DISTRIBUTION OF VIRULENCE GENES IN ENVIRONMENTAL AND CLINICAL ISOLATES OF *LEGIONELLA PNEUMOPHILA* IN BUSAN, SOUTH KOREA

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Abstract. *Legionella pneumophila* is the major causative agent of Legionnaires' disease. The distribution of *L. pneumophila* in environmental water systems of public facilities in Busan, South Korea was previously reported; however, the distribution of virulence genes in environmental and clinical isolates in Busan is unknown. Here, we investigated using PCR the distribution of three virulence loci (*dot/icm, lvh,* and *rtxA*) in isolates from Busan. A high prevalence of environmental (127/254) and clinical (3/4) isolates were observed for the simultaneous presence of all three genes, while environmental harboring *dot/icm + rtxA*, *dot/icm + lvh*, *rtxA + lvh*, and only one gene were 21%, 11%, 9% and <1-4%, respectively, The remaining clinical isolate carried *dot/icm + rtxA*. In addition, 68% of isolates from serogroup 1, the main cause of legionellosis, possessed all three virulence genes compared with 40% of isolates from serogroups 2 to 15. Prevalence of isolates with two genes was 5-14% and 6-29% in serogroup 1 and serogroups 2 to 15, respectively, whereas that of isolates with one gene was 1-2% and 0-6%, respectively. These results provide valuable information for epidemiological investigations of the relationship between environmental and clinical isolates in legionellosis outbreaks in Busan.

Keywords: *Legionella pneumophila*, *dot/icm, lvh, rtxA*, virulence gene, South Korea

INTRODUCTION

Legionella pneumophila is found in artificial water systems and is transmitted to humans through aerosols from contaminated water (Lee *et al*, 2010). There are 54 *Legionella* species; however, it is known that most cases of legionellosis are caused by *L. pneumophila* (Victor *et al*, 2002). The bacteria are able to invade, multiply within, and kill human alveolar macrophages (Horwitz and Silverstein, 1980; Rowbotham, 1980). The pathogenic cycle of *L. pneumophila* is as follows: the bacteria bind to receptors on cell surface, are taken up into phagocytes by coiling phagocytosis, and then replicate by evading phagosome-lysosome fusion (Horwitz, 1983, 1984; Bozue and Johnson, 1996). After termination of intracellular multiplication, *L. pneumophila* induces pore formation, which is essential for host cell lysis and bacterial egress, and *dot/icm* is essential for the entire process (Byrne and Swanson, 1998; Alli *et al*, 2000). Other studies showed that *lvh* and *rtxA*

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are correlated with the ability to cause disease in humans, with *lvh* being able to replace some conjugation functions of *dot/icm* and *rtxA* contributing to attachment, cytotoxicity and pore formation (Cirillo *et al*, 2001; Samrakandi *et al*, 2002). These three loci are strongly linked to legionellosis and differences in pathogenicity of *L. pneumophila* strains are related to the possession of these virulence genes (Segal *et al*, 1999; Samrakandi *et al*, 2002).

There are approximately 30 cases of legionellosis in South Korea each year and there is a trend of increasing incidence since 2006, as the annual number of cases before 2005 was 10 or fewer (Korean Centers for Disease control and prevention, n.d.). In USA and Europe pneumonia caused by *Legionella* accounts for only 2-9% of community-acquired pneumonias (Victor and Stout, 2008), the percentage is higher in South Korea where *Legionella* causes 3-16% of pneumonia cases (Lee *et al*, 2005). There has not been an outbreak of Legionnaires' disease in South Korea since the first one occurrence in 1984. However, although the number of yearly patients is small, there is still a need for studies on the pathogenicity of the *Legionella* strains currently circulating in Korea as there has been a gradual increase in detection of *Legionella* strains in domestic water systems (Lee *et al*, 2010).

There are numerous published studies on *L. pneumophila* serogroup (sg) 1 owing to sg 1 being known as the most common causative agent of legionellosis; however, there is a scarcity of studies on the other serogroups (Victor *et al*, 2002). According to previous studies on the distribution of virulence genes, *lvh* and *rtxA*, in *L. pneumophila* sg 1 would be an effective index to determine the infection potential of *L. pneumophila* sg 1 isolates (Kim *et al*, 2009). On a similar note, although numerous studies have been conducted on the genes related to the pathogenicity of *L. pneumophila*, there are only a few studies on the distribution of these virulence genes in *L. pneumophila* isolates from the environment and patients (Segal *et al*, 1999; Cirillo *et al*, 2001; Samrakandi *et al*, 2002; Huang, 2004, 2006). In addition, no study has examined the differences in gene distribution for each sg of *L. pneumophila*.

In this study, we examined the distribution of virulence genes in each *L. pneumophila* sg in environmental isolates in Busan, South Korea and, for the first time, the distribution of virulence genes in environmental and clinical isolates in Busan.

MATERIALS AND METHODS

Collection of *L. pneumophila* isolates

A total of 254 *L. pneumophila* isolates were collected from water sources (cooling towers water, hot water and cold water) in public facilities, such as public baths, large buildings, hotels, hospitals, factories, and ships were used in this study, and the serogroups were determined in a previous study (Hwang *et al*, 2016). In brief, 254 *L. pneumophila* strains were isolated from water sources on the basis of colony characteristics and presence of species-specific 16S rDNA and *mip*. Serogroups were determined by a direct fluorescent-antibody assay (DFA) method using FITC-conjugated antibodies (m-TECH™ / Monoclonal Technologies, Milton, GA). Four *L. pneumophila* sg 1 isolates from patients in Busan were also included in this study. These clinical strains were isolated from sputum of patients with primary atypical pneumonia who were referred to determine the cause of their illness. Sputum samples were treated with acidic buffer (0.2 M KCl-HCl) for 15

Table 1
Primers used in the study and amplicon size.

Gene	Primer sequence(5′→3′)	Product size
dotDCM	F : CGATTGGTCTGGTCCGATTGA R : TCTCGAATAATGGAAGCTAACAATGTC	1.9 kb
icmWX	F : TGGGTTGGTTCCTGAGGTATGA R : TGGGGCGCTGAAATTTTGATAT	1.2 kb
icmTSRQ	F : CACAGTTAAAACTTCAAGCTGAACC R : CTGCTCAGAGCTATTTTT	2.5 kb
icmLK	F : CGGAAGGCTGGGACCAATT R : CCACTCGATAATCCACGGCTTC	1.2 kb
icmJB	F : TGCCATGTTCTTTTTTGTGCTATTAC R : GAGCGTAAACCAGATCAATCCAAGTAG	2.2 kb
prpA	F : GTTTTAATCCCCCAGCAAGC R : AATATCCCTACTCATCCTCG	260 bp
lvhB3,4	F : GGCTAGGAGGTTCTTGTG R : ATTGGCCGAGATGTCCTT	1.0 kb
lvhB8,9	F : CCTCTACGCATTACAACGCC R : GTGGTGGTAAAGGGAATGCC	280 bp
lvrE	F : GGTCCAATGGGTCCAGCAGG R : AGTGGCTGATTCTGGAGTGG	420 bp
rtxA1	F : GATCCGCAAGTAGCGCTCAC R : TGTAATGCTGGCATTAGGCG	630 bp
rtxA2	F : CTGATGCTGCTACGGAACAC R : CCGCAGTCATTACACCTGCG	540 bp

minutes to reduce the numbers of other bacteria, then 0.1 ml aliquot of the sample was spread on buffered charcoal yeast extract (BCYE) with GVPC agar (Oxoid, Hamshire, UK) (Ta *et al*, 1995). Colonies were serotyped as described above.

Detection of *L. pneumophila* virulence genes by PCR

Bacteria were suspended in 50 µl of sterile water, heated at 90°C for 15 minutes, centrifuged at 15,000*g* for 10 minutes, and supernatant was used as PCR template. Primers used to amplify *dot*/*icm*, *lvh*, and *rtxA* are listed in Table 1 (Segal *et al*, 1998; Vogel *et al*, 1998; Samrakandi *et al*, 2002). PCR was carried out in 20 µl reaction mixture comprising of 20 pmol 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: 94°C for 3 minutes; followed by 30 cycles of 94°C for 20 seconds, 60°C for 10 seconds, and 72°C for 1 minute; and a final step at 72°C for 3 minutes. Amplicons were analyzed using an automated electrophoresis instrument (Advanced Analytical Technologies, Ankeny, IA).

RESULTS

The 258 *L. pneumophila* isolates comprised of 11 serogroups. The serogroup of all clinical isolates was sg 1, while serogroups of environmental isolates were distributed among 11 sgs (1-8, 12, 13,

Table 2
Serogroup distribution of *Legionella pneumophila* isolates from environment and patients in Busan, South Korea.

L. pneumophila serogroup	Number of isolates (%)	
	Environmental	Clinical
1	93 (37)	4 (100)
2	24 (9)	-
3	29 (11)	-
4	12 (5)	-
5	33 (13)	-
6	23 (9)	-
7	30 (12)	-
8	4 (2)	-
12	3 (1)	-
13	2 (1)	-
15	1 (< 1)	-
Total	254 (100)	4 (100)

L. pneumophila serogroups 9, 10, 11 and 14 were not detected.

15) (Table 2). Of the 254 environmental isolates, 50% carried all three virulence genes, *dot/icm, lvh* and *rtxA*, 21% *dot/icm + rtxA*, 11% *dot/icm + lvh*, 9% *lvh + rtxA*, and the remaining only one or no virulence genes (Table 3). Of the four clinical isolates, 3 carried all three virulence genes and 1 carried *dot/icm + rtxA*. Thus, it would appear that *L. pneumophila* isolates from patients in Busan to be community-acquired infections.

When the distribution of virulence genes carried by *L. pneumophila* isolates were analyzed according to their serogroups, sg 1 containing *dot/icm + lvh + rtxA* was the most common (68%) while sg 2-15, as a group, only 40% carried all three virulence genes (Table 4). However, the prevalence of *dot/icm* in sg 2-15 (88%) was higher than that in sg 1 (82%), whereas that of *lvh* (88%) and *rtxA* (91%) was higher in sg 1 than in sg 2-15 (61% and 80%, respectively).

DISCUSSION

It has been shown that the differences in pathogenicity of *Legionella* strains are associated with the presence of virulence genes (Segal *et al*, 1999; Samrakandi *et al*, 2002). The results of this study may help to predict the virulence of *L. pneumophila* isolates distributed in Busan. Three virulence loci, *dot/icm, lvh* and *rtxA*, play a crucial role in human infection caused by *L. pneumophila* (Samrakandi *et al*, 2002; Zink *et al*, 2002). All four clinical isolates had *dot/icm*, as did all clinical isolates from a study in Queensland, Australia (Huang *et al*, 2006). The presence of *dot/icm* is related to higher infectivity (Alli *et al*, 2003), but when examining the detection results for *lvh* and *rtxA*, we noted that isolates without *lvh* also caused human infection. Although the results from the study in Queensland, Australia showed that many clinical isolates had both *lvh*

Table 3
Distribution of virulence genes in environmental and clinical *Legionella pneumophila* isolates, Busan, South Korea.

Virulence gene			Number of isolates (%)	
dot/icm	*lvh*	*rtxA*	Environmental	Clinical
+	+	+	127 (50)	3 (75)
+	+	-	29 (11)	0 (0)
+	-	+	53 (21)	1 (25)
-	+	+	23 (9.1)	0 (0)
+	-	-	8 (3)	0 (0)
-	+	-	1 (<1)	0 (0)
-	-	+	11 (4)	0 (0)
-	-	-	2 (1)	0 (0)
Total			254 (100)	4 (100)

+, detected; -, not detected.

Table 4
Distribution of *Legionella pneumophila* virulence genes in serogroups 1 and 2-15 from environmental isolates, Busan, South Korea.

Virulence gene			Number of isolates (%)	
dot/icm	*lvh*	*rtxA*	sg 1	sg 2-15
+	+	+	63 (68)	64 (40)
+	+	-	5 (5)	24 (15)
+	-	+	7 (7)	46 (29)
-	+	+	13 (14)	10 (6)
+	-	-	1 (1)	7 (4)
-	+	-	1 (1)	0 (0)
-	-	+	2 (2)	9 (6)
-	-	-	1 (1)	1 (1)
Total			93 (100)	161 (100)

+, detected; -, not detected.

and *rtxA*, there are clinical isolates (21.6%) without *lvh* (Huang *et al*, 2006). *L. pneumophila* infections occur in environments where the bacteria can live, but various interacting factors can affect infectivity, such as bacteria concentration, pathogen virulence, transmission vector, and sensitivity of those exposed to the pathogen.

Huang *et al* (2004) reported that strains harboring two virulence genes (*lvh* and *rtxA*) are more virulent than strains that have only one gene (*lvh* or *rtxA*). The prevalence of *lvh* and *rtxA* in environmental isolates from Queensland, Australia is 57.7% and 64.4%, respectively (Huang *et al*, 2006), but the corresponding preva-

lence in the present study was higher (71% and 84%, respectively). Thus, we predict that *L. pneumophila* strains distributed in Busan should be more virulent than those in Queensland. In addition, considering the fact that environmental isolates with *lvh*, *rtxA*, and *dot/icm* were predominant, there is a high possibility of contracting legionellosis as a community-acquired infection in Busan.

L. pneumophila sg 1 is frequently present among isolates from environmental water systems, with distribution rates ranging from 75% to 90% (Doleans *et al*, 2004; Rivera *et al*, 2007; Lin *et al*, 2009). *L. pneumophila* sg 1 is the major causative agent of Legionnaires' disease, and over 90% of patients with legionellosis in USA and 70% in Europe are infected with this serogroup (Bozue and Johnson, 1996; Byrne and Swanson, 1998). Therefore, we expected a difference in the distribution of virulence genes between sg 1 and sg 2-15; our results showed that the percent sg 1 isolates with all three genes was much higher than that of sg 2-15 isolates. We posit that there is a high occurrence of legionellosis caused by sg 1 as a previous study showed that sg 1 is the dominant serogroup of *L. pneumophila* in Busan (Hwang *et al*, 2016), and our results showed that the percent strains with all three virulence gene indeed was higher among the sg 1 isolates. However, our study revealed that the prevalence of *dot/icm*, a known major virulence gene associated with *L. pneumophila* pathogenicity (Zink *et al*, 2002), was higher in sg 2-15 than in sg 1 isolates, and that strains with *dot/icm, lvh,* and *rtxA* were most common among sg 2-15 isolates. In addition, we confirmed that there was an increase in the distribution of *L. pneumophila* sg 2-15 compared with the previous study (Hwang *et al*, 2016). The Korea Centers for Disease Control reported that more Korean people carry antibodies against *L. pneumophila* sg 7-15 (Lee and Jung, 2012). This suggests that there might be a potential for human infections caused by *L. pneumophila* sg 2-15. Therefore, studies and diagnostic systems focusing on *L. pneumophila* sg 1 need to be extended to include sg 2-15.

In conclusion, this study predicts a potential risk of legionellosis in Busan, South Korea from the determination of the distribution of virulence genes in environmental and clinical isolates. However, our clinical sample size (four isolates) was too small to be representative of legionellosis isolates in Busan and there are no other studies on the distribution of virulence genes in each *L. pneumophila* serogroup. Additional studies on the distribution of *L. pneumophila* virulence genes with more clinical isolates are needed.

ACKNOWLEDGEMENTS

The work was supported by the Busan Metropolitan City Institute of Health and Environment, Korea.

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