Characterization of *Legionella pneumophila* isolates from patients in Japan according to serogroups, monoclonal antibody subgroups and sequence types

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We collected 86 unrelated clinical *Legionella pneumophila* strains that were isolated in Japan during the period 1980–2008. Most (80.2%) belonged to serogroup 1, followed by serogroups 5, 3 and 2. Interestingly, the patients with *L. pneumophila* serogroup 1 had a significantly higher male-to-female ratio (12.4) than the patients with other *L. pneumophila* serogroups (2.0) (OR, 10.5; 95% CI, 2.5–44.5). When the serogroup 1 strains were analysed by monoclonal antibody (mAb) typing, the most prevalent subgroup was Benidorm (34.9% of all isolates). Moreover, 79.7% of the serogroup 1 isolates were bound by mAb 3/1, which recognizes the virulence-associated epitope. When all 86 isolates were subjected to sequence-based typing (SBT) using seven loci, they could be divided into 53 sequence types (STs). The ST with the most isolates (seven) was ST1, to which most isolates from patients and environments around the world belong. However, six of the seven ST1 isolates were isolated before 1994. Other major STs were ST306 ($n=6$), ST120 ($n=5$) and ST138 ($n=5$). All ST306 and ST138 isolates, except for one isolate (ST306), were suspected or confirmed to be derived from bath water, which suggests that these strains prefer bath habitats. The sources of all ST1 and ST120 isolates remain unclear. By combining the SBT and mAb data, the 86 isolates could be divided into 59 types (discrimination index, 0.984). This confirms the usefulness of this combination in epidemiological studies.

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INTRODUCTION

Legionellosis is caused by *Legionella* species, which are environmental Gram-negative bacteria. To date, 52 species of *Legionella* have been described (Kuroki *et al.*, 2007). The species that is most commonly found in the environment and also causes the most disease is *Legionella pneumophila* (Yu *et al.*, 2002). To aid the epidemiological surveillance of legionellosis, *L. pneumophila* isolates can be divided into serogroups by antisera that recognize differences on the

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Abbreviations: EWGLI, European Working Group on *Legionella* Infections; SBT, sequence-based typing; ST, sequence type.

A supplementary table showing the sequence types, serogroups and monoclonal antibody subtypes of Japanese clinical isolates of *Legionella pneumophila* is available with the online version of this paper.

LPS molecules. At present, 15 serogroups have been identified (Brenner *et al.*, 1988). Serogroup 1 is responsible for the majority of human infections (Yu *et al.*, 2002) and its subgroups can be delineated by six monoclonal antibodies (mAbs) that recognize specific epitopes (Helbig *et al.*, 1997). *L. pneumophila* isolates can also be characterized by sequence-based typing (SBT) using the six loci (*flaA*, *pilE*, *asd*, *mip*, *mompS* and *proA*) proposed by the European Working Group on *Legionella* Infections (EWGLI; <http://www.ewgli.org/>) (Gaia *et al.*, 2005). Recently, to enhance the delineation of *L. pneumophila* strains, a seventh allele, *neuA*, has been added in SBT (Ratzow *et al.*, 2007).

In Japan, National Epidemiological Surveillance of Infectious Diseases data indicate that hot springs and public baths but not cooling towers are the major sources of *Legionella* infections (Infectious Disease Surveillance Center, 2000). Indeed, there have been four large outbreaks in public bath facilities (Kuroki *et al.*, 2009).

In our previous study (Amemura-Maekawa *et al.*, 2005), we analysed 27 epidemiologically unrelated *L. pneumophila* serogroup 1 isolates (ten from cooling towers, ten from public spas and/or hot spring baths, and seven from patients with public bath-related infections) from Japan by SBT using the six alleles proposed in 2005 by the EWGLI. The 27 isolates could be divided into 14 sequence types (STs). Notably, the 10 isolates from the cooling towers all showed the same allele types, namely *flaA* (1), *pilE* (4), *asd* (3), *mip* (1), *mompS* (1) and *proA*(1), whereas the public bath-derived isolates were more diverse.

Here, to further characterize clinical *L. pneumophila* isolates from Japan and to confirm the usefulness of the mAb- and SBT-based classification methods, we analysed 86 isolates by both typing methods.

METHODS

***L. pneumophila* strains.** We analysed 86 clinical isolates of *L. pneumophila* that were isolated in Japan during the period 1980–2008. Of these, 42 clinical *Legionella* isolates were from the *Legionella* Reference Center, which collects *Legionella* isolates obtained in six representative prefectural/municipal public health institutes of each district in Japan. The remaining 44 isolates were from the collection of the National Institute of Infectious Diseases, Department of Bacteriology I (NIIB). Of the 86 isolates, 84 were from unrelated cases. The remaining two isolates were obtained from the same patient but belonged to different serogroups (NIIB 2136 and NIIB 2137; Supplementary Table S1 in JMM Online). Nosocomial cases were defined as those in patients who had been hospitalized, and travel-associated cases were defined as those in patients who had spent at least one night away from home before onset of the symptoms. The incubation period was set to 2–10 days, but this depended on the discretion of the physician who notified a patient with legionellosis. Because previous studies (Infectious Disease Surveillance Center, 2000; Kuroki *et al.*, 2009) have indicated that the major sources of *Legionella* infections are public baths without taking into consideration whether or not the infected individuals had engaged in previous travelling or not, the relationship between travelling and legionellosis has not been under active consideration.

Outbreaks were defined as two or more cases for which there was strong epidemiological evidence of a common source of infection, with or without microbiological evidence. The 86 isolates included one representative isolate from each of the 12 *Legionella* outbreaks; all of the outbreaks were community-acquired, of which four were major outbreaks (Kuroki *et al.*, 2009).

Serogrouping and mAb subgrouping. Serogrouping of the isolates was performed by slide agglutination tests using a monovalent serum for *L. pneumophila* serogroups 1–15 (Denka Seiken). Serogroup 1 and 5 isolates were then subtyped serologically by using mAbs as described previously (Helbig *et al.*, 1997).

DNA manipulation, sequencing and sequence typing. Genomic DNA was extracted by using a High Pure PCR Template Preparation kit (Roche Diagnostics) and amplified by using the GeneAmp PCR System 9700 (Applied Biosystems) and previously described reaction mixtures and conditions (Amemura-Maekawa *et al.*, 2005; Gaia *et al.*, 2005). Both strands of the amplicons were sequenced by a model 3100, 3130 or 3130xl ABI Sequencer (Applied Biosystems). The nucleotide sequences obtained were confirmed and the allele numbers were determined using the online Sequence Quality Tool of the EWGLI website (Underwood *et al.*, 2006). Putative novel variants were submitted to the site 'Sequence Quality Tool' for verification and assignment of new allelic numbers. New combinations of allelic numbers were also submitted to the curators via the EWGLI website for assignment of new ST numbers. In this study, the isolates that failed amplification of *neuA* (indicated allele number as '0') were not given ST numbers but were allocated arbitrary numbers that start with J (J1, J2, etc.). A minimum spanning tree was generated by BioNumerics software (version 5.1; Applied Maths) using as parameters the categorical coefficient of similarity and the priority rule of the highest number of single-locus variants.

RESULTS AND DISCUSSION

Patient age, gender and sources of isolates

The mean and median age of the 85 legionellosis patients (72 males, 10 females and 3 unknown cases) was 59.2 and 60 years, respectively (range 0–81; excluding the three patients whose ages were unknown). It has been shown previously that many more males than females contract legionellosis (Infectious Disease Surveillance Center, 2008; Neil & Berkelman, 2008; Ng *et al.*, 2008), although the reasons for this are unclear. There were seven nosocomial cases (8.2%), eight travel-associated cases (9.4%), 49 community-acquired cases (57.6%) and 21 cases for which the source could not be identified (24.7%). The source of infection for 35 of the 85 patients was suspected to be public bathing facilities ($n=30$, 35.3%), many of which apparently had circulation systems and 25 of which used hot-spring waters, domestic baths ($n=2$), a shower ($n=1$), a humidifier ($n=1$) and a cooling tower ($n=1$). The sources of the infection for 16 of these 35 cases were indeed confirmed to be public baths ($n=15$) and a humidifier ($n=1$) by comparing the PFGE DNA patterns (Amemura-Maekawa *et al.*, 2005) of the clinical isolates to those of environmental isolates from the suspected origin of the infection at prefectural/municipal public health institutes. The sources of infection of the remaining 50 cases (61.2%) are unknown. In addition, 12 isolates (14.1%) were

derived from 12 individual outbreaks, of which 9, 1, 1 and 1 outbreaks occurred at public bath facilities (hot-spring waters were used at eight of the nine facilities and tap water was used at the remaining one), a nursing home (Maesaki *et al.*, 1992), the cooling tower of a waste-processing plant (Isozumi *et al.*, 2005) and the spa of a cruise ship (Kura *et al.*, 2006) (Supplementary Table S1), respectively.

Serogroups

The majority of the isolates ($n=69$, 80.2 %) belonged to serogroup 1, including 11 of 12 outbreak-derived isolates. Two, five, one, seven (including the remaining one outbreak-derived isolate), one and one isolate(s) belonged to serogroups 2, 3, 4, 5, 6 and 9, respectively (Supplementary Table S1). The majority of the male patients (87.5 %) were infected with serogroup 1 strains whereas only 40 % of the female patients were infected with serogroup 1 strains. Thus, the serogroup 1-infected patients were significantly more likely to be male (male:female ratio=12.4) than the patients infected with isolates belonging to other serogroups (male:female ratio=2.0; OR, 10.5; 95 % CI, 2.5–44.5). The reasons for this difference remain to be elucidated.

mAb subgroups

Of the 69 serogroup 1 isolates, 79.7 % ($n=55$) had the virulence-associated epitope that is recognized by mAb 3/1 and is not present on any other serogroups (Helbig *et al.*, 2002). Thus, of the 86 *Legionella* isolates, 64.0 % were mAb 3/1-positive, which is similar to the frequency of 66.8 % reported by the pan-European study. The 69 serogroup 1 isolates belonged to the Benidorm (44.9 %), Allentown/France (17.4 %), OLDA (14.5 %), Philadelphia (10.1 %), Knoxville (7.2 %), Oxford (4.4 %) and Bellingham (1.5 %) mAb subgroups. The distribution of mAb subgroups in Japanese isolates differs from that in pan-European isolates, which most frequently have the Philadelphia subgroup (28.5 %) and then the Benidorm subgroup (20.0 %) (Helbig *et al.*, 2002). Eleven of the 12 outbreak-derived isolates were mAb 3/1-positive (the single serogroup 5 outbreak-derived isolate lacked this marker).

STs

The 86 clinical isolates could be divided into 53 STs (discrimination index, 0.979) (Hunter & Gaston, 1988), though amplification of the *neuA* target failed for eight isolates of serogroups 2, 4 and 5 (Table 1 and Supplementary Table S1). The minimum spanning tree illustrates the distribution of the STs (Fig. 1). The tree has seven clonal complexes. The ST with the largest number of isolates was ST1 (seven isolates). ST1 is the most prevalent ST in the world (Borchardt *et al.*, 2008; Cazalet *et al.*, 2008; Harrison *et al.*, 2009; Kozak *et al.*, 2009; Reimer *et al.*, 2010), although a Canadian study has reported that the prevalence

of ST1 clinical isolates has decreased dramatically during the past 12 years (Tijet *et al.*, 2010). Indeed, in our study, six of the seven known clinical ST1 strains were isolated before 1994, and it is now unusual to isolate clinical ST1 strains; thus, it is striking that the majority of environmental isolates from cooling tower water still belong to ST1 (Amemura-Maekawa *et al.*, 2005; Cazalet *et al.*, 2008). The next major STs were ST306 (six isolates), ST120 (five isolates) and ST138 (five isolates). Notably, all of the ST138 and ST306 isolates but one (ST306) were suspected or confirmed to be derived from public bath water, which suggests that these strains prefer bath habitats. Both ST138 and ST306 are unique to Japan according to data submitted to the EWGLI SBT database as of 12 January 2010. In contrast, the sources of all but one of the Japanese isolates belonging to ST1 and ST120 remain unclear (one isolate was suspected to come from shower water). Four isolates were ST23 strains, two of which (Supplementary Table S1, NIIB 292 and NIIB 374) were derived from two large public bath facility-associated outbreaks that affected hundreds of people (Nakamura *et al.*, 2003; Okada *et al.*, 2005). In Europe, clinical isolates often belong to ST23 (Borchardt *et al.*, 2008; Cazalet *et al.*, 2008). These major STs (ST1, ST23, ST120 and ST306), except for ST138, belonged to clonal complexes, and these STs were distinct from each other, with the exception of ST23 and ST120 (Fig. 1). ST430 and J2 had three isolates each, and the other seven STs had two isolates each (Table 1). The remaining 39 STs each had one isolate. Thirty-four of the 53 STs were unique to Japan as of 12 January 2010, including two SBT profiles that could not be assigned to STs because they failed *neuA* amplification (Table 1). With regard to the 12 outbreak-derived strains, two belonged to each of ST23, ST138 and ST139, and the remaining six belonged to different STs (ST2, ST36, ST89, ST142, ST434 and J4). Notably, for ST142, there was not only an outbreak-derived isolate but also another unrelated non-outbreak-derived isolate. Similarly, ST2, ST36 and ST89 isolates have also been found in patients elsewhere in Japan (unpublished results) and/or abroad, as Philadelphia 1 was typed to ST36 (Ratzow *et al.*, 2007).

The discrimination index (0.979) in our investigation was higher than that described in previous reports which were based on isolates from England and Wales (0.901; Harrison *et al.*, 2009), USA (0.946; Kozak *et al.*, 2009) and Canada (0.964; Reimer *et al.*, 2010). This may reflect the different infection sources in Japan: the main infection source was public bath water (Supplementary Table S1), whereas those of outbreaks in Europe were hot- or cold-water systems and cooling towers (Ricketts *et al.*, 2007). (There have been no reports for the dataset of the infection sources of sporadic cases in Europe, as far as we know.) The water of Japanese public baths is often obtained from hot springs. The characteristics of hot spring water, namely chemical features such as pH and temperature, are highly variable, whereas the water from hot- or cold-water systems and cooling towers tends to

Table 1. Sequence types of 86 Japanese clinical isolates of *Legionella pneumophila*

ST, Sequence type: seven-allele profile; J2 and J5, arbitrary numbers allocated to each unique six-allele profile without *neuA*; Others, STs each presented by only a single isolate.

ST	No. of isolates	%	mAb (no. of isolates)
Serogroup 1	69	80.2	
1	7	8.1	OLDA (6), Oxford (1)
306*	6	7.0	Benidorm
138*	5	5.8	Benidorm (4), Allentown/France (1)
120	5	5.8	Benidorm
23	4	4.7	Allentown/France (2), Philadelphia (1), Oxford (1)
122*	2	2.3	Benidorm
42	2	2.3	Benidorm
118*	2	2.3	Philadelphia
123*	2	2.3	Benidorm
139*	2	2.3	Allentown/France (1), Benidorm (1)
142*	2	2.3	Allentown/France
Others†	30	34.9	(Not shown)
Other serogroups (serogroup)	17	19.8	
430* (3)	3	3.5	
J2 (5)	3	3.5	Dallas (2), Cambridge (1)
J5 (5)	2	2.3	Dallas
Others‡ (2, 3, 4, 5, 6, 9)	9	10.5	(Not shown)
Total	86	100.0	

*Described only in Japan as of 12 January 2010.

†Nineteen of 30 STs only in Japan as of 12 January 2010.

‡Seven of nine STs were described only in Japan as of 12 January 2010.

have rather similar characteristics due to similar water treatment procedures in addition to the environmental selective pressure. Thus, reflecting the wider array of *Legionella*-suitable environmental niches in Japan, the Japanese clinical isolates may be more genetically variable than Western isolates. This idea is supported by previous reports from our laboratory, which showed that Japanese isolates derived from public baths differ genetically from Japanese isolates derived from cooling towers (Amemura-Maekawa *et al.*, 2005, 2008).

Combining sequence typing and mAb subgrouping

Some STs were composed of isolates belonging to the different mAb subgroups (and vice versa). Thus, six of the seven ST1 isolates were OLDA subgroup ($n=6$) and the remaining ST1 isolate was an Oxford subgroup, while two of the four ST23 isolates were Allentown/France subgroup and the other two were Philadelphia and Oxford subgroups. All six ST306, all five ST120 and four of the five ST138 isolates were Benidorm subgroup. Moreover, in six of the seven STs, each of which consisted of two isolates, both isolates belonged to the same subgroup. By combining the SBT and subgrouping data, we could divide the 86 isolates into 59 types (discrimination index, 0.984) (Table 1 and Supplementary Table S1).

Importance of *Legionella* isolation

Based on the Infectious Diseases Control Law, legionellosis in Japan has been classified as a category IV notifiable infectious disease and has been monitored by the National Epidemiological Surveillance of Infectious Diseases (NESID) since 1999. Consequently, in Japan, physicians must notify the authorities about legionellosis cases. In 2008, 884 legionellosis cases were reported, which represents a remarkable fivefold increase in the previous 5 years (Infectious Disease Surveillance Center, 2008). However, this increase is thought to be due to the widespread use of a highly accurate urine antigen assay, which is easier to perform than the laborious and time-consuming process of isolating *Legionella* from the patients. From January 2003 to September 2008, 2460 legionellosis cases were reported, of which only 97 cases were diagnosed by the isolation of *Legionella* (Infectious Disease Surveillance Center, 2008). We collected 29 isolates during the same period. Therefore, our study may not entirely faithfully delineate the strains responsible for legionellosis in Japan. Health workers should isolate the bacterium from the patient and identify the infection source by genotyping the organism and comparing this genotype to those of environmental isolates from the surroundings of the patient.

The legionellosis incidence in Japan did not vary in a seasonal fashion (Infectious Disease Surveillance Center,

Fig. 1. Minimum spanning tree showing how the *L. pneumophila* clinical isolates, with seven determined alleles, are distributed in terms of their STs. The ST number is shown in the circle and the size of the circle indicates the number of isolates. Short thick lines connect single-locus variants, thin lines connect double-locus variants, black broken lines connect triple-locus variants and grey broken lines connect more than three different STs. The grey parts of the circles (pie charts) indicate the clinical isolates associated with bath water. The clonal complexes that were generated with single-locus variants and double-locus variants are indicated by the shaded backgrounds.

2003) until 2005, which is when the number of legionellosis cases started peaking in July (Infectious Disease Surveillance Center, 2008). This seasonality relates to humidity: rainfall increases microbial contamination in source waters or water distribution systems (Fisman *et al.*, 2005; Ng *et al.*, 2008). However, the epidemiological profile of sporadic legionellosis remains poorly understood. It has recently been proposed that legionellosis can be acquired from puddles on asphalt roads on rainy days (Sakamoto *et al.*, 2009a) and from the air-conditioning systems of motor cars (Sakamoto *et al.*, 2009b). It is likely that there are many as yet unrecognized infection sources of *Legionella*. It is possible that groups of isolates with particular genotypes inhabit distinct infection sources including unrecognized sources.

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