Case Report

Detection and quantification of *Legionella pneumophila* DNA in serum: case reports and review of the literature

Bram M. W. Diederer¹, Caroline M. A. de Jong¹, Jan A. J. W. Kluytmans², Anneke van der Zee¹ and Marcel F. Peeters¹

Correspondence

Bram M. W. Diederer
bramdiederer@gmail.com

¹Laboratory of Medical Microbiology and Immunology, St Elisabeth Hospital, PO Box 747, 5000 AS Tilburg, The Netherlands

²Laboratory of Microbiology and Infection Control, Amphia Hospital, PO Box 90158, 4800 RK Breda, The Netherlands

Received 6 December 2005

Accepted 5 January 2006

Legionella pneumonia can be difficult to diagnose. Existing laboratory tests all have shortcomings, especially the ability to diagnose all *Legionella* spp. at an early stage. Detection of *Legionella* DNA in serum can be a valuable tool for the diagnosis of Legionnaires' disease (LD). This report describes two patients with LD diagnosed by PCR using serum samples. In addition, quantification of *L. pneumophila* DNA using real-time PCR during the course of illness was carried out. The results obtained mirrored both the clinical condition and C-reactive protein values during the course of the illness. Quantification of *Legionella* DNA in serum using real-time PCR could be a valuable tool to monitor the effects of antimicrobial therapy in patients with LD.

Introduction

Legionnaires' disease (LD) is an acute pneumonia caused by *Legionella* spp. These Gram-negative bacilli are ubiquitous in both man-made and natural aquatic reservoirs. Although currently more than 48 *Legionella* spp. have been described, more than 90 % of culture-confirmed clinical cases are caused by *Legionella pneumophila* (Yu *et al.*, 2002). *Legionella* spp. are responsible for 1–5 % of cases of community-acquired pneumonia (CAP) (Breiman & Butler, 1998). Clinically and radiographically, LD cannot be distinguished from pneumonia caused by other microbial pathogens. Because of the high mortality rate in patients with LD requiring hospitalization, early diagnosis to enable adequate antimicrobial treatment is potentially life-saving (Fields *et al.*, 2002). Diagnosis of LD can be quite difficult and is based on phenotypic (culture, serological testing and antigen detection in urine) and genotypic (PCR) methods. Isolation of *Legionella* from respiratory secretions is considered to be the gold standard in case definition, but is not very sensitive (10–80 % sensitivity), and a positive result is not available until at least 3 days after incubation. Another major limitation of sputum culture is that <50 % of patients with LD produce sputum (Murdoch, 2003). Serological testing has a high sensitivity and a high specificity, but is of limited clinical value, as it may take up to 9 weeks for patients to develop detectable antibodies. For the detection of *L. pneumophila* serogroup 1, urinary antigen tests have sensitivities in the

range of 70–100 % and specificities approaching 100 %, but these assays only detect *L. pneumophila* serogroup 1 reliably (Murdoch, 2003; Yzerman *et al.*, 2002). Genotypic methods utilize PCR to detect the presence of *Legionella*-specific DNA in respiratory secretions, urine and serum, and show varying degrees of sensitivity and specificity (Murdoch, 2003). Diagnostic PCR assays have principally targeted specific regions within 16S (Hayden *et al.*, 2001; Cloud *et al.*, 2000) and 5S (Lindsay *et al.*, 2004) rRNA genes and the macrophage inhibitor potentiator (*mip*) gene (Ballard *et al.*, 2000; Lindsay *et al.*, 1994). PCR techniques have the potential to provide a rapid diagnosis of LD with the use of readily obtainable specimens such as serum and urine (Lindsay *et al.*, 2004; Matsiota-Bernard *et al.*, 2000; Murdoch *et al.*, 1996).

In this report, we describe two patients with LD diagnosed using *Legionella*-specific real-time PCR on serum samples. In addition, a serial quantification of *L. pneumophila* DNA during the course of the illness was carried out. To our knowledge, this is the first time that the sequel of quantifying *L. pneumophila* DNA during LD has been described.

Case 1

In September 2005, a 67-year-old white male was admitted to the pulmonology department of the Amphia Hospital, Breda, The Netherlands, with a history of malaise, cough, fever and shortness of breath. He was a smoker with a mean consumption of 20 cigarettes per day. He did not take any medication and had not travelled recently. Vital signs on

Abbreviations: CAP, community-acquired pneumonia; CRP, C-reactive protein; ICU, intensive care unit; LD, Legionnaires' disease.

arrival included a blood pressure of 155/85 mm Hg, a heart rate of 117 beats min^{-1} , a respiratory rate of 30 min^{-1} and a temperature of 40.7 °C. Examination of the chest revealed decreased breath sounds and crackles in the right lung base, and a chest radiograph showed extensive consolidation of the right lung. A complete blood count revealed a white blood cell count of 12 300 cells μl^{-1} and a C-reactive protein (CRP) value of 535 mg l^{-1} . Antibiotic therapy with oral amoxicillin/clavulanic acid (625 mg every 8 h), started by his general practitioner, was changed to intravenous amoxicillin/clavulanic acid (1000/200 mg q.i.d.) plus erythromycin (1000 mg q.i.d.). Bacterial cultures for bacteria and fungi (sputum and blood) remained negative. Two days after admission, his condition deteriorated and he was transferred to the intensive care unit (ICU) where he underwent intubation as a result of hypoxia and respiratory distress.

One day after admission at the ICU (day 2), a sputum and a serum sample were tested for *Legionella* using real-time PCR. The serum (200 μl) was processed with the MagNA Pure Total Nucleic Acid kit (Roche Diagnostics). For the detection of *Legionella* in serum samples, an assay was used targeted at specific regions within the 5S rRNA gene (Lindsay *et al.*, 2004) and detected in real-time using a TaqMan probe, Leg5S [6-carboxyfluorescein (FAM)-5'-CCGCGCCAATGATAGTGTGAGGC-3'-6-carboxytetramethylrhodamine (TAMRA)]. Real-time PCR was performed on an AbiPrism 7900HT Sequence Detection System (Applied Biosystems). DNA was amplified according to the following parameters: after incubation for 2 min at 50 °C and denaturation for 10 min at 95 °C, amplification consisted of 50 cycles of denaturation for 15 s at 95 °C and 1 min of annealing and extension at 65 °C. Random serum samples from two healthy volunteers were included as negative controls after every four samples. In each run, a no-template negative control was added. Sensitivity controls consisted of tenfold dilutions of *L. pneumophila* DNA ranging from 1000 to 10 fg. As an internal control, *Phocid herpesvirus 1* was added to the samples to monitor processing as well as PCR inhibition (Diederer *et al.*, 2005a).

Both samples (sputum and serum) tested positive for *Legionella*. In addition, an immunochromatographic membrane test (Binax NOW; Binax) to detect *L. pneumophila* serogroup 1 soluble antigens in urine was positive. Culture was carried out as follows: samples were plated on buffered charcoal yeast extract (BCYE) agar (Oxoid) and on BCYE agar with cefamandole, polymyxin and anisomycin (Oxoid). *Legionella*-specific media were incubated for 10 days at 35 °C in a humidified atmosphere. Colonies that grew on BCYE agar but failed to grow on blood agar were expected to be *Legionella* spp. Further identification was carried out by determination of the *L. pneumophila* serogroups using a slide agglutination test (Dryspot Legionella Latex Test; Oxoid). A lower respiratory tract sample (bronchoalveolar lavage) produced *L. pneumophila* serogroup 1. We collected consecutive serum samples to quantify *L. pneumophila* using a *Legionella*-specific PCR. The threshold cycle value (Ct) is

inversely proportional to the log of the amount of target DNA initially present and was calculated by using SDS software version 2.0 (Applied Biosystems). The results of *Legionella*-specific real-time PCR during the course of the illness are outlined in Fig. 1. Antibiotic therapy was changed to ciprofloxacin (400 mg b.i.d.) plus erythromycin (1000 mg q.i.d.) on day 3 after admission to the ICU. His clinical condition, however, did not improve significantly in the first days after ICU admission. Despite appropriate treatment, he remained critically ill with unstable systemic blood pressure and development of renal failure. Ct values mirrored the clinical condition and CRP values during the course of illness; for the first 4 days, Ct values were stable at ~29 in *Legionella* real-time PCR. On the fifth day after admission to the ICU department, the patient's condition stabilized, but on day 6 he deteriorated again and became haemodynamically unstable, despite inotropic drug support with serum urea and creatinine at 23.6 mmol l^{-1} (reference values 2.9–7.5 mmol l^{-1}) and 539 $\mu\text{mol l}^{-1}$ (reference values 55–95 $\mu\text{mol l}^{-1}$), respectively. This severe deterioration

Fig. 1. Results of CRP measurements (a) and real-time *Legionella* PCR (b) of serum from patient 1. The threshold cycle value (Ct) is inversely proportional to the log of the amount of target DNA initially present and was calculated by using SDS software version 2.0 (Applied Biosystems).

coincided with a decrease in Ct (an increase in bacterial load) on day 6. In addition to the haemodynamic support, rifampicin (600 mg b.i.d.) and activated protein C (Xigris) were added. The patient showed a remarkable improvement during the following days and arterial oxygenation improved with radiographic resolution of infiltrates. Ct values increased during the following days and all samples tested after day 8 were negative in *Legionella*-specific real-time PCR. Two weeks after admission to the ICU, he was transferred to the medium-care department.

Case 2

A 42-year-old white male was admitted to the pulmonology department of the St Elisabeth Hospital, Tilburg, The Netherlands, with a history of cough, shortness of breath and mental confusion in October 2005. He smoked 25 cigarettes per day. Vital signs on arrival included blood pressure of 140/80 mm Hg and a temperature of 37.6 °C. Examination of the chest revealed crackles in the left lung base and a chest radiograph showed consolidation of the left lung base. A complete blood count showed a white blood cell count of 6800 cells μl^{-1} and a CRP value of 315 mg l^{-1} . Antibiotic therapy with oral ofloxacin (400 mg b.i.d.) was started. A serum sample taken on the first day after admission tested positive in real-time PCR for *Legionella* with a Ct of 30. An immunochromatographic membrane test (Binax NOW; Binax) to detect *L. pneumophila* serogroup 1 soluble antigens in urine was positive. Pleural fluid obtained on day 3 after admission was cultured on *Legionella*-specific media and produced *L. pneumophila* serogroup 1. The patient responded after initiating therapy with ofloxacin; his condition gradually improved and CRP values dropped from 227 mg l^{-1} on day 2 to 70 on day 7. The results of *Legionella*-specific real-time PCR showed an almost linear increase in Ct values (indicating a logarithmic decrease in bacterial DNA) over the time course of infection (Fig. 2) and mirrored the clinical condition and CRP values during the acute stage of infection. The patient was discharged home 14 days after admission.

Discussion

The main disadvantage of testing sputum samples is that <50 % of patients with LD produce sputum. This has prompted investigation of the use of PCR for testing other sample types. The first report on the detection of *Legionella* DNA in serum was by Lindsay *et al.* (1994) using a conventional *mip* gene-based PCR assay using Southern blotting with a *Legionella*-specific probe. All patients with confirmed LD (five in total) tested positive in the acute and convalescent sera. This study included one convalescent serum sample that was positive for *Legionella* DNA 37 days after the onset of the patient's symptoms. Murdoch *et al.* (1996) tested urine and serum samples from 28 patients with LD and 24 patients with pneumonia due to organisms other than *Legionella* spp. The majority of patients in this study were infected with organisms other than *L. pneumophila*

Fig. 2. Results of CRP measurements (a) and real-time *Legionella* PCR (b) of serum from patient 2. The threshold cycle value (Ct) is inversely proportional to the log of the amount of target DNA initially present and was calculated by using SDS software version 2.0 (Applied Biosystems).

serogroup 1. *Legionella* DNA was detected in serum samples in 12 of 28 patients (43 %) with LD using a conventional 5S rRNA gene-based PCR assay. If used at the time of specimen collection, PCR of both urine and serum combined would have detected an additional 11 cases in the acute phase of disease. *Legionella* DNA was not detected in samples from the control patients. In another report, Murdoch *et al.* (1999) used a guinea pig model and detected *Legionella* DNA in 55 % of leukocyte samples and 28 % of urine samples. The sensitivity of the PCR was highest for samples collected within 3 days of inoculation. Matsiota-Bernard *et al.* (2000) detected *L. pneumophila* DNA in 12 serum samples from 41 patients with LD (29 %) and no *Legionella* DNA in 10 patients with pneumonia due to organisms other than *Legionella*. In a recent report, a *Legionella*-specific 5S rRNA gene PCR on patient serum was positive in 80.5 % of cases (54/67 patients), with a peak positivity at 6–10 days after disease onset (Lindsay *et al.*, 2004). This peak corresponded to the urinary antigen peak, suggesting that circulating DNA coincides with the urinary antigen filtered from the blood by the kidneys. Unlike urinary antigen, the number of PCR positives decreased very slowly, perhaps as a result of the slow release of DNA from *Legionella* spp. surviving in macrophages. Murdoch & Chambers (2000)

described the use of PCR in a patient with CAP caused by *Legionella dumoffii* in New Zealand. *Legionella* DNA was detected in serial peripheral leukocytes, serum and urine samples when first collected. Buffy coat and urine samples remained positive for up to 56 days after the onset of symptoms, whereas serum samples were positive from 10 to 16 days after the onset of symptoms.

In New Zealand and Australia, there have been multiple cases of *Legionella longbeachae* CAP associated with exposure to soil and *L. longbeachae* is now recognized as the second most common cause of legionellosis (Cameron *et al.*, 1991; Steele *et al.*, 1990). Although CAP due to *Legionella non-pneumophila* spp. is rare in other parts of world, when specifically sought, unsuspected community-acquired infections may be uncovered. Recently, we described a patient who developed CAP due to *L. longbeachae* diagnosed with PCR analysis of sputum and serum (Diederer *et al.*, 2005b). We concluded that, since most laboratory tests for *Legionella* cannot detect infections caused by *Legionella non-pneumophila* spp., culture on *Legionella*-selective media or PCR should be considered when diagnosing severe pneumonia in patients with an unknown aetiology.

The decision to order diagnostic tests for *Legionella* infection is usually limited to at-risk patients or patients with severe pneumonia and should consist of a urinary antigen test combined, if available, with *Legionella* PCR on sputum and serum. This is, in our view, the best initial testing strategy that will detect all *Legionella* spp. and provide results within a time frame that will affect clinical management. Here, we describe two patients with LD diagnosed using *Legionella*-specific real-time PCR on serum samples. The association between disease progression and viral load is well established for infections with a number of viruses, particularly human immunodeficiency virus, hepatitis B virus and hepatitis C virus (Hodinka, 1998). This is the first study in which real-time PCR has been used to monitor *L. pneumophila* DNA longitudinally in patients with LD. Detection of *Legionella* DNA in serum may reflect changes in bacterial load over time and may allow the assessment of the response of the patient to treatment. Further investigation of PCR-based diagnosis using serum samples is needed to establish its role in diagnosis and monitoring of patients with LD.

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