

## CASE REPORT

### Diagnosis of Legionnaires' disease by urinary antigen and DNA detection: A case report

Y NORMAZNAH, MD, PhD, K SANIAH, MIMLS, A NOOR RAIN, PhD and P SABIHA, MD, MRCPath

Institute for Medical Research, Kuala Lumpur and \*Faculty of Medicine, University Technology Mara, Shah Alam.

#### Abstract

A 38-year-old male patient who was admitted to a private hospital in Kuala Lumpur presented with fever, symptoms of respiratory infection and diarrhoea. On admission, he was febrile, toxic looking, dehydrated with hypotension and tachycardia. No clinical signs of respiratory infection were detected on admission. Initially he was treated as a case of septicaemia with fluid therapy and intravenous antibiotic (Perfloxacin). Subsequently, he was noticed to have pneumonia in the right lower zone of the lung. His sputum, stool and blood were sent for culture and the results were negative. Sputum culture for *Legionella* and serological tests for *Mycoplasma* and *Legionella* were also reported negative. Sandwich ELISA performed on his urine sample detected *Legionella pneumophila* antigen. *L. pneumophila* mip gene was also detected in his urine by polymerase chain reaction. The patient was commenced on Erythromycin and he responded favourably to the treatment. The present case shows that *L. pneumophila* should not be overlooked as one of the causative agents of pneumonia and rapid techniques of urinary antigen and DNA detection should be utilized to make an early diagnosis of the infection.

**Key words:** pneumonia, *Legionella pneumonia*, urinary antigen, DNA, polymerase chain reaction.

#### INTRODUCTION

*Legionella* species may cause a wide range of infections in humans. The clinical manifestations of *Legionella* infections include pneumonia with or without extrapulmonary involvement known as Legionnaires' disease, a non-pneumonic flu-like febrile illness also known as Pontiac fever and asymptomatic infections. Legionnaires' disease varies in severity from a mild pneumonia to an adult respiratory distress syndrome accompanied by major extrapulmonary manifestations. Infections caused by *Legionella pneumophila* may occur sporadically as well as in well-defined epidemic clusters. The organisms are transmitted to human primarily through aerosolized particles originating from aquatic environment including cooling towers, humidifiers and decorative fountains<sup>1,2</sup>. Other modes of transmission include aspiration of contaminated water and direct inoculation by respiratory therapy equipment. However, direct person-to-person spread has not been documented.

The incidence of Legionnaires' disease would be expected to vary with the degree of environmental exposure of the susceptible population to this pathogen. The incidence of the disease varies geographically<sup>3</sup>. Although there are very few reports of legionellosis occurring in Southeast Asia, *Legionella* has been shown to be present in the environment. In Singapore, environmental surveillance of *L. pneumophila* showed that 36% of cooling towers, 15-19% of decorative fountains and waterfalls and 2% of spa pools were positive<sup>4</sup>. In Malaysia, a survey on cooling towers in Kuala Lumpur showed that *L. pneumophila* serogroup 1 and 7 were the commonest serogroups isolated<sup>5</sup>. Studies have suggested that most of the pneumonia cases were caused by the *L. pneumophila* serogroup 1<sup>6</sup>. Therefore, the local community is exposed to the risk of infection caused by this organism. This paper reports a case of pneumonia caused by *L. pneumophila* or Legionnaires' disease admitted to a private hospital in Kuala Lumpur.

Address for correspondence and reprint requests: Dr. Y Normaznah, Biotechnology Unit, Medical Research Resource Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur.

## CASE REPORT

A 38-year old male patient was admitted to a private hospital in Kuala Lumpur with the presenting symptoms of fever, vomiting and diarrhoea. The symptoms developed one day prior to admission. He also complained of cough and sore throat which developed two days earlier. Fever was associated with chills and cough was with expectoration of purulent sputum.

On clinical examination, he was febrile (39°C), toxic-looking with no anaemia. The lips were dry. The pulse rate was 114/min and blood pressure was 70/50 mm Hg. On admission, examination of the heart and lungs were normal. Abdominal and neurological examinations were also normal.

Initially he was treated in the high dependency ward for septicaemia with fluid therapy to correct the hypotension. The patient was given intravenous antibiotic (Perfloxacin). Since he did not improve, he was transferred to the intensive care unit. He was then noticed to have diminished breath sounds in the base of the right chest posteriorly. Chest x-ray confirmed the presence of pneumonia in the lower zone of the right lung. His sputum, stool and blood were sent for culture and sensitivity. All the culture results were negative. Serological tests for *Mycoplasma* and *Legionella* antibodies were requested and the results were also reported negative. Sputum culture for *Legionella* was also requested and the result was negative. Urine sample of the patient was collected and sent to the Institute for Medical Research for *Legionella* urinary antigen testing. *L. pneumophila* antigen was detected as positive in the patient's urine by sandwich ELISA using in-house monoclonal antibodies. The results of the test were conveyed to the consultant physician in-charge of the patient. The patient was commenced on intravenous Erythromycin 1 gm 6 hourly. The patient showed favourable response to the treatment. He improved clinically and the fluid therapy for the hypotension was stopped. Subsequent follow-up could not be done as the patient requested a transfer to a government hospital.

### Microbiology

With a clinical diagnosis of septicaemia, blood, urine and stool specimens were collected from the patient for culture and sensitivity testing. The results of the tests were reported negative. Despite the intravenous antibiotic (Perfloxacin), the patient did not show clinical improvement. On the second day of admission, when he was

diagnosed to have right lower lobe pneumonia, sputum was collected for *Legionella* culture and sensitivity testing. The culture was reported as negative. IFAT (indirect fluorescent antibody technique) performed on the patient's serum for *Mycoplasma* and *Legionella* antibodies was also found to be negative. Sandwich ELISA using four different monoclonal antibodies following the method of Zheng *et al*<sup>7</sup> carried out on the patients's urine detected *L. pneumophila* antigen.

Sandwich ELISA performed on the serum samples also detected *L. pneumophila* antigen. The levels of antigen were noted to be lower in the serum compared to urine. The results of the sandwich ELISA on the patient's urine and serum samples are shown in Table 1. The urine and the first serum samples were collected on the fourth day after the onset of the illness, while the second serum sample was sent by the government hospital and was taken about two weeks after the onset of the illness. Bacterial genomic DNA was extracted from the patient's urine sample using the GeneClean II Spin protocol (BIO 101, USA). The DNA was then used in the polymerase chain reaction (PCR) for amplification of the macrophage infectivity potentiator (*mip*) gene of *L. pneumophila* using a pair of specific primers. *L. pneumophila* (reference strain 11920) obtained from the PHL (Public Health Laboratory), Colindale, United Kingdom) was used as a positive control. The PCR amplification gave a positive product of 630 bp size (Fig. 1). For confirmation of the gene, the PCR product was purified with the QIAquick PCR purification kit (Qiagen) and DNA sequencing was subsequently performed on the purified DNA by BigDye chemistry with the ABI Prism 377 Sequencer (PE Applied Biosystem). The DNA sequence analysis using the BLAST software demonstrated 99% identity with the *L. pneumophila* serogroup 1 in the GenBank database (Accession number AJ 496265).

## DISCUSSION

The accurate diagnosis of *Legionella* pneumonia has important implications for treatment of the infection. Many first line antibiotics commonly used to treat typical bacterial pneumonia (i.e. beta-lactams) are ineffective against *Legionella* species<sup>8</sup>. This is at least partially due to the fact that *Legionella* bacteria are intracellular pathogens. There is thus a great need for rapid diagnosis of legionella infections.

**TABLE 1: Optical density (OD) values of four different monoclonal antibodies using sandwich ELISA**

Clinical sample	Mab 1F4.10E	Mab 1C7.2B	Mab 2B2.11E	Mab 2B2.10F
Urine	2.007	0.464	0.493	0.490
First serum	0.541	0.629	0.418	0.466
Second serum	0.428	0.381	0.338	0.365

Mab- monoclonal antibody

Cut-off OD values for urine:	1F4.10E	0.286
	1C7.2B	0.282
	2B2.11E	0.133
	2B2.10F	0.138
Cut-off OD values for serum:	1F4.10E	0.212
	1C7.2B	0.380
	2B2.11E	0.281
	2B2.10F	0.540

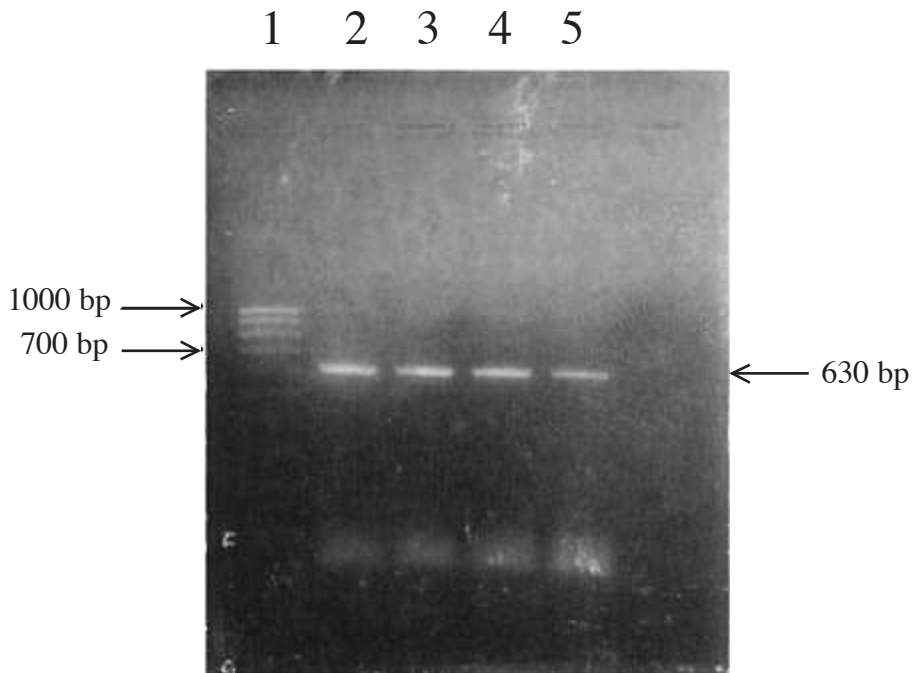


FIG 1: PCR of *mip* gene of *L. pneumophila*. Lane 1- 100 bp ladder marker, lanes 2 &3- positive control, lanes 4&5- duplicate of DNA extracted from patient's urine sample.

Currently, culture is regarded as the "gold standard" for detection of *Legionellae*. However, culture of *Legionella* bacteria has inherent problems. There is a broad range (50-99%) of reported sensitivity of culture in the literature<sup>9,10</sup>. This discrepancy is most likely due to differences in the types and qualities of the specimens used in each study. Collection of specimens in saline (bronchoalveolar specimens, bronchial washes) lowers the sensitivity of culture because saline inhibits the growth of *Legionellae*<sup>10</sup>. Sputum samples may be contaminated with oropharyngeal organisms thus masking the presence of *Legionella spp.* Another contribution to a lowered sensitivity of culture is the use of samples collected from patients being treated with antibiotics.

Due to delay in detecting rising titres of *Legionella* antibody with respect to the onset of the illness, diagnosis should not rely mainly on antibody detection. Therefore, newer methods including antigen detection in serum and urine have been developed to improve the diagnosis. Detection of soluble antigen can be done using various immunological methods with either monoclonal or polyclonal antibodies. Monoclonal antibodies can detect specific antigen, and therefore serve as an excellent reagent for specific diagnosis of this bacterial infection. Some of the methods have been commercialized. Among the commercially available methods, ELISA and RIA (radioimmunoassay) have demonstrated high sensitivity and specificity<sup>11</sup>. A subsequent study demonstrated that immunochromatography gave higher sensitivity and specificity<sup>12</sup>. In this study, four in-house monoclonal antibodies used in the antigen detection assay were produced by hybridoma technology following the method of Kohler and Milstein<sup>13</sup>. All of the monoclonal antibodies were of IgM class. Characterization by the indirect ELISA, sandwich ELISA and immunoblotting showed that the monoclonal antibodies had specific reactions with antigens of *L. pneumophila*. Monoclonal antibody 1F4.10E reacted with a specific protein antigenic fraction of a reference strain of *L. pneumophila* (Knoxville 1) with approximate molecular weight size of 33.0 kDa, while others recognized non-protein epitopes since immunoblot primarily detected protein antigens. The cut-off value for each of the monoclonal antibodies was determined by running the test on control serum and urine samples collected from healthy staff of the Institute for Medical Research and blood donors. The values above the cut-off levels of the

monoclonal antibodies were considered positive. The in-house sandwich ELISA using the four monoclonal antibodies detected *L. pneumophila* antigens in the urine and serum samples of the patient. The ELISA values were noted to be higher in the urine compared to serum, particularly for the monoclonal antibody 1F4.10E. A previous study also reported higher antigen levels in urine than in serum<sup>14</sup>. The PCR carried out on the DNA extracted from the patient's urine gave a positive amplification product of the *L. pneumophila mip* gene of 630 bp size and was confirmed by DNA sequencing. With the absence of positive culture result, PCR is useful to support the diagnosis of *Legionella* infection<sup>15</sup>. Erythromycin is the antibiotic recommended for treatment of *Legionella* infection<sup>8</sup>. The patient showed clinical improvement after intravenous Erythromycin was instituted.

In view of the reported presence of *L. pneumophila* in our local environment<sup>5</sup>, the present case reminds us that *L. pneumophila* should be considered as one of the causative agents of pneumonia, particularly if the patient does not respond to the conventional antibiotic treatment for typical pneumonia. Besides culture, rapid techniques including urinary antigen detection and PCR should also be utilized to make an early diagnosis of the infection and thus appropriate treatment can be instituted.

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#### REFERENCES

1. Zuravleff JJ, Yu VL, Shonnard JD, *et al.* *Legionella pneumophila* contamination of a hospital humidifier: demonstration of aerosol transmission and subsequent subclinical infection in exposed guinea pigs. *Am Rev Respir Dis* 1983;128:657-61.
2. Hlady WG, Mullen RC, Mintz CS, *et al.* Outbreak of Legionnaires' disease linked to a decorative fountain by molecular epidemiology. *Am J Epidemiol* 1993;138:555-62.
3. Bhopal RS, Fallon RJ. Variation in time and space in non-outbreak Legionnaires' disease in Scotland. *Epidemiol Infect* 1991;106:45-61.

4. Nadarajah M, Goh KT. Isolation of *Legionella pneumophila* from hospital cooling towers. Ann Acad Med Singapore 1986; 15:6-8.
5. Ngeow YF, Tan CH, Lim SY. *Legionella* species isolated from cooling towers in Kuala Lumpur. Med J Malaysia 1992;47:15-9.
6. Plouffe JF, File Jr TM, Breiman RF, *et al.* Reevaluation of the definition of Legionnaires' disease: use of the urinary antigen assay. Clin Infect Dis 1995;20:1286-91.
7. Zheng H, Tao Z, Reddy MVR, *et al.* Parasite antigen in sera and urine of patients with bancroftian and brugian filariasis detected by sandwich ELISA with monoclonal antibodies. Am J Trop Med Hyg 1987; 36(3):554-60.
8. Klein NC, Cunha BA. Treatment of Legionnaires' disease. Semin Respir Infect 1998;13:140-6.
9. Breiman RF, Butler JC. Legionnaires' disease: clinical, epidemiological and public health perspective. Semin Respir Infect 1998;13:84-9.
10. File TM, Jr., Tan JS, Plouffe JF. The role of atypical pathogens: *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila* in respiratory infections. Infect Dis Clinics of North America 1998;12:569-92.
11. Kashuba AD, Ballow CH. Legionella urinary antigen testing: Potential impact on diagnosis and antibiotic therapy. Diag Microbiol Infect Dis 1996; 24:129-39.
12. Helbig JH, Uldum SA, Luck PC, *et al.* Detection of *Legionella pneumophila* antigen in urine samples by the BinaxNOW immunochromatographic assay and comparison with both Binax Legionella urinary antigen enzyme immunoassay (EIA) and Biotest Legionella urinary antigen. J Med Microbiol 2001; 50:509-16.
13. Kohler G, Milstein C. Continuous culture of fused cells secreting antibody of predefined specificity. Nature 1975;256:495-7.
14. Bibb WF, Arnow PM, Thacker L, *et al.* Detection of soluble *Legionella pneumophila* antigens in serum and urine specimens by enzyme-linked immunosorbent assay with monoclonal and polyclonal antibodies. J Clin Microbiol 1984;20:478-82.
15. Lindsay DS, Abraham WH, Fallon RJ. Detection of *mip* gene by PCR for diagnosis of Legionnaires' disease. J Clin Microbiol 1994; 32:3068-9.