Detection of *Legionella pneumophila* in the Water Samples of Food Industries and Hospitals in Bangladesh

Nazmun Naher[1], Sangita Ahmed[1]*, and Md. Latiful Bari[2]

Abstract

Background: The human pathogen *Legionella pneumophila* causes a serious pneumonia-like respiratory disease called Legionnaires' disease, mainly in elderly and immunocompromised individuals. This pathogen can be found in the water distribution systems of large constructions with cooling towers which is a common phenomenon at present in Bangladesh due to its rapid economic growth. But there is a dearth of information on the incidence of *Legionella* in Bangladesh. Therefore, the current study aimed to investigate the presence of *Legionella pneumophila* in hospital and industrial water distribution systems in Dhaka, Bangladesh. Methods: A total of 114 water samples collected from two hospitals and five food industries were inoculated on the *Legionella*-specific medium Buffer Charcoal Yeast Extract (BCYE) agar medium before and after the treatment with acid, heat, or a combination of both. Samples producing *Legionella*-like colonies on BCYE agar medium were screened by *Legionella* Latex Test Kit, and the metagenomic DNAs obtained from these samples were analyzed by PCR using *L. pneumophila*-specific 16S rRNA primers. Results: Among 114 samples, *Legionella*-like colonies were observed in 30 water samples which demonstrated no agglutination in the Latex agglutination test. PCR analysis showed the presence of *L. pneumophila* in seven water samples, four in the potable water, chiller water, and cooling tower water of two different food industries, and three in ICU tap water, cooling tower water, and Fan Coil Units of two different hospitals. Sequence analysis of amplicons revealed that all seven sequences had 100% similarity with *L. pneumophila*. Conclusion: The presence of *L. pneumophila* in the water samples of local hospitals and food industries indicates that these habitats might serve as a potential site for Legionnaires' infection in Bangladesh. The results also showed that PCR, contrary to the conventional culture methods, could be more efficient and rapid in the identification of *L. pneumophila*.

Keywords: Legionnaires' disease; Water distribution system; *Legionella pneumophila*; PCR-based detection.

Abbreviations: BLASTN, Basic Local Alignment Search Tool; DNA, Deoxyribonucleic acid; ICU, Intensive Care Unit; µl, microliter; LPFP, *Legionella pneumophila* specific forward primer; LPRP, *Legionella pneumophila* specific reverse primer; NCBI, National Center for Biotechnology Information; ng, nanogram; PCR, Polymerase Chain Reaction; rpm, revolutions per minute; VBNC, Viable but nonculturable.

Introduction

Legionella pneumophila is a Gram-negative respiratory pathogen that causes pneumonia, with a mortality rate of 10% for elderly and immunocompromised patients (Alarcon Falconi *et al*., 2018). This pathogen is fastidious and ubiquitous in aquatic environments although its presence is at a very low or undetectable level in natural and artificial environments as well as buildings and mechanical equipment (WHO 2002, ASHRAE 2000). But the incidence of legionellosis, a serious type

Significance | PCR-based rapid detection of slow-growing *Legionella pneumophila*

*Correspondence: **Dr. Sangita Ahmed**, Professor, Department of Microbiology, University of Dhaka, Dhaka -1000, Bangladesh. **Contact no.**: +8801766946585; **Email**: sangita@du.ac.bd

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Author Affiliation:
[1] Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh.
[2] Centre for Advanced Research in Sciences, University of Dhaka, Dhaka-1000, Bangladesh.

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nocompromised patients (Alarcon Falconi *et al*., 2018). This pathogen is fastidious and ubiquitous in aquatic environments although its presence is at a very low or undetectable level in natural and artificial environments as well as buildings and mechanical equipment (WHO 2002, ASHRAE 2000). But the incidence of legionellosis, a serious type of pneumonia, has increased significantly in recent years due to the extended use of water in large technical systems like hospitals, hotels, and industries (Falkinham, 2020; Tercělj-Zorman *et al*., 2004; Weiss *el al*., 2017). The potential risk sites in these facilities are premise plumbing systems, cooling towers, evaporative condensers, and hot and cold water systems with a temperature range of 20-45°C (Moens 2002). In these facilities, the bacteria attach, settle, grow and multiply in high numbers through biofilms (Pereira *et al*., 2021) and might cause infection when susceptible individuals inhale the aerosol from the water distribution systems (Buse*et al*., 2012). Therefore, the disease is a serious public health concern (Flannery*et al*., 2006), and considering the threat to public health of *L. pneumophila*, the World Health Organization (WHO) and the U.S. Centers for Disease Control and Prevention (CDC) emphasize routine monitoring of this pathogen (CDC, 2005; Parr *et al*., 2015).

There are quite a very few studies on the incidence of *legionellosis* in Bangladesh although cases of atypical pneumonia are being reported continuously throughout the world (Erdoğan and Arslan, 2013; Matsumoto *et al*., 2006; Stamm and Stankewicz, 2022). The detection of *Legionella* was reported from natural water (Haque *et al*., 2016; Vasanthabharathi and Jayalakshmi, 2018), hospital tap water, hotel, and clinical samples (Jonas *et al*., 1995). In Bangladesh, the number of large industries and big hospitals is in increasing trend requiring the installation of air conditioners, cooling towers, hot and cold water systems, and decorative fountains at their premises. It is imperative to study whether the water distribution systems of these developments serve as the habitats for *L. pneumophila,* as the ambient weather condition of Bangladesh, with temperatures ranging from 20–45°C is favorable for the proliferation of this pathogen. Therefore, this study aimed to investigate the presence of *L. pneumophila* in water samples of different food industries and hospitals in Bangladesh.

Methodology

Sample collection

A total of 114 water samples were collected from two hospitals and five different food industries in Dhaka city from June 2019 to November 2019. Water samples were collected from 14 different points of two hospitals and five food industries, respectively (Table 1). Samples were collected in a sterile, non-transparent plastic bottle, maintained at average temperature, and immediately transported to the laboratory within 2 hours. For chlorine treated sample, 0.5 ml of 0.1N sodium thiosulfate was added to each 1.0 liter of water sample to neutralize the chlorine. The collected water samples were cultured following the detection method described in water quality-Enumeration of *Legionella* ISO 11731:2017.

Detection of *Legionella* using a culture-based method

Each water sample was filtered and concentrated by pouring the sample into a sterile 47 mm funnel assembly containing 0.45 µm polycarbonate filters. The filter was taken off aseptically and inserted into 5 ml of sterile water, and the tube was then vortexed for one minute to loosen the bacteria into the water. This concentrate (5ml) was cultured on Buffered Charcoal Yeast Extract agar medium (Difco, Germany) either directly (untreated) or after acid treatment (KCl–HCl solution, pH- 2.2), heat treatment (30 min at 50 °C) or combined acid and heat treatment. The plates were incubated at 35 °C in a 2.5% CO_2 incubator and were examined daily for up to seven days for the growth of *Legionella*. Presumptive *Legionella* colonies were identified based on colony morphology, and colonies that appeared round, glistening, and convex with frosted glass appearance (CDC, 2005) were subjected to a latex agglutination test by *Legionella* Latex Test Kit (LK04-Hi, Himedia) as per the manufacturer's instructions.

Isolation of total DNA

One liter of water was filtered with a 0.22 µ membrane filter, and the filter was washed with 10 ml of sterile deionized water. The wash-off water containing the retentate of the filter was centrifuged at 13000 rpm for 5 min, and the pellet was resuspended in 0.1 ml of sterile deionized water. Following incubation at 100 °C for 5-10 minutes in a water bath, the tube was immediately chilled on ice for 30 minutes. The suspension was then centrifuged for 5 minutes at 12000×g at 4 °C, and the supernatant containing the resulting metagenomic DNA was transferred to a new sterile microfuge tube and stored at -20 °C for further use.

Legionella pneumophila-specific 16SrRNA PCR

Metagenomic DNAs from the water samples were used to amplify the *Legionella*-specific 16SrRNA gene fragment following the method described by Jonas *et al*., 1995. The primers used in these PCR reactions were LPFP: (5'–AGGGTTGATAGGTTAAGAGC-3'); and LPRP: (5'–CCAACAGCTAGTTGACATCG-3').

PCR was performed in a final reaction volume of 25 µl containing 12.5 µl of master mix (OneTaq quick load 2×Master mix, New England Biolabs), 0.5 µl of each forward and reverse primer, 2 µl of metagenomic DNA, and 9.5 µl of nuclease-free water. Amplification was carried out in a Thermal Cycler (Veriti, Applied Biosystems, USA) with initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 1.5 min, annealing at 57 °C for 1.5 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. After amplification, the PCR products were processed for gel documentation and stored at -20 °C for further use.

Table 1. Collection of water samples from Hospital and Food industry water systems at Dhaka city.

Sources of water samples				
Sl. no.	**From Hospital**	**No. of samples**	**From Food industry**	**No. of samples**
1.	ICU Tap water	2	Reservoir water / Holding Tank	10
2.	Reservoir water / Holding Tank	4	Cooling Tower water	35
3.	Reverse Osmosis Dialysis water	3	Softener water	10
4.	Oxygen Flowmeter water	2	Potable water/ Drinking water	5
5.	Sterilize water for utensils	2	Bottle washing water	5
6.	Fan Coil Unit water	1	Hot Water Tank/ Preheated water	10
7.	ICU drinking water	2	Chiller water	5
8.	Cooling Tower water	1	Air condition water	10
9.	Softener water	1		
10.	Inlet water	1		
11.	Air Handling water	1		
12.	Potable water/ Drinking water	1		
13.	Hot Water Tank/ Preheated water	1		
14.	Chiller water	2		

Figure 1. Growth on BCYE agar media- a) *Legionella pneumophila* ATCC 33152, b) sample with presumptive growth of *Legionella*, c) sample with no growth of *Legionella* like colony (right)

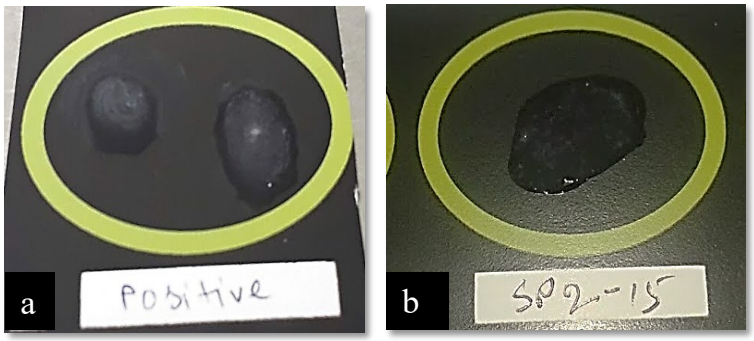

Figure 2. Observation on the latex agglutination assay. (a) Positive agglutination of *Legionella pneumophila* ATCC culture and (b) No agglutination for presumptive isolates.

The amplified DNA fragments were subject to horizontal gel electrophoresis in a 1.5% agarose gel slab and the gel was stained with ethidium bromide. The gel was visualized in a gel documentation system (Infinity Vilber Lourmat, France) after destaining. With strict adherence to the manufacturer's instructions, a PCR cleaning kit (Favorgen, Taiwan) was used to clean the amplified PCR products, and about 0.5-100 ng of the purified product was sent to Macrogen (Korea) for sequencing. The sequences of 16S rRNA gene fragments were submitted to the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) GenBank and the blast-n analysis was performed for identification (Shokraei *et al.*, 2019). A maximum likelihood phylogenetic tree was constructed based on these sequences using Molecular Evolutionary Genetics Analysis (MEGA) software version 11.

Results

Detection of *Legionella pneumophila* using culture method

A total of 114 water samples, treated with acid or heat or a combination as well as untreated water, were inoculated on Buffered Charcoal Yeast Extract Agar medium (BCYE) and round, glistening, convex, frosted glass colonies (typical characteristics for *Legionella* as described in the Procedures for the recovery of *Legionella* from the environment, CDC 2005), were isolated from 30 samples (Fig. 1).

Detection of *Legionella* by Latex agglutination kit

In the Latex agglutination assay, none of the presumptive *Legionella* isolates showed agglutination, while the positive control showed direct agglutination (Fig. 2).

Detection of *Legionella pneumophila* by Polymerase Chain Reaction

Detection of a 386-bp amplicon by PCR is considered a positive result for *Legionella* (Cloud *et al*., 2000). Among the 30 samples tested, the *Legionella pneumophila*-specific PCR product was detected in the 7 samples (Fig. 3). A comparison among the sequence of these PCR products in the GenBank database of the NCBI showed 100% homology of all seven isolates with *L. pneumophila*. The isolates were designated with accession numbers by NCBI GenBank after necessary verifications, which are MZ102255, MZ102256, MZ102257, MZ102258, ON924465, ON924466, and ON924467 (Table 2). Three isolates were obtained from hospital ICU tap water, cooling tower water, and Fan Coil Unit water of two different hospitals, while the remaining four were obtained from food industry cooling tower water, potable water, and chiller water.

A maximum likelihood tree was constructed based on the LP16S rRNA sequences of the seven *L. pneumophila* (Fig. 4). The 16S rRNA sequences of three reference strains of *L. pneumophila* obtained from the NCBI database were included in the analysis. The phylogenetic analysis demonstrates proximity among the sequences of the seven *L. pneumophila* with those available in the database, including the ATCC strain MZ59157.1. The sequences were distributed in two different lineages with divergences from a single root.

Discussion

Legionellosis is not a commonly reported disease in Bangladesh which might be because of overwhelmed medical sectors with other common diseases, lack of available, cost-effective diagnosis methods, and lack of awareness about the disease among the people. There are also only a few scientific reports on *L. pneumophila* in clinical trachea samples in Bangladesh (Jahan *et al*., 2015). Although the reports of atypical pneumonia are increasing day by day, the role of *L. pneumophila* in this connection is rarely investigated here in Bangladesh (Marchello *et al*., 2016; Matsumoto et al., 2006). Therefore, this study was designed to investigate the prevalence of *L. pneumophila* in water samples in Bangladesh, initially focusing on the hospital and food industry, which holds potential sites for the growth of this pathogen. Screening of 114 water samples collected from 14 sites of two hospitals and five food industries revealed the presence of seven *L. pneumophila.* To the best of our knowledge, this is the first report on detecting *L. pneumophila* from the industrial water system in Bangladesh.

The culture method using the selective BCYE agar medium was considered the gold standard for detecting *L. pneumophila*. However, we did not find any *L. pneumophila* using the selective growth medium in the current study. Borges *et al.,* (2012) reported a similar observation where they found *Legionella*-like colonies on BCYE agar and none was *Legionella*, as further revealed by 16SrRNA sequencing. These reports suggest that the presence of other microorganisms or disinfectants in a water sample inhibits the growth of *Legionella* sp. on the BCYE medium (Gilpin and Gilpin, 2014). The presence of *L. pneumophila* in a viable but non-cultural (VBNC) state in the environment also interferes with its detection using a culture-based method (Ahmadrajabi *et al*., 2016). The same might be the case for the current study, as in Bangladesh, disinfectants are regularly used to maintain water quality, and the presence of a large number of heterotrophic bacteria is also typical in water samples (Islam *et al*., 2010; Islam *et al*., 2020 ; Mahbub *et al*., 2011; Shahidul *et al*., 2014). The *L. pneumophila* ATCC33512 showed confluent growth on the BCYE medium in the current study, possibly because it was a pure culture and did not have any competitive flora or inhibitory factors. Borges *et al.,* (2012) reported the growth of the members of *Chitinophagaceae* on the BCYE agar media with typical gray colonies like *L. pneumophila*. It might be worthwhile to investigate whether the isolates that showed similar colony morphology on BCYE medium in the current study belong to *Chitinophagaceae*, which might have interfered with the study.

Table 2. Detection of *Legionella pneumophila* by PCR

Sources of water sample	Number of *L. pneumophila* detected by PCR	GeneBank accession number
Food industry		
Cooling tower water	2	MZ102257 MZ102258
Potable water	1	ON924467
Chiller water	1	ON924466
Hospital		
ICU tap water	1	MZ102255
Cooling tower water	1	MZ102256
Fan Coil Unit water	1	ON924465

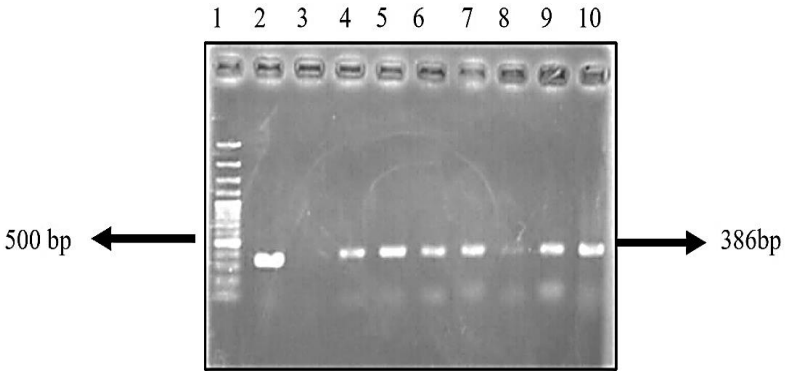

Figure 3. **Detection of *Legionella pneumophila* (LP) specific 16srRNA gene (386bp) in water samples.** (Lane 1: 100 bp DNA ladder, Lane 2: Positive control *Legionella pneumophila* ATCC33512, Lane 3: Negative control, Lane 4-7: Cooling tower water, Potable water, Chiller water samples from food industry, Lane 8-10: Fan Coil Unit water, ICU tap water, Cooling tower water from hospital).

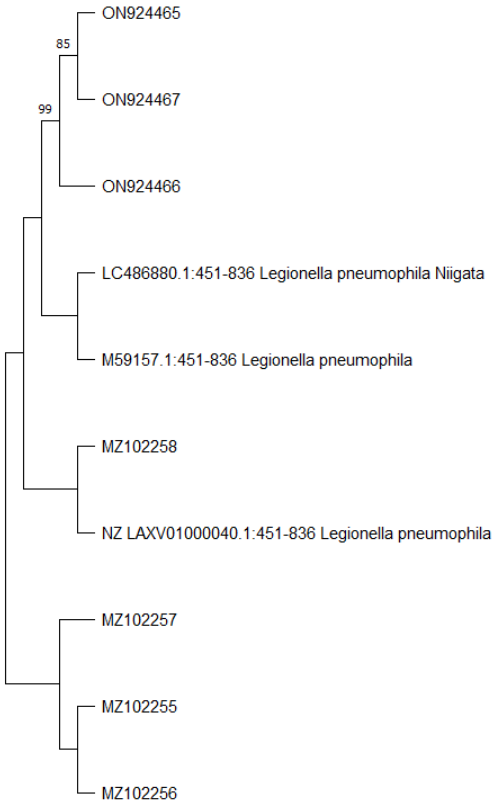

Figure 4. **A maximum likelihood phylogenetic tree was constructed based on LP16SrRNA sequences.** Sequencings were Aligned by ClustalW algorithm Bootstrap method using Molecular Evolutionary Genetics Analysis (MEGA) software version 11. The values above nodes indicate the bootstrap values. Bootstrap values below 75 are not displayed.

In contrast to the culture-based method, the *Legionella*-specific PCR assay, targeting the 16S rRNA gene of *L. pneumophila* was found to be more sensitive for detecting this pathogen, as seven *L. pneumophila* have been detected in the current study using the PCR assay. This finding is in agreement with other studies where compared to the culture-based methods, PCR assay exhibited higher efficacy in the detection of *L. pneumophila*, even those in the VBNC state (Ahmadrajabi *et al*., 2016; Shishir and Hoq, 2020; Rafiee *et al.*, 2014; Wellinghausen *et al.*, 2001). This finding also emphasizes formulating a new growth medium, which would be more selective and sensitive for detecting this pathogen from an environmental sample.

In the current study, 43% of *L. pneumophila* were detected in cooling tower water samples of one hospital and two food industries, which is in alignment with global data of cooling tower systems being the major reservoir of *Legionella* (Ishimatsu *et al*., 2001; Lam *et al*., 2011; Pagnier *et al*., 2009; Van den Hoek *et al*., 2006). *L. pneumophila* was also detected in the tap water of the ICU and Fan Coil Unit of the hospital. These data, therefore, indicate that the water distribution systems of hospitals in Bangladesh might be a credible source of transmission of this pathogen among admitted patients. However, as there are no reported cases of Legionnaires' disease in Bangladesh, it is difficult to establish a link between the presence of this pathogen in hospitals and the actual disease. However, atypical pneumonia should be investigated extensively for the etiological agents. To investigate any such possibility, implementing a routine diagnosis of *L. pneumophila* in hospital-acquired pneumonia patients in Bangladesh and regular surveillance of the hospital water system is essential.

Phylogenetic analysis of the LP16SrRNA sequences displays proximity among the sequences of the seven *L. pneumophila* obtained in the current study with those available in the database. However, no specific association was observed between the sequences and sample collection sites. The sequence of MZ102258, detected in a food industry cooling tower, was homologous to the reference sequence NZ LAXV01000040.1, which was detected in a cooling tower in China, suggesting a possible link. A more extensive study of the genome sequences of this pathogen is crucial to identify the route of transmission of *L. pneumophila* in Bangladesh.

This study demonstrates that water distribution systems of hospital and food industries are colonized by *L. pneumophila* in Bangladesh and present a possible threat to the people, which emphasizes regular surveillance and decontamination of water supply in these premises. Observations from the study form the basis of more comprehensive research on the prevalence of *L. pneumophila* in other potential sources in Bangladesh.

Conclusion

From the present investigation, it can be concluded that *Legionella pneumophila* might be prevalent in the water distribution systems in Bangladesh, which needs further exploration. The data from the current study suggest that the PCR method, instead of the conventional culture-based technique, would be more effective and rapid for the detection of this pathogen from water samples.

Author Contribution

The manuscript has been read and approved by all authors. The research work has been conceptualized and supervised by Professor SA and MLB. NN designed and conducted the research work, analyzed the data, and was involved in writing and editing the script. The corresponding author is the sole contact for the editorial process. She is responsible for communicating with other authors about progress, submission of revisions, and final approval of proofs.

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Competing financial interests

The authors declare that they have no competing financial interests.

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