Original Article

Bath Water Contamination with *Legionella* and Nontuberculous Mycobacteria in 24-Hour Home Baths, Hot Springs, and Public Bathhouses of Nagano Prefecture, Japan

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SUMMARY: Bath water samples were collected from 116 hot springs, 197 public bathhouses, and 38 24-hour home baths in Nagano Prefecture, Japan, during the period of April 2009 to November 2011, for determining the presence and extent of contamination with *Legionella* and nontuberculous mycobacteria. Cultures positive for *Legionella* were observed in 123 of the 3,314 bath water samples examined. The distribution and abundance of *Legionella* and/or combined contamination with *Legionella* and nontuberculous mycobacteria were investigated to clarify the contamination levels. The abundance of *Legionella* was demonstrated to correlate considerably with the levels of combined contamination with *Legionella* and nontuberculous mycobacteria. *Legionella* spp. were obtained from 61% of the water samples from 24-hour home baths, but only from 3% of the samples from public bathhouses and hot springs. This is despite the fact that a few outbreaks of Legionnaires' disease in Nagano Prefecture as well as other regions of Japan have been traced to bath water contamination. The comparatively higher rate of contamination of the 24-hour home baths is a matter of concern. It is therefore advisable to routinely implement good maintenance of the water basins, particularly of the 24-hour home baths.

INTRODUCTION

The first occurrence of Legionnaires' disease was reported as a consequence of the airborne transmission of *Legionella pneumophila* through cooling towers, showers, and other aerosolizing devices in 1976 (1,2), and successive detections of *Legionella* spp. have been documented worldwide to date (3–8). In Japan, several outbreaks caused by *L. pneumophila* associated with bath water in hot springs or public bathhouses (9,10), as well as nontuberculous mycobacteria associated with bath water in 24-hour home baths and public bathhouses (11,12), have been documented.

There are 1,347 facilities of hot springs in Nagano Prefecture, Japan, the second largest number after Shizuoka Prefecture. In addition, there are 1,095 public bathhouses in Nagano Prefecture, the fifth largest number among the 46 prefectures in Japan [<http://www.joy.hi-ho.ne.jp/ma0011/Ken-m.htm>]. Efficient usage of limited water resources in Japan has been a long-standing recommendation. With the aim of water conservation and convenience, a 24-hour home bath was first invented in 1984 and has since been in use chiefly in homes. The 24-hour home bath has been

principally adopted into household bathrooms for private use. It is therefore quite different from the Spa pools of Europe, which have become increasingly popular as they provide health benefits through massage, relaxation, and pain relief and are largely equipped in sports complexes, health clubs, hotels, holiday complexes, as well as cruise ships [http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1200471667418]. The 24-hour home bath continuously filters and circulates bath water for reuse and permits configuration of the water-temperature from 36°C to 45°C, depending on the bather's choice. However, these 24-hour home baths are ineffective in complete disinfection of the entire circulating water mass. This can be partly explained by its cleaning mechanism, which depends on the biological regulation of microorganisms, and is chiefly explained by the formation of biofilms adhering to the surface of the filter, which enable the proliferation of intracellular *Legionella* or mycobacteria. This drawback is taken care of in several 24-hour home-bath units through a physical cleaning device that disinfects the water after it passes through a filtering section. The device uses heat, ultraviolet, or ozone radiations for disinfection [<http://24furo.v.wol.ne.jp/user/03/01.htm>].

In remarkable contrast to Western countries, the incidence of Legionnaires' disease had remained extremely low in Japan (13,14), with only a few reported cases, for which bath water was determined to be the direct source of infection (15). However, in 2008, 896 cases of legionellosis were reported (16), representing a 5-fold increase in incidence over the previous 5 years (Infecti-

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ous Disease Surveillance Center, 2008), such that the incidence of legionellosis in Japan become comparable to that in Europe and the USA.

More than 1,700,000 units of the 24-hour home baths have been adapted into largely average homes in Japan by the year 2012, according to the association of 24 Hour Bath System [<http://24furo.v.wol.ne.jp/member/04/index.htm>]. However, *Legionella* contamination of home bath waters has been reported previously, and such baths have been suspected to be a potential source of infections caused by *Legionella* spp. (17,18). Infections cause by nontuberculous mycobacteria associated with bath water have also been reported (11,12,19,20). In the present study, we investigated the presence or inhabitation of *Legionella* spp. and *Mycobacterium* spp., both of which are well known facultative intracellular parasites, in the 24-hour home baths, public bath-houses, and hot springs of Nagano Prefecture, Japan.

MATERIALS AND METHODS

Collection of water samples: A total of 3,314 water samples were collected in Nagano Prefecture, Japan, during the period of April 2009 to November 2011. The samples included 38 water samples from 38 24-hour home-baths, 922 samples from 116 hot springs (sampling performed 2 times a year, and 2 samples collected each time), and 2,354 samples from 197 public bathing facilities (sampling performed 3 times a year, and 2 samples collected each time). The samples were collected in 1,000-ml sterile polypropylene containers. Sodium thiosulfate was prospectively incorporated into the samples. The containers were maintained at 4°C during transport to the laboratory and tested within 12-hours. Figure 1 shows the districts in Nagano Prefecture from which the water samples were collected.

Quantitative cultures of *Legionella* spp.: The water samples were concentrated in a biological safety cabinet by pouring the 500-ml water samples into a sterile 47-mm filter funnel assembly containing Millipore cellulose acetate membrane filters with a pore size of 0.45 μ m (Millipore Co., Tokyo, Japan); a vacuum source and side-arm flasks required for operating the apparatus were also employed. The filters were then aseptically removed from the holders using sterile filter forceps, folded from the outside, and placed into sterilized screw-cap containers with 5 ml of sterile water.

The screw-cap containers were subsequently subjected to vortexing for 1 min to free bacteria and organic material from the filter, and thus, 100 fold concentrated water samples were obtained. If a sample was concentrated using more than 1 filter, the additional filters were added to the same container. The concentrated water samples were placed in a water bath at 50°C for 20 min for heat treatment. Subsequently, 0.1 ml each of fold filter-concentrated water samples were inoculated onto GVPC (Sysmex-biomerieux Co., Tokyo, Japan) or WYO α (Eiken Chemical Co., Ltd., Tokyo, Japan) media and incubated at 37°C for 5–7 days. The suspected *Legionella* colonies were spread onto B-CYE α (Sysmex-biomerieux Co.) and Sheep Blood agar (Nippon Becton Dickinson, Tokyo, Japan) media and incubated at 37°C. Colonies that showed growth on B-CYE α but not Sheep Blood agar media were sub-

The numerical figure indicates the number of bath water samples collected.

Fig. 1. Districts of Nagano Prefecture, Japan, from which all of the bath water samples were collected.

jected to further examination. DNA extraction from the suspected *Legionella* colonies was conducted according to the Manual for the Detection of Pathogenic Microorganisms from the National Institute of Infectious Diseases, Japan [<http://www.nih.go.jp/niid/ja/lab-manual.html>].

After DNA extraction, the PCR assay was performed using *Legionella*-specific 16S rRNA primers (LEG primers; 21) in order to confirm the genus. Furthermore, the following examinations were conducted: characteristic culture and growth properties on B-CYE α media, distinctive cellular morphology on Gram-staining, and confirmation of positive reactions for oxidase, catalase, β -lactamase, and hippurate hydrolysis.

The total cell counts of *Legionella* spp. in the original water samples were calculated from the colony numbers obtained on GVPC or WYO α media, as detailed above.

Qualitative cultures of *Mycobacterium* spp.: The water samples positive for *Legionella* spp. were also examined for *Mycobacterium* spp. using qualitative culture techniques. Cellulose acetate Millipore membrane filters with pore sizes of 0.45 μ m were employed for a further 2-fold concentration of the 100 fold filter-concentrated water samples in screw-cap containers in order to obtain 200 fold concentrated water samples. These concentrated water samples were mixed with equal volumes of 3% NaOH and incubated at room temperature for 15 min. Then, 100 μ l of the samples were inoculated (in duplicate) onto 3% Ogawa (Kyokuto Seiyaku Co., Ltd., Tokyo, Japan) media. The

tubes were incubated at 37°C in a slanting position with a partially unscrewed cap. After evaporation of the liquid phase in 4–5 days, the caps were tightly screwed, and the tubes were incubated at 37°C for 2 months in a standing position. The cultures were observed daily during the first week and weekly thereafter. The colonies obtained were microscopically examined subsequent to Ziehl–Neelsen staining. Acid-fast colonies were subcultured on new tubes containing Dubos media, and kept in culture until characterization. All isolates with positive acid-fast staining were identified using the DDH-Mycobacteria kit (Kyokuto Seiyaku Co., Ltd.).

RESULTS AND DISCUSSION

Isolation of *Legionella* spp. from water samples: A total of 3,314 water samples were collected from all over Nagano prefecture, Japan (Fig. 1) and subjected to quantitative culture for *Legionella* spp.; positive *Legionella* cultures were obtained from 123 samples. The distribution of *Legionella* cell numbers (colony-forming-units [CFU]/100 ml) among these 123 water samples is shown in Fig. 2. Approximately a quarter (32 of 123) and one-third (45 of 123) of the water samples yielded *Legionella* counts of ≤ 10 CFU/100 ml and 11–100 CFU/100 ml, respectively. Among the water samples with *Legionella* contamination, 46 samples yielded counts of ≥ 100 CFU/100 ml, sometimes reaching 10^4 CFU/100 ml.

Isolation of *Legionella* spp. in respective water samples: The breakdown of the 123 *Legionella*-positive water samples collected from Nagano Prefecture, Japan, is shown in Table 1; 28 of 922 (3%) hot spring, 72 of 2,354 (3%) public bathhouse, and 23 of 38 (61%) 24-hour home bath water samples were positive for *Legionella* species. The overall prevalence of *Legionella*

in this survey was 4% (Table 1). Fisher’s exact test using the data from Table 1 revealed that 24-hour home baths were significantly contaminated with *Legionella* spp. compared with hot springs or public bathhouses, with a p -value of $< 2.2 \times 10^{-16}$ obtained using two-sided tests. This result is in good agreement with the results of investigations conducted in other regions of Japan (22,23).

Occurrence of nontuberculous mycobacteria in *Legionella*-positive water samples: Nontuberculous mycobacteria were detected in 30 of the 123 *Legionella*-positive water samples tested (Table 2), including 7 of 28 hot spring, 15 of 72 public bathhouse, and 8 of 23 24-hour home bath water samples. Of note, water samples from 24-hour home baths, which are intended for household-limited use, were shown to be contaminated with nontuberculous mycobacteria at a considerable frequency (35%); this frequency is almost similar to that for hot springs (25%) and public bathhouses (21%), both of which are equipped not for individual use but for the general public.

Statistical analyses using R revealed a significant association between *Legionella* cell counts (CFU/100 ml) and combined contamination with *Legionella* and nontuberculous mycobacteria. In addition, the application of multiple comparison analyses using the chi-square test for measures repeated 3 times with the significance level at 0.01667 (0.01667/3) revealed that the greater the *Legionella* cell counts, the higher the percentages of combined-contamination with nontuberculous mycobacteria in the water samples ($p = 0.0001$ and $p = 0.006$ between *Legionella* cell counts of 1–10 and 1,001–10,000 CFU/100 ml, and 11–100 and 1,001–10,000 CFU/100 ml, respectively, using Fisher’s exact test), as clearly demonstrated in Fig. 3.

Distribution of nontuberculous *Mycobacterium* spp. identified in *Legionella*-positive bath water samples: A total of 30 nontuberculous *Mycobacterium* spp. were identified in the 30 *Mycobacterium*- and *Legionella*-

Numeric characters indicate the numbers of water samples from respective type of bathtub-water.

Fig. 2. Quantitative cultures for *Legionella* spp. from bath water samples.

Table 1. Frequency of isolation of *Legionella* spp. from bath water samples in Nagano Prefecture, Japan

Bath water sample examined	No. of samples	No. of <i>Legionella</i> positives	Isolation percentage (%)
Hot springs	922	28	3
Public bathhouses	2,354	72	3
24-hour home baths	38	23	61
Total	3,314	123	4

Table 2. Frequency of isolation (%) of nontuberculous mycobacteria among the 123 *Legionella* positive samples

Bath water sample examined	No. of nontuberculous mycobacteria/No. of <i>Legionella</i> positive samples	Frequency of isolation of nontuberculous mycobacteria (%)
Hot springs	7/28	25
Public bathhouses	15/72	21
24-hour home baths	8/23	35
Total	30/123	24

positive water samples using the DDH-Mycobacteria kit and tabulated (Table 3) according to Runyon's grouping. More than half (18 of 30) of the isolates belonged to Runyon group III; a majority of them (12 of 18) were identified as *Mycobacterium avium*. Indeed, *M. avium*, accounting for almost half of the 30 isolates identified, was the most frequently isolated species in Japan (24,25), followed by *M. gordonae*, in turn followed by *M. nonchromogenicum* and *M. fortuitum*.

In Japan, infections due to the *M. avium-intracellulare* complex, which are called MAC diseases, are of great clinical importance because the frequency of the pathogenic *M. avium-intracellulare* complex is second only to the *M. tuberculosis* complex among all *Mycobacterium* species. The fact that *M. avium* isolates were most frequently encountered, although a small number of causes ($n = 30$) were examined is consistent

with the present state of nontuberculous mycobacterial infections in Japan. It is likely that bath water is a possible source of MAC infection, at least in Japan (26). Indeed, the *M. avium* complex was the most common cause of infections due to nontuberculous mycobacteria not only in Japan (24,25) but also worldwide (27,28).

High-level contamination of bath water with both *Legionella* and nontuberculous *Mycobacterium* spp.: future issues: Legionnaires' disease caused by exposure to 24-hour home baths carrying water contaminated with *L. pneumophila* serogroup-5 (SG 5) was observed in a 79-year-old Japanese male patient (29) the *Legionella* isolates from the sputum of the patient and the 24-hour home bath water sample were confirmed to be indistinguishable from each other using pulsed-field gel electrophoresis analysis. A large outbreak of legionellosis, resulting in 295 causes of illness and 7 deaths, occurred in a new hot spring bath facility in Hiuga City of Miyazaki Prefecture, Japan; 34 cases were definitely diagnosed as Legionnaires' disease caused by *L. pneumophila* SG 1 (30). Another instance of large-scale infections due to bath water-associated *Legionella* was also reported (31), with a special focus on *Legionella* concentrations in the water sample, which ranged from 90 to 140,000 CFU/100 ml. These observations authenticate the current Japanese safety regulation levels for *Legionella* in bath water, which is set below the detection limit (10 CFU/100 ml) of culture techniques, and should be appropriate for the prevention of bath water-associated legionellosis. In the present study, the 32 *Legionella*-positive and 3,191 culture-negative samples were observed to conform to the reference level.

In the case of nontuberculous mycobacteria, 3 cases of skin disorders due to *M. avium* infection have been reported from private homes; 24-hour home bath water was suspected as the source of infection in these cases (12). Similarly, isolate of the *M. avium-intracellulare* complex obtained from the household plumbing of a patient and from the patient's sputum were reported in 2007 to be well matched, as analyzed by restriction fragment length polymorphism analyses, strongly indicating that household water was the source of MAC infection (20). The cleaning procedures of the 24-hour home bath systems in Japan are dependent on filtration using the

Fig. 3. Frequency of dual inhabitation of both nontuberculous mycobacteria and *Legionella* spp.

Table 3. Species distribution by Runyon groups of 30 nontuberculous mycobacterial isolates from *Legionella* positive bath water samples

Classification	No. of isolates	Species identified (No. of species identified)
Runyon's I (Photochromogens)	2	<i>Mycobacterium marinum</i> (1) <i>Mycobacterium simiae</i> (1)
Runyon's II (Scotochromogens)	7	<i>Mycobacterium gordonae</i> (5) <i>Mycobacterium scrofulaceum</i> (1) <i>Mycobacterium szulgai</i> (1)
Runyon's III (Non-photochromogens)	18	<i>Mycobacterium avium</i> (12) <i>Mycobacterium gastri</i> (1) <i>Mycobacterium intracellulare</i> (1) <i>Mycobacterium nonchromogenicum</i> (3) <i>Mycobacterium triviale</i> (1)
Runyon's IV (Rapid growers)	3	<i>Mycobacterium abscessus</i> (1) <i>Mycobacterium fortuitum</i> (2)

membrane filter, the surface of which is easily amenable to biofilm formation; these biofilms could be considered amplifiers of a certain amoeba that enables the growth of facultative intracellular organisms. In fact, cultures of biofilms from plumbing and plumbing fixtures have been demonstrated to yield a higher recovery of nontuberculous mycobacteria than culture of the collected water samples (32). The methods used in the present study, therefore, likely underestimate the actual number of culture-positive specimens, at least for nontuberculous mycobacteria.

A recent report observed that *Legionella* contamination in bath water could be controlled by observing physicochemical parameters, having a good filtration system, and frequent cleaning of the basin (33). However, this filtration system is difficult to apply to the Japanese 24-hour home baths without the addition of chlorine because of the unique disinfection or bacteriological cleaning mechanism, which depends on the biological regulation of microorganisms. The WHO guidelines of 2006 recommend that hot tub facilities should undertake a program of control measure verification (34), such as ensuring that *Legionella* levels are maintained at < 1 CFU/100 ml in facilities where microbial testing for *Legionella* is undertaken, in addition to daily or weekly inspection and maintenance of cleaning operations. Such routine screenings for *Legionella* in bath water samples from hot springs, public-bathing facilities, and 24-hour home baths have not yet been made mandatory in Japan. Such measures, however, will not only help prevent outbreaks by monitoring the contamination levels and prompting corrective actions when the levels are high but will also help in confirm the effectiveness of maintenance measures in place. In fact, eradication of bath water contamination with *Legionella* and nontuberculous mycobacteria should prevent infection; this, however, is not a realistic goal. A more realistic goal would be the control of *Legionella* proliferation below a determined reference level (31,34). We reaffirm the recent Japanese guidelines on water quality (31) and confirm that *Legionella* cell counts in bath water samples from public baths, 24-hour home baths, and hot springs should be limited to below the detection limit of culture techniques (i.e., 10 CFU/100 ml). To conclude, we believe that the present study potentially offers valuable suggestions regarding the risk of *Legionella* and nontuberculous mycobacterial infections resulting from environmental or man-made water systems that are applicable in not only Japan but also worldwide.

Conflict of interest None to declare.

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