Laboratory diagnosis of legionnaires’ disease due to * Legionella pneumophila* serogroup 1: comparison of phenotypic and genotypic methods

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Laboratory results of 67 cases of legionnaires’ disease caused by * Legionella pneumophila* serogroup (Sg) 1 spanning a 6-year period were analysed by both phenotypic and genotypic methods. The methods compared were urinary antigen enzyme immunoassay (EIA), an immunofluorescent antibody (IFA) test, direct fluorescent antibody (DFA), culture and a 5s rRNA PCR with Southern blotting confirmation. Urine was available in 53 cases, of which 35 (66 %) were positive, with an antigen peak observed at 5 – 10 days after onset of disease symptoms. The IFA test was positive in 62 (92.5 %) cases, with 56 (90.3 %) cases producing a greater than fourfold rise in titre and 6 (9.7 %) giving presumptive high titres of \( > 1 : 128 \). There were two antibody peaks, one at 10 – 15 days and another at > 25 days after onset. In 23 cases where samples were available, DFA and culture were respectively positive in 5 (22 %) and 10 (48 %) cases. There was a peak in culture-positives 5 – 10 days after onset of disease. A * Legionella*-specific 5s rRNA PCR on patient serum was positive in 54 (80.5 %) cases, with a peak in PCR positivity at 6 – 10 days after disease onset. In 22 of the 67 cases, the full panel of diagnostic methods was available for comparison. The relative sensitivity and specificity of the urinary antigen EIA and the serum PCR was 100 %. The IFA gave relative sensitivity and specificity values of 93.8 and 95 %. DFA and culture, although 100 % specific, produced only low sensitivities, of 19 and 42.8 %, respectively. This study has shown that urinary antigen and serum PCR are valuable tests in the acute phase of disease, with excellent sensitivity and specificity values. At present, the * Legionella* species causing infection requires to be verified by IFA serology and/or culture, but this could become unnecessary as new antigen and * L. pneumophila* Sg 1-specific PCR tests become available.

INTRODUCTION

The majority of cases of legionnaires’ disease (LD) in Europe are caused by * Legionella pneumophila* serogroup (Sg) 1 (Joseph, 2002). However, diagnosis of LD can be difficult, as clinical features are often indistinguishable from other causes of pneumonia. There are a number of laboratory-based methods, both phenotypic and genotypic, to help in diagnosis.

Urinary antigen detection, serum antibody titration and culture are the phenotypic methods currently used. Urinary antigen is detected in the acute phase of disease by an enzyme immunoassay (EIA) and has become the favoured method of laboratory diagnosis. However, a recent report of two commercially available EIA kits (Binax and Biotest) showed a significantly reduced sensitivity in nosocomial cases (Helbig et al., 2003). Serology involves titration of a * Legionella*-specific antibody response by an immunofluorescent antigen (IFA) test or an ELISA-based test, but serological cross-reactions with other micro-organisms have been reported (Wilkinson et al., 1981; Fallon & Abraham, 1992; Musso & Raoult, 1997). However, the isolation of the * Legionella* organism from an ill patient is still seen as the gold standard in case definition (McDade et al., 1977).

Genotypic methods utilize PCR to detect the presence of * Legionella*-specific DNA in respiratory secretions (Jaulhac et al., 1992; Cloud et al., 2000), urine (Maiwald et al., 1995) and serum (Lindsay et al., 1994; Murdoch & Chambers, 2000), and show varying degrees of sensitivity and specificity.

Abbreviations: DFA, direct fluorescent antibody; EIA, enzyme immunoassay; IFA, immunofluorescent antibody; LD, legionnaires’ disease.
Currently, a confirmed case of LD, as defined by the European Working Group on Legionella Infections (EWGLI), is either a positive culture or positive urinary antigen or greater than fourfold rise in titre to \textit{L. pneumophila} Sg 1. A positive PCR and single high titre of $\geq 1:128$ are only presumptive of a case of LD.

In this study, we looked at 67 cases of LD caused by \textit{L. pneumophila} Sg 1 over a 6-year period and compared the phenotypic and genotypic methods used in the laboratory for the diagnosis of LD.

**METHODS**

**Study population.** The patients were 67 adults (48 males and 19 females) between the ages of 30 and 80 (mean age 55.7 years), and all were laboratory-confirmed cases of LD as defined by EWGLI (http://www.ewgli.org). Of the 67 cases, 69\% were travel associated, 10\% were community acquired, 7\% were nosocomial and in 14\% a possible route of infection was difficult to ascertain and was classified unknown.

**Phenotypic methods.** The urinary antigen was detected by a variety of methods over the 6-year period, including an in-house ELISA and the Binax and Biotest EIA kits. The commercial assays were used according to the manufacturers’ instructions. The in-house urinary antigen ELISA, serology IFA, direct fluorescent antibody (DFA) test and culture were performed as described previously (Fallon, 1981; Wilkinson et al., 1981; Birtles et al., 1990). All the phenotypic methods were performed in duplicate. Culture was performed on respiratory secretions including bronchoalveolar lavage, tracheal aspirates, sputum and post-mortem lung samples. Samples of heated (56°C for 30 min) and unheated respiratory secretions were plated onto BCYE and BMPA media (Oxoid) and incubated at 37°C for up to 10 days.

**Genotypic methods.** DNA was extracted from 200 μl patient serum with Biogene nucleospin blood columns (Clontech). A positive control was prepared by heating a suspension of \textit{L. pneumophila} Sg 1 ATCC 33152$^\text{t}$ to 100°C for 10 min and then freezing. The resulting supernatant was used as a positive control in the 5S rRNA PCR at a concentration of 100 pg ml$^{-1}$. The DNA was amplified in a control PCR to determine the efficiency of the DNA extraction procedure. The \textit{β}-globin gene was amplified using two 20-mer primers ($5’$-CAACTT CATTCCAGTTTACCC-3’ and $5’$-GAAGAGCCAAGGACAGTAC-3’). The \textit{β}-globin gene product is 140 bp long. The presence of the \textit{β}-globin gene was an indication that the DNA extraction was successful and that there were no major inhibitors present in the DNA sample. Only \textit{β}-globin PCR-positive samples were tested in the 5S rRNA PCR. A Legionella 5S rRNA-specific PCR was performed, as described previously (Lindsay et al., 2002), on all patient sera from the 67 cases. Briefly, the serum DNA was added to a standard PCR mixture containing 20 mM Tris/HCl, pH 8.3, 100 mM KCl, 2.5 mM MgCl$_2$, 500 ng of each primer ($5’$-ACTATAGCGATTTGGAACCA-3’ and $5’$-GGCATGACCTACTTTGCGCAT-3’), 0.25 mM dNTPs and 1.25 U Taq polymerase. The tubes were subjected to 35 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s (Hybaid Omnigene thermocycler). The specificity of the PCR was confirmed by Southern blotting with a 50-mer digoxigenin-labelled \textit{Legionella}-specific probe ($5’$-CTCGAATCTCA GAAGTCACATTTCGGCCGCAATGTAGTGAGCCTTC-3’).

**RESULTS AND DISCUSSION**

**Legionella antigen in urine**

A total of 69 urine samples were available from 53 cases, of which 35 (66\%) were positive in at least one sample by urinary antigen ELISA (Table 1). Over the 6-year period, three ELISA assays were used. The in-house assay detected only mAb 3/1 (Dresden panel) strains (Philadelphia, Knoxville, Allen-town, Benidorm and France) of \textit{L. pneumophila} Sg 1. The Binax and Biotest ELISA detect all subgroups of \textit{L. pneumophila} Sg 1, although they are optimal for mAb 3/1 strains and less sensitive at detecting non-mAb 3/1 strains (Camperdown, Oxford, Heysham, Bellingham and OLDA). They also detect other \textit{L. pneumophila} serogroups and \textit{Legionella} species to varying degrees (Domínguez et al., 1998). When urinary antigen results and date of onset were compared, an antigen peak was found at 5–10 days after onset of disease symptoms (Fig. 1). The antigen peak diminished rapidly after this time, and was barely detectable 21 days after onset.

In recent years, urine has become the main sample of choice for the laboratory diagnosis of LD, as it is produced in the acute phase of disease. However, as this and other studies have shown, relying solely on urinary antigen can lead to missed diagnoses (Helbig et al., 2003; Murdoch, 2003a). The availability, timing of urine collection and \textit{Legionella} species present can all affect the outcome of these tests. In a recent study comparing Binax and Biotest kits, sensitivity and specificity were respectively 94 and 95\% in travel-associated cases, where the majority of cases were mAb 3/1 \textit{L. pneumo- phila} Sg 1. However, this dropped to 44 and 46\% for

**Table 1. Laboratory results of 67 cases of LD caused by \textit{L. pneumophila} Sg 1**

<table>
<thead>
<tr>
<th>Test</th>
<th>Cases (n)*</th>
<th>Samples tested (n)</th>
<th>Positive cases (n) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary antigen ELISA</td>
<td>53</td>
<td>69</td>
<td>35 [66]</td>
</tr>
<tr>
<td>IFA serology</td>
<td>67</td>
<td>154</td>
<td>62 [92.5]*</td>
</tr>
<tr>
<td>DFA</td>
<td>23</td>
<td>30</td>
<td>5 [22]</td>
</tr>
<tr>
<td>Culture</td>
<td>23</td>
<td>30</td>
<td>10 [48]</td>
</tr>
<tr>
<td>Serum PCR/Southern blotting</td>
<td>67</td>
<td>154</td>
<td>54 [80.5]</td>
</tr>
<tr>
<td>Respiratory PCR/Southern blotting</td>
<td>10</td>
<td>15</td>
<td>8 [80]</td>
</tr>
</tbody>
</table>

*Number of cases where samples were available for testing.

†Comprised 56 cases with a greater than fourfold rise in titre and six cases with a single high titre.
nosocomially acquired infections, where over 50 % of cases were attributed to non-mAb 3/1 strains or non-Sg 1 (Helbig et al., 2003). As these tests detect other \( L. \) pneumophila serogroups and species, a positive urinary antigen should be verified by culture or convalescent serology to identify the species and/or serogroup.

**Antibody response**

Serology was available on 154 sera from all 67 cases and tested by a validated IFA method for \( L. \) pneumophila Sg 1 (Wilkinson et al., 1981). In total, 62 (92.5 %) cases produced a positive serological response, with 56 (90.3 %) showing a greater than fourfold rise in titre to \( L. \) pneumophila Sg 1 and 6 cases (9.7 %) producing a single high titre (Table 1). The IFA serology results showed two peaks of antibody activity (Fig. 1). The first peak was at 11–15 days post-onset and a second occurred at >25 days after onset of disease. Only six cases were IFA negative.

A fourfold rise is considered to be diagnostic for a case of legionellosis. A single high titre is presumptive. However, in the six cases with a single high titre, the diagnosis was confirmed by either urinary antigen or culture. The peaks of antibody activity we describe probably reflect the IgM and IgG peak responses to the \( L. \) pneumophila antigen. The IFA test used a swine anti-human pan-immunoglobulin, containing IgM and IgG (Nordic Immunologicals), to detect any Legionella-specific antibodies in serum. The negative cases may represent the small number of LD cases described previously in immunocompromised and immunocompetent patients where no antibody response was produced or detected (Harrison et al., 1987; McWhinney et al., 2000).

**DFA and culture**

Only 23 of the 67 cases produced samples suitable for culture (Table 1). Of the 23, 5 (22 %) were DFA positive. This agreed with a previous report that stated that DFA had a broad sensitivity range (25–70 %) when compared with culture (Edelstein, 1993). Of the 23 cases, 10 (48 %) were culture positive. The monoclonal subtypes of \( L. \) pneumophila Sg 1 identified were Benidorm \( (n = 5) \), Philadelphia \( (2) \), Knoxville \( (2) \) and OLDA \( (1) \). This breakdown of culture subtypes followed a Europe-wide trend, as shown in a recent study of culture-confirmed cases of LD; mAb 3/1-positive strains accounted for 66.8 % of all the \( L. \) pneumophila Sg 1 strains isolated and mAb 3/1-negative (including OLDA) accounted for a much lower percentage (11.7 %). In 44 cases, no samples were available for culture, which equates to 66 % of the 67 cases.

Isolation is still very important in epidemiological studies and should be encouraged in order to identify the source of infection. Unfortunately, as previous studies have shown, more than 50 % of LD patients produce no suitable specimens for culture (Lieberman et al., 1996). In this study, if a specimen was available, our recovery rate was 48 %. Other studies have estimated sensitivity of between < 10 and 80 % (Sopena et al., 1998; Chambers et al., 1999). Recovery rates are affected by specimen availability, timing of collection and previous antibiotic therapy. Fig. 1 shows the best time to collect samples for culture, i.e. 5–10 days after disease onset. A recent review reported that culture rates were higher when bronchoscopic specimens were used rather than sputum and the samples were collected in the acute phase of disease (Murdoch, 2003a). However, in the future, with the availability of sequence-based typing, there may be no requirement for the actual culture of the organism, as species identification could be done from DNA in blood, urine or respiratory secretions.

**5S rRNA PCR and Southern blotting of patient serum**

A total of 54 (80.5 %) cases were serum PCR positive in at least one specimen from each case. Only 13 (19.5 %) cases were PCR negative in all samples. All PCRs were verified by Southern blotting, as the 5S rRNA primers have been shown to amplify DNA from other organisms (Maiwald et al., 1995). The Legionella-specific probe used in Southern blotting was checked against all known gene sequences in GenBank and found to have only 20 % similarity to sequences from organisms other than the Legionellaceae. In addition, a total of 100 sera and over 500 routine respiratory secretions that showed no laboratory or clinical evidence of Legionella infection was tested previously to determine the specificity of the 5S rRNA PCR/Southern blotting technique. All non-Legionella samples were negative after PCR/Southern blotting (Lindsay et al., 2002). The relationship between date of onset of disease and PCR reactivity was compared (Fig. 1). The results showed a peak in Legionella-specific DNA in the blood, 6–10 days after onset of disease symptoms. This peak
corresponded with the urinary antigen peak, which suggests that circulating DNA coincides with the urinary antigen filtered from the blood by the kidneys. However, unlike urinary antigen, the number of PCR positives decreased very slowly and Legionella-specific DNA was still detectable at >25 days after onset of disease symptoms, perhaps as a result of the slow release of DNA from Legionella surviving in macrophages. In this study, the overall sensitivity of the 5S rRNA PCR was 63 % of the total sera tested. Legionella-specific DNA has previously been detected in serum, but with low sensitivities of 30 and 43 % (Murdock et al., 1996; Matsiota-Bernard et al., 1997). As seen in this study and others (reviewed by Murdoch, 2003b), these sensitivity levels can be increased if samples are obtained early in the course of the illness and when multiple samples are tested.

Comparison of phenotypic and genotypic techniques

In 22 of the 67 cases, the full panel of diagnostic methods was available for comparison of relative sensitivity and specificity of each technique. Relative specificity was calculated from a group of samples sent to the laboratory as part of atypical pneumonia screens. The relative specificity and sensitivity of the urinary antigen EIA and the serum PCR was 100 % (Table 2). The relative sensitivity and specificity of the IFA were respectively 93-8 and 95 %. The DFA and culture, although 100 % specific, only produced low relative sensitivities, of 19 and 42-8 %, respectively (Table 2). This study has shown that urinary antigen and serum PCR are valuable tests in the acute phase of disease, with excellent sensitivity and specificity scores. However, at present, the Legionella species causing infection needs to be verified by convalescent IFA serology or culture. In this study, PCR was more sensitive than culture, and we are currently devising a 16S rRNA PCR-ELISA that is specific for L. pneumophila Sg 1. This would eliminate the need for convalescent antibody or culture verification and may, in time, allow PCR to become confirmatory instead of just presumptive of a case of LD.

Table 2. Relative sensitivity and specificity of each diagnostic method (from 22 of the 67 cases)

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive (n) [%]</th>
<th>Relative sensitivity (%)*</th>
<th>Relative specificity (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary antigen EIA</td>
<td>17 [77:2]</td>
<td>100 [17/17]</td>
<td>100 [20/20]</td>
</tr>
</tbody>
</table>

*Values in square brackets are no. positive/no. positive in two or more other assays.
†Values in square brackets are no. negative/no. negative in three other assays.

REFERENCES


