DISTRIBUTION OF *LEGIONELLA PNEUMOPHILA* SEROGROUPS ISOLATED FROM WATER SYSTEMS OF PUBLIC FACILITIES IN BUSAN, SOUTH KOREA

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Abstract. *Legionella pneumophila* is the major causes of legionellosis worldwide. The distribution of *L. pneumophila* was investigated in water systems of public facilities in Busan, South Korea during 2007 and 2013-2014. *L. pneumophila* was isolated from 8.3% of 3,055 samples, of which the highest isolation rate (49%) was from ships and the lowest 4% from fountains. Serogroups of *L. pneumophila* isolated in 2007 were distributed among serogroups (sgs) 1-7 with the exception of sg 4, while those of isolates during 2013 and 2014 included also 11 sgs (1, 2, 3, 4, 5, 6, 7, 8, 12, 13, 15). *L. pneumophila* sg 1 was predominated among isolates from fountains (75%), hotels (60%), buildings (44%), hospitals (38%), and public baths (37%), whereas sg 3 and sg 7 was the most prevalent from ships (46%) and factories (40%), respectively. The predominated serogroup of *L. pneumophila* isolates from hot and cooling tower water was sg 1 (35% and 46%, respectively), while from cold water was sg 3 (29%). These results should be useful for epidemiological surveys to identify sources of outbreaks of legionellosis in Busan, South Korea.

Keywords: *Legionella pneumophila*, public facility, serogroup, type of water source, South Korea

INTRODUCTION

Numerous outbreaks of legionellosis have been reported all over the world. Legionellosis disease is pneumonia caused by *Legionella* spp, ubiquitous gram-negative bacteria found in natural and man-made aquatic environments (Diederen, 2008; Mekkour *et al*, 2013a). Recent studies showed high isolation rates of *Legionella* spp in environmental sources worldwide, with 31.5% in Morocco (Mekkour *et al*, 2012), 31.1% in Iran (Rafiee *et al*, 2014), and 22.1% in Kuwait (Al-Matawah *et al*, 2012). More than 50 species of *Legionella* have been recognized, of which at least 24 are associated with human infection (Mekkour *et al*, 2013b). *L. pneumophila* causes about 90% of reported cases of legionellosis and the infection occurs mainly by inhalation of contaminated aerosols generated from water systems (Lee *et al*, 2010; Mekkour *et al*, 2013b).

In South Korea, statistics showed that the patients with legionellosis have been steadily increased since its first outbreak in 1984 (Kim *et al*, 1985). Legionellosis

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occurs throughout the year regardless of season (http://stat.cdc.go.kr). Thus, surveillance of water supply systems having the potential of being contaminated, as well as cooling water towers, at various public facilities should be performed all year round. Recently, *Legionella* spp has been isolated from environmental sources in South Korea and the distribution of *Legionella* spp was different depending on geographical region, with 10.9% in Seoul, 2.5% in Gyeonggi, 20.0% in Chungcheong, and 2.0% in Jeju (Lee *et al*, 2010). Accordingly, studies on the regional distribution of *Legionella* spp isolated from the environment are warranted.

Busan is the second largest city in South Korea with approximately 3.6 million residents and is becoming reputed as an international convention place. Good hygiene of public facilities in Busan is important for public health. To date, there is no report on the isolation and distribution of *L. pneumophila* from public facilities in Busan. In order to obtain data on *L. pneumophila* distribution, we have isolated *L. pneumophila* from environmental water systems of public facilities in Busan, such as large buildings, public baths, hospitals, hotel, factory, and ship, and investigated serological distribution of isolates and distribution according to type of facility. The most important task in epidemiological investigations of infectious disease is to identify the source of infection rapidly. This can prevent the propagation and relapse of the disease. It is necessary to gather data about the distribution of *Legionella* in Busan to find the source of infection in the outbreak of legionellosis in the city. This study will provide useful epidemiological information not only for the prevention of legionellosis, but also for tracking the source of infection.

MATERIALS AND METHODS

Sampling

A total of 3,055 water samples were collected from water systems in Busan, South Korea public facilities such as large buildings, public baths, hospitals, and hotels in 2007 and from 2013 to 2014, and from factories and ships from 2013 to 2014 only. Aliquots of 1,000 ml of water samples were kept in sterile bottles.

Identification of *L. pneumophila*

L. pneumophila was isolated as described previously (Barbaree *et al*, 1988; Orrison *et al*, 1981). In brief, 1 liter aliquots of water samples were filtered through 0.45 mm membranes, which then were resuspended in 20 ml aliquots of distilled water. The suspensions (0.1 ml) were plated onto buffered charcoal yeast extract (BCYE) supplemented with GVPC agar (Oxoid, Hamshire, UK). Plates were incubated at 37°C under a humidified atmosphere for 10 days and then suspected *L. pneumophila* colonies were selected and sub-cultured on BCYE agar with and without L-cysteine (Oxoid) (Lee *et al*, 2010). Colonies growing only on BCYE with L-cysteine then were assayed for *L. pneumophila*-specific 16S rDNA and *mip* by PCR (Jaulhac *et al*, 1992; Segal *et al*, 1998). The 16S rDNA was amplified using primers F (5′-AGGGTTGATAGGTTAAGAGC-3′) and R (5′-CCAACAGCTAGTTGACATCG-3′) and *mip* using primers F (5′-GGTGACTGCGGCTGTTATGG-3′) and R (5′-GGCCAATAGGTCCGCCAACG-3′). The 20 μl of reaction mixture contained 20 pmol of each primer and Maxime PCR PreMix Kit (INtRON Biotechnology, Gyeonggi-do, South Korea). Thermocycling was performed in a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) as follows: 95°C for 5 minutes; followed by 30 cycles of 95°C

Table 1
Distribution of *Legionella pnuemophila* isolates collected from water systems of public facilities in 2007 and from 2013 to 2014, Busan, South Korea.

Source	2007		2013, 2014		Total	
	No. of samples	No. of isolates (%)	No. of samples	No. of isolates (%)	No. of samples	No. of isolates (%)
Building	508	35 (6.9)	428	31 (7.2)	936	66 (7.0)
Public bath	346	42 (12.1)	375	40 (10.6)	721	82 (11.4)
Hospital	590	25 (4.2)	469	28 (6.0)	1,059	53 (5.0)
Factory	-	-	149	10 (7.0)	149	10 (7.0)
Hotel	26	6 (23.0)	21	9 (43.0)	47	15 (32.0)
Ship	-	-	49	24 (49.0)	49	24 (49.0)
Fountain	14	3 (21.0)	80	1 (1.0)	94	4 (4.0)
Total	1,484	111 (7.5)	1,571	143 (9.1)	3,055	254 (8.3)

–, not collected.

for 1 minute, 60°C for 1 minute and 72°C for 1 minute. Amplicons (386 and 630 bp of 16S rDNA and *mip*, respectively) were analyzed by 1% agarose gel-electrophoresis (Advanced Analytical Technologies, Ankeny, IA).

Serogrouping of *L. pneumophila* isolates

L. pneumophila serogroups were determined using a direct fluorescent-antibody assay (DFA) method employing FITC-conjugated antibodies (m-TECH™ / Monoclonal Technologies, Milton, GA) for identifying *L. pneumophila* serogroups 1-15 according to the manufacturer's protocol. In short, isolates were suspended in phosphate-buffered saline (PBS, pH 7.6) and heated at 100°C for 15 minutes or 1 hour (Kim *et al*, 2010). The suspension then was diluted 1,000 folds in PBS and used as antigen.

RESULTS

***L. pneumophila* isolates**

Based on colony characteristics and presence of species-specific 16S rDNA and *mip* (data not shown), 254 (8.3%) samples containing *L. pneumophila* were isolated from 3,055 samples, with isolation rate of 49% (24/49) from ships (cabin, bridge, and galley), 32% (15/47) hotels (air conditioning system, and bath), 11.4% (82/721) public baths, 7.0% (66/936) buildings (air conditioning system), 7.0% (10/149) factories (air conditioning system, tank, and humidity controller), 5.0% (53/1,059) hospitals (patient's room and bathroom), and 4% (4/94) fountains (Table1). When classified according to water source, isolation rate was 10.4% (120/1,156 samples) from cooling tower water, 7.2% (86/1,198) hot water and 6.8% (48/701) cold water (Table 2).

Serological distribution of *L. pneumophila*

Of *L. pneumophila* samples isolated in 2007 ($n = 111$) the major serogroup was sg 1 (48.6%) (Fig 1A), but among the 143 strains isolated from 2013 to 2014 the proportion of sg 1 was reduced to 27.3 % and there was the appearance of sg 8, sg 12, sg 13, and sg 15 (Fig 1B). When analyzed based on facilities, *L. pneumophila* serogroup sg 1

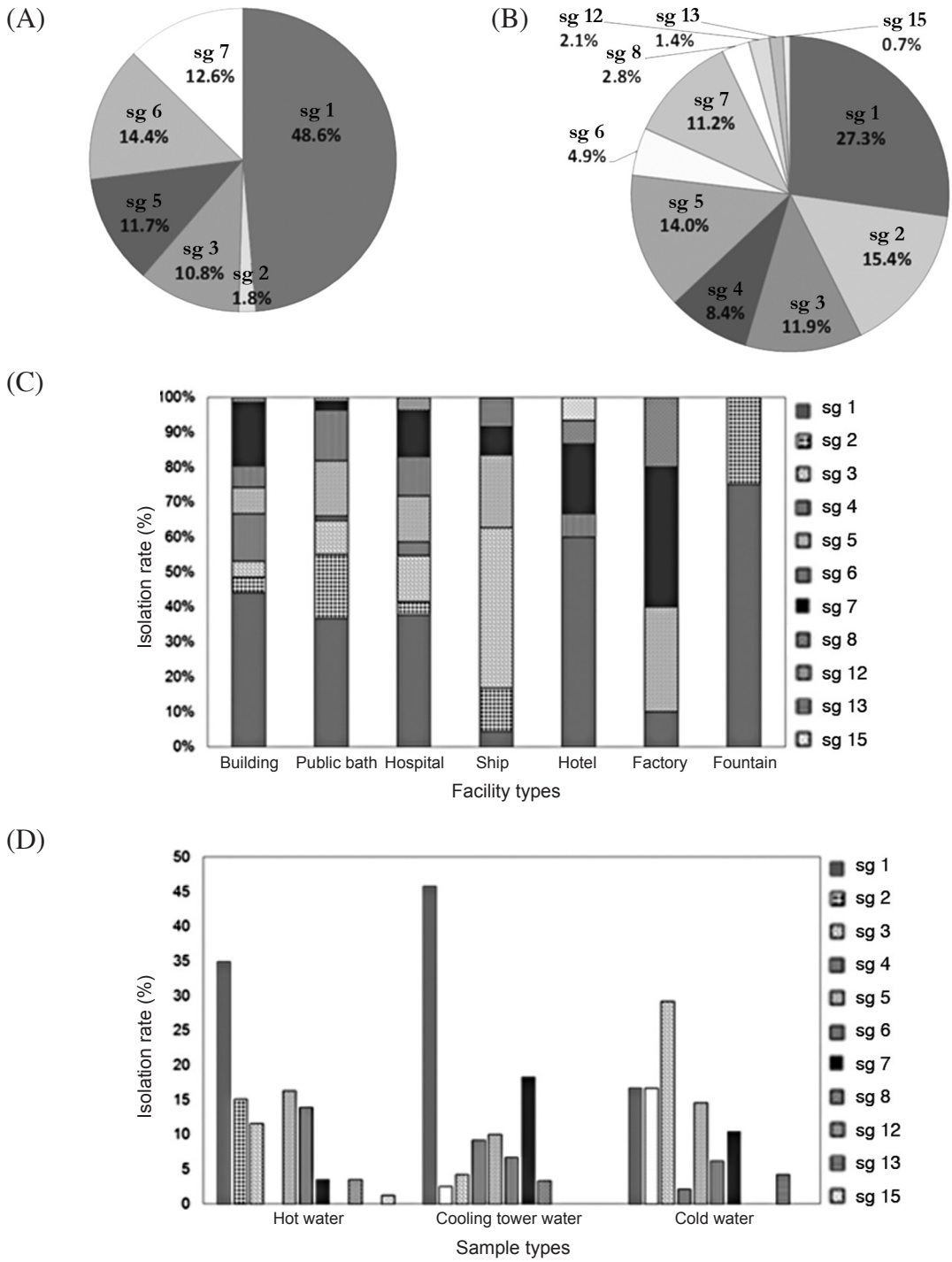

Fig 1–Distribution of *Legionella pneumophila* serogroups from water systems in public facilities, Busan, South Korea. (A) in 2007 ($n = 111$), (B) from 2013 to 2014 ($n = 143$), (C) according to facility types ($n = 254$), (D) according to water sources ($n = 254$). *L. pneumophila* serogroups were determined using a direct fluorescent-antibody assay. Sg, serogroup.

Table 2
Distribution of *Legionella pnuemophila* isolates from water sources in 2007 and from 2013 to 2014, Busan, South Korea.

Source	2007		2013, 2014	
	No. of samples	No. of isolates (%)	No. of samples	No. of isolates (%)
Hot water	766	32 (4.2)	432	54 (12.5)
Cooling tower water	616	68 (11.0)	540	52 (9.6)
Cold water	102	11 (11)	599	37 (6.2)
Total	1,484	111 (7.5)	1,571	143 (9.1)

was predominant in fountains (75%), hotels (60%), buildings (44%), hospitals (38%), and public baths (37%), whereas sg 3 (46%) and sg 7 (40%) was predominant in ships and factories, respectively (Fig 1C); and when analyzed based on water source, *L. pneumophila* sg1 was predominant in cooling tower water (46%) and hot water (35%), while sg 3 (29%) was predominant in cold water (Fig 1D).

DISCUSSION

In this study, we report for the first time the isolation and distribution of *L. pneumophila* from public facilities in Busan, South Korea. The isolation rate of *L. pneumophila* in Busan (8.3%) was lower than that reported in other countries: 60% in Italy (Borella *et al*, 2005), 58.9% in China (Lin *et al*, 2009), 26% in Germany (Zietz *et al*, 2001), and 20.0% in Japan (Edagawa *et al*, 2008).

Cooling tower water in this study showed the highest isolation rate (10.4%) of *L. pneumophila*, compared with 58.9% and 48.9% from cooling tower water of air-conditioning systems in Shanghai, China (Lin *et al*, 2009) and in Greece (Mouchtouri *et al*, 2010), respectively. Hot water showed an isolation rate of 7.2% in this study, while it is 33.3% in Italy (Mouchtouri *et al*, 2007), 30.0% in Finland (Zacheus and Martikainen, 1993), 26.0% in Germany (Zietz *et al*, 2001) and 20.0% in Japan (Edagawa *et al*, 2008). The main reason for the low distribution of *Legionella* in cooling tower water and hot water in Busan, compared with other areas, is probably due to the increase in stringency in surveillance for *Legionella* spp in public facilities nationwide by the Korea Centers for Disease Control and Prevention (KCDC) since 2006. Moreover, as most of the water used for cooling tower water and hot water in Busan is supplied from waterworks storing chlorine-sterilized water, this could have a beneficial effect in limiting growth of *Legionella* spp (and other microorganisms).

With respect to *Legionella* contamination in cruise ships and ferries, previous studies by Goutziana *et al* (2008) reported *Legionella* is present in 66.7% of the water system facilities within the ships and by Azara *et al* (2006) that 42% of water system facilities within ships are contaminated with *Legionella*. In line with these reports, we show an isolation rate of 49.0% in ships. Thus, it is not surprising that there have been reported cases in which passengers aboard ships are infected with *Legionella* (Joseph *et al*, 2005; Ricketts and Joseph, 2005). Therefore, thorough

inspection should be conducted on the water system facilities within domestic ships to check for *Legionella* contamination. Moreover, inspection standards for foreign ships should be also tightened to prevent *Legionella* contamination from other countries while the ship is docked in domestic harbor.

Our results reveal distinct difference in the distribution of *L. pneumophila* serogroups between isolates in 2007 and those from 2013 to 2014, with more serogroup diversity of *L. pneumophila* strains obtained in the more recent survey. Distribution of *L. pneumophila* serogroups isolated in 2007 was similar to previous reports (Kim *et al*, 2009, 2010). The increase in *L. pneumophila* serogroup diversity among isolates of 2013 and 2014 may be due to influx of new species from overseas or change in dominant species in Busan. *L. pneumophila* sg 1 was the most prevalent serogroup from water source of buildings, public baths, hospitals, hotels, and fountains, but not factories and ships. A previous study reported that sg 6 predominated in ships, while sg 1 was not identified (Goutziana *et al*, 2008). Our results also reveal that the predominated serogroup of isolates in hot water and cooling tower water was sg 1, but sg 3 in cold water. These results demonstrate that the distribution of *L. pneumophila* serogroups is dependent on the source and sample type.

It is well documented that *L. pneumophila* sg 1 is the most frequently isolated strain from environmental water system, with distribution rate ranging from 75% to 90% and causes more than 90% of clinical cases (Azara *et al*, 2006; Diederen, 2008; Mekkour *et al*, 2013b). Similarly, sg 1 was also identified as the major strain in this study. The distribution of *L. pneumophila* serogroups from 2013-2014 Busan survey showed a similar pattern to that reported in China (Lin *et al*, 2009), with sg 1 being the most predominant (82.0%) among the environmental isolates, followed by sg 2 (18.0%), sg 5 (10.6%), and sg 3 (10.0%). However, serological typing of *L. pneumophila* strains isolated from water systems of residential facilities in Kuwait revealed that sg 3 accounted for 80.4% of total isolates, followed by sg 1 (13.0%) and sg 4, sg 7, and sg 10 (all 2.1%) (Al-Matawah *et al*, 2012). In addition, biofilms developed within a warm spring of a French thermal spa contained *L. pneumophila* strains consisting of sg 12 (55.0%), sg 1 (25.9%) and sg 10 (18.5%) (Chaabna *et al*, 2013). Taken together, these results indicate geographical differences in the distribution of *L. pneumophila* serogroups. These dara will be very useful for future epidemiological investigations.

In conclusion, this is the first study to identify differences in serological distribution among *L. pneumophila* strains isolated from environmental water systems of public facilities in Busan. This study also reveal differences in *L. pneumophila* prevalence and serogroup in relation to time period, facility, and water sample type. The study provides useful fundamental data for programs of continuous monitoring of *L. pneumophila*, which will make a significant contribution to the prevention of legionellosis and to epidemiological surveys in the event of legionellosis outbreaks in Busan. It is important that surveillance of the water systems of public facility should be continuously performed in other cities not only of South Korea but globally to prevent outbreak of Legionnaires' disease.

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REFERENCES

Al-Matawah QA, Al-Zenki SF, Qasem JA, Al-Waalan TE, Ben Heji AH. Detection and quantification of *Legionella pneumophila* from water systems in Kuwait residential facilities. *J Pathog* 2012; 2012: 138389.

Azara A, Piana A, Sotgiu G, *et al*. Prevalence study of *Legionella* spp. contamination in ferries and cruise ships. *BMC Public Health* 2006; 6: 100.

Barbaree JM, Morrill WE, Fields BS, Martin WT, Sanden GN. Detection and recovery of *Legionella* in water. *Toxic Assess* 1988; 3: 479-90.

Borella P, Montagna MT, Stampi S, *et al*. *Legionella* contamination in hot water of Italian hotels. *Appl Environ Microbiol* 2005; 71: 5805-13.

Chaabna Z, Forey F, Reyrolle M, *et al*. Molecular diversity and high virulence of *Legionella pneumophila* strains isolated from biofilms developed within a warm spring of a thermal spa. *BMC Microbiol* 2013; 13: 17.

Diederen BMW. *Legionella* spp. and Legionnaires' disease. *J Infect* 2008; 56: 1-12.

Edagawa A, Kimura A, Tanaka H, *et al*. Detection of culturable and nonculturable *Legionella* species from hot water systems of public buildings in Japan. *J Appl Microbiol* 2008; 105: 2104-14.

Goutziana G, Mouchtouri VA, Karanika M, *et al*. *Legionella* species colonization of water distribution systems, pools and air conditioning systems in cruise ships and ferries. *BMC Public Health* 2008; 8: 390.

Jaulhac B, Nowicki M, Bornstein N, *et al*. Detection of *Legionella* spp. in bronchoalveolar lavage fluids by DNA amplification. *J Clin Microbiol* 1992; 30: 920-4.

Joseph C, Wijngaarden j, Mshar P, *et al*. Cruiseship-associated legionnaires disease, November 2003-May 2004. *MMWR Morb Mortal Wkly Rep* 2005; 54: 1153-55.

Kim JA, Jung JH, Kim SJ, Jin YH, Oh YH, Han GY. Molecular epidemiological relationship of the pathogenicity of *Legionella* spp. isolated from water systems in Seoul. *Korean J Microbiol* 2009; 45: 126-32.

Kim SH, Kim MJ, Kee HY, *et al*. Surveillance of *Legionella* contamination on water supply systems of public utilizing facilities in Gwangju, Korea. *J Bacteriol Virol* 2010; 40: 19-28.

Kim JS, Lee SW, Shim HS, *et al*. An outbreak of legionellosis in ICU of K Hospital, Korea. *Korean J Epidemiol* 1985; 7: 44-58.

Lee HK, Shim JI, Kim HE, Yu JY, Kang YH. Distribution of *Legionella* species from environmental water sources of public facilities and genetic diversity of *L. pneumophila* serogroup 1 in South Korea. *Appl Environ Microbiol* 2010; 76: 6547-54.

Lin H, Xu B, Chen Y, Wang W. *Legionella* pollution in cooling tower water of air-conditioning systems in Shanghai, China. *J Appl Microbiol* 2009; 106: 606-12.

Mekkour M, Ben Driss EK, Cohen N. Prevalence of *Legionella pneumophila* in production networks and distribution of domestic hot water in Morocco. *World Environ* 2012; 2: 11-5.

Mekkour M, Ben Driss EK, Jarraud S, Tai J, Squinazi F, Cohen N. Investigation of *Legionella pneumophila* serogroup 1 population in Morocco by monoclonal antibody subtyping. *Int J Emerg Sci* 2013a; 3: 43-51.

Mekkour M, Ben Driss EK, Tai J, Cohen N. *Legionella pneumophila*: an environmental organism and accidental pathogen. *Int J Sci Technol* 2013b; 2: 188-96.

Mouchtouri VA, Goutziana G, Kremastinou J, Hadjichristodoulou C. *Legionella* species colonization in cooling towers: risk factors and assessment of control measures. *Am J Infect Control* 2010; 38: 50-5.

Mouchtouri V, Velonakis E, Tsakalof A, *et al*. Risk factors for contamination of hotel water distribution systems by *Legionella* species. *Appl Environ Microbiol* 2007; 73: 1489-92.

Orrison LH, Cherry WB, Milan D. Isolation of *Legionella pneumophila* from cooling tower water by filtration. *Appl Environ Microbiol* 1981; 41: 1202-05.

Rafiee M, Mesdaghinia A, Hajjaran H, Hajaghazadeh M, Miahipour A, Jahangiri-Rad M. The efficacy of residual chlorine content on the control of *Legionella* spp. in hospital water systems. *Iran J Public Health* 2014; 43: 637-44.

Ricketts KD, Joseph CA. Legionnaires' disease in Europe 2003-2004. *Euro Surveill* 2005; 10: 256-9.

Segal G, Purcell M, Shuman HA. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. *Proc Natl Acad Sci USA* 1998; 95: 1669-74.

Zacheus OM, Martikainen PJ. Occurrence of legionellae in hot water distribution systems of Finnish apartment buildings. *Can J Microbiol* 1993; 40: 993-9.

Zietz B, Wiese J, Brengelmann F, Dunkelberg H. Presence of *Legionellaceae* in warm water supplies and typing of strains by polymerase chain reaction. *Epidemiol Infect* 2001; 126: 147-52.