Detection of Legionella DNA by PCR of whole-blood samples in a mouse model

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INTRODUCTION

Legionella pneumophila is one of the leading causes of bacterial pneumonia, particularly in susceptible individuals or the immunocompromised (El-Solh et al., 1997; Marston et al., 1997; Sepeña et al., 1999). Legionella pneumonia is not always easy to diagnose, since clinical and radiographic features are often indistinguishable from those of pneumonias caused by other pathogens. Although serological examination has been one of the methods traditionally used for the diagnosis of Legionella pneumonia, paired sera are usually required. Moreover, as many as 25 % of patients with Legionella pneumonia may fail to exhibit diagnostic antibody titres (Harrison & Taylor, 1988). Culture of sputum or other respiratory samples such as a transtracheal aspirate (TTA) is another traditional method for the detection of Legionella species, reported to be specific and considered the ‘gold standard’ test (Yu, 1995). However, the sensitivity of the culture method has been reported to be as low as 10–60 % (Breiman & Butler, 1998; Waterer et al., 2001). In addition, it is frequently difficult to obtain respiratory samples from patients with Legionella pneumonia, since most patients have a non-productive cough; physicians frequently need to obtain samples by invasive methods such as TTA or bronchoalveolar lavage (BAL). Furthermore, culture often fails to isolate the pathogens when patients have already been treated with antibiotics, even when such agents are not clinically potent.

Recently, enzyme immunoassay (EIA) for the detection of Legionella antigen in urine has been used for diagnosis of Legionella pneumonia, with a reported sensitivity of 63–77 % (Benson et al., 2000; Dominguez et al., 1998; Stout & Yu, 1997). PCR has also been used as a rapid diagnostic method, employing samples of BAL fluid (BALF) samples, cultures of blood and BALF and detection of Legionella urinary antigen. Blood PCR was positive until 8 days after infection, while BALF PCR became negative on day 4. These results indicate that PCR using blood samples may be a useful, convenient and non-invasive method for the diagnosis of Legionella pneumonia.

METHODS

Bacteria. L. pneumophila serogroup 1 ATCC 33152T (Philadelphia 1T) was used for the animal experiments and for assessment of the sensitivity of PCR detection. The bacterial strains used for evaluation of specificity of PCR detection are summarized in Table 1.

Bacterial inoculum. L. pneumophila ATCC 33152T was stored until use at –80 °C in Muller–Hinton broth containing 30 % glycerol. A portion of the stock was cultured on buffered charcoal yeast extract (BCYE)-α agar (Oxoid) for 4 days at 37 °C. A single colony grown on BCYE-α agar was inoculated into 5 ml buffered yeast extract (BYE)
Table 1. Bacterial strains used for examination of PCR specificity

<table>
<thead>
<tr>
<th>Strain</th>
<th>PCR result</th>
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</thead>
<tbody>
<tr>
<td>L. pneumophila ATCC 33152(^T) (serogroup 1)</td>
<td>+</td>
</tr>
<tr>
<td>L. pneumophila ATCC 33153 (serogroup 1)</td>
<td>+</td>
</tr>
<tr>
<td>L. pneumophila ATCC 33154 (serogroup 2)</td>
<td>+</td>
</tr>
<tr>
<td>L. pneumophila ATCC 33155 (serogroup 3)</td>
<td>+</td>
</tr>
<tr>
<td>L. pneumophila ATCC 33156 (serogroup 4)</td>
<td>+</td>
</tr>
<tr>
<td>L. pneumophila ATCC 33215 (serogroup 5)</td>
<td>+</td>
</tr>
<tr>
<td>L. pneumophila ATCC 33216 (serogroup 6)</td>
<td>+</td>
</tr>
<tr>
<td>Legionella micdadei ATCC 33218(^T)</td>
<td>+</td>
</tr>
<tr>
<td>Legionella baeumanae ATCC 33217(^T)</td>
<td>+</td>
</tr>
<tr>
<td>Legionella dumoffii ATCC 33229(^T)</td>
<td>+</td>
</tr>
<tr>
<td>Legionella longbeachae ATCC 33469 (serogroup 1)</td>
<td>+</td>
</tr>
<tr>
<td>L. longbeachae ATCC 33484 (serogroup 2)</td>
<td>+</td>
</tr>
<tr>
<td>Legionella gormanii ATCC 33227(^T)</td>
<td>+</td>
</tr>
<tr>
<td>Legionella jordanis ATCC 33623(^T)</td>
<td>+</td>
</tr>
<tr>
<td>Klebsiella pneumoniae (clinical isolate)</td>
<td>–</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa NUS10 (clinical isolate)</td>
<td>–</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae MAC</td>
<td>–</td>
</tr>
<tr>
<td>M. pneumoniae M129</td>
<td>–</td>
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<tr>
<td>M. pneumoniae FH</td>
<td>–</td>
</tr>
<tr>
<td>Chlamydia pneumoniae TW-183(^T)</td>
<td>–</td>
</tr>
<tr>
<td>C. pneumoniae KGpm-15</td>
<td>–</td>
</tr>
</tbody>
</table>

DNA extraction and PCR. DNA was extracted from blood samples with a Nucleo Spin blood kit (Clontech) and from BALF with a Nucleo Spin tissue kit (Clontech). Bacterial DNA was extracted with a DNeasy tissue kit (Qiagen). A 106-bp region of the Legionella 16S rRNA-encoding gene was amplified using the 20mer primers LSP-GCG-5\(^\prime\)sense, 5\(^\prime\)-GCCGCTACCGCGCCATTAC-3\(^\prime\); designed in this study) and Cpr3.2 (antisense, 5\(^\prime\)-CCCAACTAATGCTGATCG-3\(^\prime\); Jonas et al., 1995). PCR included initial denaturation at 94 °C for 4 min followed by 35 cycles of annealing at 56 °C for 1 min, extension at 72 °C for 1 min and denaturation at 94 °C for 1 min and a final step of extension for 10 min at 72 °C. For analysis of the PCR product, 2% agarose gel electrophoresis was performed with 10 μl of the reaction solution and the DNA fragment was confirmed using ethidium bromide staining.

Detection of Legionella antigen in urine. Legionella antigen in urine was detected with Legionella urinary antigen EIAs from Binx and Biostest AG.

RESULTS AND DISCUSSION

PCR sensitivity and specificity

We first confirmed that the primers used in our study could detect as little as 20 fg purified L. pneumophila DNA prepared in Tris/EDTA (Fig. 1). In another control study, it was possible to detect as little as 200 fg L. pneumophila DNA in 200 μl blood obtained from healthy volunteers. PCR assays using whole-blood samples have been considered difficult because of the presence of inhibitors in blood. PCR detection of Legionella in the serum or buffy coat has been reported previously (Aebischer et al., 1999; Lindsay et al., 1994; Murdoch & Chambers, 2000; Murdoch et al., 1996, 1999), but not in whole-blood samples. Recently, commercially available kits for DNA extraction have facilitated PCR on whole-blood samples. The PCR product was detected in L. pneumophila serogroups 1–6, Legionella longbeachae serogroups 1 and 2 and five other Legionella species tested. On the other hand, PCR products were not detected for seven other bacterial strains unrelated to Legionella (Table 1). PCR amplification using primers for the mip gene has been widely used in previous studies.

Figure 1. Sensitivity of the PCR. L. pneumophila serogroup 1 ATCC 33152\(^T\) DNA was purified and prepared in TE and 2 ng–2 fg was used for PCR. Lanes: M; molecular size marker; 1, 2 ng; 2, 200 pg; 3, 20 pg; 4, 2 pg; 5, 200 fg; 6, 20 fg; 7, 2 fg; 8, negative control (water).
used, since these primers can detect most *Legionella* species, with the exception of *Legionella stentoria* (Ratcliff et al., 1998; 2001). For our PCR assay, we chose primers that amplify 106 bp of the 16S rRNA gene, which may have certain advantages compared with methods reported previously. Firstly, amplification of the 16S rRNA gene may be more sensitive than that of the mip gene (Engleberg et al., 1989; Iwamoto et al., 1994) because multiple copies of the 16S rRNA gene exist in bacteria. Furthermore, amplification of 5S rRNA failed to detect several clinically relevant *Legionella* species, such as *Legionella jordanis* (Brieland et al., 1994). Our primers could detect most of the relevant species of *Legionella*, including *L. jordanis*, with high specificity.

**Features of experimentally induced pneumonia**

In previous studies (Brieland et al., 1994; Winn et al., 1982), animal models of *Legionella* pneumonia were established by incision of the trachea. In our mouse model of *Legionella* pneumonia, bacteria were inoculated intratracheally in a less invasive technique than those used previously. Therefore, the characteristics of infection in our mouse model resembled those of patients with *Legionella* pneumonia more closely than have other animal models. Acute pneumonia was confirmed by pathological examination of resected murine lungs stained with haematoxylin and eosin, as evident by cellular infiltration in alveoli and effusion. Body weight decreased gradually from 28.4 ± 1.0 g (mean ± SD) at baseline to 20.9 ± 1.5 g at day 4 after infection. However, body weight subsequently recovered gradually and was 24.4 ± 1.9 g on day 13. As expected, acute pneumonia was associated with leukocytosis (basal leukocyte count, 2250; day 5, 11 077 cells ml⁻¹), but the count decreased to 3090 cells ml⁻¹ on day 12 (Fig. 2). Our results were similar to those of Brieland et al. (1994) with regard to the severity of pneumonia. Their results in mice infected with *Legionella* showed that *L. pneumophila* grew exponentially in the lung during the 24–48 h post-inoculation and was gradually eliminated from the lungs during days 3–7 post-inoculation. Based on changes in body weight and leukocyte counts in our model, we concluded that pneumonia was most severe on days 3 to 5 after inoculation. In addition, the pathological changes in the lungs and inflammatory response were serious by 72 h after inoculation.

**Evaluation of diagnostic methods in a mouse model**

In the present study, we evaluated PCR on blood samples by comparing the results of this assay with the results of PCR on BALF samples, culture of blood and BALF samples and urinary antigen detection in a mouse model of *Legionella* pneumonia. Previous studies have reported the usefulness of PCR as a diagnostic test for *Legionella* pneumonia using respiratory specimens (Cloud et al., 2000; Hirakata et al., 1996; Jaulhac et al., 1998; Jonas et al., 1995; Lo Presti et al., 2000; Weir et al., 1998), with a sensitivity of >90% (Matsiota-Bernard et al., 1994). However, cough is non-productive in most patients with *Legionella* pneumonia, and clinical symptoms deteriorate rapidly and severely. Therefore, it is often difficult to collect respiratory specimens using invasive techniques such as BALF. Consequently, specimens that could be collected non-invasively might be preferable. *L. pneumophila* was isolated from cardiac blood samples obtained on days 1 and 2, but could not be detected on or after day 3. On the other hand, the pathogen was recovered from BALF on days 1, 2 and 3, but was not detected on or after day 4. PCR assays of BALF samples were positive only on days 1, 2 and 3 after inoculation, while blood PCR remained positive until day 8 after infection and became negative on day 9. *Legionella* antigen in urine was positive from days 1–12 (Fig. 2) and was detected continuously up to day 30 by both the Biotest and Binax EIA. Therefore, in clinical cases, in addition to urinary antigen detection, whole-blood PCR may allow the diagnosis of *Legionella* pneumonia during the first several days after onset of the disease.

We performed further experiments to examine the relationship between positive urinary antigen and illness (data not shown). In these studies, we first established an oral-administration *Legionella* model by intraoesophageal inoculation of

![Fig. 2. Serial changes in body weight (●) and leukocyte count (●) in a mouse model of Legionella pneumonia. A/J mice were inoculated intratracheally with *L. pneumophila* (10^7 c.f.u. per mouse) and body weight and peripheral leukocyte numbers were measured on the days indicated after infection. Data represent means ± SD. WBC, Whole-blood count; p.i., post-inoculation. Moreover, blood and BALF samples were cultured and used for PCR and urine samples were examined for Legionella antigen; results are shown.](http://jnm.sgmjournals.org)
In conclusion, in the present study, we have demonstrated that PCR gives the possibility of identifying antigen detection is that PCR gives the possibility of 
disease. Another advantage of PCR compared with urinary infections. These results, however, suggest that a person who 
drinks contaminated water may have a positive urinary antigen test even in the absence of clinical features of the 
disease. Another advantage of PCR compared with urinary antigen detection is that PCR gives the possibility of 
identifying Legionella to the species level in future by using the multiplex PCR method (Clark et al., 1993) or sequencing (Ratcliffe et al., 2001).

ACKNOWLEDGEMENTS

We thank Dr Naoyuki Miyashita, Department of Microbiology, Kawasaki Medical School, for supplying C. pneumoniae strains.

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