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Prevalence of Antibodies in Response to *Legionella* Species, Analysis of a Healthy Population from Jeollanam-do Province, Korea

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Seroepidemological investigation of antibodies to *Legionella* species in 500 healthy individuals from a single geographical location in Korea was conducted by indirect fluorescent antibody assay (IFA). Considering an antibody titer of ≥1:128 as positive reaction, 15.2% of total sera were positive. In males and females older than 40 years old, levels of IgM and IgG were 1.2% and 14%, respectively. The sera with antibody titers of ≥1:128 to *Legionella* species accounted for 85 sera, and 9 sera of these were reacted to more than one *Legionella* species. Reactivity to *L. bozemanii, L. micdadei, L. longbeachae, L. pneumophila* sg 6, and *L. gormanii* were 32.9%, 20%, 15%, 10.6%, and 8%, respectively. However, *L. pneumophila* sg 1, sg 2, and sg 3 did not react to any sera. Serological analysis revealed that the level of antibody in response to *L. bozemanii* was more prevalent than *L. pneumophila.* Our results suggest that the antibodies of non-*L. pneumophila* species, such as *L. bozemanii,* may be highly prevalent in healthy population within Korea. Although conclusions based on the findings of this study must be cautiously considered given that the population sampled were sourced from a single province, we have added to the knowledge base of serodiagnosis of infections due to non-*L. pneumophila* species in Korea.

***Keywords*:** antibody, *Legionella* species, healthy population, Korea

Since *Legionella pneumophila* was first recognized as the primary causative pathogen for Legionnaires' disease (LD) in 1977 (Fraser *et al.*, 1977), epidemiolgical studies of LD have been reported from all over the world (Klaucke *et al*., 1984; Ratshikhopha *et al*., 1990; Greig *et al*., 2004; Kobayashi *et al*., 2004; Ricketts *et al*., 2005; Ozerol *et al*., 2006). Moreover, according to some studies (Ernct *et al.*, 1998; Harris *et al.*, 1998), infections due to non-*L. pneumophila* are more significant in patients receiving immunosuppressive therapy.

In Korea, community and hospital-acquired legionellosis have been occasionally reported since the first recognized outbreak of Pontiac fever by *L. gormanii* in 1984 (Kim *et al.*, 1985; Choi, 1998; Seog *et al.*, 1999). In a previous study (Lee *et al.*, 2006) on 108 Korean patients with a typical pneumonia, we used Indirect fluorescent antibody assay (IFA) to reveal that *L. gormanii* and *L. pneumophila* sg 1 accounted for 25.9% and 13% of positive sera. Therefore, to confirm LD from serological data in suspected patients in Korea, the ocurrance of antibodies in response to *Legionella* species in healthy population must be established. In this study, the prevalence of antibodies in response to *Legionella* species has been investigated in a healthy population from one geographic area, Jeollanam-do Province, Korea, between January and July in 2004.

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Materials and Methods

Samples

Sera of subjects (n=500) were obtained from Jeollanam-do Institute of Health and Environment, and Chonnam University Medical School, accompanied with information on sex and age only. The samples of all subjects, who were individuals without a history of acute respiratory illness during the previous month, were stored in portions at -70℃ until used.

Strains

Legionella strains used as the IFA antigen were *L. pneumophia* sg 1 (ATCC 33152), sg 2 (ATCC 33154), sg 3 (ATCC 33155), sg 4 (ATCC 33156), sg 5 (ATCC 33216), sg 6 (ATCC 33215), *L. bozemanii* (ATCC 33217), *L. dumoffii* (ATCC 33279), *L. feeleii* (ATCC 33849), *L. gormanii* (ATCC 33297), *L micdadei* (ATCC 33218), and *L. longbeachae* (ATCC 33462). All strains were purchased from American Tissue Culture Collection.

Indirect fluorescent antibody assay (IFA)

IFA was performed as described previously (Wilkinson *et al.*,

1979) except that sera were diluted with phosphate-buffered saline (PBS, pH 7.6). Twenty four-well slides were coated with the heat-killed antigen suspension, dried, and fixed with cold acetone. The titer of the initial serum dilution used in this study was 1:32 and subsequent two fold dilutions were carried out. Ten-microliter portions of each serum dilution were placed on a well and the slides were incubated at 37℃ for 30 min. FITC conjugate were dropped into each well after the slides were washed with PBS and dried. Slides were incubated at 37℃ for 30 min and were washed with PBS, rinsed in distilled water, and dried. A glycerol-based mounting solution (pH 9.0) was applied, and cover slips placed on the slides. Slides were read under a fluorescence microscope (Olympus BX50, Japan) equipped with a 50-W mercury vapor lamp incident-light source and appropriate filters. Antibody titers of ≥1:128 against the 12 *Legionella* antigens were considered as positive in this study. Rabbit antiserum immunized with *Legionella* was used in the positive control.

Statistics

Statistical analysis was performed with the statistical program SPSS (Version 12.0, SPSS Inc., USA).

Results

The ages of 500 healthy individuals ranged from 7 to 83 years, with a mean age of 40 years old. Of the 500 sera, 242 were males, while 248 were females. The IFA IgM antibody titers were 1.2%, 3.4%, 2.8%, and 1.4% for 1:32, 1:64, 1:128, and ≥1:256, respectively. The IgG antibody titers of 1:32, 1:64, 1:128, and ≥1:256 accounted for 7.8%, 14%, 7.0%, and 4%, respectively (Fig. 1). Overall 15.2% of sera had antibody titers of ≥1:128 and the ratio of male to female in antibody titers of ≥1:128 was 1:1.9 (Table 1 and 2). In individuals over 40 years of age, 1.6% of males and 0.8% of females exhibited IFA IgM antibody titers of ≥1:128 (Table 1), while, level of IgG antibody titers of ≥1:128 (Table 1) were 11.2% of males and 16.8% of females (Table 2).

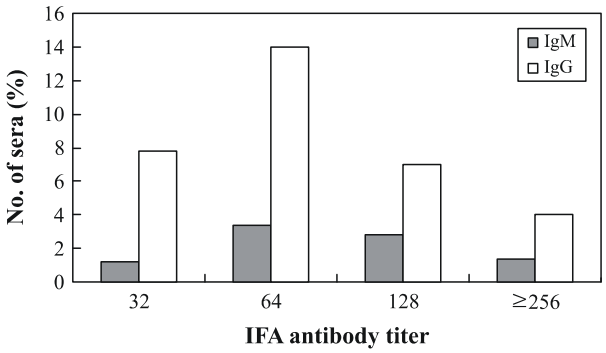

Fig. 1. Frequencies of IFA antibodies in response to *Legionella* species in a healthy population from Jeollanam-do.

Table 1. Age and sex frequencies of IFA IgM antibody titers ≥1:128

Age	No. sera of ≥1:128 in IgM antibody titers of/Total (%)		
	Male	Female	Total
<40	4/117 (3.4)	14/133 (10.5)	18/250 (7.2)
≥40	2/125 (1.6)	1/125 (0.8)	3/250 (1.2)
Total	6/242 (2.5)	15/258 (5.8)	21/500 (4.2)

Table 2. Age and sex frequencies of IFA IgG antibody titers ≥1:128

Age	No. sera of ≥1:128 in IgG antibody titers of/Total (%)		
	Male	Female	Total
<40	6/117 (5.1)	14/133 (10.5)	20/250 (8.0)
≥40	14/125 (11.2)	21/125 (16.8)	35/250 (14.0)
Total	20/242 (8.3)	35/258 (13.6)	55/500 (11.0)

Table 3. Prevalence of IFA antibody titers ≥1:128 in response to *Legionella* species (n=85)*

Sex \ Species	Lp4	Lp5	Lp6	Lb	Lg	Lm	Ll	Lf	Ld	Total
Male	2	2	4	8	2	2	6	2	1	29
Female	1	1	5	20	5	15	7	0	2	56
Total	3	3	9	28	7	17	13	2	3	85
%	3.5	3.5	10.6	32.9	8.2	20	15.3	2.4	3.5	100

Lp1, *L. pneumophila* sg 1; Lp2, *L. pneumophila* sg 2; Lp3, *L. pneumophila* sg 3; Lp4, *L. pneumophila* sg 4; Lp5, *L. pneumophila* sg 5; Lp6, *L. pneumophila* sg 6; Lb, *L. bozemanii*; Lg, *L. gormanii*; Lm, *L. micdadeii*; Ll, *L. longbeachae*; Lf, *L. feeleii*; Ld, *L. dumoffii*

* 9 sera of 85 reacted to more than one *Legionella* species.

The sera with antibody titers of ≥1:128 to *Legionella* species accounted for 85 and 9 of these sera showed reactivity with more than one etiological agent (Table 3), level to *L. bozemanii* was significantly higher than other *Legionella* species (32.9%). The antibodies to *L. micdadei, L. longbeahae,* and *L. gormanii* accounted for 20%, 15%, and 8%, respectively, while, levels for *L. pneumophila* sg 4, sg 5, and sg 6 accounted for 3.5%, 3.5%, and 10.6%, respectively. However, all sera used in this study did not react to *L. pneumophila* sg 1, sg 2, and sg 3 in comparison with other countries. Reactivity to *L. bozemanii, L. gormanii,* and *L. micdadeii* were higher in females than males.

Discussion

Prevalence of antibody in response to *L. pneumophila* subjects above 46 years of age in populations samples from four geographic regions within United States, revealed the frequency of IFA antibody titers ≥1:128 to be 1.3% (Storch *et al.*, 1979). There was no statistically significant difference among the four sampled regions.

Antibody titers of ≥1:128 were shown in 15% of hotel employees in Bloomington (Macdade *et al.*, 1977), while among volunteer control subjects from an industry in Burlington, Vt., 15% had titers of ≥1:128 (Broome *et al.*, 1979). As part of a LD outbreak investigation, 122 individuals were tested in a sero-survey of control subjects in Bristol, Tenn., from which IFA titers of ≥1:128 were observed in 5.7% of the tested population (Dondero *et al.*, 1979).

In another study, antibody titers of ≥1:128 were found among 4% of Veteran Administration employees in an office building near Wadsworth Medical Center (Haley *et al.*, 1979). Of 517 sera from a rural community in an agricultural area of northeast Iowa, USA, 13.2% (n=68) exhibited titers of ≥1:128 against *L. pneumophila* sg 1 (Helms *et al.*, 1980), among which 14.9% were males and 11.6% were females. In addition, the frequency of males above 40 years of age was significantly greater than that of females. However, as shown in Table 1 and 2, the prevalence of IgM or IgG antibody titers of ≥1:128 was higher in females (19.4%) than males (10.8%). In subjects older than 40 years of age, the frequency of IgM or IgG antibody titers ≥1:128 were higher in females (17.6%) than males (12.8%). The frequency of IgM or IgG in males younger than 40 years old was lower (8.5%) when compared with those older than 40 years of age (12.8%). The same comparative analysis for younger and older than 40 years of age showed female accounted for (21.1% and 17.6%, respectively). These results are similar to a study carried out on healthy New Zealanders (Bettelheim *et al.,* 1982). Out of 500 subjected sampled from five areas, 2.8% and 4.0% exhibited IgG and IgM titers of ≥1:128, respectively. In Korean study, antibody titers of ≥1:32 against *Legionella* antigens accounted for 22% of subjects younger than 40 years of age, compared to 17% for individuals older than 40 years of age (Chong and Lee, 1987).

In this study, the frequency of IgM for both male (3.4%) and female (10.5%) younger than 40 years old were higher compared to those older than 40 years of age (1.6% and 0.8%). However, both male (5.1%) and female (10.5%) subjects younger than 40 years of age exhibited lower IgG frequencies compared to those in the older age group (11.2% and 16.8%). These results are similar to those reported by Bettelheim *et al.* (1982). In a seroepidemiologic survey conducted on 600 sera from healthy and non-hospitalized Michigan residents, the researchers used Hemagglutination (HA) test to conduct a comparative seasonal study between late winter and late summer (Edson *et al.,* 1979). Analysis showed a statistically significant difference among the prevalent in the winter group (15.2%) compared with the summer population sample (29.8%). The prevalence of HA antibody titers of ≥1:16 against *L. pneumophila* in subjects younger than 40 years old, were higher (57.4%) in comparison to the older age group (42.6%), while parallel comparative analysis in females revealed the older age group ranked lower (40.8%) than subjects younger than 40 years of age (59.2%). However, these investigations did not distinguish between IgM or IgG in their analysis.

Therefore, given the fact that the majority of seroepidemiological studies show higher HA frequencies in younger (< 40 years old) subjects compared to older individuals (≥ 40 years old), results should further be analyzed according to spatial and temporal factors, in addition to other socioeconomic characteristics such as occupation, and underlying diseases. However, given that only age and sex information was made available to, we could not ascertain epidemiological relationships with other factors.

In a previous serological study, we reported that from 165 patients with atypical pneumonia, 13% reacted to *L. pneumophila* sg 1, while reactivity to *L. gormanii, L. longbeachae, L. bozemanii*, and *L. micdadeii* were 25.9%, 16.7%, 15.7%, and 13.9%, respectively (Lee *et al.,* 2006). When we analyzed the distribution of *Legionella* species reacting to paired sera from 58 patients for LD, the frequency of antibodies in response to *L. pneumophila* sg. 1*, L. gormanii* and *L. bozemanii* were 22%, 17%, and 7%, respectively (Lee *et al.,* 2007a). This implies that other species, as well as *L. pneumophila,* may be important causative agents of legionellosis in Korea. However, this conclusion must be cautiously considered given we did not investigate the prevalence of antibodies against non-*L. pneumophila* species. Here, we have attempted to rectify this shortcoming by ascertaining the prevalence of antibodies against *Legionella* species in a healthy Korean population.

According to Klein *et al.* (1979), the upper limit of normal value is defined as the level of antibody titer which does not exceed 15% in this study. The presence of antibodies in response to *L. gormaniii* were calculated to be 8.2% in tested sera. Therefore *L. gormaniii* is a very important agent of legionellosis in Korea, even though the healthy group used in this study may not be representative of the general population. However, the prevalence of antibodies in response to *L. bozemanii* were very high (32.9%) and it is similar to data from other studies.

Fotos *et al.* (1985) studied the frequency of antibodies to *Legionella* species in sera from a dental clinic and a regional population, reporting that 38% of the 65 positive sera reacted with *L. bozemanii*. However, they did not conduct comparative analysis on *L. bozemanii* between the clinic and regional population. Other studies (e.g., Collins *et al.,* 1984) have investigated the prevalence of antibodies in response to 10

Legionella species, including *L. bozemanii,* by microagglutination (MA) analysis from 128 cystic fibrosis patients and 103 normal children and young adults. When MA titers of ≥ 1:64 were considered positive, the prevalence of antibodies in response to *L. bozemanii* was 35.6% and 3.2% in patients and normal groups, respectively. In a survey of domestic animals looking at detecting serological responses against *Legionella* species by IFA, titers of ≥ 1:128 against *L. bozemanii* showed 2% in porcine sera, 44% in Equine sera, and 16% in Ovine sera (Barth *et al.,* 1983).

As mentioned here, many studies have reported prevalence of antibodies to *L. bozemanii*. This may be due to the possibility of exposure to antigens such as viruses, *Mycoplasma, Chlamydia*, or *Pseudomonas*. Furthermore, the upper limit of normal value to *L. bozemanii* in Korea or other countries may be serologically high due to exposure against this organism over an extended period of time. However, given the lack of historical information on the underlying diseases, or other socio-economic or demographic parameters (except age and sex), we cannot make conclusive statements. Therefore, further studies may be needed to investigate whether other factors can influence the prevalence of antibodies in order, to establish antigenic specificity in response to other species, such as *L. bozemanii.* The isolation of other species, including *L. bozemanii,* from environmental water systems should also be studies.

To date, more than 20 of above 50 *Legionella* species have been isolated from patients, and greater than 90% of all reported cases of LD in the USA have been attributed to *L. pneumophila* sg 1 (Fields *et al.,* 2000). However, cases attributed to non-*L. pneumophila* species have only occasionally been reported. Three species, *L. micdadei, L. bozemanii*, and *L. dumoffii*, have been reported as major causative agents in patients with immunosuppressant treatment, transplants, and cardiac disorders (Gläsier *et al.,* 2005). Cases attributed to infections by *L. longbeachae* have been reported in other countries such as Australia and Japan (Korman *et al.,* 1998; Kubota *et al.,* 2007). McNally *et al.* (2000) reported that 4% of patients with pneumococcal bacteremia exhibited seorological evidence of *L. bozemanii* infection. In 508 cases of culture-confirmed community-acquired LD, *Legionella* species isolated were *L. longbeachae* (3.9%), and *L. bozemanii* (2.4%), followed by *L. micdadei, L. dumoffii, L. feeleii, L. wadsworthii*, and *L. anisa* (Yu *et al.,* 2002). Evidently, non-*L. pneumophila* species are gradually being reported as important agents in community acquired pneumonia as well as nosocomial infections.

Distribution analysis of *Legionella* species from environmental water systems has shown that *L. pneumohila* sg 1 exist in most cooling towers, while *L. midadei*, *L. gormanii*, and *L. anisa*, and *L. pneumophila* were isolated from Spas. In particular, *L. bozemanii* were more prevalent in cold and hot water systems than in cooling towers (Bartram *et al.,* 2007). Furthermore, *L. longbeachae* has been associated with exposure to potting composts in Australia, USA, and Japan (Steele *et al.,* 1990; Koide *et al.,* 2001). In Korea, the only major species isolated from environmental water systems is known to be *L. pneumophila* sg 1. However, *L. busanensis* has apparently been isolated from cooling tower water (Park *et al.,* 2003), while non-*L. pneumophila* species such as *L. pneumophila* sg 6, *L. micdadei*, *L. bozemanii*, *L. erythra*, and *L. anisa* have been isolated with the help of developing genetic methods for identification of *Legionella* species (data not shown). In addition, isolates from environmental water systems within Korea were very different to *L. pneumophila* sg 1 from other countries, which were extracted by pulsed field gel electrophoresis and sequence-based type (Ju *et al.,* 2007; Lee *et al.,* 2007b). Therefore, future research in Korea should attempt to substantiate the hypothesis that other species, such as *L. bozemanii,* are widespread in the environmental water systems. Comparative spatial analysis looking in to the prevalence of antibodies to these organisms should address the paucity of data in this field.

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