

# ISOLATION OF *LEGIONELLA* FROM COOLING TOWERS AND POTABLE WATER SYSTEMS IN HOSPITAL AND NON-MEDICAL BUILDINGS IN A UNIVERSITY CAMPUS

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**ABSTRACT:** Six cooling towers and eleven sources of potable water in a university campus in Kuala Lumpur were surveyed for the presence of *legionellae*. One non-medical building cooling tower and three hot water systems from one ward of the hospital tested positive for *Legionella*, two of which contained *Legionella pneumophila* serogroup 1. The identification of *Legionella* was based on isolation, immunofluorescence and polymerase chain reaction (PCR). (JUMMEC 2003-2005; 8: 23-27)

**KEYWORDS:** *Legionella* isolation, hot water system, cooling towers

## Introduction

*Legionella* spp. are Gram-negative bacteria that occur ubiquitously in soil and aquatic environments (1). The most well-known of these is *Legionella pneumophila*, which is the causative agent of Legionnaires' Disease, an atypical form of pneumonia characterized by a non-productive cough coupled with pneumonia symptoms. Ever since its discovery in an air-conditioning system in 1976, this organism, along with other members of its genus, has been found with increasing frequency in both natural and man-made environments, especially in air-conditioning cooling towers and potable water systems (1-3). These organisms are dispersed in the form of aerosolized water droplets which, if inhaled by the susceptible, may be brought into the lungs where they cause disease.

Because of the *Legionella* organism's association with outbreaks of community and nosocomial pneumonia as well as a milder, self-limiting form of infection called Pontiac Fever, there has been an increasing need to monitor water systems for their presence, especially in hospital environments, where equipment relying on potable water sources, for example, humidifiers and nebulizers, which provide vehicles of transmission for these organisms, are used on a regular basis.

Until recently, there have been very few reports of Legionnaires' Disease in South-East Asia; however, several cases have been reported of late in Singapore (4) and China (5), in conjunction with the develop-

ment of this region. In Malaysia, however, the last reported survey of the prevalence of *Legionellae* was carried out in 1990 (6). This paper describes a study carried out in 2002 as a follow-up of the study in a university campus in Kuala Lumpur.

## Methods

### Media

A commercial Buffered Charcoal Yeast Extract Agar with  $\alpha$ -ketoglutarate (Oxoid) supplemented with cysteine and ferric ions (Oxoid) and added with the selective agents glycine, vancomycin, polymyxin and cycloheximide (Oxoid) was used for the isolation of *Legionella* spp.

### Samples

Water samples were collected from air-conditioning cooling towers and potable water systems from different sites in the university campus, including the university hospital according to the standard procedures of the American Public Health Association (APHA 1998). One litre of water was collected from

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each cooling tower as well as hot water from taps and showers, using sterile bottles. These water samples were kept at 4°C and processed within 24 hours. Temperature and pH readings for the water samples were also determined by using a handheld temperature probe and pH indicator paper, respectively.

### **Water processing**

The water samples were filtered by negative pressure through 47 mm polycarbonate membrane filters of 0.22 µm pore size. The filter membranes were subsequently washed in 10 ml sterile distilled water to dislodge any trapped organisms. The suspension was then centrifuged at 4,000 rpm for 10 minutes. The supernatant was poured out, and the sediment was re-suspended in 1 ml of the original water sample.

### **Acid buffer treatment**

The water samples were subjected to acid buffer treatment as described by Bopp *et al* (7). The HCl-KCl buffer was prepared by mixing 3.9 ml of 0.2 M HCl with 25 ml of 0.2 M KCl, producing a solution of approximately pH 2.2. The 1 ml suspensions were diluted 1:10 with the acid buffer and incubated at room temperature for 15 minutes. 0.1 ml of each treated water sample was then removed from the acid suspension and inoculated onto BCYE- $\alpha$  agar by the spread plate method. The plates were incubated in a moist chamber at 37°C for up to 14 days.

### **Screening suspect Legionella isolates**

Colonies which appeared after three to five days of incubation, and were greyish-white in colour with round edges and shiny appearance were presumed to be *Legionella*. These suspect isolates were plated onto Horse Blood Agar (HBA) as well as BCYE- $\alpha$  agar and incubated at 37°C. *Legionella*-like organisms were identified by positive growth on BCYE- $\alpha$  agar but absence of growth on HBA. Verification of *Legionella* was carried out by a direct immunofluorescence assay (*Legionella* Direct Fluorescent Test System, Scimed Corp., USA) and DNA amplification.

### **DNA extraction and amplification**

A loopful of each suspected *Legionella* isolate was suspended in 200 µl of TBE buffer (5 mM Tris, 5 mM boric acid, 0.1 mM EDTA), pH 8.0. The suspensions were centrifuged at 10,000 g for 30 minutes to pellet the bacterial cells. The cells were then lysed with lysis buffer containing 0.5% (v/v) Nonidet P-40, 0.5% Tween 20 (v/v) and 0.1 mg/mL Proteinase K at 60°C for one hour. The enzymes were subsequently deactivated by boiling the mixture at 100°C for 10 minutes. The extracts were then stored at -20°C until use.

Amplification of the DNA was carried out using primers that amplified a 386-bp region of the *Legionella* 16S rRNA, as described by Jonas *et al* (8). The two 20-base oligonucleotides (5'-AGGGTTGATAGGTTAAGAGC-3' (JFP) and 5'-CCAACAGCTAGTTGACATCG-3' (JRP)) were complementary to positions 451 to 470, and 836 to 817 respectively.

Polymerase chain reaction (PCR) was performed in a 40 µl reaction mixture containing 1X PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 3.0 mM MgCl<sub>2</sub>, 200 µM of dNTP (dATP, dCTP, dGTP, dTTP), 1 µM of each primer, 2 U of Platinum Taq DNA polymerase (Invitrogen, USA) and 4 µl of each extracted template DNA. Thermal cycling was performed on a PTC-100™ programmable thermal controller (MJ Research, Inc. USA). The cycling conditions began with a 5-minute hot start at 95°C to activate the DNA polymerase and was followed by 35 cycles consisting of 95°C for 45 s, 57°C for 45 s, and 72°C for 60 s, ending with a final extension at 72°C for 10 minutes. The mixture was held at room temperature until analysis by gel electrophoresis.

Ten µl of each amplified product was mixed with 5 µl of loading buffer and electrophoresed on 1.5% agarose gel containing ethidium bromide, in TBE buffer at 90V for 45 minutes. The gel was viewed on a UV trans-illuminator. The migration distance of the amplified fragment was compared to that of the Gene Ruler 100-bp marker to determine its approximate size.

## **Results**

Out of the 17 water samples collected, four yielded *Legionella*-like organisms. All four isolates grew on BCYE- $\alpha$  after three to eight days of incubation, forming shiny grayish-white colonies with round edges. These were presumed to be *Legionella* by their inability to grow on HBA, which did not contain cysteine. DNA amplification yielded bands of 386-bp size (Figure 1), confirming their identity as *Legionella* spp., and direct fluorescence assay using monoclonal antibodies determined that two of the four isolates were *L. pneumophila* serogroup 1. The remaining two were non-reactive with the *L. pneumophila* serogroup 1 antiserum but were identified as *Legionella* by PCR.

Table 1 shows the breakdown of isolation based on sampling site. Out of a total of 17 sites around the university campus, six were from non-hospital ground-based cooling towers from various faculties that could be accessed without written permission. The remaining 11 were from different sites in the university hospital where potable water was available, namely the taps and showers in ward bathrooms. One of the six



**Figure 1.** Gel electrophoresis picture of 16s rRNA gene PCR for *Legionella* spp. Lanes 21-24: water samples positive for *Legionellae*; lanes 25-27: negative controls; lane 28: 100-bp marker. The specific 16s rRNA amplicon appears as a 386-bp band. The second (649-bp) band in lanes 22 and 23 is the product of amplification of a virulence gene in *Legionella pneumophila* (not described in this study).

cooling towers tested positive for *Legionella*. The other three samples that yielded *Legionellae* were two hot water taps and a shower head from a paediatrics ward of the hospital. This gives an overall isolation rate of 23.5% (16.7% from the water cooling towers and 27.3% from hospital warm water supplies).

The mean pH and temperature of the water samples obtained from the cooling towers and wards are as shown in Table 1. Hot water systems in the hospital were targeted for sampling because, as previously reported, (a) *Legionellae* can multiply in waters of temperatures that are not favourable to many other pathogens, and (b) these organisms are found more frequently and in larger numbers in hot water systems than cold water systems.

## Discussion

The isolation of *Legionella* from natural waters without association with disease (3) was the first indication that this organism was not merely an accidental contaminant in the air-conditioning cooling tower involved

**Table 1.** Breakdown of isolation based on sampling site

Sample Type	Cooling Towers	Ward Hot Water Supply
Sampling location	<ul style="list-style-type: none"> <li>• Pathology department</li> <li>• Business faculty 1</li> <li>• Business faculty 2</li> <li>• Symphonic Band headquarters</li> <li>• Hospital 1</li> <li>• Hospital 2</li> </ul>	Adult Oncology Unit <ul style="list-style-type: none"> <li>• Bone marrow transplant bathroom shower head</li> <li>• Water tap 1</li> <li>• Water tap 2</li> </ul> Milk kitchen <ul style="list-style-type: none"> <li>• Preparation area tap</li> <li>• Wash area 1</li> <li>• Wash area 2</li> </ul> Paediatrics 4 ward <ul style="list-style-type: none"> <li>• Shower head</li> <li>• Water tap (dirty utility room)</li> <li>• Water tap (clean utility room)</li> </ul> Intensive Care Unit (ICU) <ul style="list-style-type: none"> <li>• Water tap 1</li> <li>• Water tap 2</li> </ul>
Total no. sampled	6	11
No. positive for <i>Legionella</i>	3	3
Mean pH of water	4.7	4.6
Mean temperature (°C)	31.2	46.6

in the 1976 Philadelphia outbreak, but actually dwelled in aquatic habitats. Since then, there have been reports of *Legionella* being found in potable water systems such as plumbing systems (3,9), and luxury items such as fountains, spas (10) and misting devices (11).

In our study, we have pinpointed certain areas in a university hospital as harbouring *Legionella*. Two of the four sites were found to be contaminated with *L. pneumophila* serogroup 1, which is responsible for 50-70% of Legionnaires' Disease cases (8), and the two other sites by non-serogroup 1 *Legionella*. The detection rate of 23.5% is somewhat lower than the 40% previously reported from this country and its neighbour (6); however, the lower detection rate may be accounted for by the filtration isolation technique, which has a reported success rate of 53%, and the acid buffer treatment which, while necessary to eliminate other microorganisms in the water, has been reported to reduce the recovery rate by 30% (12). With the development of molecular biology, it may be more prudent to couple these tools with traditional culture methods to increase detection accuracy and sensitivity in future studies.

It has long since been established that *Legionella* infests waters as intracellular parasites of amoebae and protozoa. This carries serious implications since it has recently been shown that not only does this provide *Legionella* with sanctuary from harsh environmental conditions (13) – and thus increases its resistance against elimination by chemical methods – it also enhances its infectivity (14). In the hospital environment especially, its proliferation in plumbing systems is encouraged by the relatively high temperature of the water which, while hot enough to eliminate most other pathogens, is usually not high enough to kill *Legionella* spp. as they thrive in temperatures of about 32-42°C (1,3), although they cannot survive temperatures of over 60°C. This study found that the *Legionellae* were able to survive in hospital hot water systems with an average water temperature of 46°C.

Many previous studies have reported heavy contamination of *Legionella* in the plumbing systems of hospitals and other health care facilities (3,15), and these organisms have been associated with nosocomial outbreaks (5,7,12,14). The presence of *Legionella* in the hot water supply to the paediatrics ward poses a potential infection hazard as immunocompromised patients in the ward would be at risk for Legionnaires' Disease; furthermore, *Legionella pneumophila* has been associated with bacteremic coinfection (16).

Until recently there has been very little concern about this disease in this region, and there is no data regarding the extent of endemic *Legionella* infections in Malaysia (6); however, the case reports from Singapore (4) and China (5) serve as warning of the health issues Malaysia may face in the future, especially since there is a fast-growing trend towards the use of misting devices in both places of residence and public locations. If legionellosis is to be avoided, especially in health care establishments, then monitoring of water supplies and chemical disinfection with oxidizing agents or thermal disinfection (17), coupled with regular scouring of the cooling towers to remove the slime and sediments that would harbour *Legionellae* should be carried out as a preventive measure, and not as corrective action after disease occurs.

## References

1. Yee RY, Wadowsky RM. Multiplication of *Legionella pneumophila* in unsterilized tap water. *Appl Environ Microbiol* 1982; 43:1330-34.
2. Hsu SC, Martin R, Wentworth BB. Isolation of *Legionella* species from drinking water. *Appl Environ Microbiol* 1984; 48:830-32.
3. Wadowsky RM, Yee RB, Mezmar L, et al. Hot water systems as sources of *Legionella pneumophila* in hospital and non-hospital plumbing fixtures. *Appl Environ Microbiol* 1982; 43:1104-110.
4. Goh KT, Ed. Surveillance of Legionellosis and *Legionella* bacteria in Singapore, 1999. *Epidemiol Bull* 2000; 26:39-44.
5. Chen YS, Lin WR, Liu YC, et al. Residential water supply as a likely cause of community-acquired Legionnaires' Disease in an immunocompromised host. *Eur J Clin Microbiol Infect Dis* 2002; 21:706-09.
6. Ngeow YF, Tan CH, Lim SY. *Legionella* species isolated from cooling towers in Kuala Lumpur. *Med J Malaysia* 1992; 47:15-19.
7. Bopp CA, Sumner JW, Morris GK, et al. Isolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective medium. *J Clin Microbiol* 1981; 13:714-19.
8. Jonas D, Rosenbaum A, Weyrich S, et al. Enzyme-linked immunoassay for detection of PCR-amplified DNA of *Legionellae* in bronchoalveolar fluid. *J Clin Microbiol* 1995; 33:1247-52.
9. Tobin JO'H, Beare J, Dunnill MS. Legionnaires' Disease in a transplant unit: isolation of the causative agent from shower baths. *Lancet* 1980; 2:118-121.

10. Hershey J, Burrus B, Marcussen V, et al. Legionnaires' Disease Associated with a Whirlpool Spa Display – Virginia, September-October, 1996. MMWR 1997; 46: 83-6.
11. LaMaire W, Jackson H, McFarland L. Epidemiologic Notes and Reports: Legionnaires' Disease Outbreak Associated with a Grocery Store Mist Machine – Louisiana, 1989. MMWR 1990; 39:108-110.
12. Boulanger CA, Edelstein PH. Precision and accuracy of recovery of *Legionella pneumophila* from seeded tap water by filtration and centrifugation. Appl Environ Microbiol 1995; 61:1805-09.
13. Gao LY, Harb OS, Kwaik YS. Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. Infect Immun 1997; 65:4738-46.
14. Cirillo JD, Falkow S, Tompkins LS. Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. Infect Immun 1994; 62:3254-61.
15. Sabria M, Yu VL. Hospital-acquired *Legionellosis*: solutions for a preventable infection. Lancet Infect Dis 2002; 2:368-73.
16. Tan MJ, Tan JS, File TM Jr. Legionnaires' Disease with bacteremic coinfection. Clin Infect Dis 2002; 35:533-9.
17. Kim BR, Anderson JE, Mueller SA, et al. Literature review – efficacy of various disinfectants against *Legionella* in water systems. Water Res 2002; 36:4433-44.