

Legionella species and serogroups in Malaysian water cooling towers: identification by latex agglutination and PCR-DNA sequencing of isolates

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ABSTRACT

In this study, we investigated the distribution of *Legionella* species in water cooling towers located in different parts of Malaysia to obtain information that may inform public health policies for the prevention of legionellosis. A total of 20 water samples were collected from 11 cooling towers located in three different states in east, west and south Malaysia. The samples were concentrated by filtration and treated with an acid buffer before plating on to BCYE agar. *Legionella* viable counts in these samples ranged from 100 to 2,000 CFU ml⁻¹; 28 isolates from the 24 samples were examined by latex agglutination as well as 16S rRNA and *rpoB* PCR-DNA sequencing. These isolates were identified as *Legionella pneumophila* serogroup 1 (35.7%), *L. pneumophila* serogroup 2–14 (39%), *L. pneumophila* non-groupable (10.7%), *L. busanensis*, *L. gormanii*, *L. anisa* and *L. gresilensis*. *L. pneumophila* was clearly the predominant species at all sampling sites. Repeat sampling from the same cooling tower and testing different colonies from the same water sample showed concurrent colonization by different serogroups and different species of *Legionella* in some of the cooling towers.

Key words | latex agglutination, *Legionella*, Malaysia, *rpoB* PCR, water cooling towers, 16S rRNA PCR

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INTRODUCTION

The genus *Legionella* was first established after a severe outbreak of pneumonia in Philadelphia, USA, in 1976, which resulted in 239 cases and 34 deaths (Fraser 2005). To date, 50 species of *Legionella* have been described of which at least 21 are associated with human infections (Coscolla & Gonzalez-Candelas 2007). *Legionella pneumophila* serogroup 1 alone is believed to be responsible for 60–80% of legionnaires' disease; serogroups 2–14 account for another 15–35% and other *Legionella* species are associated with the remaining infections (Yu *et al.* 2002).

Legionnaires' disease is identified as a community-acquired pneumonia that can present as sporadic infections or outbreaks (Kusnetsov *et al.* 1997; Liang *et al.* 2006; Joseph & Ricketts 2007). Transmission is via the inhalation or aspiration of aerosols contaminated with legionellae mostly

from water cooling towers and other man-made water systems. Travel-associated legionnaires' disease has been increasingly reported in Europe and the USA where there has been a dramatic increase in the incidence of legionellosis since 1990 (CDC 2007; Neil & Berkelman 2008; Ricketts *et al.* 2008).

Many epidemiological studies have shown the link between environmental and clinical strains of *Legionella*, and environmental surveillance programmes can provide useful data for risk assessment of outbreaks (Huang *et al.* 2004; Harrison *et al.* 2007). Even though no outbreaks of legionnaires' disease have been documented in Malaysia, *Legionella* organisms have been shown to be present in the environment (Ngeow *et al.* 1992). *Legionella* were found in water cooling towers and in hospital warm water supplies

(Ngeow *et al.* 1992; Chin *et al.* 2003–2005). As these environmental reservoirs may be the source of clinical disease, it is of interest to know the distribution of *Legionella* species and serogroups in them. In this study, *Legionella* isolates from water cooling towers in various parts of Malaysia were serogrouped by latex agglutination with antisera and speciated by 16S rRNA and *rpoB* DNA sequencing. The 16S rRNA and *rpoB* gene sequences obtained were also used to study phylogenetic relationships among the *Legionella* isolates.

MATERIALS AND METHODS

Reference strains of *Legionella* species

Three *L. pneumophila* (serogroups 1, 5, 12) and eight non-pneumophila reference strains (five *L. longbeachae*, two *L. micdadei* and one *L. gormanii*) were included in this study as the positive and negative controls in the latex agglutination test and PCR-DNA sequencing of isolates of *Legionella*-like organisms. These strains were kindly provided by Professor P.M. Hawkey at the University of Leeds, UK, and Dr Elizabeth L. Hartland at the University of Melbourne, Australia.

Water sampling

Water samples were collected in 2005 and 2006. Samples from cooling towers 1–6 were collected and processed by the Public Health Laboratory in the state of Johor in south peninsular Malaysia. Isolates from these samples were supplied by the National Public Health Laboratory. Samples from towers 7–11 were collected by the authors from cooling towers located in different buildings in west peninsular Malaysia (Table 1). Water samples (500 ml) were collected from each cooling tower by immersing a sterilized 1-litre bottle approximately 10 cm below the surface of water. The water sample was then kept on ice, delivered to the laboratory and processed within 48 hours of collection, as described in the Australian/New Zealand Standard (AS/NZS 3896:1998) for waters: examination for legionellae including *L. pneumophila*.

Water processing

Each water sample was pressure filtered through a 0.45 µm cellulose nitrate membrane (Millipore) and eluted with 10 ml of sterile phosphate buffered saline (PBS pH 7.2 ~ 7.3). This was followed by 20 minutes of centrifugation at 4,000 rpm. The resulting sediment was resuspended in 1 ml sterile distilled water.

Acid treatment of concentrated filtrate

The 1 ml of bacterial suspension was acid treated with 9 ml of HCl-KCl pH 2.2 (0.1 M Tris HCl, 0.1 M KCl) for 30 minutes, after which 100 µl of the treated suspension was plated on BCYE-GVPC medium (Oxoid Ltd, Basingstoke, UK).

Identifying and quantifying *Legionella*-like organisms on BCYE-GVPC medium

After incubation at 37°C for up to 10 days in 5% CO₂, glassy-looking colonies resembling *Legionella* were selected and sub-cultured onto Horse Blood Agar (FC Bio Sdn. Bhd., Malaysia) and BCYE-GVPC media. Colonies growing on BCYE-GVPC agar but not on HBA were presumptively identified as *Legionella*-like organisms. The number of suspected *Legionella* colonies on each BCYE-GVPC agar was counted and CFU ml⁻¹ were calculated by multiplying the total number of colonies by the dilution factor and dividing by the volume of inoculum.

Characterization of *Legionella*-like organisms using latex slide agglutination test

A few colonies from each positive water sample were used for latex slide agglutination with test kits (Dryspot *Legionella* Latex test; Oxoid Ltd, Basingstoke, UK) for serogroup (SG) 1, SG 2–14 and *Legionella* species. The *Legionella* species kit contains rabbit antisera against *L. anisa*, *L. longbeachae*, *L. gormanii*, *L. jordanis*, *L. dumoffii*, *L. micdadei* and *L. bozemanii*. *Legionella*-like organisms that were latex agglutination negative were subsequently analysed by 16S rRNA and *rpoB*

Table 1 | *Legionella* isolates identified by latex slide agglutination, 16S rRNA and *rpoB* PCR-DNA sequencing

Source of <i>Legionella</i>	Latex test	16S rRNA sequencing	<i>rpoB</i> sequencing
<i>South peninsular Malaysia</i>			
Tower 1 (Polyclinic)	Colony a	SG 1	<i>L. pneumophila</i>
	Colony b	–	<i>L. pneumophila</i>
Tower 2 (Healthcare centre)	Colony a	SG 1	NP
	Colony b	SG 2–14	<i>L. pneumophila</i>
	Colony c	SG 2–14	<i>L. pneumophila</i>
Tower 3 (Office Building)	Colony a	SG 1	NP
	Colony b	SG 1	<i>L. pneumophila</i>
Tower 4 (Healthcare centre)		SG 2–14	<i>L. pneumophila</i>
Tower 5 (Office Building)	Colony a	SG 1	NP
	Colony b	SG 2–14	<i>L. pneumophila</i>
	Colony c	SG 1	<i>L. pneumophila</i>
<i>East Malaysia</i>			
Tower 6 (Healthcare centre)	Colony a	SG 2–14	<i>L. pneumophila</i>
	Colony b	Species	<i>L. pneumophila</i>
	Colony c	Species	<i>L. anisa</i>
<i>West peninsular Malaysia</i>			
Tower 7 (Healthcare centre) 100–1,000 CFU ml ⁻¹		SG 2–14	<i>L. pneumophila</i>
Tower 8 (Factory) 100 CFU ml ⁻¹		SG 1	<i>L. pneumophila</i>
Tower 9 (Office tower) 200–2,000 CFU ml ⁻¹	Sample a	–	<i>L. busanensis</i>
	Sample b colony a	SG 2–14	<i>L. pneumophila</i>
	Sample b colony b	SG 2–14	<i>L. pneumophila</i>
Tower 10 (Office tower) 200–2,000 CFU ml ⁻¹	Sample a	SG 2–14	<i>L. pneumophila</i>
	Sample b	Species	<i>L. dumoffi</i> or <i>L. longbeachae</i> or <i>L. gormanii</i>
	Sample c	SG 2–14	<i>L. pneumophila</i>
	Sample d	Species	<i>L. pneumophila</i>
Tower 11 (Healthcare centre) 180 CFU ml ⁻¹	Sample a	SG 1	<i>L. pneumophila</i>
	Sample b	–	<i>L. gresilensis</i>
	Sample c	SG 1	<i>L. pneumophila</i>
	Sample d	SG 2–14	<i>L. pneumophila</i>
	Sample e	SG 1	<i>L. pneumophila</i>

SG, serogroup; –, no agglutination with any antiserum used; NP, not performed.

PCR-sequencing. All except three of the latex agglutination positive *Legionella* strains and all reference strains were similarly examined by PCR-sequencing.

Characterization of *Legionella*-like organisms by 16S rRNA and *rpo B* genes PCR-DNA sequencing

Genomic DNA extraction

Chromosomal DNA was extracted from isolates using DNAzol[®] Genomic DNA Isolation Reagent (Molecular Research Centre, Inc.) according to the manufacturer's instructions.

PCR primers and reactions

The 16S rRNA PCR primers used were previously described by Miyamoto *et al.* (1997). PCR was performed using 20 ng of template DNA, 10 μ M of each primer, 1 \times reaction buffer, 2.2 mM MgCl₂, 0.4 μ M deoxyribonucleoside triphosphate mix and 1 U of *Taq* polymerase per 25 μ l reaction. The thermocycling conditions used were: 95°C for 4 minutes, 35 cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute and 72°C for 5 minutes. The PCR reaction mixture and thermocycling parameters to amplify *rpo B* genes were as described by Ko *et al.* (2002).

DNA sequencing

PCR amplicons for partial 16S rRNA (600 bp) and *rpo B* (360 bp) genes were purified using GenElute PCR clean-up kit (Sigma-Aldrich) prior to DNA sequencing. All DNA sequencing reactions were performed by using dye primer and dye terminator chemistries in LI-COR[®] NEN 4200 Global IR2 DNA Sequencing System and ABI[®] PRISM DNA Sequencers.

Phylogenetic analyses

Multiple sequences of all isolates and reference strains of *Legionella* were aligned using Clustalw2 (Larkin *et al.* 2007) prior to plotting phylogenetic trees to reveal the DNA relatedness among them. Phylogenetic trees were constructed by the neighbour-joining method (NJ) in MEGA version 4 (Tamura *et al.* 2007). *Coxiella burnetii*, the

aetiological agent of Q fever, was employed as the outgroup in both trees. The clusters in both trees were defined as groups of isolates sharing more than 97% homology in DNA sequences. Branch bootstrap values were evaluated with 1,000 replications.

RESULTS

Legionella counts

The viable counts of *Legionella*-like organisms ranged from 100 to 2,000 CFU ml⁻¹ water in the cooling towers screened. Repeat samples were taken from four cooling towers. In the absence of water treatment, repeat samples from the same cooling tower gave CFU counts that were mostly within 1 log different (data not shown).

Latex slide agglutination

All reference strains were correctly identified by the latex agglutination test as either *L. pneumophila* or non-*pneumophila* species. As shown in Table 1, the isolates were identified as *L. pneumophila* serogroup 1 in ten water samples, *L. pneumophila* serogroups 2–14 in eleven and *Legionella* species in three. *Legionella*-like isolates from three samples were not serogroupable. Serogroup 1 comprised 35.7% of all isolates. In some instances, different serogroups were identified among different colonies taken from the same sample of water and among different samples from the same cooling tower.

16S rRNA and *rpo B* DNA sequencing of isolated *Legionella*-like organisms

All reference strains of *L. pneumophila* and non-*pneumophila* *Legionella* were correctly identified by both 16S rRNA and *rpo B* PCR-DNA sequencing (results not shown). Similarly, both PCR-DNA sequencing examinations gave concordant results for 22 isolates identified as *L. pneumophila* and one *L. anisa* (Table 1). Two *Legionella* species, *L. busanensis* and *L. gresilensis*, were identified by the 16S rRNA DNA sequencing but were not amplified using the *rpo B* primers. Of the four isolates that agglutinated with *Legionella* species reagent in the latex

agglutination test, surprisingly, two turned out to be *L. pneumophila* by both PCR-DNA sequencing tests. The remaining two were identified to be *L. gormanii* by *rpo B* PCR-DNA sequencing and *L. anisa* by both 16S rRNA and *rpo B* PCR-DNA sequencing (Table 1). Mixed populations of *L. pneumophila* and other *Legionella* species were found in six of the cooling towers.

Phylogenetic relationship of *Legionella* isolates based on 16S rRNA and *rpo B* DNA sequences

The 16S rRNA phylogenetic tree established three clusters of *Legionella* species (Figure 1). Cluster A consisted of only *L. pneumophila*, with intra-species homology of 99.4–100%, supported by a robust bootstrap value of 98%. Cluster B consisted of *L. anisa*, *L. longbeachae* and *L. gormanii*, sharing 99.1–99.3% homologous sequences with a low bootstrap value of 66–58%. Cluster A and B share 98.9% of homology in sequence. Cluster C consisted of *L. micdadei*, *L. busanensis* and *L. gresilensis*, sharing sequence homology of 97.1–98.9% with a relatively high bootstrap value of 84%. Cluster A and C share 97.2% of homologous sequences.

The *rpo B* phylogenetic tree displayed four distinctive clusters of *Legionella* species (Figure 2). Cluster A consisted of *L. pneumophila* species with intra-species homology of 97.2–100%. Cluster B consisted of only one species, *L. gormanii*, which shared 85% sequence homology with cluster A. The species *L. anisa* and *L. longbeachae* formed clusters C and D, respectively. Clusters C and D share 81.1% and 82% homologous sequences with cluster A, respectively. All strains of *Legionella* within clusters B, C and D showed 100% intra-group homology and all three clusters were supported by 100% bootstrap values.

DISCUSSION

Legionella pneumophila has been classified as a re-emerging pathogen that has been causing increasing numbers of human infections in recent years (Neil & Berkelman 2008). In Malaysia, seroprevalence studies have indicated substantial population exposure to this organism and sporadic infections have been reported (Ngeow &

Asia-CAP study group 2005; Normaznah *et al.* 2005). In this study, the isolation of *L. pneumophila* from all 11 cooling towers examined reconfirmed the widespread presence of this organism in the environment. The overall 75% predominance among isolates is also similar to that reported in a 1992 study on 30 cooling towers in the capital city of Kuala Lumpur, in which 9 out of 12 *Legionella* isolates were identified as *L. pneumophila* (Ngeow *et al.* 1992).

The frequent occurrence of *L. pneumophila* in water cooling towers may be related to its enhanced resistance to chlorine disinfection or its ability to persist for long periods of time in the environment despite susceptibility to chlorine (Kuchta *et al.* 1985; García *et al.* 2008). The latter has been attributed to the bacterium's existence inside amoebae and within biofilms, both of which provide protection from chlorination (Bichai *et al.* 2008). Older cooling towers, especially those with corrosion and sedimentation of sand and minerals are more prone to biofilm formation and *Legionella* proliferation. In Malaysia, the warm humid weather could be another factor favouring the long-term survival of the organism in the environment.

Latex slide agglutination tests are commonly used in the diagnostic laboratory because they are simple and rapid. However, the sensitivity and specificity of the agglutination test depend on the surface antigens of bacteria and the antisera used. In this study, the Dryspot *Legionella* Latex test was negative in one *L. pneumophila* isolate and wrongly diagnosed two *L. pneumophila* isolates as non-*pneumophila* species, thus giving a sensitivity of only 85.7% (18 out of 21) for the identification of *L. pneumophila*, when compared with PCR-DNA sequencing. There is a possibility that the isolate that was agglutination negative belonged to serogroup 15 or a hitherto unknown serogroup, but the two isolates that agglutinated with the *Legionella* species reagent were probably examples of cross-reactivity caused by inadequate adsorption of polyclonal antisera. For the four non-*pneumophila* species, the Dryspot *Legionella* latex test showed positive agglutination with *L. anisa* and *L. gormanii* but not with *L. busanensis* and *L. gresilensis* because the latter two species were not represented in the *Legionella* species reagent in the kit. This limitation illustrates the importance of conducting *Legionella* studies locally so that the usefulness of commercially available

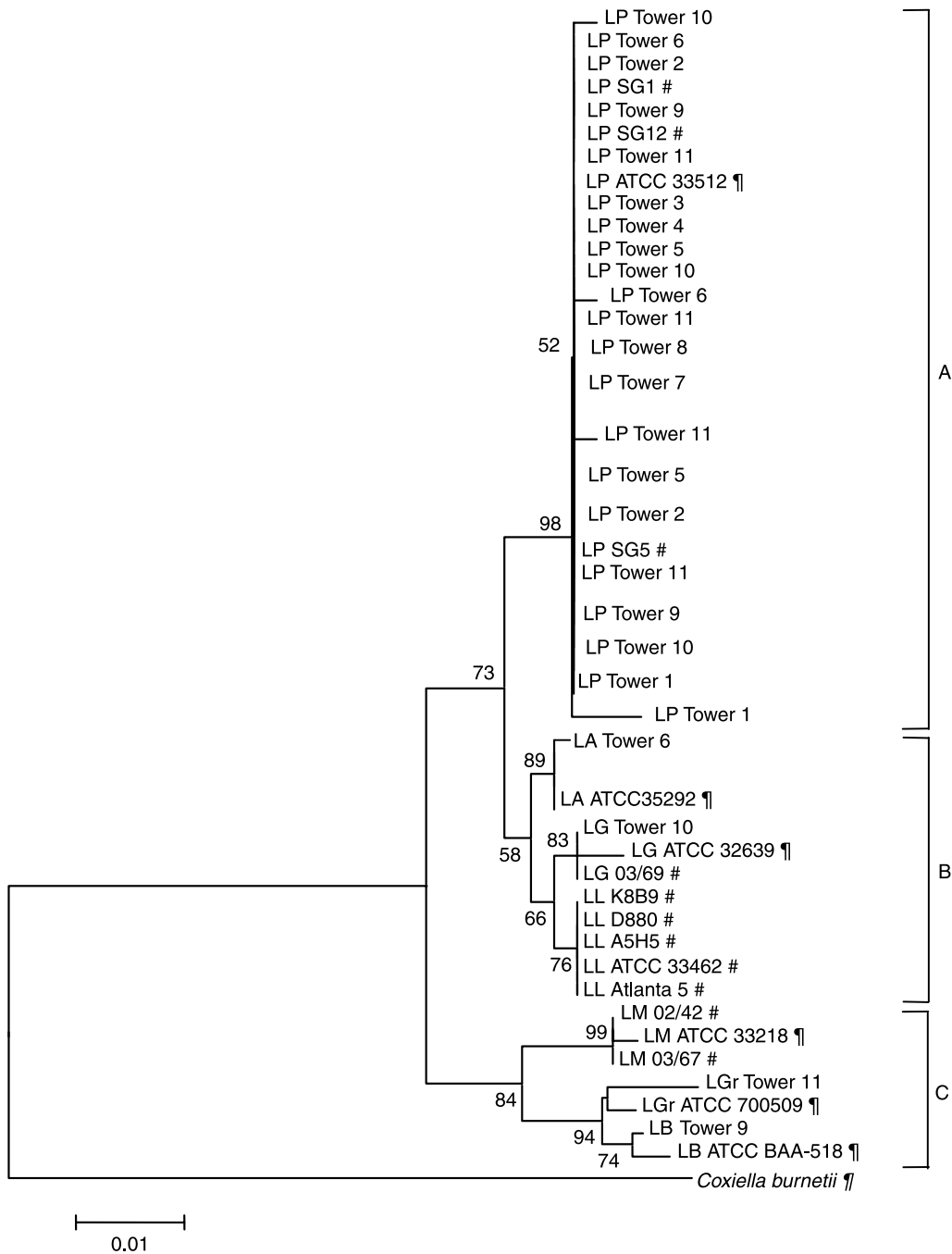


Figure 1 | Phylogenetic tree of *Legionella* species from partial 16S rRNA gene sequence generated by the NJ method in MEGA version 4. *Coxiella burnetii* was used as the outgroup. Bootstrap values presented at corresponding branches were evaluated from 1,000 replications. Values below 50% are not indicated. Species of *Legionella* are abbreviated (LP, *L. pneumophila*; LA, *L. anisa*; LG, *L. gormanii*; LL, *L. longbeachae*; LB, *L. busanensis*; LGr, *L. gresilensis*). Designations of strains are shown after species abbreviation. 16S rRNA gene sequences for bacteria with ¶ were obtained from GenBank and reference strains are indicated by symbol #. Scale bar represents 1 substitution per 100 nucleotides.

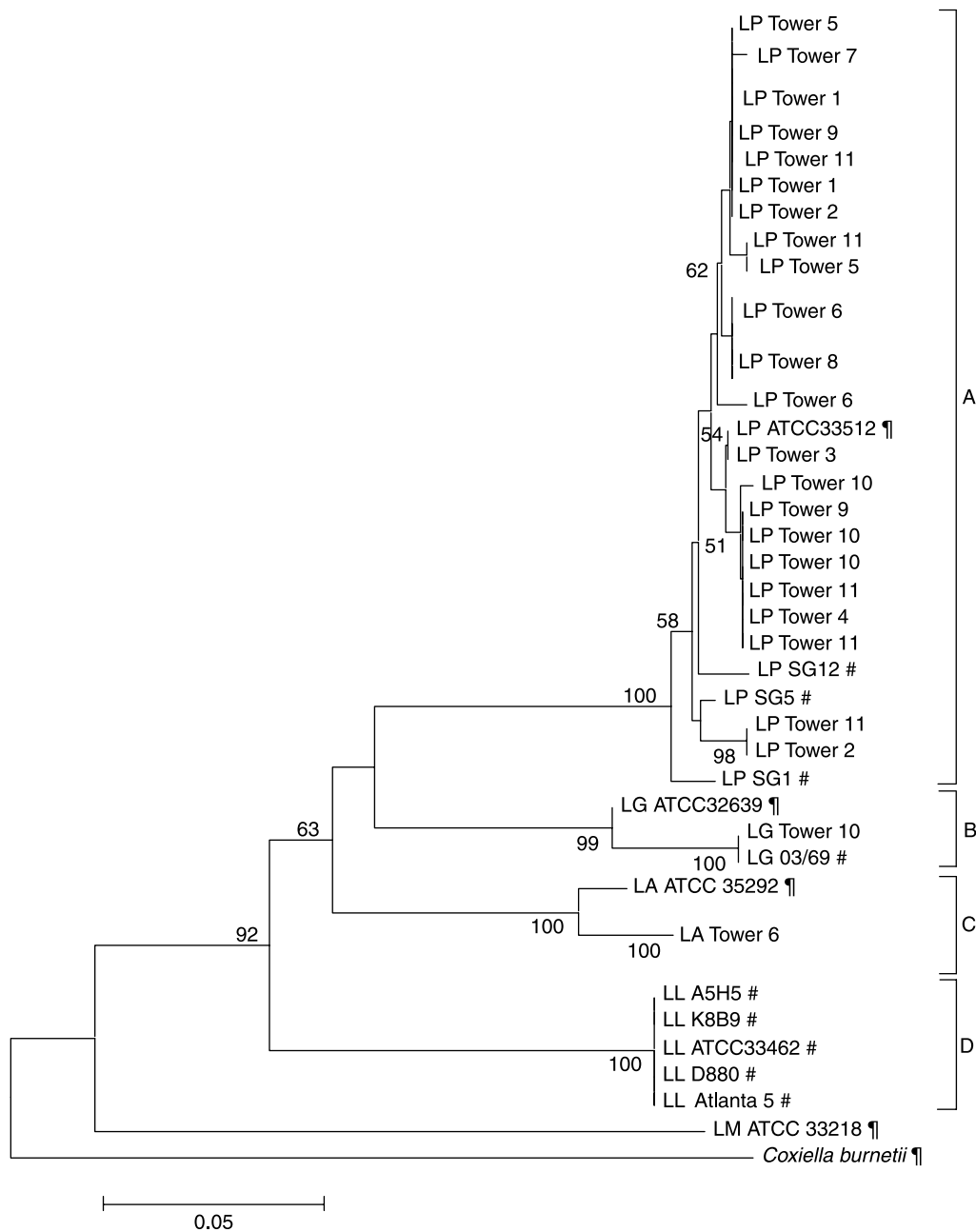


Figure 2 | Phylogenetic tree of *Legionella* species from partial *rpoB* gene sequence generated by the NJ method in MEGA version 4. *Coxiella burnetii* was used as the outgroup. Bootstrap values presented at corresponding branches were evaluated from 1,000 replications. Values below 50% are not indicated. Species of *Legionella* are abbreviated (LP, *L. pneumophila*; LA, *L. anisa*; LG, *L. gormanii*; LL, *L. longbeachae*). Designations of strains are shown after species abbreviation. *RpoB* gene sequences for bacteria with # were obtained from GenBank and reference strains are indicated by the symbol #. Scale bar represents 5 substitutions per 100 nucleotides.

Legionella identification kits can be assessed for their ability to identify locally prevalent strains.

The partial 16S rRNA and *rpoB* DNA sequencing results concurred in all *L. pneumophila* isolates but differed in three of the four *Legionella* species.

L. busanensis and *L. gresilensis* were identified by the 16S rRNA sequencing but not by *rpoB* gene sequencing. On the other hand, *rpoB* sequence analysis identified *L. gormanii* which was not identified by the partial 16S rRNA gene sequence analysis. Both DNA analyses can be

used to complement latex slide agglutination to give a more complete characterization of *Legionella* isolates. An added advantage is the potential detection of novel species from DNA sequence alignment.

The phylogenetic trees of 16S rRNA and *rpoB* genes were constructed to determine the DNA relatedness of the *Legionella* isolates from different locations. Both trees displayed high intra-species homology for *L. pneumophila* (99.4–100% with 16S rRNA and 97.2–100% with *rpoB*) but, with the exception of three *L. pneumophila* isolates in Tower 10 (Figure 2), there was no distinct clustering by location. This lack of clustering could be the result of the introduction of different strains at different times from different sources or the divergence in genetic evolution over a long period of time in the same location. There was clear separation of *L. pneumophila* from the other species (Figures 1 and 2). Although there were very few non-pneumophila *Legionella* isolates in this study, they clustered with their corresponding reference strains as shown by *L. gormanii* (Tower 10) and *L. gormanii* (03/69) from Australia as well as *L. gresilensis* (Tower 11) and *L. gresilensis* ATCC 700509 (Figure 1). The 16S rRNA phylogenetic tree revealed a closer relationship among the *Legionella* species (97.2–98.9%) compared with the *rpoB* gene phylogenetic tree (81.1–85%). This might have been the reason why *L. busanensis* and *L. gresilensis* from towers 9 and 11 were amplified and identified by the 16S rRNA PCR but not by the *rpoB* PCR.

In summary, although there have been no documented outbreaks of *Legionella* infection in Malaysia, the high rate of contamination in water cooling towers is a cause for concern. Routine screening for *Legionella* in water cooling towers is not yet mandatory in the country but is encouraged, as it will not only help to prevent outbreaks by monitoring the level of contamination and prompting corrective actions when levels are high, but will also help to confirm or refute the effectiveness of maintenance measures in place.

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