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Nosocomial Legionnaires' Disease Outbreak in Tehran

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Abstract: The study took place during the summer time of 2007. A 20 years old university hospital with 600 beds equipped with central air conditioning. No special disinfection program was achieved for the hospital water supplies at the time of investigation. The hospital is supplied by city water and sewages organization and is treated with standard chlorination. To analyze the first nosocomial outbreak of Legionnaires' disease in a major university hospital of Iran. Seventy Broncho Alveolar Lavage specimens were obtained from patients with pneumonia. In addition 20 water samples of various hospital points were screened for the presence of *Legionella* species and free-living amoebae. Six nosocomial cases occurred over an 8 weeks period, between the first and last case detection. *Legionella* isolates from the patients matched the water sample isolates. *L. pneumophila* were grown up from only 3 out of 70 samples, while the bacteria *mip* gene were detected from additional three cases. *L. pneumophila* (serogroup 1) were isolated from two hospital sites. Since, *Legionella* positive patients had been admitted to the hospital at least 2 weeks prior to sampling, the cases could be assumed as hospital acquired Legionnaire's disease, originated from hospital water supplies which should be treated for effective disinfection.

Key words: Nosocomial, legionnaires' disease, outbreak, PCR

INTRODUCTION

Hospital-acquired Legionnaires' disease has been reported from many parts of the world since the first outbreak of Legionnaires' disease in 1976 (Fields *et al.*, 2002). Pneumonia caused by *Legionella* has a poor prognosis unless it is diagnosed early and treated with specific antibiotics. In the warm and cold water systems of large buildings such as hotels and hospitals, growth conditions are favorable for *Legionellae*. Among the bacteria responsible for atypical pneumonias, *Legionellae* are the main agents involved in severe forms of the disease (Brooks *et al.*, 2001). Within members of the family Legionellaceae, *L. pneumophila* is involved in more than 95% of cases of severe atypical pneumonia (Balows and Duerden, 1998). *Legionella* may be widely spread through hospital water systems and may have been primarily isolated in potable hot water systems and cooling towers of hospitals and other institutions (Anaissie *et al.*, 2002). These bacteria are present at the highest concentrations in biofilms within hot water systems and openings of water outlets (Meyer, 2005). Aerosol inhalation and ingestion or aspiration of potable water from cooling towers containing the organism has been more frequently associated with community outbreaks (Sala *et al.*, 2007). However, this route has also been

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implicated in nosocomial cases (Kowska *et al.*, 2003). Isolation of causative agent from bronchoalveolar lavage (BAL) fluid specimens is fastidious and time consuming. Although legionellosis occurs in persons with no underlying disease, the risk is significantly increased in people with illnesses such as chronic obstructive pulmonary disease, malignancy, immunosuppressive therapy, or organ transplantation (England and Fraser, 1981). Therefore, hospitals are points of congregation for persons at risk for infection, besides providing suitable conditions for amplification of *Legionella*. The goal of this study was to determine whether legionellosis was occurring in the hospital and if so, whether these were nosocomial infections. To answer this question, all respiratory tract secretions submitted to the clinical laboratory over an eight weeks period were tested for *Legionella* contamination. In addition, water samples from the warm and cold water system of the hospital building were cultured for *Legionella*. Isolates from patients were then compared with those from hospital water samples and typing.

MATERIALS AND METHODS

Setting

A twenty-year-old teaching hospital with 600-beds and three Intensive Care Units (ICU) with 25 beds. Three cooling towers which located in open area provide necessary water for hospital air condition system. There is no especial program for water supplies disinfection at the time of the outbreak.

Outbreak

Sample collection of suspected patients was taken placed during 8 weeks, between 7 July and 31 August 2007. *Legionella pneumophila* genome was diagnosed from two patients during the first week of July, followed by four other patients over seven week's period. The samples of positive patients were reconfirmed by PCR technique using mip gene primers. After the first detection, 20 samples of hospital water supplies from rooms and sections where patients stayed were tested and *Legionella pneumonia* were isolated from two points. The risk factors for *Legionella* infections such as alcohol consumption, smoking, chronic obstructive pulmonary disease, aging, history of recent surgery and immunosuppression and also chemical injuries were recorded for each case. The clinical characteristics of the patients were shown in table. All cases were detected within the inpatient group who were admitted at least four weeks prior to BAL sampling. All patients had a history of either using ventilator or taking shower connected to the hospital water supplies.

BAL Sampling

A retrospective study of 70 patients suffering of pneumonia was conducted. All patients were adults with pneumonia diagnosed by temperature of 38°C or higher and pulmonary infiltrates on chest X-ray who admitted to hospital at least 2 weeks prior to study. Patients were eligible for the study if bronchoscopy was to be performed and if the patients agreed to it as a part of the endoscopic procedure. Patients without respiratory infection and pulmonary infiltrates were excluded from the study. Patients with mentioned criteria were subjected to BAL sampling irrespective of empiric therapy for infection pneumonia prior to study. The standard Broncho Alveolar Lavage (BAL) fluid sampling procedures were achieved in hospital bronchoscopy section, by using an Olympus BF10 or P10 fiber-optic bronchoscope. The bronchoscope apparatus were washed twice with soap solution and clean water in disinfected in Sidex or Korosolex for 20 min before next use. The BAL samples were then transferred to laboratory immediately. Each BAL sample was homogenized, divided into two portions of 2 mL each. One portion of each sample kept frozen at -80°C until it was used for DNA amplification and the other portion processed for standard culturing and identification.

Water Sampling

Water samples were obtained from shower heads (hot and cold), outlets and ventilator equipments of hospital sections and rooms where patients stayed. All water samples were prepared from main supplies, according to methods previously described by Doust *et al.* (2008b). The temperature of cold water samples were between 8-15°C and of hot water samples between 40-55°C. The hospital was provided with potable water connected to city water network. Each sample of 1 L water were collected from each point aseptically. Samples were concentrated aseptically by membrane filtration, using a three-piece PVC manifold (Millipore) and cellulose type HA membranes with a pore size of 0.45 µm (Millipore Corporation) (Hajia *et al.*, 2004). The concentrated samples were removed by cutting the membranes into pieces, placing them in sterile containers containing 10 mL of original sample and shaking vigorously for 15 min until the membranes appeared to be clean.

Culturing Procedures

Standard protocols Buffered Charcoal Yeast Extract (BCYE) agar formulated in laboratory and enriched with L-cystein and iron salts, polymyxin B, anisomycin and vancomycin (PAV) were then added (Doust *et al.*, 2002). The samples then were subjected to heat treatment procedure which is believed to be more effective, before inoculated onto BCYE plates. All inoculated plates were incubated at 35-37°C for up to 14 days and examined daily for evidence of growth. Colonies resembling *Legionella* were subcultured on BCYE with and without L- cystein. Suspicious *Legionella* colonies that failed to grow in the absence of L-cystein were typed by direct immuno-fluorescence with a commercial monoclonal antibody test. A commercially available latex agglutination test kit (Oxoid, UK) was used for the final confirmation of presumptive *Legionella* colonies.

Molecular Detection

A thermic-lysis procedure was used to release the bacterial DNA extraction (Patterson *et al.*, 1997). Briefly, a total of 400 µL of *Legionella* suspension was boiled for 5 min in a 1.5 mL µ centrifuge tube and chilled on ice for 5 min; this procedure was repeated once. After 2 min of centrifugation at 1100 x g, 20 µL of the supernatant was tested for specific DNA amplification. A set of primers and detection probe were chosen from the *mip* gene of *L. pneumophila* which was described previously by Doust *et al.* (2008b) and Julhac *et al.* (1992). The *mip* gene, which codes for a virulence protein, seemed to be a promising target for the diagnosis of legionellosis. Two 20-base oligonucleotides (L.pm-1 = 5'-GGTGA CTGCGGCTGTTATGG-3', L.pm-2= 5'-GGCCAATAGGTCCGCCAACG-3'). A 25-base internal probe, (L.pm-3 =5'-CAGCAATGGCTGCAACCGATGCCAC-3') located at nucleotides 885 to 912 from the coding strand, was labeled with [γ -³²P] ATP as described previously and was used as detection probe.

The samples were submitted to 40 cycles of amplification in a 50 µL volume containing 1 U of Taq polymerase 0.1 µM (each) primer, 0.2 mM (each) the four deoxynucleotides, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂ and 0.2 mg of gelatin per mL. After an initial denaturation step at 95°C for 5 min, each amplification cycle was performed as follows; 2 min for annealing at 62°C, 1.5 min for primer extension at 72°C and 1.5 min for denaturation at 94°C. The sample preparation, PCR amplification and electrophoresis were performed in three different rooms. Ten microliter of each sample containing amplified DNA was electrophoresed through a 1.5% agarose gel in 0.5x TEB buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1.25 mM disodium EDTA [pH 8.3]), after staining with ethidium bromide photographed under UV light. DNA extracted from *L. pneumophila* Philadelphia strain was used as positive control and double distilled water as the negative one. The restriction analysis was used to check product specificity according the method previously described by Doust *et al.* (2007). The *kpn*I restriction enzyme was used for control of 630 bp PCR products specificity. Two bands approximately 400 and 230 bp were produced after 12 h incubation of PCR product with *kpn*I restriction enzyme.

RESULTS

Using the primer set and the detection probe described here we were able to detect the Legionnaire's disease bacterium in 6 (17%) who were admitted to the hospital during two to four weeks prior to BAL sampling. All 70 patients had pneumonia and were suspected having Legionnaires' disease. Over 36% of patients' natraemia were over 130 and 63% bellow 130 mM L⁻¹. Male/Female rate of the patients under investigation were 64/36%. The most dominant clinical findings were observed as; 67% dry cough, 17% productive cough, 70% chills and fever and 16% abdominal pains. Clinical and laboratory backgrounds of patients with Legionnaires' disease bacterium within BAL specimens are shown in Table 1. The case No. 1 had bronchiectasis, breath shortness, pulmonary infections, case No. 2 complained of chest pain and breath shortness, case No. 3 had homeostasis, chest pain, breath shortness and cases No. 4-6 suffered from breath shortness on admission. All patients' age was between 40 and 65 years old, 66% male and 34% female. The blood natremia of the patients with proved Legionnaires' disease was between 120 and 125 mM L⁻¹ (Table 1). Sub clinical findings of the patients are detailed in Table 2. All patients were treated with erythromycin protocol. The sensitivity of this assay has been evaluated for *L. pneumophila* serogroup 1-14, *L. micdadei* and *L. bozemani* serogroup 1. *Legionella pneumophila* of serogroups 1 were isolated from three cases. The amplification product of 600 bp was observed in all 6 patients (Fig. 1). The pattern of restriction enzyme showed specificity of PCR products (Fig. 2). Amplification of *Legionella* specific sequences by using primers from the *mip* gene sequences permitted the detection of DNA all *L. pneumophila* strains of serogroups 1-14. Non *L. pneumophila* serogroup 1 or *L. micdadei* and *L. bozemani* might be included in PCR positive and culture negative cases. Since, *L. micdadei* and *L. bozemani* serogroup 1 had been also detected by the primer and probe system. DNA from gram positive and gram negative species tested and also from human leukocyte were not amplified by this procedure. Using the primers from the *mip* gene sequence, also reported the detection of DNA from *L. pneumophila* serogroup 1-14 strains but not those from some other *Legionella* species (Julhac *et al.*, 1992). *L. micdadei*, a non *L. pneumophila* species is known to be responsible for Legionellosis and use of DNA amplification to detect this species in biological fluids is therefore a great interest. Considering the data, we decided to use the primers constructed of the *mip* gene and reported here, to assess the feasibility of *Legionella* detection in BAL fluid specimens.

Table 1: Brief description of patients with Legionnaires' disease

Cases	Clinical signs on sampling	Signs an symptoms	Age/Sex	Sodium concentration (meq/desiL)	Results	
					PCR	Culture
I	Elderly, pneumonia	Fev. and Chi., Npc., Wl.	71/F	130	+	-
II*	Diab. mellitus, pneumonia	Fev. and Chi., Npc., Wl., appetite	45/F	126	+	+(30 cfu)
III	Drug abuser, pneumonia	Fev. and Chi., Npc., Ap., Polpol.	43/M	133	+	+(42 cfu)
IV	Elderly, pneumonia	Pc.	81/F	143	+	-
V	Chronic lung injury due to sulfur mustard	Npc. and W.	39/M	130	+	+(50 cfu)
VI	Chronic lung injury due to sulfur mustard	Npc. and W.	45/M	130	+	-

**Mycobacterium tuberculosis* was also detected along with *Legionella*. Fev: Fever, Chi: Chills, Npc: Non productive coughs, Pc: Productive coughs, Wl: Weight losses, Ap: Abdominal pains, Pol: Polyarthritits, W: Weakness

Table 2: Para-clinical findings of patients

Clinical cases	IF/Urine AG	SGPT/SGOT	Temperature/Pulse	Serum phosphorus
I	+/+	67/75	38/118	1.5
II*	+/-	63/70	39.5/120	1.7
III	+/+	65/72	38/115	2.0
IV	+/+	60/71	37.5/114	2.3
V	+/+	62/74	38/117	1.8
VI	+/+	66/74	39/119	2.0

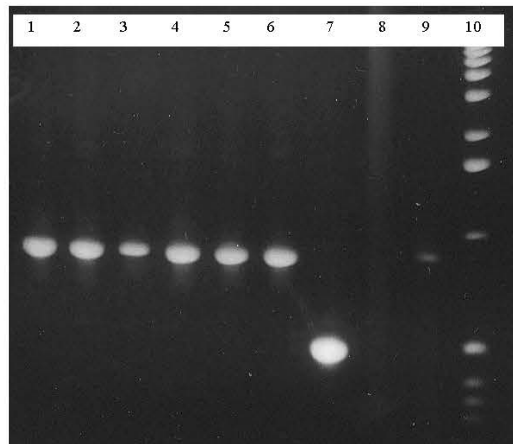


Fig. 1: Agarose gel of PCR products from patients BAL specimens. No. 1-6 represent BAL fluids from six patients, No. 7; non *L. pneumophila*, No. 8; Control negative, No. 9; *L. pneumophila* 1, No. 10; DNA ladder

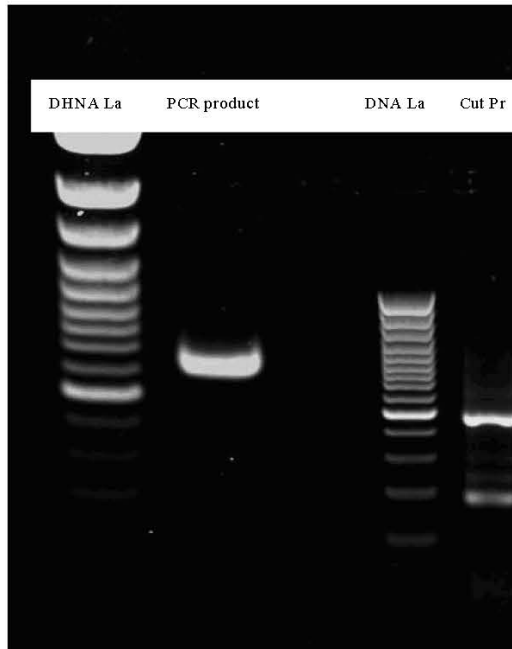


Fig. 2: Specificity of PCR products of patient's sample by restriction enzyme kpn1

DISCUSSION

This study reports the first nosocomial outbreak of Legionnaire's disease within a university hospital of Iran. This is the first outbreak of Legionnaires' disease reported from Iran. The clinical aspects of the patients and also some of the risk factors (age the history of Sulphur Mustard exposure history) are unique in this outbreak. Ozerol *et al.* (2005) also reported an outbreak of Legionnaires'

disease from Turkey. *Legionella* contamination have been reported within clinical and environment samples of Iran and also from some nearby countries (Aslani *et al.*, 1997; Doust *et al.*, 2008b). We detected Legionnaires' disease (Ld) bacterium within 9% of nosocomial pneumonia cases during study period by standard culture method and PCR (Juhac *et al.*, 1998). No Ld case was detected in second group also with pneumonia, which were selected randomly from the outpatient cases. The largest outbreak of nosocomial Legionnaires' disease occurred in Los Angeles with at least 218 confirmed cases from 1977-1982. Since then, more than 300 reports of nosocomial Legionnaires' disease have appeared in public health reports (Fields *et al.*, 2002). Present report complete the Legionnaires' disease related events across the world especially middle-east region, were usually are visited by considerable number of foreign tourists. The relation of at least 39 species and more than 60 serogroup of *Legionella* have been described within human infections. Legionnaires' disease, a sever form of nosocomial and community-acquired pneumonia, is cause mainly by *L. pneumophila*. Other species, mainly *L. micdadei* account for approximately 15% of cases of *Legionella pneumonia* and are more often reported in cases of Pontiac fever. *L. pneumophila* serogroups 1 is responsible for 80% of reported human cases of *Legionella* infections and is usually the only species isolated during routine testing (Brundrett, 1992).

Water samples of the mentioned hospital were collected after the first case of Legionnaires' disease and *L. pneumophila* was diagnosed (Doust *et al.*, 2008b). The multi-points of hospital water samples were checked for *Legionella* and samples of two points proved to be contaminated with *L. pneumophila* by standard culture and PCR methods. Interestingly, predominant samples of the hospital water supplies were contaminated with different species of free-living amoeba which coexist with *Legionella* (Doust *et al.*, 2008a) During present investigation we examined water supplies of the 20 year-old hospital yielded results indicating some samples demonstrated the presence of *Legionellae* with evidence of exposure in 3-6 individuals out of 70 tested. We managed to reduce the *Legionella* colonization of hospital sites by flushing water outlets for 30 min and superheating water at 65°C. To prevent contamination in future, hyperchlorization and ultraviolet radiation systems were introduced into the hospital infection committee. Hospital water system is frequently colonized with *Legionella* species, once present in such systems, they are able to survive and multiply, particularly when hot water temperatures are kept low to minimize the risk of scalding for patients (Qasem *et al.*, 2008). The presence of different species of free-living amoebae within the hospital water distribution system, indicated the risk of *Legionellae* release in the water source of hospital despite of routine chlorination, this data are supported by report of Singh and Coogan (2005) who detected *Legionella*-laden amoebae in dental unit waterlines. The hospital water system was connected to the municipal water networks. An association between *Legionella* infection and exposure to contaminated water has been described previously by Garcia-Nuñez *et al.* (2007) and Spalekova and Bazovska, (2003). Legionnaires' disease is thought to be acquired by inhalation of aerosols containing *Legionella* or by microaspiration of contaminated water that are rarely reported. Nosocomial Legionellosis is more frequent in immunocompromized patients, the patients with previous disease and transplant recipients. One or more Legionnaires' disease infection risks were noticed in majority of our nosocomial pneumonia cases even without Legionnaire's disease bacterium. Interestingly enough, the only new risk factor for one patient, was war chemical agent inhalation, during last 15 years with present respiratory injuries.

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