therefore wished to evaluate the effect of these modifications on the sensitivity and specificity of the IFA test for legionellosis as currently performed in the Center for Disease Control (CDC) Special Immunology Laboratory (SIL-IFA) compared to the test described by McDade et al. (original IFA). To validate the new test, we used sera from the original Philadelphia outbreak of LD and sera from several subsequent legionellosis outbreaks investigated by CDC, because these sera constitute the best available group of positive control sera. Similarly, negative control sera were selected from cases of pneumonia of other etiologies that had been documented both by seroconversion and by association with an epidemic. As a result of this study, we concluded that the SIL-IFA is at least as sensitive and specific as the original test with no evidence to suggest the occurrence of falsely elevated titers due to heat-labile serum...
factors, rheumatoid factors, or to antibodies directed against *Mycoplasma pneumoniae*, *Chlamydia psittaci*, *Francisella tularensis*, or *Coxella burnetii*.

**MATERIALS AND METHODS**

**Patient selection.** Fifty-four sets of sera (one set per patient, ≥2 sera per set) were available from cases of epidemic legionellosis; 34 were from the 1976 Philadelphia LD epidemic (12, 16), and 20 were from five subsequent outbreaks: Kingsport, Tenn. (LD, 5); Burlington, Vt. (LD, 2); Memphis, Tenn. (LD, 6); James River, Va. (Pontiac Fever, 11); and Atlanta, Ga. (LD, 3). Single serum specimens were tested for an additional 17 cases from the Philadelphia, Kingsport, and Burlington outbreaks, for an overall total of 71 legionellosis cases. Cases were defined during the initial investigations by a combination of clinical, epidemiological, and serological data and by the absence of other apparent etiological agents. The quantity of each serum was not always sufficient to perform all tests. Therefore, analyses are based on the number of cases with results for the tests being compared.

An additional 71 sets of sera from sporadic cases were used in tests of immunoglobulin class, rheumatoid factor, and heat lability. These cases were selected on the basis of a fourfold rise in titer to ≥256 against the *L. pneumophila* serogroup 1 antigen in the SIL-IFA.

Control sera from 87 patients with pneumonia of etiologies other than *Legionella* were obtained during investigations of outbreaks due to *M. pneumoniae*, *F. tularensis*, *C. burnetii*, and *C. psittaci*. Diagnosis was based on seroconversion to the etiological agent and epidemiological association with the outbreak.

**IFA test.** The SIL-IFA was performed as described previously (23, 24). Briefly, heat-killed antigens in 0.5% buffered normal yolk sacs were prepared from the 2- to 3-day growth of *L. pneumophila* strains on charcoal-yeast extract agar (CYE; 10), serogroup 1 (Philadelphia 1 strain), serogroup 2 (Tongus 1), serogroup 3 (Bloomington 2), serogroup 4 (Los Angeles 1), and polyvalent. The polyvalent antigen contained the four monovalent antigens for serogroups 1 through 4 at double the dilution that was used individually and buffered normal yolk sacs in a final concentration of 0.5%. Sera to be tested were diluted in 0.01 M phosphate-buffered saline (PBS), pH 7.6, or in an immunosorbert extract from a strain of *E. coli* O13:K92: H4, as described previously (23). Conjugates included fluorescein isothiocyanate-labeled antihuman immunoglobulin with reactivity for IgG, IgM, and IgA (polyimmunoglobulin; Biological Products Div., CDC) and fluorescein isothiocyanate-labeled anti-IgG, anti-IgM, and anti-IgA (Calbiochem-Behring Corp., San Diego, Calif.). The specificity of the conjugates was verified by the modified immunoglobulin-coated Sepharose 4B bead technique of Scales et al. (21).

That the antigen could be prepared reproducibly was verified by testing 20 control sera (titer range, <64 to 1,024) against heat-killed antigens prepared from five successive CYE agar subpassages of the serogroup 1, Philadelphia 1 strain. The first subpassage was inoculated with an infected hen yolk sac suspension supplied by George Gorman. The strain had not previously been cultured in vitro. IFA titers were the same (± a twofold dilution factor) against the five antigens.

**Interpretation of titers.** IFA titers were determined as previously described (24) and were interpreted in two ways. Serum titers from a patient were defined as positive by a low interpretation, i.e., low criteria, if they showed a seroconversion to ≥64 or at least one convalescent titer of ≥128 (16). They were considered positive by a high interpretation, i.e., high criteria, if they showed seroconversion to ≥128 (confirmed) or at least one titer of ≥256 (presumptive; 2, 3, 5, 6). For comparing the SIL to the original IFA, the original test titers obtained at the time of the Philadelphia investigation were used (16). SIL tests were not done in duplicate as were the original tests because of the recently demonstrated high reproducibility of the SIL test (0.98, manuscript in preparation).

**Heat-labile serum factors.** Thirty-two convalescent sera from sporadic cases of LD were tested in the IFA against the serogroup 1 antigen after they had been heated in a 56°C water bath for 30 min.

**Rheumatoid factor.** Sera from 46 sporadic cases of LD were tested for rheumatoid factor by the microtiter latex agglutination technique (kit no. 966648; Calbiochem-Behring Corp.) of Reimer et al. (20).

**RESULTS**

**Comparison of SIL-IFA and original IFA.** Sera from 46 LD cases in the 1976 Philadelphia epidemic were available for repeat tests by the SIL method. Since this outbreak was caused by *L. pneumophila* serogroup 1 and since serogroup 1 antibodies were used in the original tests (16), the SIL tests were done with the serogroup 1 antigen and with the polyimmunoglobulin conjugate. As shown in Fig. 1, a linear relationship existed when titers obtained in the two test systems were plotted. The Kendall rank correlation coefficient (15) was 0.660, *P* < 0.001. Discrepancies greater than twofold (the variation that is generally acceptable in serological tests) showed a tendency for SIL titers to be higher than the original titers, both when all titers were included in the analysis and when only the peak convalescent titer from each patient was analyzed. However, this tendency may have been the result of selecting the lowest of duplicate titers in the original test (16). The original test showed 68 and 82% of the 46 cases to have positive titers by the high and low criteria, respectively; the SIL test showed 79 and 85% of the same cases to be positive. These data suggest that the SIL-IFA is at least as sensitive as the original test.

**Comparison of serogroup 1 antigen and polyvalent antigen.** There was no significant difference in the mean titer obtained when the Philadelphia outbreak sera were tested against the serogroup 1 SIL antigen and when they were
tested against the polyvalent antigen containing serogroups 1 through 4 strains \( P = 0.675 \); two-tailed, Wilcoxon matched-pairs signed-ranks test; 22). Figure 2 demonstrates the linear distribution of titers obtained against the two antigens. Among 20 sets of sera from the five remaining legionellosis outbreaks, three seroconversions (to \( \geq 128 \)) were detected with the polyvalent antigen that were not detected with the monovalent, serogroup 1 antigen. Subsequent tests against monovalent antigens of serogroups 2, 3, and 4 showed that the three cases (two from the James River outbreak and one from the Atlanta outbreak) had maximal peak titers against the serogroup 4 antigen. Polyvalent and serogroup 4 antigen titers agreed within one doubling dilution.

**Specificity of the SIL test.** Table 1 shows the results of testing 87 paired sera from patients with tularemia, psittacosis, Q fever, or *Mycoplasma pneumonia* in the SIL-IFA against the polyvalent antigen. The specificity of the test with the low criteria for positivity was 90%; use of the high criteria increased the specificity to 99%. When the cross-reactive sera were diluted in immunosorbent and retested, none was positive by the high criteria, and only two were positive (presumptively, only) by the low criteria. Therefore, the specificity of the test was 100% (or 92%, low criteria) with immunosorbent.

**Effect of immunosorbent on LD IFA titers.** To determine the effect of immunosorbent

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**Fig. 1.** Scattergram of IFA titers obtained with 1976 Philadelphia LD epidemic sera against *L. pneumophila* serogroup 1 antigens from original test and in modified SIL test. Numbers inside data points represent number of sera with the specified titers. Numbers within shaded areas were sera with identical titers (solid lines) or titers that differed by no more than one twofold dilution factor (broken lines) in the two tests. (A) All sera, \( n = 74 \); (B) peak convalescent sera only, \( n = 40 \).

**Fig. 2.** Scattergram of IFA titers (SIL test) obtained with 1976 Philadelphia epidemic sera against *L. pneumophila* serogroup 1 antigen and polyvalent antigen (serogroups 1 through 4). Numbers inside data points represent number of sera with the specified titers. Numbers within shaded areas were sera with identical titers (solid lines) or titers that differed by no more than one twofold dilution factor (broken lines) against the two antigens. (A) All sera, \( n = 63 \); (B) peak convalescent sera only, \( n = 28 \).
on LD epidemic case titers, we tested sera from the six outbreaks with and without immunosorbent against the serogroup 1 antigen. As depicted in the scattergrams of Fig. 3, several titers were lowered (>1-tube dilution factor) when immunosorbent was used as the serum diluent. Titers of acute phase sera were significantly lower with sorbent than they were without sorbent (P = 0.00002, Wilcoxon matched-pairs signed-rank test [22]). In contrast, peak convalescent titers did not differ significantly with sorbent (P = 0.91). Therefore, the immunosorbent tended more often to inhibit nonspecific, acute-phase titers. When the same analysis was done with titers obtained against the polyvalent antigen, both the peak (P = 0.0004) and the acute-phase titers (P = 0.00002) were lowered significantly. Reasons for this discrepancy are not readily apparent.

Comparison of interpretative criteria for positivity. Data obtained in the preceding analyses were compiled and tabulated (Table 2) to determine the optimal cutoff levels for positive titers. Comparable levels of sensitivity and specificity were obtained in interpreting as positive those serum sets that seroconverted to ≥128 or that had single or standing titers of at least 256 (high criteria; no immunosorbent added; sensitivity = 79%, specificity = 99%) and, in the presence of immunosorbent, serum sets that seroconverted to ≥64 or that had single or standing titers of at least 256 (sensitivity = 76%, specificity = 100%). For reasons to be discussed, these estimates of sensitivity might have been lower if all the Philadelphia epidemic sera had been available for testing.

Immunglobulin classes. Previous studies suggested that the antibody response to legionellosis could be in one or in several immunoglobulin classes (19, 23), but that IgM could be the more specific class in legionellosis cases (19). In the present study, we found no evidence for loss of specificity with the polyclononal conjugate (see above), but we wished to clarify the possible loss of sensitivity by use of class-specific conjugates. Forty-three sets of epidemic legionellosis sera and 71 sets of positive (with polyclononal conjugate) sporadic-case sera were tested with class-specific conjugates against the serogroup 1 or polyvalent antigen, respectively. By using each class-specific conjugate at its optimal titer (as determined by checkerboard titrations with immunoglobulin-coated beads and with LD reactive sera), we were reasonably sure that titer levels were comparable. To eliminate the possibility of small quantitative differences that might influence the interpretation of presumptive titers that were minimally positive, however, we included in the analysis only seroconversions to ≥64 and to ≥128. The results are summarized in Table 3. Had an IgM-specific conjugate been used alone, 24% or 35% of the positive epidemic titers and 21% or 28% of the positive sporadic-case titers would have been considered negative by the low or high criteria, respectively. Similarly, by using only the IgG-specific conjugate, 21% or 19% of the epidemic positive sera and 6% or 9% of the sporadic-case positive sera would have been missed. The IgA conjugate failed to detect 41% or 65% of the epidemic and 41% or 51% of the sporadic-case positive titers. Inclusion of presumptive titers in the analysis (not shown) gave similar results.
**TABLE 1. IFA tests of sera from outbreaks of pneumonia other than legionellosis**

<table>
<thead>
<tr>
<th>Serum sets</th>
<th>No. (%) with positive LD IFA titers* by interpretation:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of pairs</td>
<td></td>
</tr>
<tr>
<td>Tularemia</td>
<td>7</td>
<td>6' (86)</td>
</tr>
<tr>
<td>Psittacosis</td>
<td>21</td>
<td>6' (29)</td>
</tr>
<tr>
<td>Mycoplasma pneumonia</td>
<td>50</td>
<td>21' (42)</td>
</tr>
<tr>
<td>Q fever</td>
<td>9</td>
<td>2 (22)</td>
</tr>
</tbody>
</table>

* Against L. pneumophila polyvalent antigen.
1 Of 35 (40%), 22 were inhibited by immuno-sorbent to <64.
2 Inhibited by immuno-sorbent to <64. Totals are 8 (9%) for seroconversion to ≥64 and 1 (1%) for seroconversion to ≥128.
3 Of 9 (10%), 2 were inhibited by immuno-sorbent to <64.
4 Of 21, 10 were inhibited by immuno-sorbent to <64.
5 Of 6, 1 was inhibited by immuno-sorbent to 64.

**TABLE 2. Effect of cutoff levels for positive IFA titers**

<table>
<thead>
<tr>
<th>Estimated parameter*</th>
<th>SC ≥ 64 or S ≥ 64</th>
<th>SC ≥ 64 or S ≥ 128 (low criteria)</th>
<th>SC ≥ 64 or S ≥ 256 (high criteria)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS Sorbent PBS Sorbent PBS Sorbent PBS Sorbent PBS Sorbent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>97 88</td>
<td>85 82</td>
<td>79 76</td>
</tr>
<tr>
<td>Specificity</td>
<td>60 85</td>
<td>80 92</td>
<td>91 100</td>
</tr>
</tbody>
</table>

* Estimates based on testing 34 sets of sera from Philadelphia LD epidemic against the serogroup 1 antigen (sensitivity) and 87 sets of sera from outbreaks of pneumonia of other etiologies (see Table 1) against the polyvalent antigen (specificity). Sensitivity may be greater than that shown (see text).
1 SC, Seroconversion; S, single or standing titer.

**Effect of heat on IFA titers.** Thirty-two convalescent-phase sera from sporadic cases of legionellosis had the same titers (all ≥ 128) against the serogroup 1 antigen with the polyimmunoglobulin conjugate before and after they were heated at 56°C for 30 min. Titers obtained with class-specific conjugates changed after heat treatment in only one serum and with only one conjugate; its IgM titer was lower after heating, but still remained above the level of diagnostic significance (high criteria).

**Lack of relationship of rheumatoid factor titers and L. pneumophila IFA titers.** A statistical analysis (Kendall rank correlation coefficient [15]) of titers obtained in testing 46 sporadic-case sera against the L. pneumophila serogroup 1 antigen with the polyimmunoglobulin conjugate and in testing the same sera for rheumatoid factor showed a lack of correlation (n = 110, P = 0.267), suggesting that the presence of anti-immunoglobulin antibody in patients' sera does not have a significant influence on the LD IFA test.

**DISCUSSION**

In the absence of sufficient numbers of culture-confirmed cases of legionellosis with matching, appropriately timed serum specimens, it is difficult, if not impossible, to provide an accurate measure of the sensitivity of serology as a diagnostic aid. However, a study of epidemic legionellosis provides an alternative source of documentation for positive controls. The sensitivity of the IFA test as currently used by CDC was estimated to be at least that of the original test, on the basis of a comparison of titers against the Philadelphia 1 antigen from LD cases in the 1976 Philadelphia epidemic: 85 versus 82% when the low criteria for positivity were used and 79 versus 68% when the high criteria were used. Since the sera tested were not from a random sample of LD cases in the epidemic, we could not calculate sensitivity directly. In the original Philadelphia investigation (16), 101 of 111 appropriately timed specimens would have been considered positive by the low criteria or 87 of 111 would have been considered positive by the high criteria for a sensitivity of 91% or 78%,
respectively. Therefore, the sensitivity of the SIL test may well be above 78%. This is greater than that reported by Edelstein et al., who found 10 of 14 cases diagnosed by culture or by direct immunofluorescence staining to have positive IFA tests (8). However, the number of cases available for analysis was small.

For estimating specificity, we used sera from patients in outbreaks of pneumonia caused by *M. pneumoniae, F. tularensis, C. burnetti*, and *C. psittaci*. In other studies alleging a high fraction of cross-reactions, it has been difficult to determine what the actual diagnosis has been, and methods for performing and interpreting the serological tests have varied (7, 13, 19). The specificity of the SIL test with a polyvalent antigen containing serogroups 1 through 4 was 99 to 100% by the high criteria.

In addition to comparing the results of the SIL-IFA test and the original test with outbreak sera, we compared the polyvalent with the serogroup 1 antigen, the use of immunosorbent versus PBS as serum diluent, and the class-specific versus polyimmunoglobulin (reactive with IgG, IgM, and IgA) conjugates in the SIL test. In the first comparison, no significant differences in mean titers were obtained. However, three seroconversions were detected with the polyvalent antigen (and with the monovalent serogroup 4 antigen, subsequently) that were not detected with the serogroup 1 antigen. In the second comparison, there was also no significant difference in convalescent titers with and without sorbent against the serogroup 1 antigen, although acute-phase titers were lowered significantly. The net result was a more easily interpreted test when seroconversions occurred with a minimally positive peak titer. A loss of sensitivity was observed, however, when the same comparison was made with the polyvalent antigen because a significant decrease also occurred in the peak convalescent titer level. These findings are unexplained at this time. For this reason, and because the specificity of the test is high without immunosorbent (which is difficult to prepare in large volume), we do not recommend using it routinely. The third comparison confirmed our previous conclusions from testing sporadic-case sera (23). Specific LD titers are the result of IgG, IgM, and IgA classes, individually or in various combinations. Conjugates that react with only one immunoglobulin class failed to detect from 6 to 65% of sera that had positive LD titers.

In the final phase of this study, we looked for the possible influence of heat-labile serum factors or anti-antibodies on IFA titers against *L. pneumophila* antigens. We found no evidence for the association of heat-labile factors or of rheumatoid factors with positive LD titers.

In summary, the IFA test as currently used for the serodiagnosis of legionellosis at CDC appears to be at least as sensitive and specific as the test originally described by McDade et al. The antigen can be prepared reproducibly as heat-killed suspensions of *L. pneumophila* strains, harvested from CYE agar media, and used either individually or combined (polyvalent, up to four strains). Conjugates must detect IgG, IgM, and IgA because the immune response in patients with legionellosis can be in one or several classes. Neither rheumatoid factor nor complement appears to cause false-positive legionellosis titers with the conjugate used in this study. And finally, using an immunosorbent as serum diluent slightly improves the specificity of the test (from 99 to 100% among the control sera tested from defined outbreaks) but also appears to lower slightly its sensitivity against the polyvalent antigen. Therefore, there seems to be no distinct advantage in using it routinely. Instead, sorbent could be conserved for retesting sera which give equivocal titers when a stringent test for specificity is required.

Insufficient numbers of sera from legionellosis cases caused by *L. pneumophila* serogroups 2 through 6 (9, 17, 18) and *Legionella*-like organisms (4) are available for an analysis such as

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**Table 3. Immunoglobulin classes in immune response to legionellosis**

<table>
<thead>
<tr>
<th>Serum sets</th>
<th>No. tested</th>
<th>Seroconversion to</th>
<th>Antigen</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
<th>IgG + IgM</th>
<th>IgG + IgA</th>
<th>IgM + IgA</th>
<th>IgG + IgM + IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Four outbreaks</td>
<td>43</td>
<td>≥64</td>
<td>Serogroup 1</td>
<td>5 (12)</td>
<td>2 (5)</td>
<td>1 (2)</td>
<td>5 (12)</td>
<td>1 (2)</td>
<td>3 (7)</td>
<td>12 (28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥128</td>
<td></td>
<td>8 (19)</td>
<td>3 (7)</td>
<td>0</td>
<td>6 (14)</td>
<td>1 (2)</td>
<td>2 (5)</td>
<td>6 (14)</td>
</tr>
<tr>
<td>Sporadic cases</td>
<td>71</td>
<td>≥64</td>
<td>Polyvalent</td>
<td>12 (17)</td>
<td>3 (4)</td>
<td>0</td>
<td>13 (18)</td>
<td>2 (3)</td>
<td>1 (1)</td>
<td>37 (52)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥128</td>
<td></td>
<td>17 (24)</td>
<td>3 (4)</td>
<td>0</td>
<td>14 (20)</td>
<td>2 (3)</td>
<td>3 (4)</td>
<td>28 (39)</td>
</tr>
</tbody>
</table>

* The difference in number tested and total number detected represents serum sets that were negative by the restrictive criteria used for this analysis.

* Philadelphia, Kingsport, Burlington, Memphis.
the one reported herein for serogroup 1. It should be noted, however, that both patients with culture-confirmed serogroup 6 LD and from whom sera were available had impressive seroconversions in IgG, IgM, and IgA classes against the \textit{L. pneumophila} serogroup 6 IFA antigen (18). Furthermore, titers against the expanding number of defined species in the genus \textit{Legionella} (1, 14, 18a) are typically of the same magnitude and in the same range as those observed against serogroup 1 (unpublished data). Studies are now in progress to determine whether using additional antigens, perhaps in polyvalent pools, would improve the serodiagnosis of legionellosis.

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LITERATURE CITED


values and probability levels for the Wilcoxon rank sum test and the Wilcoxon signed rank test. American Cyanamid Co., Pearl River, N.Y.
