CHAPTER SIX

Diagnosis of *Legionella* infection in Legionnaires' disease

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ABSTRACT

Since 1977, the diagnostic tools for Legionnaires' disease have been culture and serological investigation. Both methods require considerable time to produce results and have low to reasonable sensitivity. Since the introduction of urinary antigen tests in the mid 1990s, underdiagnosis has diminished and mortality has declined, thanks to early diagnosis. To obtain the most accurate diagnosis, culture, serological investigation, and urinary antigen testing should all be performed. In the last decade, much effort has been directed toward the development of assays detecting *Legionella* nucleic acid. Thus far, only widely varying results with small patient series have been reported. Furthermore, these assays are labour intensive and complicated. As a result, these assays are not yet suitable for the average medical microbiological laboratory.

INTRODUCTION

*Legionella pneumophila* was discovered as the causative agent of Legionnaires' disease in 1977 (1). The discovery led to the description of a new genus: *Legionella*, the sole member of the family *Legionellaceae* (2). In later years, it became clear that *Legionella* spp. were responsible for 2-5% of the cases of community-acquired pneumonia (3, 4). Thus far, 48 species and 70 serogroups have been distinguished (5, 6). *Legionella pneumophila* consists of 15 serogroups, of which serogroup 1 is the most common, followed by serogroups 4 and 6. In the USA, 91% of isolates from Legionnaires' disease patients are typed as *Legionella pneumophila* serogroup 1 (4). This is in contrast to the situation in Australia and New Zealand, where 30% of the cases of Legionnaires' disease are caused by *Legionella longbeachae* (7). In this review, we describe various microbiological methods used in the diagnosis of Legionnaires' disease: culture, serological investigation, detection of urinary antigen, and detection of *Legionella* DNA. We chose to describe tests that could be used in an average-sized hospital-based
laboratory of medical microbiology. Only tests with a specificity of at least 99% are described, which is a prerequisite, given the relatively low incidence of Legionnaires' disease (8). Ideally, the test specificity was studied in a population of patients with pneumonia caused by pathogens other than Legionella spp. We also focus on several clinically relevant aspects of these methods: availability of clinical specimens, usefulness of the methods early in the course of disease, lag time to available results, and test sensitivity.

CULTURE

In 1977, bacilli were isolated from guinea pigs that had been infected with lung specimens of Legionnaires' disease patients collected on autopsy. In addition, bacilli were isolated by successive passage in embryonated eggs. In both instances, the Giminez method of staining had been used (1). Subsequently, the bacilli were grown on supplemented Mueller-Hinton agar (9). Later, a charcoal yeast extract agar supplemented with L-cysteine and ferric pyrophosphate was developed (10). The addition of N-(2-acetamido)-2-aminoethanesulfonic acid (11), alpha-ketoglutarate, and semiselective antibiotics (12) has led to the current type of medium in use, which is enriched with buffered charcoal yeast extract (BCYE) and is available commercially.

Specimens used for culture of Legionella spp. include sputum or bronchoalveolar lavage specimens, bronchial aspirates, lung biopsy specimens, and blood. Several techniques have been developed to enhance the sensitivity of culture. Among them are preheating steps and acid washing procedures, which reduce overgrowth by other microorganisms (13, 14). Isolation of Legionella spp., which has a specificity of 100%, is the gold standard for diagnosis of Legionnaires' disease. However, according to experts, isolation of Legionella spp. from respiratory secretions is not a very sensitive diagnostic test (25-75%) (8, 15). This is mostly due to inexperienced laboratory personnel, as evidenced by the finding that two-thirds of otherwise well-qualified clinical microbiology laboratories in the USA in 1989 were unable to grow a pure Legionella culture (16).

Table 1 shows different sensitivities of culture found in several published studies. Apart from low sensitivity, culture has the disadvantage of delay, because a positive result is not available until after 3 or more days of incubation. Furthermore, 25-78% of patients with Legionnaires' disease have a nonproductive cough (16), which excludes a culture-based diagnosis unless invasive methods are used to obtain clinical specimens. Regarding this aspect, it is important to mention that sputum specimens that contain many squamous epithelial cells or few polymorphonuclear leukocytes normally are rejected by microbiological laboratories, even though they may contain culturable Legionellae (17). The abovementioned drawbacks for isolation of Legionella spp. have spurred the development and use of easy-to-perform urinary antigen tests, which are now the
mainstay of diagnosis in Europe (18). The decrease in culture-based diagnosis in the last decade, due to the introduction of urinary antigen tests, limits the recognition of non-\textit{Legionella pneumophila} serogroup 1 disease and impairs the investigation of outbreaks because fewer \textit{Legionella} isolates are provided for further examination (19).

For species other than \textit{Legionella pneumophila}, culture remains, for the time being, the mainstay of microbiological diagnosis. It is important to know that these species may grow at a slower rate and may therefore be detectable only after 10 days (20). The addition of albumin to the BCYE may be necessary to obtain detectable growth (21), since commercially available BCYE agars may not support the growth of non-pneumophila species (22).

**SEEROLOGICAL INVESTIGATION**

The first serological test to identify antibodies against \textit{Legionella pneumophila} was the indirect immunofluorescent antibody test (IFAT), developed by the Centers for Disease Control (CDC) in Atlanta in 1977 (I). Live, yolksac grown organisms were used as antigen in the initial test. The CDC later changed to an ether-killed and, subsequently, to a heat-killed preparation for the IFAT antigen. Each of these assays showed different test characteristics and cutoff values.

Several simpler tests were developed as well. Among them was a microagglutination test (MAT) developed in 1978 (23), followed by a rapid version in 1982 (24). Additionally, numerous (mostly experimental) ELISA assays based on several different antigen-extraction methods have been developed (23, 25-28).

The reported sensitivities of these assays vary substantially, from 41 to 75\% (Table 2). This variation may be due to differences in the antigen preparation, the reference strain used, or the valence (mono- or polyvalent) of the antigen used; cross-reactions; differences in the ability of the assay to detect IgM (29), IgG, or both classes of antibodies (30); and differences in the study population. Apart from these differences, the few studies on the development of antibodies over time show consistent results. Few patients develop antibodies against \textit{Legionella} spp. early in the course of disease. Although 80\% of diagnostic titers were seen within 4 weeks after the onset of disease, seroconversion after 2 months or more has been reported. Moreover, antibodies were still detectable 48 months after disease onset in 33\% of patients (31). This observation implies that single high or standing titers detected in sera from patients with pneumonia may be the result of a past infection with \textit{Legionella} spp. Unfortunately, determining the class of antibodies is of no help in differentiating between acute and past infection. In some studies, IgM antibodies are found predominantly early in the course of the disease (32). In other studies, there is a mix of IgM and IgG (33). Furthermore, patients have been described in whom only IgG was demonstrated in serum (34).
Table 1: Sensitivity of culture of *Legionella* spp. in patients with Legionnaires' disease (LD)

<table>
<thead>
<tr>
<th>Gold standard</th>
<th>Sensitivity</th>
<th>Patient material cultured</th>
<th>No.</th>
<th>Population</th>
<th>Reference</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive DFA test or seroconversion</td>
<td>28%</td>
<td>Respiratory secretions, lung tissue</td>
<td>29</td>
<td>Prospective inclusion of hospitalized LD patients</td>
<td>(71)</td>
<td>1980</td>
</tr>
<tr>
<td>Positive DFA test</td>
<td>45%</td>
<td>Respiratory secretions, lung tissue</td>
<td>56</td>
<td>Prospective inclusion of notified sporadic LD patients</td>
<td>(72)</td>
<td>1981</td>
</tr>
<tr>
<td>Fourfold rise in titer or positive UAg or DFA test</td>
<td>11% (compared with rise in titer); 59% (compared with UAg or DFA test)</td>
<td>Sputum; sputum or lung autopsy material</td>
<td>27</td>
<td>Prospective inclusion of hospitalized LD patients</td>
<td>(46)</td>
<td>1990</td>
</tr>
<tr>
<td>Positive DFA or UAg test</td>
<td>18%</td>
<td>Sputum</td>
<td>11</td>
<td>Prospective inclusion of hospitalized LD patients</td>
<td>(65)</td>
<td>1994</td>
</tr>
</tbody>
</table>

DFA, direct fluorescent antibody (immunofluorescent microscopy); UAg, urinary antigen

Table 2: Sensitivity of IFAT, MAT, and ELISA assays in patients with Legionnaires' disease (LD)

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Antigen preparation</th>
<th>Gold standard</th>
<th>Sensitivity</th>
<th>No.</th>
<th>Population</th>
<th>Reference</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFAT</td>
<td>Whole cell</td>
<td>Epidemic criteria</td>
<td>46%</td>
<td>136</td>
<td>Patients from an outbreak of community-acquired LD</td>
<td>(1)</td>
<td>1977</td>
</tr>
<tr>
<td>IFAT</td>
<td>Formolized yolk sac antigen</td>
<td>DFA or culture</td>
<td>60%</td>
<td>119</td>
<td>Selected sample of LD patients</td>
<td>(24)</td>
<td>1987</td>
</tr>
<tr>
<td>MAT</td>
<td>Formalin-killed suspension</td>
<td>DFA or culture</td>
<td>63%</td>
<td>119</td>
<td>Selected sample of LD patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>EDTA</td>
<td>IFAT</td>
<td>IgM: 58%; IgG: 41%</td>
<td>11</td>
<td>Selected sample of LD patients</td>
<td>(25)</td>
<td>1982</td>
</tr>
<tr>
<td>ELISA</td>
<td>LPS</td>
<td>Culture or MAT</td>
<td>IgM: 75%; IgG: 70%</td>
<td>32</td>
<td>Selected sample of LD patients</td>
<td>(73)</td>
<td>1994</td>
</tr>
</tbody>
</table>

IFAT, immunofluorescent antibody test; MAT, microagglutination test; ELISA, enzyme-linked immunofluorescent assay; EDTA, ethylene-diamino-tetra-acetate; LPS, lipopolysaccharide

Low titers of antibodies against *Legionella* spp. have been found in healthy volunteers, (35-37), blood donors, outpatients, and hospitalized patients (24, 38-40). Such titers seem to indicate previous exposure to *Legionella* spp. (41). Subclinical seroconversion is known to occur sporadically or during outbreaks (42).
The characteristics of the first urinary antigen tests were published in the late 1970s (43). Since then, numerous publications have followed, each describing a different antigen detection technique and together providing evidence that confirms the value of urinary antigen detection for the diagnosis of Legionnaires' disease (44-52). The urinary antigen test appeared to give positive test results 1-3 days after the onset of disease, while a small proportion of patients remained positive for almost a year (53). The most important feature of the assay appeared to be its >99% specificity, which is a requirement when testing for a relatively rare disease. Furthermore, a moderate-to-high sensitivity for *Legionella pneumophila* infections was demonstrated, ranging from 56 to 99% (Table 3). All studies showed a higher sensitivity for *Legionella pneumophila* serogroup 1. The differences in sensitivities found were attributed initially to differences in the proportion of clinical material from pneumonia patients with infection caused by *Legionella pneumophila* serogroup 1 versus infection caused by other serogroups. Moreover, variation was attributed to differences in the type of patient material used. This is especially noteworthy since only two prospective studies have been published (46, 48). When the use of urinary antigen tests was evaluated in an outbreak situation, the sensitivity appeared to be associated with the clinical severity of the disease (54). While 40-53% of the urinary antigen tests were positive in Legionnaires' disease patients with mild disease, 88-100% of the tests were positive in patients with severe Legionnaires' disease. Another association between sensitivity and defined subpopulations was demonstrated in patients with travel-associated, community-acquired, and nosocomial Legionnaires' disease: sensitivities in these groups were 94%, 76-87%, and 44-46%, respectively (55). In fact, the observed differences reflect a tendency of the urinary antigen assays to detect mainly monoclonal antibody (Mab)-3/1- and Mab-2-positive strains, which are the predominant strains in travel-associated Legionnaires' disease. This finding points out one of the limitations of diagnosis of Legionnaires' disease by urinary antigen testing (56). Another important limitation is the low sensitivity of urinary antigen tests to detect serogroups other than *Legionella pneumophila* serogroup 1. Different assays have been evaluated, with the sensitivity for detection of other serogroups ranging from 14 to 69% (56-59).

DETECTION OF *LEGIONELLA* NUCLEIC ACID

Potentially, an assay to detect *Legionella* nucleic acid could detect all known *Legionella* species. Various PCR tests have been developed which aim at different parts of the genome: the macrophage infectivity potentiator (*mip*) gene, the 5S ribosomal DNA gene, the 16S ribosomal DNA gene, the 23S-5S spacer (Table 4).
Table 3. Overview of the sensitivity of the urinary antigen test in patients with Legionnaires' disease (LD)

<table>
<thead>
<tr>
<th>Test</th>
<th>Gold standard</th>
<th>Serogroups</th>
<th>No.</th>
<th>Study population</th>
<th>Sensitivity</th>
<th>Ref</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>Positive culture</td>
<td>1, 4, 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23</td>
<td>Retrospective sample of hospitalized LD patients</td>
<td>57%</td>
<td>(50)</td>
<td>1998</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in IFAT titer</td>
<td>1, 3, 8, 12</td>
<td>120</td>
<td>Selected sample of LD patients</td>
<td>77%</td>
<td>(47)</td>
<td>1990</td>
</tr>
<tr>
<td>EIA</td>
<td>Culture</td>
<td>1</td>
<td>51</td>
<td>Selected sample of LD patients</td>
<td>84%</td>
<td>(47)</td>
<td>1990</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in IFAT titer or single high titer</td>
<td>1, 4, 6</td>
<td>27</td>
<td>Prospectively included hospitalized LD patients</td>
<td>70%</td>
<td>(46)</td>
<td>1990</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in IFAT titer or single high titer</td>
<td>1</td>
<td>20</td>
<td>Prospectively included hospitalized LD patients</td>
<td>84%</td>
<td>(46)</td>
<td>1990</td>
</tr>
<tr>
<td>RIA</td>
<td>Positive culture or 4-fold rise in IFAT titer</td>
<td>1, 3, 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68</td>
<td>Prospectively included hospitalized LD patients</td>
<td>56%</td>
<td>(48)</td>
<td>1995</td>
</tr>
<tr>
<td>RIA</td>
<td>Positive culture</td>
<td>1</td>
<td>35</td>
<td>Prospectively included hospitalized LD patients</td>
<td>80%</td>
<td>(49)</td>
<td>1997</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in IFAT titer or single high titer</td>
<td>1</td>
<td>59</td>
<td>Selected sample of LD patients</td>
<td>78%</td>
<td>(49)</td>
<td>1997</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in IFAT titer</td>
<td>unknown</td>
<td>65</td>
<td>Selected sample of LD patients</td>
<td>64% (89%&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>(51)</td>
<td>1998</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in IFAT titer</td>
<td>unknown</td>
<td>59</td>
<td>Selected sample of LD patients</td>
<td>74% (92%&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>(52)</td>
<td>2001</td>
</tr>
<tr>
<td>ICT</td>
<td>Positive culture or 4-fold rise in IFAT titer</td>
<td>1, 2, 3, 4, 5, 6, 10</td>
<td>187</td>
<td>Selected sample of LD patients</td>
<td>80%</td>
<td>(53)</td>
<td>2001</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in IFAT titer</td>
<td>1, 2, 3, 4, 5, 6, 10</td>
<td>187</td>
<td>Selected sample of LD patients</td>
<td>79%</td>
<td>(53)</td>
<td>2001</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in IFAT titer</td>
<td>1, 2, 3, 4, 5, 6, 10</td>
<td>187</td>
<td>Selected sample of LD patients</td>
<td>83%</td>
<td>(53)</td>
<td>2001</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture, 4-fold rise in IFAT titer or single high titer, or other positive UAg test</td>
<td>1</td>
<td>167</td>
<td>Selected sample of LD patients</td>
<td>99%</td>
<td>(74)</td>
<td>2001</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in MAT titer or seroconversion in ELISA</td>
<td>1</td>
<td>58</td>
<td>LD patients of 1 outbreak</td>
<td>71% (74%&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>(54)</td>
<td>2002</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in MAT titer or seroconversion in ELISA</td>
<td>1</td>
<td>58</td>
<td>LD patients of 1 outbreak</td>
<td>69% (79%&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>(54)</td>
<td>2002</td>
</tr>
<tr>
<td>ICT</td>
<td>Positive culture or 4-fold rise in MAT titer or seroconversion in ELISA</td>
<td>1</td>
<td>58</td>
<td>LD patients of 1 outbreak</td>
<td>72% (81%&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>(54)</td>
<td>2002</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture or 4-fold rise in MAT titer or seroconversion in ELISA</td>
<td>1</td>
<td>58</td>
<td>LD patients of 1 outbreak</td>
<td>81%</td>
<td>(55)</td>
<td>2003</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture</td>
<td>1, 3, 4, 5, 6, 8, 10, 12, 13</td>
<td>317</td>
<td>Selected sample of LD patients</td>
<td>81%</td>
<td>(55)</td>
<td>2003</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive culture</td>
<td>1, 3, 4, 5, 6, 8, 10, 12, 13</td>
<td>317</td>
<td>Selected sample of LD patients</td>
<td>77%</td>
<td>(55)</td>
<td>2003</td>
</tr>
</tbody>
</table>

<sup>a</sup>Including cases of *non-pneumophila* LD

<sup>b</sup>After concentration of urine
<table>
<thead>
<tr>
<th>Gene targeted</th>
<th>Type of clinical specimen</th>
<th>Gold standard</th>
<th>Sensitivity</th>
<th>Study population</th>
<th>Control population</th>
<th>Reference Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>73S rRNA</td>
<td>BAL specimens</td>
<td>Positive culture</td>
<td>70%</td>
<td>Selected sample of LD patients</td>
<td>Panel of control strains</td>
<td>(75) 1987</td>
</tr>
<tr>
<td>Mip</td>
<td>BAL specimens</td>
<td>DFA, positive culture, serology</td>
<td>11%</td>
<td>Selected sample of LD patients</td>
<td>Panel of control strains</td>
<td>(75) 1987</td>
</tr>
<tr>
<td>Mip</td>
<td>BAL specimens</td>
<td>DFA, positive culture, serology, urinary antigen test</td>
<td>72%</td>
<td>Selected sample of LD patients</td>
<td>Patients with pneumonia of other origin</td>
<td>(75) 1999</td>
</tr>
<tr>
<td>16S rDNA</td>
<td>BAL specimens</td>
<td>Culture</td>
<td>100%</td>
<td>Selected sample of LD patients</td>
<td>Panel of control strains</td>
<td>(75) 1997</td>
</tr>
<tr>
<td>5S rRNA</td>
<td>Urine</td>
<td>Culture, serology</td>
<td>69%</td>
<td>Selected sample of LD patients</td>
<td>Panel of control strains</td>
<td>(75) 1999</td>
</tr>
<tr>
<td>5S rRNA</td>
<td>BAL specimens</td>
<td>Culture</td>
<td>100%</td>
<td>Selected sample of LD patients</td>
<td>Panel of control strains</td>
<td>(75) 1999</td>
</tr>
<tr>
<td>Mip</td>
<td>Biopsy specimens</td>
<td>Culture</td>
<td>17%</td>
<td>Selected sample of LD patients</td>
<td>Panel of control strains</td>
<td>(75) 1999</td>
</tr>
<tr>
<td>16S rDNA</td>
<td>BAL specimens</td>
<td>Culture</td>
<td>100%</td>
<td>Selected sample of LD patients</td>
<td>Panel of control strains</td>
<td>(75) 1997</td>
</tr>
<tr>
<td>Mip</td>
<td>Sputum</td>
<td>Culture</td>
<td>94%</td>
<td>Selected sample of LD patients</td>
<td>Panel of control strains</td>
<td>(75) 1997</td>
</tr>
</tbody>
</table>
The *mip* gene is genus specific, whereas the 16S and 5S rRNA genes are specific for *Legionella pneumophila*. Few laboratories use PCR for the diagnosis of Legionnaires' disease. The sensitivity of the test varies from 11 to 100%, and many publications report specificities of lower than 99%. The PCR assay can be performed on different specimens: BAL samples, pharyngeal swabs, nasopharyngeal swabs, peripheral blood mononuclear cells, urine, and serum (60-67). The most recent publications on realtime PCR are promising, reporting high sensitivities and specificities (68-70). However, in order for PCR to become established as a reliable diagnostic tool in Legionnaires' disease, the reproducible specificity needs to rise to values higher than 99%.

**COMBINING DIAGNOSTIC TOOLS TO ACHIEVE OPTIMAL SENSITIVITY**

Clinically, there are no clear clues to differentiate Legionnaires' disease from other types of pneumonia. However, early diagnosis of Legionnaires' disease is associated with lower mortality as a result of timely administration of antibiotics to which *Legionella* spp. are sensitive. From the discovery of the organism to the mid 1990s, culture and serological investigation were the only diagnostic tools available at the average medical microbiological laboratory. The low sensitivity of culture and the late seroconversion in many patients undoubtedly led to underdiagnosis during that period. Furthermore, both methods required from several days to many weeks before a diagnostic result was obtained. The availability of commercial urinary antigen tests in the 1990s led not only to more Legionnaires' disease patients being diagnosed but also to a lower mortality, possibly as a result of obtaining a diagnosis earlier in the course of disease. Still, the overall sensitivity of the urinary antigen test is only 70% for cases of pneumonia caused by *Legionella pneumophila* serogroup 1. For other serogroups, this figure is much lower, which means that, at present, optimal sensitivity for diagnosis of Legionnaires' disease will be achieved by using a combination of culture, serological investigation, and urinary antigen detection. For public health purposes, both culture and serological investigation remain important diagnostic tools.

In the future, it is likely that an easy-to-perform PCR test with high sensitivity and a specificity above 99% will become available on a wider scale. It would not be surprising if such a test eventually becomes accepted as the new gold standard for diagnosis of Legionnaires' disease.

**REFERENCES**


