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

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Received 13 June 2002
Accepted 23 December 2002

A detection system for *Legionella* DNA in blood samples based on the PCR was developed and evaluated in A/J mice with experimentally induced *Legionella* pneumonia. Primers were designed to amplify a 106 bp DNA fragment of the 16S rRNA gene specific to *Legionella* species. The PCR system could detect clinically relevant *Legionella* species including *Legionella pneumophila*, *Legionella micdadei*, *Legionella bozemanae*, *Legionella dumoffii*, *Legionella longbeachae*, *Legionella gormanii* and *Legionella jordanis*. The sensitivity of the PCR system was 20 fg extracted DNA. In the mouse model, the blood PCR was compared with results obtained by PCR on bronchoalveolar lavage 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.

INTRODUCTION

Legionella pneumophila is one of the leading causes of bacterial pneumonia, particularly in susceptible individuals or the immunocompromised (El-Solh *et al.*, 2001; Marston *et al.*, 1997; Sopena *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.

Abbreviation: BALF, bronchoalveolar lavage fluid.

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) (Cloud *et al.*, 2000; Jaulhac *et al.*, 1998; Jonas *et al.*, 1995; Matsiota-Bernard *et al.*, 1994; Weir *et al.*, 1998) or pleural effusion (Breiman & Butler, 1998; Hirakata *et al.*, 1996; Lo Presti *et al.*, 2000). However, PCR detection of *L. pneumophila* using non-invasive specimens is preferable, particularly by family physicians and non-pulmonologists.

The aim of the present study was to examine the efficacy of PCR on blood samples as a rapid diagnostic method for *Legionella* pneumonia and to compare the results with those of PCR on BALF, cultures of blood and BALF samples and EIA to detect urinary antigen in a mouse model of *Legionella* pneumonia.

METHODS

Bacteria. *L. pneumophila* serogroup 1 ATCC 33152^T (Philadelphia 1^T) 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 33152^T was stored until use at −80 °C in Müller–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

Strain	PCR result
L. pneumophila ATCC 33152^T (serogroup 1)	+
L. pneumophila ATCC 33153 (serogroup 1)	+
L. pneumophila ATCC 33154 (serogroup 2)	+
L. pneumophila ATCC 33155 (serogroup 3)	+
L. pneumophila ATCC 33156 (serogroup 4)	+
L. pneumophila ATCC 33215 (serogroup 5)	+
L. pneumophila ATCC 33216 (serogroup 6)	+
Legionella micdadei ATCC 33218^T	+
Legionella bozemanae ATCC 33217^T	+
Legionella dumoffii ATCC 33279^T	+
Legionella longbeachae ATCC 33469 (serogroup 1)	+
L. longbeachae ATCC 33484 (serogroup 2)	+
Legionella gormanii ATCC 33297^T	+
Legionella jordanis ATCC 33623^T	+
Klebsiella pneumoniae (clinical isolate)	–
Pseudomonas aeruginosa NUS10 (clinical isolate)	–
Mycoplasma pneumoniae MAC	–
M. pneumoniae M129	–
M. pneumoniae FH	–
Chlamydia pneumoniae TW-183^T	–
C. pneumoniae KKpm-15	–

broth (Difco Laboratories) and incubated under shaking at 130 r.p.m. for 24 h at 37 °C. A portion of the culture was inoculated into 5 ml fresh BYE broth and incubated until the OD_{595} of the culture broth reached 0·5. The bacterial suspension was centrifuged at 3000 r.p.m. for 15 min at 4 °C and the pellet was washed twice and resuspended in physiological saline. The bacterial suspension was adjusted to 1×10^9 c.f.u. ml^{-1} by turbidimetry.

Animal model of *Legionella* pneumonia. Male, 8-week-old, specific-pathogen-free A/J mice (Japan SLC Inc.) were used for all experiments. The mice were housed in a pathogen-free environment and received sterile food and water. These mice were inoculated intratracheally with *L. pneumophila* as described previously (Yanagihara *ihara et al.*, 1997). Briefly, mice were anaesthetized with pentobarbital and then 0·1 ml bacterial suspension (approx. 10^5 c.f.u. per mouse) was inoculated through the mouth into the trachea using the outer sheath of an intravenous needle (Top Co.). Body weight was measured on the days indicated after infection. The experimental protocol was approved by the Animal Care and Use Committee of Nagasaki University.

Sample collection. Urine samples were collected from mice housed in metabolic cages (Tecniplast). For sampling blood and BALF, five mice were selected at random on the days indicated and anaesthetized. An incision was made through the skin of the chest to expose the trachea, lungs and heart. Cardiac blood samples (about 800 µl per mouse) were obtained using a 26-gauge needle and 1 ml syringe containing a small amount of EDTA. A portion of blood was used for leukocyte counts. BALF was also obtained from these mice. For this purpose, the trachea was intubated with an intravenous catheter (3 Fr. Atom Co.), which was connected to a 1-ml syringe. The lungs were washed three times with 0·6 ml cold Ca^{2+}- and Mg^{2+}-free PBS (Gibco).

Recovery of *L. pneumophila* from BALF and blood samples. BALF and blood samples were cultured on BCYE-α agar containing vancomycin, polymyxin B and amphotericin B (WYO α agar; Eiken) and 5 % sheep-blood agar (Nissui Pharmaceutical) for 7 days.

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-S (sense, 5′-GCGGCTACCTGGCCTAATAC-3′; designed in this study) and Cp3.2 (antisense, 5′-CCAACAGTAAGTTGACATCG-3′; 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 Binax and Biotest 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

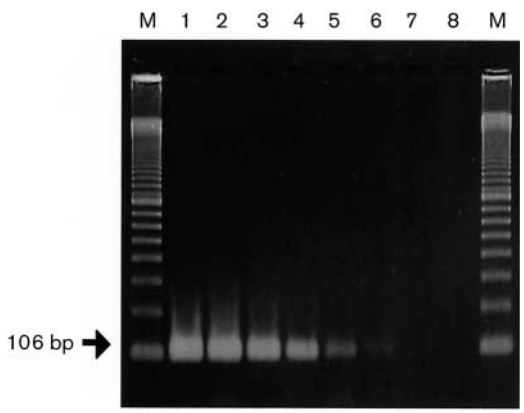

Fig. 1. Sensitivity of the PCR. *L. pneumophila* serogroup 1 ATCC 33152^T DNA was purified and prepared in TE and 2 ng–2 fg was used for PCR. Lanes: M, molecular size marker; 1, 2 ng; 2, 200 pg; 3, 20 pg; 4, 2 pg; 5, 200 fg; 6, 20 fg; 7, 2 fg; 8, negative control (water).

used, since these primers can detect most *Legionella* species, with the exception of *Legionella geestiana* (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

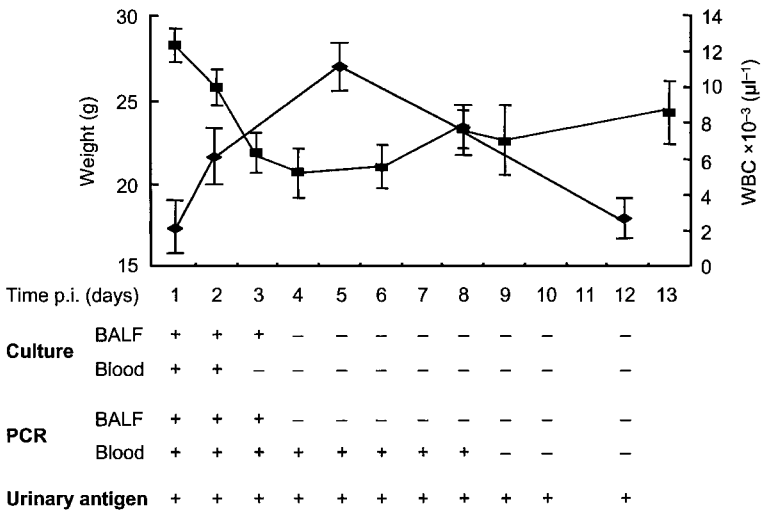

Fig. 2. Serial changes in body weight (■) and leukocyte count (◆) in a mouse model of *Legionella* pneumonia. A/J mice were inoculated intratracheally with *L. pneumophila* (10^5 c.f.u. per mouse) and body weight and peripheral leukocyte numbers were measured on the days indicated after infection. Data 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.

6×10^6 c.f.u. *L. pneumophila* to A/J mice. The leukocyte count did not increase and weight loss was not observed in this model. Urinary antigen became positive at 6 h after inoculation and remained positive for 2 weeks after onset of infection. Blood PCR was negative throughout the test period. The chance of detecting urinary antigen would be minimal, as healthy humans are not tested for *Legionella* 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 (Ratcliff *et al.*, 2001).

In conclusion, in the present study, we have demonstrated that *Legionella* DNA could be detected by PCR in whole blood in a mouse model, suggesting that whole-blood PCR is a useful method for the diagnosis of *Legionella* pneumonia. Before whole-blood PCR can be applied to human samples, its specificity must be confirmed by Southern blotting or sequencing. Several studies have reported the usefulness of urine PCR (Helbig *et al.*, 1999; Maiwald *et al.*, 1995; Socan *et al.*, 2000) as a diagnostic method. Further prospective studies of a large number of patients are required to examine the sensitivity and specificity and clinical impact of whole-blood PCR and to compare the assay with PCR on BALF and urine samples.

ACKNOWLEDGEMENTS

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

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