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Comparison of Two Commercial Enzyme-Linked Immunosorbent Assays with an Immunofluorescence Assay for Detection of 
*Legionella pneumophila* Types 1 to 6

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Members of the genus *Legionella* are characterized as gram-negative, motile, freshwater-dwelling bacteria that were responsible for a pneumonia outbreak among American Legion members in 1976. Because clinicians routinely order serologic testing for *Legionella pneumophila* serogroups 1 to 6 as a screen for possible *L. pneumophila* infections, we evaluated the Wampole Laboratories *L. pneumophila* type 1 to 6 immunoglobulin G (IgG) and IgM combined enzyme-linked immunosorbent assay (ELISA) and the Zeus Scientific *L. pneumophila* type 1 to 6 IgG-IgM-IgA multispecific combined ELISA systems and compared them to an IgG-specific immunofluorescence assay (IFA) for *L. pneumophila* serogroups 1 to 6. The Centers for Disease Control and Prevention recommends that the positive titer cutoff for an IFA be 1:256. Regardless of where the positive IFA cutoff titer is placed, however, the sensitivity of both commercial assays was below what would be acceptable for a screening assay. With a 1:256 IFA titer as the positive cutoff, the agreement, sensitivity, and specificity of the Wampole ELISA were 74.6, 21.4, and 98.4%, respectively. The agreement, sensitivity, and specificity of the Zeus ELISA were 72.6, 10.5, and 100.0%, respectively. We recommend that any laboratories attempting to replace an IFA type 1 to 6 screen with an alternative ELISA carefully investigate the sensitivity of the replacement assay.

Legionellae are motile, gram-negative, rod-shaped bacteria that grow in freshwater environments (5). Strains of the organism were first isolated in the 1940s (13), but the bacterium did not gain notoriety until it was found to be responsible for a pneumonia outbreak among American Legion members in 1976 (3, 6). Three years later, the genus *Legionella* was established (3). Consisting of 48 species and 70 serogroups (1, 2, 7, 8), legionellae are spread by inhalation of aerosolized biofilm droplets containing the bacteria and multiply intracellularly. The organisms invade the lungs and other organs and cause Legionnaires’ disease (14). Legionellae can also cause subclinical infections, nonpneumonic disease, and extrapulmonary inflammatory disease.

While *Legionella pneumophila* serogroup 1 causes 80% of all reported cases of legionellosis, another 14 serogroups actually make up the *L. pneumophila* group (9). Historically, serologic testing for serogroups 1 to 6 has been performed as a screen for possible *L. pneumophila* infections, although the Centers for Disease Control and Prevention (CDC) does not consider infections with serogroups other than type 1 to be reportable. Because many physicians prefer to screen for other *L. pneumophila* serotypes than type 1, we continue to perform this test. An enzyme-linked immunosorbent assay (ELISA) screen is generally preferred over immunofluorescence assay (IFA) testing because it is less expensive and less subjective and is thought to be more sensitive than IFA testing. Since evaluation of commercial kits for *L. pneumophila* serology has not been performed (5), we compared two Food and Drug Administration-cleared commercial test systems to a Food and Drug Administration-cleared IFA for *L. pneumophila* types 1 to 6. Additionally, since we have offered an in-house *L. pneumophila* type 1 to 6 immunoglobulin G (IgG)-specific ELISA based on a commercially available antigen in the past, we included that assay in the evaluation.

**MATERIALS AND METHODS**

**Clinical samples.** Included in this study were 222 serum samples submitted to our reference laboratory for *L. pneumophila* antibody (types 1 to 6) IgG testing by IFA. These samples were deidentified and tested for *L. pneumophila* types 1 to 6 by three ELISA systems: an in-house ELISA developed for IgG, the Wampole Laboratories IgG and IgM combined ELISA, and the Zeus Scientific, Inc., IgG-IgM-IgA multispecific combined ELISA. Samples with discrepant results were retested in duplicate if the discrepant result was observed in only one ELISA system. For example, if the sample had a positive result by IFA, Zeus, and Wampole but had a negative result by the in-house assay, that sample was retested. However, if a sample had a positive result by IFA but had a negative result by Zeus, Wampole, and the in-house ELISA, that sample was not retested. All sera were received at our laboratory on ice and were stored at 2 to 8°C until testing was completed.

**L. pneumophila** type 1 to 6 IgG IFA. Samples were tested for *L. pneumophila* type 1 to 6 IgG by an IFA procedure. Samples were diluted 1:128 in sample diluent (0.5% fetal bovine serum [Sigma, St. Louis, Mo.] and 0.1% Thimerisol [Sigma] in phosphate-buffered saline [PBS; Bio-Rad Laboratories, Hercules, Calif.]). Fourfold serial dilutions to 1:2,048 were then made into sample diluent from the initial 1:128 screening dilution. Twenty-five-microliter volumes of the 1:128, 1:512-, and 1:2,048-diluted samples were added to separate wells of MARDX (MARDX, Carlsbad, Calif.) and incubated for 30 min at room temperature in a moist chamber. After incubation, the IFA slides were washed in a Coplin jar of PBS (Sigma) and rinsed...
with deionized water. Anti-human IgG fluorescein-labeled conjugate (MARDX) was added to each well, and the slides were incubated for 30 min at room temperature in a moist chamber. The slides were then washed in PBS and rinsed with deionized water. Excess water was removed, and mounting medium was used to apply a coverslip to the slides. Wells were viewed at a final magnification of 100× (numerical aperture of 0.85 mm) with an Olympus (Tokyo, Japan) BH-2 transmitting fluorescence microscope with a 100-W mercury lamp. Results were determined on the basis of the fluorescence intensity of antibodies against *L. pneumophila* organisms in each well. A fluorescence of 2− to 4+ signified moderate-to-maximum intensity, and 1+ fluorescence signified definite but dim intensity. Samples with a fluorescence intensity of <2+ were considered negative, while samples were considered positive if antibodies against the majority of *L. pneumophila* organisms stained at ≥2+. The CDC has determined that titers of single specimens of ≥1:256 are significant indicators of Legionella infection. Therefore, we designated an IFA titer of 1:256 positive and a titer of 1:128 equivocal.

**TABLE 1. Reference ranges for the *L. pneumophila* type 1 to 6 IgG IFA, *L. pneumophila* type 1 to 6 IgG ELISA, Wampole *L. pneumophila* type 1 to 6 IgG-IgM ELISA, and Zeus *L. pneumophila* type 1 to 6 IgG-IgM-IgA ELISA.**

<table>
<thead>
<tr>
<th>Test system</th>
<th>Reference range <strong>a</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pneumophila</em> type 1 to 6 IgG IFA (ARUP)</td>
<td>≤1:128 ≤1:256 ≥1:128</td>
</tr>
<tr>
<td><em>L. pneumophila</em> type 1 to 6 IgG ELISA (ARUP)</td>
<td>&lt;0.89 &gt;1.10 0.90–1.10</td>
</tr>
<tr>
<td><em>L. pneumophila</em> type 1 to 6 IgG-IgM ELISA (Wampole)</td>
<td>≤0.90 ≥1.10 0.91–1.09</td>
</tr>
<tr>
<td><em>L. pneumophila</em> type 1 to 6 IgG-IgM-IgA ELISA (Zeus)</td>
<td>≤0.90 ≥1.10 0.91–1.09</td>
</tr>
</tbody>
</table>

**a** ARUP (Associated Regional and University Pathologists) IFA values are titers. ARUP ELISA values are index values. Wampole ELISA values are immune status ratios. Zeus ELISA values are OD ratios.

**RESULTS**

**Comparison of in-house ELISA to IFA.** Use of the CDC-recommended titer of 1:256 as the cutoff resulted in a calculated agreement, clinical sensitivity, and clinical specificity of 81.7, 82.1, and 81.4%, respectively, for 222 samples tested by the in-house ELISA (Table 2). Ten (17.2%) of 58 samples with positive IFA results had negative results by the in-house ELISA, and 21 (16.3%) of 129 samples with negative IFA results had positive results by the in-house ELISA.

Of the 222 samples tested, there were 53 with equivocal results that were not included in the calculations. Twenty-five samples (47.2%) had positive results by ELISA and equivocal results by IFA. Seven samples had negative results by ELISA and equivocal results by IFA. Two samples had positive IFA results by ELISA and equivocal results by IFA. Sixteen samples (30.2%) had equivocal results by ELISA and negative results by IFA. Three samples had equivocal results by both ELISA and IFA (Table 2).

**Comparison of Wampole ELISA to IFA.** With the positive IFA cutoff set at ≥1:256, agreement, clinical sensitivity, and clinical specificity were 74.6, 21.4, and 98.4%, respectively, for 222 samples tested by the Wampole ELISA (Table 3). Forty-four (75.9%) of 58 samples with positive results by IFA had negative results by Wampole ELISA. Two of 129 samples had no longer be produced for use in the reference laboratory; hence, we returned to the use of the *L. pneumophila* type 1 to 6 IFA.

**TABLE 2. Agreement, sensitivity, and specificity of the in-house Legionella ELISA compared to the Legionella IFA.**

<table>
<thead>
<tr>
<th>ELISA result</th>
<th>No. of IFA results <strong>a</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>46</td>
</tr>
<tr>
<td>Negative</td>
<td>21</td>
</tr>
<tr>
<td>Equivocal</td>
<td>2</td>
</tr>
</tbody>
</table>

**TABLE 3. Agreement, sensitivity, and specificity of the Wampole Legionella ELISA compared to the Legionella IFA.**

<table>
<thead>
<tr>
<th>ELISA result</th>
<th>No. of IFA results <strong>a</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>12</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
</tr>
<tr>
<td>Equivocal</td>
<td>2</td>
</tr>
</tbody>
</table>

**a** Agreement, sensitivity, and specificity were 74.6, 21.4, and 98.4%, respectively.
negative results by IFA but positive results by Wampole ELISA.

Of the 222 samples tested, there were 41 samples with equivocal results that were not included in the calculations. Eight samples had positive results by Wampole ELISA and equivocal results by IFA. Twenty-four samples (58.5%) had negative Wampole ELISA results and equivocal results by IFA. Three samples had equivocal results by both Wampole ELISA and IFA. Two samples had equivocal Wampole ELISA results and positive results by IFA. Four samples had equivocal results by Wampole ELISA and negative results by IFA (Table 3).

Comparison of Zeus ELISA to IFA. With the positive IFA cutoff set at $\geq$1:256, the agreement, clinical sensitivity, and clinical specificity were 72.6, 10.5, and 100.0%, respectively, for 222 samples tested by the Zeus ELISA (Table 4). Fifty-one (87.9%) of 58 samples with positive results by IFA had negative results by Zeus ELISA.

Of the 222 samples tested, there were 36 samples with equivocal results that were not included in the calculations. Five samples had positive results by Zeus ELISA and equivocal results by IFA. Thirty samples (83.3%) had negative results by Zeus ELISA and equivocal results by IFA. One sample had an equivocal Zeus ELISA result and a positive IFA result (Table 4).

Comparison of commercial ELISA systems. With the Wampole assay randomly chosen to be the “gold standard,” the Zeus Legionella ELISA had an agreement, clinical sensitivity, and clinical specificity of 94.8, 47.6, and 100.0% for 222 samples (Table 5). Eleven (50.0%) of 22 samples with positive results by the Wampole assay had negative results by the Zeus ELISA. Ten samples had equivocal results by one of the commercial assays. Linear regression analysis of the data yielded an $R^2$ value of 0.83, with the majority of the discrepant Zeus ELISA results occurring at or near the cutoff of the Wampole assay (Fig. 1).

DISCUSSION

Serologic testing for L. pneumophila serogroups is often the primary method of screening for possible L. pneumophila infections. High sensitivity is paramount in a screening assay, since the assay should detect the greatest possible number of samples positive for L. pneumophila antibodies. Theoretically, the assay should also detect equivocal samples as positive so that any cases of possible infection are caught in the screen. However, only 8 of 35 serum samples with equivocal IFA results (titer of 1:128) were found positive by the Wampole assay, and only 5 of 35 equivocal samples were found positive by the Zeus assay. This poor correlation was also observed among positive IFA samples. Even when the CDC-recommended positive IFA titer of 1:256 was used as the cutoff, the sensitivity of the Wampole assay was 21.4% while the sensitivity of the Zeus assay was 10.5%. These statistics were further examined by comparing the Wampole ELISA results to the Zeus ELISA results. With the Wampole assay randomly chosen as the gold standard, the Zeus assay had a calculated clinical sensitivity of 47.6%. This was an improvement over the Zeus clinical sensitivity with IFA as the gold standard but was still far below the acceptable level for a screening assay.

Some uncertainty exists with regard to the cutoff titer for the positive range of IFA. On the basis of previous validations with epidemic sera, the CDC considers single samples with titers of $\geq$1:256 to be presumptive of Legionella infection (15). But according to recent studies, even a titer of 1:256 may not distinguish between Legionnaires’ disease and pneumonia due to other causes. Plouffe et al. reported that in a study of 68 samples, only 10% of the samples that were culture confirmed or showed a fourfold rise in the antibody titer had acute-phase Legionella titers of $\geq$1:256 (10). Although the IFA clearly is not the best gold standard test, it is the only test that has been validated with clinical manifestations. Regardless of where the positive IFA cutoff titer is placed, however, the sensitivities of both commercial assays were below what would be acceptable for a screening assay. Additionally, as discussed above, the commercial assay results did not agree with each other.

With the in-house ELISA as a model for comparison, the possibility exists for an assay to achieve a higher sensitivity; the sensitivity of the in-house ELISA was 82.1% at a positive IFA

### TABLE 4. Agreement, sensitivity, and specificity of the Zeus Legionella ELISA compared to the Legionella IFA

<table>
<thead>
<tr>
<th>Zeus ELISA result</th>
<th>No. of IFA results*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
</tr>
<tr>
<td>Negative</td>
<td>51</td>
</tr>
<tr>
<td>Equivocal</td>
<td>1</td>
</tr>
</tbody>
</table>

* Agreement, sensitivity, and specificity were 72.6, 10.5, and 100.0%, respectively.

### TABLE 5. Agreement, sensitivity, and specificity of the Wampole Legionella ELISA compared to the Zeus Legionella ELISA

<table>
<thead>
<tr>
<th>Zeus ELISA result</th>
<th>No. of Wampole ELISA results*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
</tr>
<tr>
<td>Equivocal</td>
<td>1</td>
</tr>
</tbody>
</table>

* Agreement, sensitivity, and specificity were 94.8, 47.6, and 100.0%, respectively.
cutoff titer of 1:256. While many components of the commercial assays differed from the components of the in-house assay, one possible reason for the discrepancies in results is the type of antigen that was bound to the wells of the microtiter plate used in the ELISA systems. Although specific details were not available, it is possible that strains of similar serogroups were chosen to formulate the Cambrex antigen that was used in the in-house ELISA and the antigen that was used on the MARDX IFA slides. This would help explain why results of the in-house ELISA matched those of the IFA. Details regarding antigen preparations were likewise unavailable in the Wampole and Zeus package inserts, but because the results of the two commercial ELISAs and the IFA were so different, it appears that different strains of the various serogroups were used. It is possible that if a different combination of Legionella strains was used as the antigen in a commercial L. pneumophila serogroup 1 to 6 ELISA, greater sensitivity might be achieved.

Additional factors also illustrate the difficulty in Legionella antibody testing. First, it has been argued that the use of L. pneumophila serotypes 1 to 6 in a screening assay may not always be helpful in making a definitive diagnosis of Legionella infection. It has been suggested that screening for L. pneumophila types 1 to 6 should be avoided since samples that are positive for a polyvalent antigen do not produce consistent results when tested with a monovalent antigen (4). Since 65 to 70% of the cases of Legionnaires’ disease can be detected with assays specific for L. pneumophila type 1 (4, 11), use of a type 1 antigen would alleviate the need for polyvalent antigens covering various serotypes and would still allow fairly high sensitivity in the detection of L. pneumophila infections. Second, testing of a single sample, rather than paired samples, may indicate falsely elevated antibody levels. This is due to the presence of high background antibody levels in specimens obtained from patients in North America (10). The issue of high Legionella antibody titers in the general public (up to 20%) illustrates the necessity of follow-up testing of convalescent-phase samples from patients with acute-phase samples that are positive for Legionella infection. We included equivocal samples (16%) in the panel for an accurate representation of the high background seroprevalence of Legionella antibody titers in the general population.

Despite the many reasons why screening for L. pneumophila types 1 to 6 has limited utility, we continue to offer this testing because of clinician demand for detection of Legionella serotypes other than type 1. Since neither the Wampole nor the Zeus assay was found to be an acceptable screening assay and since the commercial antigen is no longer available, the Legionella type 1 to 6 IgG IFA appears to be a better screen for Legionella infections. As other commercial assays or Legionella antigen sources become available, repeat evaluation of the Zeus and Wampole assays, along with the MARDX IFA slide, may help determine if the assay or antigen serogroup combination is acceptable in a screening assay. Laboratories attempting to replace an IFA type 1 to 6 screen with an alternative ELISA should carefully investigate the sensitivity of the replacement assay.

REFERENCES