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Environmental surveillance and molecular characterization of *Legionella* in tropical Singapore

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Abstract. Legionnaires' disease is often acquired by inhalation of legionellae from a contaminated environmental source. In recent years, Singapore has seen an increase in the use of aerosol-generating fixtures such as mist fans and spa pools. Poorly maintained and designed water fixtures could pose a public health threat to the community. In this study, we provided an update on the prevalence of *Legionella* in mist fans (N=28), household water heaters with storage tanks (N=19) and instantaneous heaters (N=30); and extended the survey to spa pools (N=29) and aerosol-generating fixtures in nursing homes (N=116). The prevalence of *Legionella* were 21.1% in water heaters with storage tanks, 24.1% in spa pools, 14.2% in mist fans and 3.3% in instantaneous heaters. *Legionella* was not detected in nursing homes. A total of 37 isolates were subjected to molecular characterization using Sequence-Based Typing (SBT) protocol from the European Working Group on *Legionella* Infections (EWGLI). This is the first study on the use of SBT protocol on environmental strains isolated from tropical South East Asia. The *Legionella* flora was very heterogenous. The overall diversity of the allelic profile was found to be 0.970 (95% CI 0.946 – 0.994). All known STs of our isolates have been associated with clinical cases in EWGLI database. The phylogenetic analysis showed that our novel environmental isolates were clustered with clinical STs that were previously reported in Europe, Japan, United Kingdom and United States etc. (in EWGLI database), suggesting that *Legionella* found in the environment of Singapore may potentially cause human disease.

INTRODUCTION

Legionnaires' disease is often acquired by inhalation or aspiration of legionellae from a contaminated environmental source (Borella *et al.*, 2004). Legionellae are ubiquitous in the environment. Man-made water systems in public buildings including cooling water systems, spa pools as well as hot and cold water systems have previously been associated with *Legionella* outbreak (Gilmour *et al.*, 2007; Modi *et al.*, 2008). The operative temperature range (25 – 45ºC) of these systems is favourable for *Legionella* growth. In many countries, including Singapore, outbreak prevention and control measures have been implemented through legislation and education. However, reported cases of Legionnaires' disease continue to increase in many temperate countries. In tropical Singapore, an average of 35 cases per year have been reported, since 1989 (Ministry of Health, 2009).

There are more than 40 species of *Legionella*, and majority of the clinical cases are attributed to *L. pneumophila* serogroup 1. *Legionella* from the environment have been found to be pathogenic and non-pathogenic (Fields *et al.*, 2002). Previous studies (Harrison *et al.*, 2009), has shown that clinically important strains were rarely found in managed water system, and environmental strains were

also rarely associated with clinical cases. Risk assessment of a water body based only on the quantity of *Legionella* is thus inadequate. Knowledge of the *Legionella* flora in the environment is critical for a pragmatic understanding of the risk associated with *Legionella* present in water systems.

In recent years, Singapore has seen an increase in the use of aerosol-generating fixtures such as mist fans, jacuzzi pools and indoor water fountains. Although chlorinaminated city water is used in all these fixtures, poorly maintained and designed water fixtures could pose a public health threat to the community. Previous local surveys had revealed *Legionella* risk in mist fans, cooling towers and home water heaters (Goh *et al.*, 2005; Kek *et al.*, 2006; Lim *et al.*, 2008). In this study, we provided an update on the prevalence of *Legionella* in mist fans, household water heater tanks and instantaneous heaters; and extended the survey to spas and nursing homes. Molecular characterization of *Legionella* isolates derived from the environment was also performed. A Sequence-Based Typing (SBT) protocol (Gaia *et al.*, 2005) from the European Working Group on *Legionella* Infections (EWGLI) was employed to characterize isolates derived from the various water fixtures. The protocol enables the differentiation of *L. pneumophila* strains and allows comparison with a global collection of SBT data deposited in a web-accessible database (Gilmour *et al.*, 2007). This study is part of our regular national environmental risk assessment for *Legionella*.

MATERIALS AND METHODS

Sample collection and *Legionella* isolates

A swab sample and 500 ml of water sample were collected from each of the 28 mist fans from 22 commercial and community outlets. A fan is considered positive if any of the samples (swab or water) was tested positive. To survey household heaters, 19 water heaters with storage tanks from 15 households and 30 instantaneous heaters from 30 households were sampled by collecting 500 ml of water through the outlet taps. For surveillance of spa outlets, a water sample from the pool and a swab sample from the nozzle were collected from 29 pools from 20 spa outlets. Among the 29 pools, 12 were small tubs with water changed after each use. The rest were larger pools that are used by multiple patrons before any change of water. A pool is considered positive when either the water or swab sample was found positive. Surveys at nursing homes were performed by taking 116 swabs and 116 water samples from faucets, showerheads and water coolers of 34 homes. The samples were processed and colony isolated by a commercial laboratory, according to the International Standard method (ISO 11731: 1998 or AS/NZ3896: 1998 Detection and Numeration of *Legionella* in Water). Briefly, 200 ml of each water sample was filtered through a membrane, and the residue was resuspended in 20 ml of sterile Ringer's solution. 0.1 ml of serial dilutions of the concentrated sample were plated onto GVPC (Glycine, Vancomycin, Polymyxin B, Cicloheximide) selective media and incubated at 36±1°C for up to 10 days. The detection limit is 1 CFU/ml. For each sample, the first three *Legionella* colony, randomly picked from the primary isolation plate, was used for analysis and characterization. Serogrouping was done by latex beads agglutination. Isolates from cooling towers were obtained from commercial testing laboratories that collected them as part of their service to routine monitoring of centralized air-conditioned premises from 2002 – 2006. Positive *Legionella* isolates were stored in glycerol, at –80°C.

Molecular confirmation and typing of isolates

Glycerol stocks of *L. pneumophila* positive isolates were streaked on Buffer Charcoal Yeast Extract Agar containing 0.1% alpha-ketoglutarate and 0.04% L-

cysteine (BCYEα agar; Oxoid Ltd., Basingstoke, Hampshire, England) and a single colony was then propagated on BCYEα for DNA extraction. DNA extraction was performed using DNeasy Blood & Tissue Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instruction. Polymerase chain reaction (PCR) was performed on the purified DNA using seven pairs of primers: *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA* and *neuA*, according to the standard method described by EWGLI (Gaia *et al.*, 2005). PCR products were sequenced and compared to previously assigned alleles using the online *Legionella* Sequence Quality Tool. For each isolate, the combination of seven alleles at each of the loci was defined as a seven-digit allelic profile by using the predetermined order *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA* and Sequence Type (ST) represented by a number. New allelic profiles encountered for the first time in this study were given EHI numbers.

Phylogenetic analysis

The concatenated sequences of *L. pneumophila* positive isolates were obtained by combining the trimmed sequences generated by EWGLI (Gaia *et al.*, 2005). Multiple alignments were accomplished with nucleotides sequences using the multiple alignments Clustal W software. Phylogenetic analysis was conducted using the Molecular Evolutionary Genetics Analysis software Version 4.1 (MEGA4) (Kumar *et al.*, 2008). A neighbour–joining tree was constructed using the Kimura 2-parameter model of substitution including transition and transversion. The reliability of the tree was assessed using bootstrap method with 1000 replicates.

Statistical analysis

The prevalence of *Legionella* in mist fans in 2003 and 2008 were compared using the independent T-test in Statistical Package for Social Sciences (SPSS) 17.0. Diversity was estimated by calculating Hunter and Gaston's modification of Simpson's index of diversity (Hunter & Gaston, 1988) as previously described.

RESULTS

Prevalence of *Legionella* in water fixtures

The results from the survey are tabulated in Table 1. *Legionella* were detected in mist fans, household water heaters with storage tanks, household instantaneous heaters and spa pools but not in nursing homes.

Table 1. Prevalence of *Legionella* in various water fixtures

Water fixtures	% (number) of fixtures positive for			
	Legionella	*L. pneumophila* SG 1	*L. pneumophila* SG 2 - 14	Other *Legionella spp.*
Mist fans(N=28)	14.2% (4)	Not detected	3.5% (1)	10.7% (3)
Water heaters with storage tank (N=19)	21.1% (4)	5.3% (1)	Not detected	15.8% (3)
Instantaneous heaters (N=30)	3.3% (1)	Not detected	Not detected	3.3% (1)
Spa pools (N=29)	24.1% (7)	13.8% (4)	6.9% (2)	3.4 (1)
Nursing Homes (Assorted fixtures[†]) (N=116)	Not detected	Not applicable	Not applicable	Not applicable

[†] Water and swab samples were collected from water tanks, faucets, showerheads and water coolers.
SG: Serogroup

The prevalence of *Legionella* in mist fans, at 14.2% is not a significant improvement from a previous survey done in 2003 (data not published), when 20.5% of 39 fans were found to be positive for *Legionella* (p=0.186). The concentration found in the mist fans in this study ranged from <1 to 89 CFU/ml of water. Among the 30 instantaneous water heaters tested, one was positive for non-pneumophila species. Four of the 19 water heaters with storage tanks were positive for *Legionella*, with one tank containing the *L. pneumophilla* species. Out of the 29 spa pools tested, 24.1% (7 pools) were tested positive for *Legionella*, with four containing serogroup 1, two containing serogroup 2-14 and one of the other species. Six of the seven positive pools were all larger pools with water changed after usage by multiple patrons. The concentration found in the water and swab ranged from 6 to 42 CFU/ml of water and 150 to 250,000 CFU/swab respectively. While *Legionella* was found only in water of two of these pools, the other four had *Legionella* in both water and nozzles. On the other hand, water samples from all the single use pools were negative for *Legionella*. However, swab from the nozzle of one of the pools was positive for *Legionella*. The level picked up by the swab in this single use pool was 150 CFU/swab. Assorted fixtures (water tanks, faucets, showerheads and drinking coolers) in nursing homes were all tested negative for *Legionella*.

Molecular characterization of isolates

A total of 37 isolates were subjected to molecular characterization using the standard method described by European Working Group on *Legionella* Infections (EWGLI) (Gaia *et al.*, 2005). The *Legionella* flora was very heterogenous. The overall diversity of the allelic profile was found to be 0.970 (95% CI 0.946 – 0.994), with 25 Sequence Type (ST) found among 37 isolates (Table 2). Seven out of the 25 STs were of serogroup 1. Only 28% of the STs had allelic profile found in the database provided by EWGLI. Interestingly all known STs of our environmental isolates were associated with clinical isolates reported in the EWGLI database. ST1, a common environmental ST that has been associated with clinical isolates, was found in three cooling towers and a household water heater with storage tank. In this study, water and swab samples collected from two spa pools, which are located 3.81 km apart, were found to be ST59. Forty-nine percent of ST59 entries in EWGLI database were clinical isolates. All the other known STs had ten or less entries in EWGLI, but were also derived from clinical isolates.

The remainders of the isolates (72%) that do not match any allelic profile in the database were found in all water fixtures except water heater tank. However, phylogenetic analysis based on the concatenated sequences did not reveal any clustering of isolates according to water fixtures (Figure 1). In addition, these novel isolates were closely related to those previously reported STs in the EWGLI database.

DISCUSSION

Water fixtures that generate mists or aerosols, intentionally or incidentally, could harbor and disperse *Legionella* - a potential public health concern in population dense Singapore. The negative findings in nursing homes demonstrated good house-keeping, which are especially critical for the health of the elderly residents. However, our study has revealed that 24.1% of spa pools were tested positive for *Legionella*. *Legionella* in water was only found in larger pools that are used by multiple patrons between changes of water. Water in these pools was drained at a frequency that ranged from daily to monthly while the frequency of chlorination dosing ranged from thrice a week to weekly. While no *Legionella* was detected in pools that had water changed after single use, the nozzles of two of such pools were found to harbour *Legionella*, though not of the *L. pneumophila* species. The findings revealed the potential risk of *Legionella* in both kinds of spa pools that are not properly

Table 2. Allelic profile of *L. pneumophila* isolated from environmental samples

Sequence type (ST[1])	Allelic Profile *flaA,pilE,asd,mip,mompS,proA,neuA*	SG	Spa	Water tank	Mist Fan	Cooling Tower	GenBank Accession number
EHI001 (ST713)	26, 8, 11, 5, 47, 30, 2	2-14	–	–	–	1	HQ190586, HQ190622, HQ190659, HQ19069, HQ190733, HQ19077, HQ190808
EHI002 (ST714)	1, 4, 3, 6, 1, 1, 9	2-14	–	–	–	1	HQ190587, HQ190623, HQ190660, HQ19069, HQ190734, HQ19077, HQ190809
EHI003	16, 4, 3, 19, 1, 30, -1	2-14	–	–	–	1	HQ190588, HQ190624, HQ190661, HQ19069, HQ190735, HQ19077, HQ190810
EHI004 (ST715)	1, 4, 3, 19, 1, 1, 2	2-14	–	–	–	1	HQ190589, HQ190625, HQ190662, HQ19069, HQ190736, HQ19077, HQ190811
EHI005 (ST716)	16, 4, 3, 19, 1, 30, 11	2-14	–	–	–	1	HQ190590, HQ190626, HQ190663, HQ190700, HQ190737, HQ190774, HQ190812
EHI006	-1, 14, 16, 12, 15, 13, 1	1	–	–	–	1	HQ190585, HQ190627, HQ190664, HQ190701, HQ190738, HQ190775, HQ190813
EHI007 (ST717)	1, 4, 3, 19, 1, 30, 1	2-14	–	–	–	1	HQ190591, HQ190628, HQ190665, HQ190702, HQ190739, HQ190776, HQ190814
EHI008	1, 4, 3, 19, 1, 30, -1	2-14	–	–	–	2	HQ190593, HQ190629, HQ190666, HQ190703, HQ190740, HQ190777, HQ190807, HQ190592, HQ190630, HQ190667, HQ190704, HQ190741, HQ190778, HQ190815
EHI009 (ST718)	7, 6, 17, 1, 48, 11, 1	1	–	–	–	3	HQ190598, HQ190631, HQ190668, HQ190705, HQ190742, HQ190779, HQ190816, HQ190599, HQ190632, HQ190669, HQ190706, HQ190743, HQ190780, HQ190817 HQ190600, HQ190633, HQ190670, HQ190707, HQ190744, HQ190781, HQ190818
EHI010 (ST719)	1, 4, 3, 19, 1, 1, 1	2-14	–	–	–	1	HQ190594, HQ190634, HQ190671, HQ190708, HQ190745, HQ190782, HQ190819

EHI011 (ST720)	16, 4, 3, 19, 1, 30, 1	1	–	–	–	1	HQ190619, HQ190635, HQ190672, HQ190709, HQ190746, HQ190783, HQ190820
EHI012 (ST721)	8, 6, 34, 9, 53, 8, 1	2-14	–	–	–	1	HQ190613, HQ190636, HQ190673, HQ190710, HQ190747, HQ190784, HQ190821
EHI013 (ST722)	7, 6, 17, 3, 1, 11, 1	2-14	–	–	–	1	HQ190601, HQ190637, HQ190674, HQ190711, HQ190748, HQ190785, HQ190822
EHI014 (ST726)	28, 21, 33, 37, 41, 1, 11	2-14	–	–	–	1	HQ190614, HQ190638, HQ190675, HQ190712, HQ190749, HQ190786, HQ190823
EHI015	-1, 21, 33, 3, 13, -1, 11	2-14	–	–	1	–	HQ190620, HQ190639, HQ190676, HQ190713, HQ190750, HQ190787, HQ190824
EHI016 (ST723)	7, 6, 31, 19, 48, 11, 1	2-14	–	–	–	1	HQ190602, HQ190640, HQ190677, HQ190714, HQ190751, HQ190788, HQ190825
EHI017 (ST724)	16, 4, 3, 19, 1, 30, 2	2-14	1	–	–	–	HQ190611, HQ190641, HQ190678, HQ190715, HQ190752, HQ190789, HQ190826
EHI018 (ST725)	16, 21, 33, 37, 41, 1, 3	2-14	–	–	1	–	HQ190612, HQ190642, HQ190679, HQ190716, HQ190753, HQ190790, HQ190827
ST1	1, 4, 3, 1, 1, 1, 1	1	–	1	–	2	HQ190595, HQ190643, HQ190680, HQ190717, HQ190754, HQ190791, HQ190828, HQ190596, HQ190644, HQ190681, HQ190718, HQ190755, HQ190792, HQ190829 HQ190621, HQ190645, HQ190682, HQ190719, HQ190756, HQ190793, HQ190830
	1, 4, 3, 1, 1, 1, 1	2-14	–	–	–	1	HQ190597, HQ190646, HQ190683, HQ190720, HQ190757, HQ190794, HQ190831

ST59	7, 6, 17, 3, 13, 11, 11	1	3	–	–	–	HQ190603, HQ190647, HQ190684, HQ190721, HQ190758, HQ190795, HQ190832, HQ190604, HQ190648, HQ190685, HQ190722, HQ190759, HQ190796, HQ190833 HQ190605, HQ190649, HQ190686, HQ190723, HQ190760, HQ190797, HQ190834
ST61	7, 6, 17, 3, 24, 11, 11	2-14	–	–	–	1	HQ190606, HQ190650, HQ190687, HQ190724, HQ190761, HQ190798, HQ190835
ST114	3, 6, 1, 6, 14, 11, 9	2-14	–	–	–	1	HQ190615, HQ190651, HQ190688, HQ190725, HQ190762, HQ190799, HQ190836
ST187	3, 10, 1, 28, 14, 9, 3	2-14	1	1	–	–	HQ190616, HQ190652, HQ190689, HQ190726, HQ190763, HQ190800, HQ190837, HQ190617, HQ190653, HQ190690, HQ190727, HQ190764, HQ190801, HQ190838
ST237	12, 8, 11, 5, 47, 12, 2	1	–	–	–	1	HQ190618, HQ190654, HQ190691, HQ190728, HQ190765, HQ190802, HQ190839
ST496	7, 6, 31, 3, 48, 15, 11	1	4	–	–	–	HQ190607, HQ190655, HQ190692, HQ190729, HQ190766, HQ190803, HQ190840, HQ190608, HQ190656, HQ190693, HQ190730, HQ190767, HQ190804, HQ190841, HQ190609, HQ190657, HQ190694, HQ190731, HQ190768, HQ190805, HQ190842, HQ190610, HQ190658, HQ190695, HQ190732, HQ190769, HQ190806, HQ190843

[1] ST: Sequence Type as designated for each seven-gene allelic profile using the EWGLI SBT database, except where prefixed by an "EHI", where it is an arbitrary number allocated to each novel isolate. Allele number '-1' represents allele types not found in the database'e, and are under validation by the EWGLI. SG: Serogroup

maintained. Spa pool associated *Legionella* infection was first reported in 1981 (Spitalny *et al.*, 1984) from the United States. Subsequently, more reports from various countries such as Japan, Sweden, France, England and Scotland have demonstrated the risk of *Legionella* infection from spa pools (Fallon & Rowbotham, 1990; Benkel *et al.*, 2000; Fields *et al.*, 2001; Gotz *et al.*, 2001; Modi *et al.*, 2008). Common errors in operation included failure to maintain adequate biocide concentrations, pH, and temperature, and failure to change water and to clean or replace sand filters. Though no *Legionella* cases have been associated

with spa pools in Singapore, the National Environment Agency had commissioned the development of a national guideline, with the aim of mitigating the risk. Working closely with the industry stakeholders, the guideline seeks to raise awareness and assist spa operators in improving the hygiene standards of spa pools. A more recent survey in 2010 suggested a successful public-private partnership, when no *Legionella* was detected in the 63 pools surveyed (Yap *et al.*, unpublished data).

The prevalence of *Legionella* in mist fans, at 14.2% was not a significant improvement from a previous survey done in 2003, when 20.5% of 39 fans were found to be positive for *Legionella (p=0.186)*. Continuous education is essential to raise the maintenance standard of mist fans, as community-wide Legionnaires' disease has previously been associated with mist machines in grocery stores in the United States (Mahoney *et al.*, 1992).

Prevalence of *Legionella* in household water heaters with storage tanks and instantaneous heaters were 21.1% and 3.3% respectively. One of the cases with storage tank was due to a faulty heater, which did not heat the water beyond 50°C. While the temperature was adequate for daily usage, the risk of *Legionella* proliferation may have been overlooked. Awareness among the public is essential, especially during the current climate of energy conservation, where the community may under-heat the water and subject themselves to the risk.

According to the relative risk assessment suggested by Miller & Kenepp (1993), >1,000 CFU/ml of *Legionella* poses very high risk; 100 -1000 CFU/ml, high risk; 10-100 CFU/ml, moderate risk; 1-10 CFU/ml, low risk; and <1 CFU/ml ,very low risk. An outbreak in an Australian aquarium was suggested to be associated with contaminated cooling tower with 3000-15000 CFU/ml of the bacteria (Greig *et al.*, 2004). In general, the concentrations of *Legionalla* detected in the various water fixtures fell within the range of 1-90 CPU/ml, suggesting that the risk of an occurrence of an outbreak due to these contaminated fixtures is moderate.

As the disease-causing potential of *Legionella* strains vary, it is essential to gain an understanding of the flora in our environment, so as to have a pragmatic assessment of risk from the environment. The heterogeneity of the *L. pneumophilla* species in our environment is consistent with findings in other countries such as the United Kingdom and United States (Harrison *et al.*, 2009; Kozak *et al.*, 2009). The overall diversity of 0.970 is equivalent to 0.954 found in United Kingdom and more than in United States (0.822). However, in contrast to these previous studies, most (72%) of our STs found no match in the EWGLI database. Similar observation was found in the study conducted by Kozak *et al.* (2009) in which 58% of the STs were found to be unique to the United States. Despite the small number of isolates (N=37) characterized in this study, 18 new sequence types were generated. This could be due to the fact that the database is largely assembled from European and Japanese isolates and our sequences are the first data from South East Asia. It would be interesting to determine if the large proportion of novel sequence type is due to a geographical variation in the *Legionella* flora.

We have found that all the known STs of our environmental isolates have been associated with clinical cases based on the EWGLI database. Unfortunately, clinical isolates from Singapore are not available for characterization. Nevertheless, the finding contrasts previous studies, where clinically significant strains are only rarely found in managed water systems (Harrison *et al.*, 2009). ST1, found in Singapore's cooling towers and a household heater tank, appears to be of clinical risk. However, the presence of functional *lag-1* gene, a virulence marker for *L. pneumophila* serogroup 1 strains, is necessary to confirm this (Kozak *et al.*, 2009). Forty-one percent of ST1's entries in the EWGLI database as in August 2010 (time of writing) were derived from clinical isolates from Japan, Netherlands, United Kingdom, Germany, Canada and others. They were contributed by both nosocomial and

community acquired diseases. ST1 was found to contribute to only 4.8% of clinical isolates in the United Kingdom (Harrison *et al.*, 2009). However, in a study conducted in Germany, the majority (19.0%) of clinical cases were of ST1 (Harrison *et al.*, 2009). The German study had a proportion of cases being nosocomial, and could suggest that ST1 may be opportunistic. Nevertheless, the phylogenetic analysis (Figure 1) showed that our novel environmental isolates were clustered with clinical STs that were previously reported in Europe, Japan, United Kingdom and United States etc. (in EWGLI database), suggesting that *Legionella* found in the environment of Singapore may potentially cause human disease.

Due to the ubiquitous nature of *Legionella* in the environment, highly discriminatory molecular typing with associated epidemiological investigations are needed to establish the link between clinical and environmental isolates so as to identify the source of infection (Scaturro *et al.*, 2005; Qasem *et al.*, 2008). Data collected from our environmental *Legionella* surveillance and characterization will be useful for future epidemiological investigation. The capacity building forms part of our preparedness to handle public health threats of environmental concern.

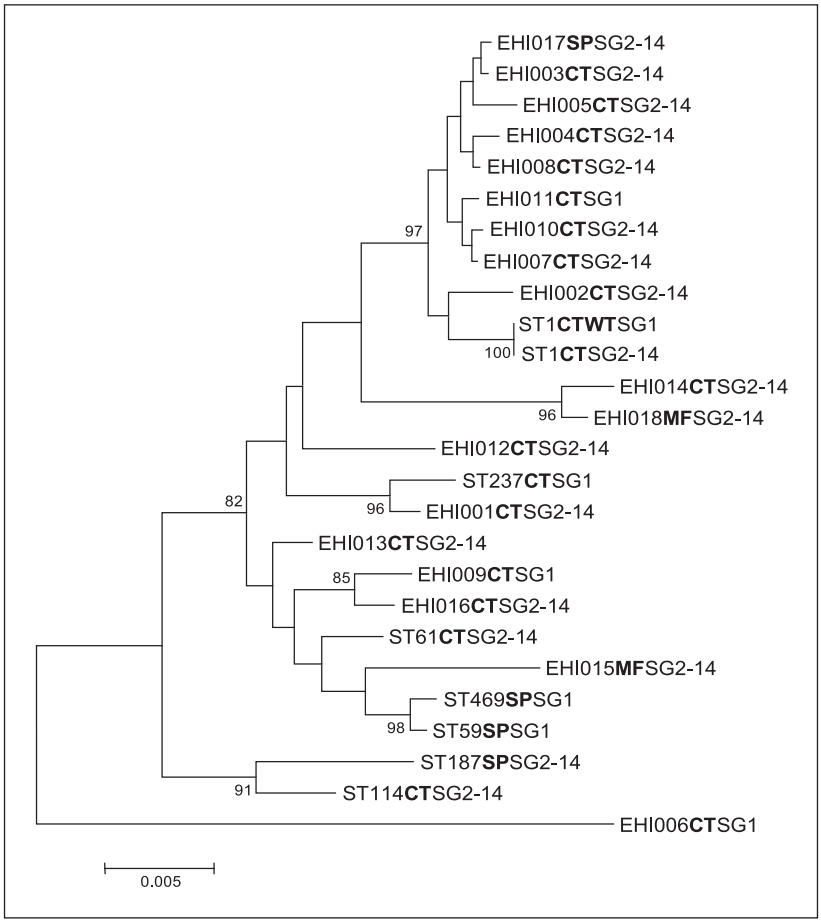

Figure 1. Phylogenetic tree of environmental *L. pneumophilia*, based on a neighbour-joining tree of the concatenated segments of seven specific gene loci (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA* and *neuA*).
CT: cooling towers; SP: spa pools; WT: water tanks; MF: mist fans

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