Detection of *Legionella* spp. in Water Samples taken from Cooling Tower and Water Holding Systems in a Hospital in Metro Manila

Dianne Melody A. De Roxas and Adelwisa R. Ortega

Department of Medical Microbiology, College of Public Health, University of the Philippines Manila

ABSTRACT

Background. Hospitals present ideal locations for transmission of Legionnaires' disease, a pneumonia-like disease caused by *Legionella* spp. In these settings, hospitalized patients may be exposed to aerosols generated by cooling towers, respiratory therapy equipment, showers and faucets, that facilitate transmission of the bacterium.

Objective. The study aimed to determine the presence of *Legionella* spp. in water samples taken from the cooling tower and water holding systems of a hospital in Metro Manila.

Methods. A total of 12 water samples were collected: 6 samples from the cooling tower, 4 from two cisterns and 2 from the water tank. The samples were concentrated, acid-treated, gram-stained and cultured. Biochemical tests were done for identification of *Legionella* spp.

Results. *Legionella* spp. was detected in 2 (16.67%) out of 12 samples, both of them from the pre-condenser sampling site of the hospital's cooling tower.

Conclusion. The study documents the presence of *Legionella* spp. in the cooling tower, a potential source of infectious aerosols that can be disseminated in the hospital and affect hospitalized patients.

Key Words: Legionella spp., cooling tower, Buffered Charcoal Yeast Extract (BCYE) agar

Introduction

Legionella refers to a genus of gram-negative bacteria that occurs ubiquitously in soil and aquatic environments.[1] In 1976, the bacterium was identified as the causative agent of an outbreak of pneumonia among people who attended an American Legion convention in Philadelphia, USA.[2] Ever since the discovery of the organism in the hotel's air-conditioning system in 1976, it has been found with increasing frequency not only in natural but also man-made environments, such as cooling towers and potable water systems.[1,3,4] In natural environments, *Legionella* is present in low density although its concentration can significantly increase in artificial habitats depending on the type of material, on the presence of biofilms and available nutrients and on the microbial condition of the water.[5]

Given the particular traits of these pathogens, some environments are particularly at risk and among these are cooling towers and potable water systems located in hospitals, which can produce aerosols and facilitate the spread of "infectious water droplets." Several studies have implicated this bacterium as the source of nosocomial or community-acquired outbreaks of Legionnaires' Disease, following inhalation of aerosols of *Legionella*-positive water generated by devices in which warm water can stagnate, such as air-conditioning cooling towers, humidifiers, shower heads and faucets.[6,7,8]

In the United States, between 8,000-18,000 cases of Legionnaires' disease occur annually,[9] of which 25% of cases reported by the Centers for Disease Control and Prevention (CDC) are acquired in the hospital.[10,11] In nosocomial outbreaks, the average mortality has been estimated to be about 15–20% of hospitalized cases.[12,13] Up to 40% case fatality rate (CFR) among nosocomial cases vs 20% CFR among community-acquired Legionnaire's disease was recorded in the US (1997).[5] More recent data from the US and Australia showed 14% CFR for nosocomial and 5-10% of community-acquired cases while in Europe, the overall CFR is about 12%.[14,15] .

In the Philippines, there is not much data on confirmed reported cases of Legionnaire's disease, much less detection of *Legionella* spp. in the environment.

The study aims to determine the presence of *Legionella* from water samples taken from the cooling tower and other water holding systems (such as water tanks and cisterns) in a hospital in Metro Manila.

Materials and Methods

The study was carried out between January and March 2014 in a public tertiary 1,500-bed capacity hospital located in Metro Manila. The hospital had one cooling tower, two water cisterns and one water tank.

Sampling sites

Cooling Tower and Water Holding systems

Cooling tower is a heat-transfer device in which warm water is cooled by evaporation in atmospheric air. It is used to provide cooling for refrigeration plant as well as to cool water for air-conditioning to buildings.

Corresponding author: Dianne Melody A. de Roxas
Department of Medical Microbiology
College of Public Health
University of the Philippines Manila
625 Pedro Gil Street, Ermita, Manila 1000 Philippines
Telephone: +632 5255874
Email: dmderoxas@gmail.com

While, cisterns and water tanks are examples of water holding systems. Water from the municipal or well supply are stored in the holding tanks or cisterns in large quantity then distributed to the building or cooling towers. The capacity of the water holding systems of the hospital are as follows: 10 000 gallons for Cistern I, 25 000 gallons for Cistern II and 6 600 gallons for the overhead water tank.

Water samples were collected in these water holding systems in order to test for the presence of *Legionella* spp. in the water. If the water in these holding systems harbored the organism, it can therefore be distributed to the water outlets such as faucets and showers that can generate an infectious aerosol. And also it can introduce *Legionella* spp. to the cooling tower, since the holding systems supply the water of the cooling tower.

Sample Collection and Processing

Water samples were collected from the cooling tower and water holding systems according to the standard procedures of the CDC.[16] One liter of water was collected from the cooling tower as well as from the cisterns and water tank using sterile bottles that contained 0.5ml of 0.1N sodium thiosulfate for each 1 liter sample to neutralize the disinfectant chlorine and were processed within 24 hours after collection.

A three-day water collection was done and temperature of each sample on collection were measured using a handheld temperature probe (Mercurial Water Bath Thermometer, Fisher USA) and recorded. A total of 12 water samples were collected: 6 samples from three sampling points of the cooling tower, 4 samples from the 2 water cisterns and 2 samples from the water tank. Table 1 shows the data on collection sites and sampling points.

Table 1. Isolation of *Legionella* spp. based on sampling points

Collection site	Sampling points	Total no. of samples	No. Positive for Legionella (%) N=12
Cooling tower	Make-up water	2	0
	Collecting basin	2	0
	Pre-condenser	2	2 (16.67%)
Cisterns	Cistern I sampling point	2	0
	Cistern II sampling point	2	0
Overhead water tank	Water tank sampling point	2	0

Sample Concentration

Isolation of *Legionella* spp. was performed by concentrating 1 L water sample using a standard filtration set (Millipore, Millipore Filter Corporation, USA) with the aid of negative pressure through polycarbonate membrane filters of 0.22μm pore size. The filter membranes were immersed in a sterile 200mL beaker containing 5 mL of the water sample and vigorously shaken for one minute to free bacteria and organic material from the filter.

Sample Treatment and Inoculation

The concentrated sample was treated with acid: 1.0 mL HCl-KCl buffer (pH 2.2) mixed with 1.0 mL of concentrated sample and incubated at room temperature (25°C to 30°C) for 10 minutes. After the acid treatment, 0.1 ml of the acid-sample suspension was inoculated into triplicate plates of GVPC agar [Buffered Charcoal Yeast Extract (BCYE) agar with addition of glycine, vancomycin, polymixin B and cycloheximide supplement, Oxoid]. The plates were placed inside a candle jar with 2.5% CO_2 then incubated at 35 °C in the incubator for 10 days.

Screening Suspect Colonies and Identification of Legionella spp.

Typical Legionella–type bacteria colonies (convex, round with entire edges and greyish-white in color) appeared after 3-5 days of incubation. These colonies, suggestive of *Legionella* spp., were then aseptically picked and plated onto a blood agar plate without L-cysteine and incubated for 24 hours. Isolates that grew on the GVPC agar with cysteine, but not blood agar, were considered as suspect colonies and underwent identification by gram staining and biochemical testing. An isolated *Legionella* spp. colony is gram negative, motile, positive for cysteine dependence, with the following biochemical test profile: negative for carbohydrates utilization, negative for citrate utilization, negative for nitrate reduction, negative for sulfur production, negative for indole test, weak positive for oxidase test and has a variable(+/-) result for catalase test.

Results

The result of the study showed that 2 out of the 12 water samples (16.67%) were positive for *Legionella* spp. according to the results of the various tests done (i.e. cysteine dependence, gram stain reaction and biochemical tests) enumerated in Tables 2 and 3, respectively. The 2 positive samples were taken from the pre-condenser sampling site of the hospital's cooling tower with an average temperature of 25.75°C. While the other sampling sites of the cooling tower, such as the collecting basin, make up water; cisterns I and II and the water tank water samples with temperature ranging from 25°C to 26.5°C were negative for *Legionella* organisms.

Discussion

Many international researches in the field of *Legionella* conclude that the issue of Legionnaires' disease has emerged as a public health problem, concerning public health professionals and individuals, the general population and also the managers of public and private organizations.[17,33,5] Cases of *Legionella* infection and prevalence of the *Legionella* in water systems such as water heaters and cooling towers have been detected and recorded in many countries around the world such as the US, UK, Saudi Arabia, Malaysia, Singapore and China [7,18-22] with the aid of improved etiological diagnostic methods and extensive surveillance of the organism in the water systems. To date, there is very

little information published on *Legionella* in the Philippines; there is a general lack of awareness regarding the health risks it poses once the organism contaminates the water.

Table 2. Result of the cysteine dependence and gram stain reaction of isolates in each water sample

Sampling points	Sample No.	Cysteine Dependence	Gram stain reaction
Pre-condenser	1	Yes	Gram negative
	2	Yes	Gram negative
Collecting basin	3	Yes	Gram negative
	4	Yes	Gram negative
Make-up water	5	No	NA
	6	No	NA
Cistern I sampling point	7	No	NA
	8	No	NA
Cistern II sampling point	9	No	NA
	10	No	NA
Water tank sampling point	11	No	NA
	12	No	NA

*Only the isolates which are dependent of cysteine were Gram stained

According to the World Health Organization (WHO), nosocomial cases usually make up a small proportion of reported cases of *Legionella* infection however, the proportion of cases that are fatal tends to be much higher with nosocomial infections than with community-acquired infections.[5] Hospitals present ideal locations for disease transmission: at risk individuals are present in large numbers; plumbing systems are frequently old and complex favoring amplification of the organism.[17] Healthcare facilities have a special responsibility for preventing Legionnaires' disease.[5]

Cooling towers are often found to harbor *Legionella* and have been implicated in many outbreaks of *Legionella* infection[7,23] Garcia-Fulgueiras et al. reported the world's biggest outbreak of *Legionella* infection in 2001 in Murcia, Spain in which 449 confirmed cases with 1% case–fatality rate (CFR), were associated with the air-conditioning cooling towers of a city hospital.[23]

The role of aerosols from contaminated aerosol-generating systems such as cooling towers, building water systems and hot tubs in the transmission of *Legionella* infection is well-established.[5] Cooling towers are a particular problem, according to Bhopal (1995) with cooling towers accounting for at least 28% of all sporadic cases of *Legionella* infection.[7]

Studies done in several countries showed that the prevalence of *Legionella* in cooling towers are as follows: 15.6% (N=18 164) of cooling tower water samples were *Legionella* positive in Singapore[21] and 58.9% of cooling towers (N=321) in China were found positive of the organism,[22]. In Saudi Arabia, the prevalence of *Legionella* in cooling towers was 5.68% (N=88).[19] While, a 16.7% prevalence (N=6) of the organism in cooling towers was documented in a study in Kuala Lumpur.[4]

These data support that cooling towers provide a conducive environment for the growth and proliferation of the organism once the water circulating in their system has been contaminated. Several factors affecting the growth and multiplication of *Legionella* are temperature, presence of protozoa, biofilms, biocides and nutrients.[5]

Yee & Wadowsky (1982) showed that naturally occurring *L. pneumophila* survives and multiplies in water at temperatures between 25 ºC and 45 ºC, with an optimal temperature range of 32–42 ºC.[3] Their study also found that *Legionellae* were most commonly isolated at temperatures between 35 ºC and 45 ºC, the temperature at which the condenser water temperature usually falls.[3] In this study, the water sample was taken at the pre-condenser site (i.e., from the water in the pipe before the condenser itself) which has a temperature that ranges from 25-26 ºC. The temperature of this site can also favor the survival and multiplication of the organism though this is not the optimal temperature range. *Legionella* organisms were isolated from the samples from the pre-condenser site, indicating that the water that will flow into the condenser is already harboring the organism; and that the organism present will probably grow and multiply inside the condenser where the temperature range is optimal.

The samples from the pre-condenser sampling site are turbid and one of them had an abundant amount of moss and iron scales from the old pipes, favoring the proliferation of the organism. The presence of biofilms is also an important factor for *Legionella* survival and growth in water

Table 3. Biochemical tests result of the suspected colonies from the cooling tower

Sampling points	Colony No.	Glu	Lac	Mal	Man	Suc	Citrate	Nitrate	Catalase	Oxidase	Sulfur	Indole	Motility
Cooling tower Pre-condenser	**1a**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**wk +**	**wk +**	**-**	**-**	**motile**
	1b	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**wk +**	**wk +**	**-**	**-**	**motile**
	1c	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**wk +**	**-**	**-**	**-**	**motile**
	2a	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**wk +**	**wk +**	**-**	**-**	**motile**
	2b	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**wk +**	**+**	**-**	**-**	**motile**
	2c	-	-	-	+	-	-	-	wk +	+	-	-	motile
Cooling tower Collecting Basin	3a	-	-	+	-	-	-	-	wk +	wk +	-	-	non-motile
	3b	+	-	-	-	-	-	-	wk +	+	-	-	motile
	4a	+	-	-	-	-	-	-	wk +	+	-	-	motile
	4b	+	-	+	-	-	-	-	-	-	-	-	non-motile

*Colonies highlighted are *Legionella spp*. according to its biochemical result

systems.[24,25] The presence of scale and corrosion in a system increases the available surface area for the formation of microniches that are protected from circulating disinfectants and also increases the concentration of nutrients and growth factors, such as iron, in the water system.[5]

The isolation of only few *Legionella* spp. organism in the pre-condenser sample and not in the samples from other sampling sites such as the cisterns and water tank may be accounted for by the filtration isolation technique and the acid treatment used to eliminate other microorganisms in the water. The concentration of the sample using filtration increased the sensitivity of the method, but not the specificity. According to several studies, filtration reduces detectable Legionellae by 16-91%.[26,27,28]

After the filtration step, the acid treatment for decontamination using acid buffer (pH 2.2 for 10 min) was done. This procedure increases the specificity for Legionella but unfortunately it can lead to a 5 to 99% decrease in isolated Legionellae.[29,27] This means that the water sample may possibly harbor the organism, but with the process of filtration and acid treatment done, it may have killed the organism and thus was not isolated.

Another possible reason for the low detection of the organism in the water samples was the viable but not culturable (VBNC) state of *Legionella*. The presence of VBNC is well known in *L. pneumophila*,[30] in which growth on agar media may be inhibited by the presence of other organisms. This state is seen especially in environmental samples.[31] A study by Steinert et al. showed that VBNC is a potential source of infection by demonstrating that VBNC *Legionella* cells could be resuscitated by co-incubation with amoebae without any loss of virulence.[32]

Culture method was used as it is generally accepted as the "gold standard" for *Legionella* detection.[31] However, this method has several limitations, such as the overgrowth of other accompanying bacteria, the VBNC state, loss of viability of bacteria after collection and during the sample treatment, and the use of antibiotics in the medium.[32] Molecular detection methods increases sensitivity and detection rates and may be considered in future studies.

Conclusion

Overall, this study documented the presence of *Legionella* spp. the cooling tower of the concerned hospital. The results indicate that the environment in the cooling system supports the growth of *Legionella.* As the cooling tower generates aerosols, the potential for spreading infectious aerosols in the hospital, which can subsequently affect hospitalized patients, is a possibility.

References

1. Yee RB, Wadowsky RM. Multiplication of Legionella pneumophila in unsterilized tap water. Appl Environ Microbiol. 1982; 43(6):1330-4.
2. Brenner DJ, Steigerwalt AG, McDade JE. Classification of the Legionnaires' disease bacterium: Legionella pneumophila, genus novum, species nova, of the family Legionellaceae, familia nova. Ann Intern Med.1979; 90(4):656-8.
3. Wadowsky RM, Yee RB, Mezmar L, et al. Hot water systems as sources of Legionella pneumophila in hospital and nonhospital plumbing fixtures. Appl Environ Microbiol. 1982; 43(5):1104-10.
4. Chin KS, Rosli AA, Wee CSL, Ngeow YF. Isolation of Legionella from cooling towers and potable water systems in hospital and non-medical buildings in a university campus. JUMMEC. 2003- 2005; 8(1):23-7.
5. World Health Organization.Legionella and the prevention of legionellosis. 2007. ISBN 92 4 156297 8 (NLM classification: WC 200); pg 15, 31-37, 89.
6. Arnow PM, Chou T, Weil D, Shapiro EN, Kretzschmar C. Nosocominal Legionnaires' disease caused by aerosolized tap water from respiratory devices. J Infect Dis. 1982; 146(4):460–7.
7. Bhopal RS, Fallon RJ, Buist EC, Black RJ, Urquhart JD. Proximity of the home to a cooling tower and risk of non-outbreak Legionnaires' disease. BMJ.1991; 302(6773):378–83.
8. Woo AH, Goetz A, Yu VL. Transmission of Legionella by respiratory equipment and aerosol generating devices. Chest. 1992; 102(5):1586–90.
9. Marston BJ, Plouffe JF, File TM Jr, et al. Incidence of community-acquired pneumonia requiring hospitalization. Results of a population-based active surveillance study in Ohio. The Community Based Pneumonia Incidence Study Group. Arch Intern Med. 1997; 157(15):1709-18.
10. Marston BJ, Lipman HB, Breiman RF. Surveillance for Legionnaires' disease: Risk factors for morbidity and mortality. Arch Intern Med. 1994; 154(21):2417–22.
11. Kool JL, Bergmire-Sweat D, Butler JC, et al. Hospital characteristics associated with colonization of water systems by Legionella and risk of nosocomial legionnaires' disease: a cohort study of 15 hospitals. Infect Control Hosp Epidemiol. 1999; 20(12):798–805.
12. Guerin JC. Epidemiology of Legionnaire's disease. From myth to reality. Rev Mal Respir, 1992; 9(1):R53–6.
13. Roig J, Rello J .Legionnaires' disease: a rational approach to therapy. J Antimicrob Chemother. 2003; 51(5):1119–29.
14. Benin AL, Benson RF, Arnold KE, et al. An outbreak of travel-associated Legionnaires' disease and Pontiac fever: the need for enhanced surveillance of travel-associated legionellosis in the United States. J Infect Dis. 2002; 185(2):237–43.
15. Howden BP, Stuart RL, Tallis G, Bailey M, Johnson PD. Treatment and outcome of 104 hospitalized patients with legionnaires' disease. Intern Med J. 2003; 33(11):484–8.
16. Center for Disease Control and Prevention. Procedures for the Recovery of *Legionella* from the Environment Manual [Online]. January 2005. Available from CDC: www.cdc.gov/legionella/specimen-collect-mgmt/procedures-manual.html.
17. Fields BS, Benson RF, Besser RE. Legionella and Legionnaires' disease: 25 years of investigation. Clin Microbiol Rev.2002; 15(3):506–26.
18. O'Mahony MC, Stanwell-Smith RE, Tillett HE, et al. The Stafford outbreak of Legionnaires' disease. Epidemiol Infect. 1990; 104(3):361–80.
19. Nasreldin Elhadi, Hatem Qutub. Detection of Legionella from teaching hospital cooling tower water of air conditioning systems in eastern province of Saudi Arabia. Asian J Med Sci. 2012; 4(2):99-102.
20. Ngeow YF, Tan CH, Lim SY. Legionella species isolated from cooling towers in Kuala Lumpur. Med J Malaysia. 1992; 47(1):15-9.
21. Lam MC, Ang LW, Tan AL, James L, Goh KT. Epidemiology and control of legionellosis, Singapore Emerg Infect Dis. 2011; 17(7):1209-15.
22. Sun H, Chen X. Prevalence of Legionella cooling tower water contamination and Legionella pneumophila antibodies among hotel workers of Jiangsu, China. Epidemiology. 2011; 22(1):S253.
23. García-Fulgueiras A, Navarro C, Fenoll D, et al. Legionnaires' disease outbreak in Murcia, Spain. Emerg Infect Dis.2003; 9(8):915-21.
24. Kramer MH, Ford TE. Legionellosis: ecological factors of an environmentally "new" disease. Zentralbl Hyg Umweltmed.1994; 195(5-6):470–82.
25. Goossens H. Legionellosis: a new disease of Western hemisphere? Verh K Acad Geneeskd Belg. 2001; 63(4):359–77.
26. Brindle RJ, Stannett PJ, CunliffeRN. Legionella pneumophila: comparison of isolation from water specimens by centrifugation and filtration. Epidemiol Infect. 1987; 99(2):241-7.
27. 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(5):1805-9.
28. Ta AC, Stout JE, Yu VL, Wagener MM. Comparison of culture methods for monitoring Legionella species in hospital potable water systems and recommendations for standardization of such methods. J Clin Microbiol. 1995; 33(8):2118-23.
29. Roberts KP, August CM, Nelson JD Jr. Relative sensitivities of environmental legionellae to selective isolation procedures. Appl Environ Microbiol. 1987; 53(12):2704-7.
30. Yamamoto H, Hashimoto Y, Ezaki T. Study of nonculturable Legionella pneumophila cells during multiple-nutrient starvation. FEMS Microbiological Ecology. 1996; 20(3):149–54.
31. Bartie, C.,Venter SN, Nel LH. Identification methods for Legionella from environmental samples. Water Res. 2003; 37 (6):1362-70.
32. Steinert, M., L. Emo¨dy, R. Amann, and J. Hacker.Resuscitation of viable but nonculturable Legionella pneumophila Philadelphia JR32 by Acanthamoeba castellanii. Appl Environ Microbiol. 1997; 63(5):2047–53.
33. Borges A, Simões M, Martínez-Murcia A, Saavedra MJ. Detection of Legionella spp. in natural and man-made water systems using standard guidelines. Journal of Microbiology Research. 2012; 2(4):95-102.