

DETECTION OF *LEGIONELLA PNEUMOPHILA* IN WATER AND BIOFILM SAMPLES BY CULTURE AND MOLECULAR METHODS FROM MAN-MADE SYSTEMS IN SÃO PAULO - BRAZIL

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Submitted: January 09, 2007; Returned to authors for corrections: March 30, 2007; Approved: September 20, 2007.

ABSTRACT

Legionella pneumophila is a pathogenic bacteria associated to aquatic habitat of natural and artificial environments. Clinical cases of legionellosis have been reported in Brazil but there is a lack of information about the incidence and concentration of this bacterium in environmental sources. Thus, the present study was designed to evaluate the occurrence of legionellae in São Paulo city, Brazil, using different methods of detection and identification. Sixty-seven water and biofilm samples from natural reservoirs and man-made systems were collected and analyzed for the presence of *Legionella* spp by culturing onto a selective medium, coculture in axenic free-living amoebae and direct fluorescent antibody (DFA) assay. Results showed that freshwater of reservoirs did not contain legionellae, *Legionella pneumophila* was isolated from man-made systems, with predominance of *Legionella pneumophila* serogroup 1 strains. Although there was no statistical difference among the proposed detection methods, the plate culture method yielded a higher number of *L. pneumophila* positive samples, followed by amoebic coculture procedure and direct fluorescent antibody assay. Results of PCR and sequencing reactions revealed that application of macrophage infectivity potentiator gene as a molecular marker was an important tool for the identification of environmental isolates of *L. pneumophila*. The agreement among the three detection methods-when all methods yielded similar results- and the prevalence of a single *Legionella* species in the sampled man-made systems could suggest that the occurrence of this bacterium had been influenced by the higher concentration of metallic ions dissociated in water of those systems than in natural reservoirs. Thus, the results of this study revealed that the water of man-made systems in Sao Paulo may serve as a reservoir for *L. pneumophila* and other microorganism, including free-living protozoans.

Key words: *Legionella pneumophila*, free-living amoebae, man-made systems

INTRODUCTION

The genus *Legionella* comprises 70 distinct serogroups from 48 species (16) and new species are frequently described (31,38,39). *Legionella pneumophila* is the etiological agent of the majority of cases of legionellosis, and the majority of Legionnaires' disease cases have been attributed to *L. pneumophila* serogroup 1 (45). In addition, at least 20 other species of *Legionella* have been associated with human infections (16). *Legionella* bacteria do exist as free-living planktonic forms in the environment (36), intracellular parasites

of protozoans (3), and/ or inhabitants of mixed-community biofilms (29). Thus, the diversity of types and the ubiquitous occurrence of legionellae in water environments or moist soil make it difficult to identify epidemic strains, and outbreaks of legionellosis have been associated mainly with contamination of man-made aquatic environments (2,16).

The metropolitan region of Sao Paulo State is constituted by Sao Paulo city and several other smaller cities around it. These cities are supplied by different water reservoirs where the presence of *Legionella* species is unknown, although the isolation of *Legionella* from man-made systems in São Paulo

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has been described (35,41). The isolation of *L. pneumophila* in Brazil was first described by Pereira Gomes *et al.* (42). Levin *et al.* (35) reported the isolation of *L. anisa* from shower water and *L. pneumophila* sg 1 from condensed water of the air conditioning system in a hospital building located downtown Sao Paulo city. Pellizari and Martins (41) demonstrated the presence of *Legionella* spp in different environmental samples from Sao Paulo city. In that study, the presence of *Legionella pneumophila* was detected in the boiler of a hospital. Moreover, Chedid *et al.* (9) described the occurrence of community-acquired pneumonia in three patients hospitalized in a southern Brazilian general university hospital. In this case *L. pneumophila* was detected by seroconversion and positive urinary antigen tests in two patients while the third patient presented negative serology but strongly positive urinary test.

The classical method for detection of *Legionella* species involves direct culture of bacteria on artificial laboratory media (18). Isolation of *Legionella* spp is based on streaking of clinical and environmental samples onto a buffered charcoal yeast extract agar base enriched with α -ketoglutarate (BCYE- α), supplemented with ferric iron and L-cysteine (13), while identification of the strains is mostly done by serological methods (23). Moreover, amoebic coculture procedure has been proposed in order to promote the intracellular multiplication of legionellae *in vitro*, and the application of natural interaction between amoebae and legionellae have been successful when conventional plate culture method has failed (15,46). Recently, the polymerase chain reaction (PCR) and nucleic acid probe detection methods have been introduced, and several molecular techniques have been either developed or adapted from earlier methods for environmental monitoring (33). Genetic markers have been proposed to identify *Legionella* strains to the species level, including 16S ribosomal RNA, macrophage infectivity potentiator (*mip*), defect organelle trafficking (*dotA*), RNA polymerase β -subunit (*rpoB*) genes or 23S-5S ribosomal intergenic spacer region (23S-5S ISR) (20,21,27,28,43). Other techniques have been proposed as alternatives to PCR and growth procedures to identify *Legionella* species, including direct fluorescent antibody (DFA) staining and latex agglutination (5). The present study aimed to compare the results obtained using the standard culture procedure, the direct fluorescent antibody (DFA) assay and the amoebic coculture procedure, in order to establish which one is the best method for the isolation and identification of *Legionella* spp from environmental samples. The *mip* gene was applied as a genetic marker to confirm the presence of potentially virulent *Legionella* strains.

MATERIALS AND METHODS

Sixty-seven water and biofilm samples were tested by direct fluorescent antibody assay (DFA), plate culture on a selective medium and amoebic coculture procedure. Environmental

isolates obtained by culture and amoebic coculture procedures were compared by PCR amplification and sequencing of the *mip* gene.

Sample collection and concentration

Environmental samples (water and biofilm) were taken from the water sources indicated in Table 1, between March 2003 and January 2005. Each superficial water sample, which consisted of 1 liter portion, was collected in sterile polypropylene containers (Nalgene Co., Rochester, NY, USA) containing 0.1 M sodium thiosulfate to neutralize residual free chlorine in the samples. Water and biofilm samples from the dental units were collected from dental water-lines supplying water to air-water syringes, high-speed handpieces and scalers. All samples were transported to the laboratory and kept at 4 to 8°C until analysis. Water samples were concentrated by filtration through 0.45 μ m pore size polycarbonate membranes (type HTTP, Millipore, Ireland). The intact membranes were aseptically removed, placed into sterile 50 mL screw-capped tubes (Corning Inc., Corning, NY, USA) and re-suspended in 10 mL of the original water samples. Each concentrated water sample was sonicated for five min to dislodge bacterial cells from the membranes using a model T7 ultrasonic bath (Thorton, São Paulo, Brazil). The cell suspension was placed in a 50°C water bath during 30 min. The heat treatment of concentrated water samples was used as a selective method to reduce the numbers of non-*Legionella* bacteria as previously described (5). These represented the prepared samples. Negative controls were done in parallel with non-contaminated environmental water and biofilm samples. All experimental samples were processed in duplicates.

Immunofluorescence techniques

The prepared samples were stained by direct fluorescent antibody (DFA) according to the manufacturer's instructions: 10 μ L of each prepared samples were transferred to a twelve-well glass slide (Perfecta, São Paulo, Brazil), air-dried and heat fixed. To each well a 10 μ L portion of one of two fluorescein isothiocyanate-labeled *Legionella* antibodies conjugates (m-Tech Alpharetta, Ga., USA) was added. The first polyclonal antibody was directed against *L. pneumophila* serogroups 1 through 14. The second polyclonal antibody was species-specific and used to identify *L. bozemanii* serogroups 1 and 2, *L. dumoffii*, *L. gormanii*, *L. micdadei*, *L. longbeachae* serogroups 1 and 2, *L. jordanis*, *L. oakridgensis*, *L. wadsworthii*, *L. feeleii* serogroups 1 and 2, *L. sainthelensi*, *L. anisa*, *L. steigerwaltii*, *L. parisiensis*, *L. spiritensis*, *L. hackeliae* serogroups 1 and 2, *L. macceachernii*, *L. jamestowniensis*, *L. cherrii*, *L. rubrilucens*, and *L. erythra*. The slides were examined using a Carl Zeiss fluorescence microscope under a dark field with 1000X objective lenses. Only strongly fluorescent, typical rod-shaped organisms were reported as DFA positive.

Plate culture method and presumptive tests

Aliquots of 100 μL of prepared samples were spread on duplicate plates of αBCYE selective medium Agar (Difco Laboratories, Detroit, Mich., USA) added of L-cysteine (0.44 mg mL^{-1}), ferric pyrophosphate (0.250 mg mL^{-1}), glycine (3.0 g L^{-1}), vancomycin (0.0025 mg mL^{-1}) and polymyxin B (0.006 mg mL^{-1}), named αBCYE -GVP selective agar medium (50). Plates were incubated at 37°C in a humidified atmosphere without CO_2 during 5 days. Colonies with the typical “ground glass” appearance of *Legionella* were subcultured on two non-selective media, sheep-blood agar and αBCYE agar without L-cysteine. Colonies that grew on αBCYE -GVP but not on non-selective media were considered putative *Legionella* strains, and were Gram stained and subcultured on a selective medium. The identification of putative *Legionella* strains as *Legionella pneumophila* or non-*Legionella pneumophila* was carried out using *Legionella* specific latex reagents (Oxoid, Hampshire, England) and direct immunofluorescence assay with polyclonal rabbit sera (m-Tech Alpharetta, Ga., USA).

Amoebic coculture procedure

Acanthamoeba castellanii (strain ATCC 30011, Manassas, VA, USA) was grown in a 25 cm^2 cell culture flask (Costar, Cambridge, Mass., USA) containing Neff's amoebae medium (120.0 mg NaCl, 3.0 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 3.0 mg CaCl_2 , 3.0 mg FeSO_4 , 142.0 mg Na_2HPO_4 , 136.0 mg KH_2PO_4 , 10.0 g Proteose Peptone, 18.0 g glucose, 1.0 L distilled water, pH 6.8). Amoebae were collected for coculture procedure by vigorous agitation of the media when cells had formed a confluent layer on the bottom of the flask (1.0×10^6 cells mL^{-1}). Amoebae were harvested and pelleted by centrifugation at 1,000 \times g for 10 min. The supernatant was removed, and the amoebae were resuspended in 20 mL of PAS buffer (Page's amoebic saline) (120 mg NaCl, 4 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 142 mg Na_2HPO_4 , 136 mg KH_2PO_4 , 1.0 L distilled water). The washing procedure was repeated and the amoebae were resuspended in 10 mL of PAS, at a final concentration of 2.0×10^5 cells mL^{-1} . Aliquots of 1 mL of this suspension were transferred to 24-well cell culture microplates (Costar, Cambridge, Mass., USA) and allowed to adhere to the wells for 1 h at 37°C. Aliquots of 500 μL of each prepared sample were added to axenic culture of *A. castellanii* in duplicate. *L. pneumophila* strain ATCC 33155 (Manassas, VA, USA) was used as a positive control at a concentration of approximately 8.0×10^7 CFU mL^{-1} . The cell suspension (100 μL) was added to each well containing amoebae in one mL of PAS to give a ratio of 40 legionellae per amoebae. After 48 h at 28°C, the samples were aspirated and wells were washed once with sterile phosphate buffered saline (PBS) solution, pH 7.0, containing 100 $\mu\text{g mL}^{-1}$ of gentamicin (Sigma, St. Louis, Mo., USA). The mixture was incubated for 2 h at 37°C to kill all extracellular legionellae. The wells were washed with 1 mL of fresh PBS without gentamicin. After a second period of incubation for 2 h

at 37°C, 100 μL of the mixture in each well were spread onto BCYE-GVP, incubated at 37°C for five days, and viable intracellular legionellae were determined by colony forming units (CFU) counting.

Isolation of free-living amoebae from water samples

In order to recover free-living amoebae from the aquatic environmental samples, a portion of water (500 mL) from each site was filtered through a 1.2 μm sterile cellulose ester membrane (Millipore, Ireland). For isolation of amoebae, the membranes were aseptically transferred to soy infusion agar plates, named Foronda's medium (overnight infusion of 2.0 g soy powder in 1.0 L of sterile distilled water, paper filtered, and then added of 1.5 g of agar). The plates were incubated at 25°C and examined for the presence and growth of free-living amoebae under an inverted microscope (200X magnification) daily for 7-14 days post-inoculation. Identification of free-living amoebae was based on characteristics described by Page (37). The size, shape, and number of pseudopodia produced and the character of the movement were noted using a hanging drop preparation.

Isolation of *Legionella* spp from environmental biofilms

Biofilms were collected from the surface of oral rinsing cups from dental units, water tank reservoir to cooling tower, evaporative condensers and cold-water tank reservoir, using sterile swabs. These swabs were transported in 5 ml water from the sampling site and inoculated on αBCYE agar with cysteine and ferric iron. The water used for biofilm transport was centrifuged for 10 min at 10,000 \times g. The pellets were resuspended in 1 ml of sterile distilled water, and an aliquot of 100 μL was spread onto αBCYE -GVP selective agar. For the amoebic coculture procedure, another aliquot of the pellet suspension (200 mL) was spread onto axenic *A. castellanii* microplates followed by plating onto a selective medium for counting of intracellular bacteria.

Statistical analysis

The χ^2 (chi-square) test was used to evaluate the positivity rate of isolation and identification of the *Legionella* spp in environmental samples by the proposed methods. The significance of the results was determined by analysis of variance. An overall significance level of 5% was adopted. All *in vitro* experiments were carried out in duplicate.

mip gene amplification and sequencing

Legionella spp strains were submitted to DNA extraction using GFX Genomic Blood DNA Purification kit (Amersham Biosciences, New Jersey, USA), according to the manufacturer's instructions. The genomic DNA was amplified by PCR using *mip* gene (686-bp nucleotides) primers Legmip-f (27-mer), 5'-GGG(AG)ATT(ACG)TTTATGAAGATGA(AG)A(CT)TGG-3'

and Legmip-r (23-mer), 5'-TC(AG)TT(ATCG)GG(ATG)CC(ATG)AT(ATCG)GG(ATCG)CC(ATG)CC-3' as previously described (43). Each amplification reaction was carried out in total volume of 25 μ L with the following mixture of components: 1 μ L of DNA template (50.0 ng μ L⁻¹), 1x reaction buffer, MgCl₂ (1.5 mmol L⁻¹), Legmip forward and reverse primers (1.0 μ mol L⁻¹ of each primer), deoxynucleosides triphosphate (200 μ mol L⁻¹), and *Platinum Taq DNA polymerase* (1.25 units) (Invitrogen Life Technologies, California, USA). Amplification cycles were preceded by denaturation of the samples for 3 minutes at 94°C, followed by 35 cycles of 60 s at 94°C, 2 min at 55°C, 2 min at 72°C and 3 min to final extension at 72°C. The amplicons were checked by gel electrophoresis on 1.5% agarose gels, using 100 bp DNA Ladder (Invitrogen Life Technologies, California, USA) as a molecular marker. The amplicons to be sequenced were purified with the Wizard[®] PCR Prep DNA Purification System (Promega Corporation, Madison, USA) according to the manufacturer's instructions. Both strands of each amplicon were sequenced with the same primer sets used in the PCR. Sequences were determined with DYEnamic ET Dye Terminator Cycle Sequencing Kit and Thermo Sequenase[™] II DNA Polymerase (Amersham Biosciences, Buckinghamshire, UK), according to the MegaBACE 1000 protocol (Amersham Biosciences, Piscataway, NJ). Lane tracking and base calling was done with Cimarron 3.12 software (Amersham Biosciences, Piscataway, NJ).

Phylogenetic analyses

Nucleotide sequences obtained with the forward and reverse primers were combined and aligned manually using BioEdit Sequence Alignment Editor version 5.0.9 (22). The consensus sequences were compared with *mip* gene sequences provided by GenBank database using the BLAST software (1) at the National Center for Biotechnology Information (NCBI). Multiple alignments were accomplished with nucleotide and amino acid sequences using the multiple alignments Clustal W software (49). Phylogenetic and molecular evolutionary analyses were conducted using MEGA3 (Molecular Evolutionary Genetic Analysis software version 3.1) (30). Phylogenetic tree of *Legionella* isolates were constructed by the neighbor-joining (NJ) method without gaps and pairwise distances were calculated using the maximum likelihood option. The Modeltest version 3.06 programs (43) was used to find the best model of evolution that fit the data, and the Tamura-Nei model of evolution (48) was used for NJ analyses. Bootstrap analysis was used to estimate the reliability of nodes. Multiple data sets were used ($n = 1,000$), and a number of dendrograms were compared.

Nucleotide sequence numbers

All *mip* gene sequences obtained from environmental strains were deposited in GenBank under accession numbers AY849371, and AY974251 to AY974258. The accession numbers in the

GenBank of NCBI for the sequences used for comparison in this study are as follows: *L. gormanii* (U91638) and *L. pneumophila* (AF022334, S42595, AJ496265, AF095220, AF095219, AF095225, AE017354, AF095215, AF095223, AJ496271, AF022326).

RESULTS

Table 1 shows the detection of *L. pneumophila* according to the source of the samples (natural reservoirs and man-made systems) and the detection method. Results showed that *Legionellae* were detected in all environments in the metropolitan region of Sao Paulo city, Brazil. The results for the presence of *L. pneumophila* and non-*pneumophila* species according to the detection method are detailed below.

Direct fluorescent antibody (DFA) staining

L. pneumophila was detected in seven water samples coming from four different sources: cooling tower (one sample), water tank reservoir to cooling tower (only in the water sample), boiler (one sample) and shower heads (four samples). All of them were also isolated by culturing onto a selective medium α BCYE-GVP carried out in parallel.

Direct culture on selective medium

The direct plate culture procedure of water samples using the α BCYE-GVP agar selective medium showed the best results in the detection of legionellae. Nine strains isolated from the water samples were identified as *L. pneumophila* by DFA staining and slide agglutination test. The overgrowth of faster-growing bacteria and fungi was minimized with the heat treatment of samples, improving the isolation of suspect colonies to distinguish legionellae from non-legionellae. The nine *Legionella* strains were isolated from four sources as follows: cooling towers (one isolate: strain BR-09), water tank reservoir to cooling tower (two isolates, being one isolate from water sample: strain BR-07, and one from biofilm sample: strain BR-08), boiler (one isolate: strain BR-10) and shower heads (five isolates: strains BR-02, BR-03, BR-04 and BR-05).

Amoebic coculture procedure

Amoebic coculture with *Acanthamoeba castellanii* (strain ATCC 30011) was applied to improve the isolation of *Legionella* spp. However, eight strains were isolated when amoebic coculture procedure was applied as a first step before plate culture, in contrast to nine strains isolated by direct culturing (Table 1). However, all strains obtained by direct inoculation onto selective medium showed a lower amount of viable cells in comparison to amoebic coculture procedure (data not shown). In addition, free-living amoebae, i.e. *Acanthamoeba* sp, *Hartmannella vermiformis* and *Vahlkampfia* sp, were isolated in all screened samples.

Table 1. *Legionella pneumophila* detection according to the source of the sample and detection method.

Source of samples	Number of samples collected	Number of samples positive to <i>L. pneumophila</i>	Number of positive samples according to the detection method***		
			Direct Fluorescent Antibody	Plate culture on selective medium	Amoebic coculture procedure
Natural water reservoir	4	0	0	0	0
Dental units*	10	0	0	0	0
Cooling towers	13	1	1	1	1
Water tank reservoiry to cooling tower*	2	2	1**	2	2
Boiler	1	1	1	1	1
Evaporative condensers*	4	0	0	0	0
Cold water tank reservoir*	2	0	0	0	0
Shower heads	31	5	4	5	4
Total of samples	67	9	7	9	8

*Water and biofilm samples were collected; ** Positive only in water sample; *** χ^2 (chi-square) test indicated that the statistical difference among detection methods was not significant ($P > 0.05$).

Molecular identification of *Legionella* environmental isolates

PCR and sequence analysis applied to the *Legionella* isolates resulted in amplicon sequences containing approximately 686 base pairs. Sequenced length of the *mip* gene ranged between 661 bp and 715 bp. Overall *mip* gene sequence similarity values of some *L. pneumophila* strains retrieved from GenBank database and all the *L. pneumophila* isolated from environmental samples ranged between 98.6 and 100%. Phylogenetic tree inferred from the *mip* gene sequences indicated that the nine isolates were grouped into three distinct clusters (Fig. 1). *Legionella* strains BR-05 to BR-10 showed 100% of similarity with *L. pneumophila* serogroup 1, while cluster 2 showed an association between *Legionella* strain BR-03 (isolated from a water sample of industrial shower) and *L. pneumophila* serogroup 8 (strain CDC F920). Finally, cluster 3 was constructed by two others *L. pneumophila* isolated from industrial shower (strains BR-02 and BR-04) and *L. pneumophila* serogroups 6, 8 and 12.

Statistical analysis

When χ^2 values were calculated, the differences in the frequency of isolation of *L. pneumophila* among the methods used in this research were not significant ($P > 0.05$). All three methods may be used to isolate and identify *Legionella* spp with 95% of reproducibility level.

DISCUSSION

Legionella species have been isolated from a wide variety of water types, such as potable water of hospitals (6), industries

and hotels (19), ground and surface water and biofilms (14). Moreover, several authors have described the isolation of *Legionella* spp from showers, cooling towers and boilers (26,33,52), which is in agreement with the findings of the present study. In contrast with previous findings reported in literature (4,51), *Legionella* spp were not detected in dental units water. One possible explanation for negative results to legionellae detection in dental units could be the presence of a large number of heterotrophic bacteria in those samples, which could secrete bacteriocins that inhibit the growth of *Legionella* species (53).

In this study, nine isolated strains were identified as *L. pneumophila* by direct plating, fluorescent antibody test and sequencing of *mip* gene. The use of the α BCYE-GVP selective medium in the isolation of *Legionella* spp from environmental samples was more sensitive than the amoebic coculture procedure. The results were consistent with previous observations (5), and indicated that *Legionella pneumophila* strains could be isolated either as intracellular pathogens of protozoan or as microbial plankton. Moreover, the presence of *L. pneumophila* in water and biofilm samples from the same cooling tower confirmed that this bacterium colonized distinct environments at the same site. This is an important aspect in the disinfection process on man-made systems.

Fliermans *et al.* (17) observed that *Legionella pneumophila* was part of the natural aquatic environment and that this bacterium was capable to survive at extreme environmental conditions. However, in the present study, *Legionella* spp was not detected from the freshwater samples from natural reservoirs, regardless the isolation and identification methods. Several biotic and abiotic factors may explain the unsuccessful detection

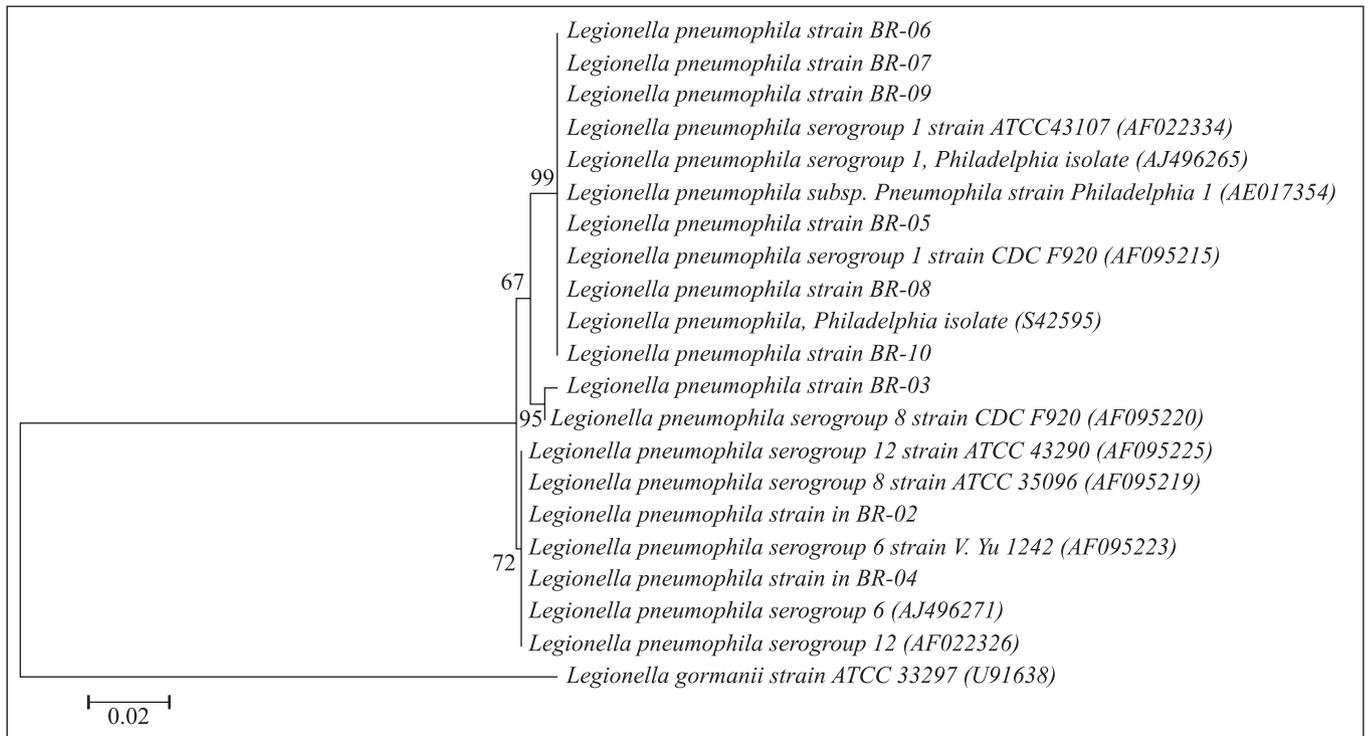


Figure 1. Consensus Phylogenetic tree showing distances based on the *mip* gene sequences (599 bp) among 09 isolates (labeled as *L. pneumophila* strain BR) from cooling towers (BR-09), water tank reservoirary to cooling tower (BR-07 isolated from water and BR-08 isolated from biofilm samples), and shower heads (BR-02, BR-03, BR-04, BR-05) sampled. *mip* gene nucleotide sequences from environmental isolates were compared with reference strains obtained from GenBank (accession numbers in parentheses). The species *L. gormanii* (ATCC 33297) was used as outgroup. Dendrogram was generated by using MEGA software packages version 3.0. Bootstrap analysis was applied to the data, and the values greater than 50% (n = 1,000 replications) are indicated at the nodes. The ratio of substitution per 100 nucleotides in dendrogram was 0.02 as demonstrated in scale bar.

of legionellae in those environments: the presence of viable but non-culturable cells, loss of viability of bacteria after collection, presence of faster-growing bacteria as potential biological competitors, and low concentration of legionellae in the samples. Similar observations were reported by other authors (8,25,40). According to Lee and West (32), legionellae are usually a minor component of a bacterial population in freshwater and moist natural environments, and the prime factor affecting their incidence appears to be water temperature. Legionellae appear to have a predilection for the warm water encountered in artificial environments, such as man-made systems.

Looking for better recovery results, Sanden *et al.* (45) observed that preincubation of water samples with free-living amoebae for several days increased the recovery of *Legionella* spp, which was related with proliferation of amoebae in the samples. The same observations were done by Bartie *et al.* (5), who showed that re-incubation of water samples concentrates with autochthonous amoebae improved the culturability of legionellae in a selective medium.

Results of amplification and sequencing of *mip* gene carried out in this study are in accordance to several authors (30,44,54). The *mip* gene encodes a 24-kDa surface protein (Mip) and has been chosen as genetic marker because it plays a crucial role in the resistance of *L. pneumophila* to intracellular killing (10). Results of PCR and sequencing procedures revealed that *Legionella pneumophila* was the only species in the sampled aquatic environments. The phylogenetic inference of *mip* gene sequences showed three different serogroup clusters related to *L. pneumophila* isolates from man-made systems, suggesting that these isolates are well adapted to environmental survival. These data are in accordance with previous reports in others regions around the world, where limited genetic variability of environmental isolates of *L. pneumophila* in the water systems and the prevalence of one genotype of this bacterium in a large proportion of man-made aquatic environments were reported (7,11,12,24,34,47). Although *Legionella* species other than *L. pneumophila* may occur in man-made systems, one explanation for the apparent spread of *L. pneumophila* observed in this

study could be the symbiotic interaction between this bacterium and free-living amoebae. It is known that amoebae are natural hosts of legionellae and play a major role in the multiplication of *L. pneumophila* in some water cultures (2,3,16,29,46). In addition, amoebae were found in the water samples of all eight sources sampled in the study. It is also possible that *Legionella* non-*pneumophila* species are less pathogenic than *L. pneumophila* due to lower infectivity or a poor intracellular growth, which could explain the major occurrence of virulent *L. pneumophila* strains in those environmental sources.

In regard to the detection procedures used in this study, no statistical significant differences were observed among them, indicating that the plate culture method, the coculture with free-living amoebae and the direct fluorescent assay are equally effective tools to monitor *Legionella* population on man-made aquatic environments. In addition, results of PCR and sequencing reactions of *mip* gene, applied in this study as a "virulence factor" gene, provided genetic evidence for the occurrence of virulent *Legionella pneumophila* strains in man-made systems which could infect both protozoan and mammalian phagocytic cells. Finally, the close clonal nature of *L. pneumophila* obtained in this study suggests that the potential sources of legionellae contamination could be the distribution system responsible for the water supply of the man-made systems or other disseminators that draw upon a public water supply.

ACKNOWLEDGEMENTS

The authors wish to acknowledge G. S. Bellicanta, R. C. Gamba and M. C. Daulisio for technical assistance in molecular biology and immunofluorescence microscopy, Dr. W. Francisco for supplying the *Legionella* reference strains used in this study and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support.

RESUMO

Deteção de *Legionella pneumophila* por métodos de cultivo e moleculares em sistemas artificiais de climatização de ambientes interiores em São Paulo - Brasil

Legionella pneumophila é uma bactéria patogênica associada à habitats aquáticos de ambientes naturais e artificiais. Casos clínicos de legionelose têm sido descritos no Brasil, mas a incidência e concentração desta bactéria em fontes ambientais ainda são pouco conhecidas. Assim, o presente estudo foi desenvolvido para avaliar a ocorrência de bactérias do gênero *Legionella* na cidade de São Paulo, Brasil, utilizando diferentes métodos de isolamento e identificação. Sessenta e sete amostras de água e biofilme de reservatórios naturais e

sistemas artificiais de climatização de ambientes interiores foram coletadas e analisadas quanto à presença de *Legionella* spp por métodos de cultivo em meio-de-cultura seletivo, cocultivo com amebas de vida livre axênicas e ensaios de imunofluorescência direta (IFD). Os resultados demonstraram que bactérias do gênero *Legionella* não foram detectadas em reservatórios naturais de água, *Legionella pneumophila* foi isolada de sistemas artificiais de climatização, com predominância de cepas de *Legionella pneumophila* sorogrupo 1. Apesar de não ter havido diferença estatística significativa entre os métodos de detecção propostos, o método de cultivo em placa produziu o melhor resultado quanto ao número de amostras positivas para *L. pneumophila*, seguido do procedimento de cocultivo com amebas e ensaio de imunofluorescência direta. Os resultados das reações de PCR e sequenciamento revelaram que a aplicação do gene potencializador de infecção em macrófagos como marcador molecular foi um importante implemento na identificação de isolados ambientais de *L. pneumophila*. A concordância existente entre os três métodos de detecção - quando todos os métodos produziram resultados similares - e a prevalência de uma espécie de *Legionella* nos sistemas artificiais amostrados poderia sugerir que a ocorrência desta bactéria tenha sido influenciada pela maior concentração de íons metálicos dissociados na água daqueles sistemas do que nos reservatórios naturais. Assim, os resultados deste estudo revelaram que a água proveniente dos sistemas artificiais de climatização de ambientes interiores pode servir como reservatório de *L. pneumophila* e outros microrganismos, incluindo protozoários de vida livre.

Palavras-chave: *Legionella pneumophila*, amebas de vida livre, sistemas artificiais de climatização

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