

Prevalence of *Legionella* spp. in various natural and processed water systems in Bangladesh and the development of an effective detection method



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**DEPARTMENT OF MICROBIOLOGY
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Prevalence of *Legionella* spp. in various natural and processed water systems in Bangladesh and the development of an effective detection method

PhD Thesis



**DEPARTMENT OF MICROBIOLOGY
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DEDICATED TO...

My beloved parents,

husband, and daughter

who treasured my life with their love and affection

Certification

It is hereby certified that the student bearing Reg. No. 48; Session: 2021-2022, has carried out the research work entitled “Prevalence of *Legionella* spp. in various natural and processed water systems in Bangladesh and the development of an effective detection method” for the fulfillment of her Ph.D. degree from the University of Dhaka, Bangladesh, under our academic supervision and co-supervision in the Department of Microbiology, University of Dhaka.

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Abstract

Legionella pneumophila is a human pathogen that causes a pneumonia-like respiratory disease known as Legionnaires' disease. This disease primarily affects elderly and immunocompromised individuals and is associated with significant morbidity and mortality. *L. pneumophila* can occasionally be found in various urban structures, including commercial buildings, hospitals, cooling towers, and other facilities equipped with condensers. Despite the country's rapid economic growth and the development of numerous factories and hospitals in recent years, there is limited information available on the incidence of *Legionella* spp. in Bangladesh. Therefore, this study aimed to investigate the prevalence of *L. pneumophila* in water systems of hospitals, industries, and hotels in Dhaka, Bangladesh, and surface water samples from different parts of the country. A total of 171 water samples were collected. Of these, 128 processed water samples were gathered from various locations, including two hospitals, five food industries, one hotel, and 43 natural water sources. Additionally, the study sought to detect the prevalence of *L. pneumophila* in tracheal aspirates from patients admitted to the Intensive Care Unit (ICU) of one hospital. Following treatment with acid, heat, or a combination of both, all water and clinical samples were inoculated on the *Legionella*-specific growth medium, Buffer Charcoal Yeast Extract agar (BCYE). Samples that produced *Legionella*-like colonies on BCYE were screened using the *Legionella* Latex Test Kit, and metagenomic DNA obtained from these samples was analyzed through molecular detection using PCR. Specifically, a 386 bp region of the 16S rRNA gene of *L. pneumophila* was targeted with specific primers. Among 171 water samples, *Legionella*-like colonies were observed in several water samples; however, none exhibited agglutination in the *Legionella* Latex agglutination test. PCR analysis revealed the presence of *L. pneumophila* in 13 (7.60%) water samples, which included potable water, chiller water from food industries and hotel, cooling tower water, ICU tap water, fan coil unit water from hospitals, and natural surface water. *L. pneumophila* was detected in 5 out of 50 tracheal aspirate samples (10%) using LP16S rRNA PCR. The PCR products were sequenced and analyzed using the National Centre for Biotechnology Information (NCBI) GenBank database. Sequencing data of the PCR products confirmed 100% similarity with *L. pneumophila* in the NCBI database. A nested PCR method was developed to further confirm the presence of *L. pneumophila* in samples that showed faint bands in the initial LP16SrRNA PCR. Primer pairs were designed using NCBI Primer-BLAST, and one pair was selected based

on GC content and low self-complementarity. This primer pair amplified a 137 bp DNA fragment within the 386 bp region of the 16S rRNA gene of *L. pneumophila*. The specificity of the selected primer pair was confirmed by ensuring no amplification occurred with closely related bacterial species, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. The nested PCR consistently detected *L. pneumophila* DNA at concentrations as low as 1 pg/μl from metagenomic samples. Using the nested PCR approach, *L. pneumophila* was detected in 30 additional samples, 21 from food industries and hotels, and 9 from natural waters and 11 from clinical samples. Sequence analysis of the PCR products confirmed 100% homology with *L. pneumophila* in the GenBank database. Overall detection rates increased from 7.6% with the LP16SrRNA PCR to 25% with the nested approach. Based on the PCR analysis, *L. pneumophila* was identified in 16 (32%) of the 50 tracheal aspirate samples of ICU-admitted patients collected from the same hospital where *L. pneumophila* was detected in the ICU tap water, suggesting nosocomial transmission. Among these clinical samples, the virulence gene *mip*, which encodes the macrophage infectivity potentiator protein, was detected in 12 samples. The presence of *L. pneumophila* in processed water samples from hospitals, the food industry, hotels, and natural waters in Bangladesh indicates that these environments could serve as potential sites for *L. pneumophila* infections. The application of the novel nested PCR-based method demonstrated greater sensitivity and specificity for detecting *L. pneumophila* in both environmental and clinical sample sources compared to conventional methods. This advancement could enable earlier and more accurate detection of *L. pneumophila*, thereby enhancing public health surveillance and prevention strategies.

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List of Abbreviations

AC	Air Condition	<i>L. pneumophila</i>	<i>Legionella pneumophila</i>
AIDS	Acquired immunodeficiency syndrome	LP16S rRNA	<i>Legionella pneumophila</i> 16S ribosomal RNA
ATCC	American Type Culture Collection	MEGA	Molecular Evolutionary Genetics Analysis
BCYE	Buffered Charcoal Yeast Extract	ND	Not Detected
BSTI	Bangladesh Standards and Testing Institution	PBS	Phosphate Buffered Saline
CAP	Community Acquired Pneumonia	qPCR	Quantitative Polymerase Chain Reaction
CDC	Centers for Disease Control and Prevention	RT-PCR	Real-Time Polymerase Chain Reaction
CFU	Colony Forming Unit	rpm	Revolutions per minute
DO	Dissolved oxygen	RtxA Toxin	Repeats-in-Toxin
DNA	Deoxyribonucleic acid	TA	Tracheal Aspirate
ECDC	European Centre for Disease Prevention and Control	TFTC	Too Few to Count
EnhC	Enhancer of infection	TNTC	Too Numerous to Count
EPA	Environmental Protection Agency	TFCC	Total Fecal Colony Count
FCU	Fan Coil Unit	TNF- α	Tumour Necrosis Factor alpha
GVPC	Glycine Vancomycin Polymyxin Cycloheximide	USA	United States of America
HAP	Hospital-Acquired Pneumonia	VBNC	Viable but non-culturable
HCl	Hydrochloric acid	WASA	Water Supply and Sewerage Authority
IL-1	Interleukin-1	WHO	World Health Organization
IL-6	Interleukin-6		
ISO	International Organization for Standardization		
KCl	Potassium Chloride		

Introduction

Chapter 01: **Introduction and Literature Review**

Introduction

1. Introduction**1.1 *Legionella* spp.**

Legionella is a small, aerobic, Gram-negative, intracellular bacterium that primarily poses a health risk to humans by causing pneumonia, known as Legionnaires' Disease (LD), a severe form of pneumonia and a flu-like illness called Pontiac fever (Mondino et al., 2020). This bacterium is characterized as a non-motile, unencapsulated bacillus that belongs to the Legionellaceae family (McDade et al., 1977).

Legionellaceae comprises over 60 species, with approximately 70 recognized serogroups (*Legionella* Guideline CDC, 2024). the most clinically significant is *Legionella pneumophila*, which is associated with the majority of Legionnaires' disease cases, a severe form of pneumonia (Fields et al., 2002). Besides *L. pneumophila*, several other *Legionella* species have been associated with Legionnaires' disease. These include *Legionella longbeachae*, *Legionella micdadei*, *Legionella bozemanii*, *Legionella dumoffii*, *Legionella feeleii*, *Legionella anisa*, *Legionella jordanis*, *Legionella wadsworthii*, *Legionella sainthelensis*, and *Legionella cincinnatiensis*. While *L. pneumophila* causes the majority of cases worldwide, *L. longbeachae* is notably more common in countries like Australia and New Zealand (Boer et al., 2004; Whiley & Bentham, 2011). *L. micdadei* and *L. bozemanii* have also been implicated in both sporadic and nosocomial infections Yu et al., 2002; Gomez-Valero et al., 2019) .

Legionnaires' disease (LD), or legionellosis, first gained attention following an outbreak in 1976 at a hotel in Philadelphia, where 221 veterans attending an American Legion convention became ill (Fraser et al., 1977). Since then, Legionnaires' disease has been identified as a severe form of pneumonia caused predominantly by *Legionella* species, with more than 90% of cases explicitly attributed to *L. pneumophila* (Mérault et al., 2011; Li et al., 2015). So far, a total of 16 serogroups of *L. pneumophila* have been identified (Zhang et al., 2017). Serogroup 1 (LP1) is recognized as the predominant strain responsible for the majority of cases of Legionnaires' disease (Jomehzadeh et al., 2019). This serogroup has been identified in numerous outbreaks and remains a significant public health concern due to its prevalence and virulence. Other serogroups have also been associated with previous outbreaks, with serogroup 6 (LP6) emerging as the second most frequently detected strain (Amemura-Maekawa et al., 2010; Messi et al., 2013).

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L. pneumophila can also lead to extrapulmonary infections, including wound infections, septic arthritis, sinusitis, endocarditis, and hospital-acquired (nosocomial) infections (Diederer, 2008). In vulnerable individuals, untreated infections may result in severe sepsis and multi-organ failure.

Pontiac fever, a self-limiting, non-pneumonic illness that mimics flu-like symptoms without involving the lower respiratory tract (ARORA et al., 2012). This illness is milder than Legionnaires' disease. The first identified cases of Pontiac fever occurred in 1968 in Pontiac, Michigan (Guidance for *Legionella* Control CDC, 2025).

The CDC, 2021 (Legionnaires' Disease and Pontiac Fever) estimates that the actual cases of Legionnaires' disease per year may be 1.8 to 2.7 times higher than those reported. The overall mortality rate for this disease is estimated to be between 5-10% (WHO, 2022). The U.S. Environmental Protection Agency (EPA) also acknowledges the public health threat posed by many species of *Legionella*. This divergence emphasizes the varying clinical presentations associated with *Legionella* infections. Globally, the burden of Legionnaires' disease is widely recognized as underreported (Bugante et al., 2011), highlighting the urgent need for proper diagnosis, improved reporting systems, and heightened awareness.

1.2 *Legionella* spp. prevalence

Legionella spp. especially *Legionella pneumophila* is often found nestled within various natural aquatic environments, forming a part of the intricate microbial flora in lakes, tranquil ponds, and flowing rivers (Riffard et al., 2001). In these natural habitats, it typically exists in low concentrations, often lying dormant and undisturbed. However, the actual threat emerges from artificial water systems, such as cooling towers, reservoir tanks, swimming pools, and chilled water systems. A total of 17 systems and matrices were classified as confirmed sources of *Legionella* (Van Heijnsbergen et al., 2015), such as cooling tower, room humidifier, air conditioning system, fountain, whirlpool and respiratory device. Such systems are standard in the food industry, hotels, hospitals, and residential establishments, increasing the potential legionellosis risk.

The infrastructure of these artificial water systems creates a conducive environment for the bacteria to settle, flourish, and multiply in alarming numbers, particularly when these

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environments are poorly maintained and infrequently sanitized. This presents a significant risk of infection when vulnerable individuals inhale aerosolized particles originating from these water systems. Stagnant water, characterized by its lack of movement and often harboring debris, becomes a veritable breeding ground for microorganisms like *Legionella*. When water sits still, it can foster diverse bacterial species, allowing them to proliferate within and along its surfaces (Lautenschlager et al., 2010; Bédard et al., 2018).

Epidemiological studies highlighted a significant link between outbreaks of legionellosis and various sources, like cooling towers (Ricketts & Joseph, 2007) and hot water systems that are commonly found in large facilities such as hotels and hospitals (Heymann, 2004). Legionellosis has frequently been associated with travel and hotel stays (Assaidi et al., 2021). In Europe, 20 per cent of Legionnaires' disease cases reported between 2011 and 2015 were travel-associated (Beauté, 2017). Earlier, between 1980 and 1998, Legionnaires' disease cases were travel-associated reported by Benin et al., (2002).

Within hospital environments, certain factors heighten the risk of infection, including recent surgeries, intubation, mechanical ventilation, aspiration, the presence of nasogastric tubes, and dependence on respiratory therapy equipment. However, the range of artificial sources that can lead to infection is broad, covering nebulizers (Lee et al., 2008), evaporative condensers (Breiman et al., 1990; Brown et al., 2001), humidifiers (Isozumi et al., 2005), whirlpool spas (Benkel et al., 2000; Sonder et al., 2008), water-baths (Franzin et al., 2002), and even household water blasters (Simmons et al., 2008). Alarming, hot springs contained *Legionella*, indicating a widespread presence of *Legionella* spp. (Zhang et al., 2017) and even rainwater collected on roads (Sakamoto et al., 2009). Specifically, *L. pneumophila* has been widely documented in the shower water of hotels and the potable water of hospitals (Marrie et al., 1992; Assaidi et al., 2021). Its prevalence also extends to dental units (Singh & Coogan, 2005) Additionally, ornamental fountains installed in hotels and hospitals can serve as breeding grounds for nosocomial Legionnaires' disease.

In the context of the food industry, particular caution is warranted for cooling towers, evaporative condensers, ice-making machines (Stout et al., 1985) and the intricate network of hot and cold water systems (Correia et al., 2016) where water temperatures can range between a tantalizing 20–45°C (Mathys et al., 2008; Assaidi et al., 2018).

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Additionally, *Legionella* spp. are associated with microbial biofilms that commonly thrive in artificial water systems. Abundant nutrients and organic matter in the poorly maintained artificial water systems facilitate these biofilms, where they rapidly proliferate and adhere to surfaces like evaporative fill materials, heat exchangers, water reservoirs, and pipelines (Pippo et al., 2018). High concentrations of *Legionella* have been isolated from the murky water sediments and biofilms found within cooling water systems (Orrison et al., 1983). In artificial water systems, such as drinking water distribution networks, *Legionella* growth is predominantly found embedded in biofilms that coat the inner surfaces of pipes and within ventilation and air conditioning systems (Lau & Ashbolt, 2009). These bacteria can form dense clusters, creating a biofilm that can infiltrate buildings through any connected water course. When significant disruptions occur in the plumbing infrastructure or shifts in water pressure, *Legionella* can be liberated from its biofilm, generating contaminated aerosols. Consequently, this biofilm holds the potential to catalyze the growth of *Legionella* alongside a multitude of other microorganisms, some harmful and others benign.

1.3 Growth Condition

Legionella spp. are highly fastidious organisms with particular growth requirements for survival and proliferation (Devos et al., 2005). *Legionella* exhibits remarkable adaptability, allowing it to survive in diverse natural and artificial aquatic habitats. The bacterium can thrive within protozoan cells or form intricate biofilms, utilizing these structures to acquire vital nutrients while shielding itself from environmental stressors such as temperature fluctuations, disinfection efforts, and predation by protozoa (Kenhove et al., 2024). Certain plastics and organic materials can also serve as nutrient sources, making modern plumbing systems hospitable to this organism. This poses a risk to people nearby, as they may inadvertently inhale or aspirate these harmful particles. Although *Legionella* is an aerobic bacterium requiring oxygen for metabolism, and low levels of dissolved oxygen (DO) can inhibit its growth, it can endure low levels of dissolved oxygen and survive in oxygen-depleted microenvironments within biofilms (Chang et al., 2007; Krause, 2022). It also demonstrates a notable resistance to chlorine disinfection, further enhancing its survival prospects.

The growth and survival of *Legionella* spp. are heavily affected by various physicochemical parameters of water, such as temperature, pH, total dissolved solids

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(TDS), and conductivity. Temperature is one of the most important factors for the proliferation of *Legionella* bacteria. The optimal growth temperature for *Legionella* spp. ranges from 25°C to 42°C, while the survival temperature range is between 20°C and 50°C (Lasheras et al., 2006; Whiley, 2016). *Legionella* grows best at pH 6.0–8.0, but can tolerate a broader range from 5.0 to 9.5 (Bartram, 2007). Extreme pH levels hinder its activity and biofilm stability. Water with high TDS and conductivity value often favours the growth of this pathogen, and it has been suggested that nutrients from TDS can support the growth of protozoa (e.g., amoebae), which act as hosts for *Legionella*'s intracellular replication (Sciuto et al., 2021). Minerals in biofilms can also protect *Legionella* from disinfectants. *Legionella* is an aerobic bacterium requiring oxygen for metabolism. Low levels of dissolved oxygen (DO) can inhibit its growth, but it can survive in oxygen-depleted microenvironments within biofilms (Krause, 2022).

1.3.1 Transmission

Water that contains *Legionella* spp. can be spread in very small droplets that people can inhale. When these droplets are breathed in, individuals may develop Legionnaires' disease or Pontiac fever. This inhalation is the most common way that people become ill from *Legionella* spp. Less commonly, illness can occur if the bacterium passes to the respiratory tract accidentally while drinking water contaminated with the pathogen. Generally, Legionnaires' disease and Pontiac fever are not spread from person to person (CDC, 2025). Most healthy individuals who are exposed to *Legionella* do not get sick. However, certain groups of people are at a higher risk of illness. This includes current or former smokers, individuals who are 50 years of age or older, and those with specific health conditions, including those with cancer, chronic lung disease, diabetes, kidney failure, liver failure, and weakened immune systems.

1.4 Pathogenesis

Humans primarily become exposed to *L. pneumophila* by inhaling contaminated aerosols (Yu, 1993). Upon inhalation, the bacteria reach the alveoli of the lungs (Figure 1.1). In the alveoli, *L. pneumophila* is phagocytosed by alveolar macrophages. However, instead of being degraded, the bacteria prevent phagosome-lysosome fusion using a Type IV secretion system Dot (defective in organelle trafficking)/ Icm (intracellular multiplication). This allows *Legionella* to form a specialized compartment known as the

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Legionella-containing vacuole (LCV). Inside the LCV, *Legionella* manipulates host cell processes by secreting over 300 effector proteins, which prevent phagosome-lysosome fusion and multiply rapidly within the vacuole, protected from host immune responses. Once replication reaches a threshold, the macrophage undergoes apoptosis or necrosis, releasing the bacteria. These free bacteria can infect neighboring macrophages or be expelled in respiratory secretions. The infection triggers a strong pro-inflammatory response, with the release of cytokines like IL-1, IL-6, and TNF- α . This leads to lung inflammation and pneumonia, the hallmark of Legionnaires' disease (Roy et al., 2002; Isberg et al., 2008; Newton et al., 2010).

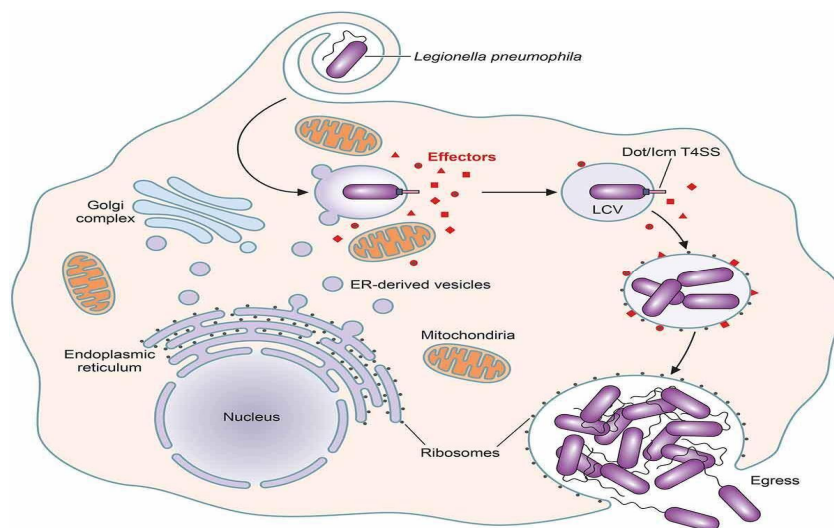


Figure 1.1 The life cycle of *Legionella pneumophila* within eukaryotic host cells. Bacterial uptake takes place by either coiling (shown) or conventional phagocytosis. Early after entry into the host cell, *L. pneumophila* loses its flagella and the *Legionella*-containing vacuole (LCV) escapes the endocytic pathway via effector-mediated recruitment of endoplasmic reticulum (ER)-derived vesicles and transient association with mitochondria. Subsequently, the LCV becomes studded with ribosomes and effectors, and exponential replication occurs (replicative phase). Upon exhaustion of host nutrients, *L. pneumophila* becomes flagellated (transmissive phase) and egresses the host cell (adapted from Chauhan et al., 2021).

The illness associated with this pathogen can be lethal, with mortality rates ranging from 3% to 33% for infected individuals. Patients typically present with a constellation of alarming symptoms, including fever, chills, persistent cough (both dry and productive),

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muscle aches, headache, loss of appetite, chest pain, gastrointestinal distress from diarrhoea to vomiting, and shortness of breath (Underwood et al., 2013). The risk of severe manifestations of Legionnaires' disease is notably higher in older adults, smokers, and individuals with compromised immune systems, underscoring the need for vigilant monitoring and prevention strategies in vulnerable populations.

1.5 Virulence factor

L. pneumophila is a facultative intracellular pathogen that primarily targets alveolar macrophages in humans and protozoa in the environment. Its capacity to evade host immune responses, persist within phagocytic cells, and modify host cell functions is due to a collection of specialized virulence factors, which include various secretion systems, proteins, and surface molecules (Newton et al., 2010; Gattuso et al., 2022).

Surface virulence factors such as outer membrane proteins, flagella, pili, and lipopolysaccharides facilitate initial adhesion to host cells and boost infectivity. Toxin production further increases infectivity, survival, and multiplication within alveolar macrophages. Certain genes may regulate biofilm formation, enhancing the pathogen's ability to persist in water supply systems (Abu Khweek & Amer, 2018). Proteins and effectors that enable *Legionella* to infect protozoa also allow it to infect mammalian cells. This pathogen has acquired numerous eukaryotic-like genes through horizontal gene transfer from host amoebae (*Acanthamoeba* spp., *Naegleria* spp., *Hartmannella* spp., *Vermamoeba vermiformis*) that govern the infection process Yang et al., 2022; Scheithauer et al., 2023).

The *mip* (macrophage infectivity potentiator) gene, encoding a critical 24-kilodalton (kDa) surface protein known as MIP, is a significant virulence factor for *L. pneumophila*. This virulence factor plays a crucial role in the pathogenesis of the bacterium by modulating the host's immune response. Specifically, MIP inhibits the recognition and phagocytosis of *L. pneumophila* by macrophages, allowing the bacteria to evade the host's immune defences. This evasion leads to enhanced cell apoptosis and necrosis, ultimately facilitating the release of bacteria from host cells and improving their overall survival rate. The *mip* gene is integral to the bacterial lifecycle and vital for the optimal intracellular survival of *L. pneumophila*. It is highly conserved across various strains of *Legionella*, underscoring its essential function in the bacterium's virulence (Ratcliff et al.,

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1998). Notably, the *mip* gene was the first identified gene linked to virulence in *Legionella pneumophila*, as demonstrated by the pioneering studies of Cianciotto & Fields (1992), highlighting its importance for effective infection of host cells. Recent advancements in molecular detection techniques have highlighted the utility of the *mip* gene as a target for detecting *L. pneumophila* using polymerase chain reaction (PCR) methods, as reported by Bagheri et al. (2021). This approach enhances the capabilities for diagnosing infections caused by this pathogen and underscores the importance of *mip* in both basic research and clinical applications.

The *Dot/Icm* genes encoded by the Type IV Secretion System (T4SS) are considered another major virulence factor of *L. pneumophila* to replicate inside host cells and cause disease (Sexton et al., 2004). A notable 22-kilobase DNA locus of *L. pneumophila* comprises 18 genes, 16 of which play a critical role in the bacterium's ability to kill macrophages, known as the *icm* genes (Segal et al., 1998). This system is essential for injecting effector proteins into host cells, thereby facilitating the pathogen's interaction with the immune system. Sid (substrate of *Icm/Dot*) effectors include SidC, SidM, and SidJ. It modulates host cell processes such as vesicle trafficking, ubiquitination, and apoptosis (Isberg et al., 2009). These effectors manipulate host cell vesicle trafficking and prevent phagosome-lysosome fusion, allowing the bacteria to replicate in a protected vacuole, known as the *Legionella*-containing vacuole (LCV).

Among other virulence factors, the protein EnhC (enhancer of infection) enhances the replication of *L. pneumophila* within macrophages and protozoa by stabilizing the bacterial envelope and facilitating intracellular survival (Luo et al., 2004). RtxA Toxin (repeats-in-toxin) contributes to cytotoxicity, adherence, and host cell invasion. It plays a role in disrupting the host cytoskeleton and promoting bacterial spread (Cirillo et al., 2001).

Other genes, such as *flaA* (flagellum subunit), *pilE*, *asd*, *mompS*, and *neuA*, demonstrated enough discrimination for epidemiological typing of *L. pneumophila* serogroup (sg) 1 (Heuner et al., 1995; Gaia et al., 2003). They assist in assigning a sequence type (ST) for outbreak tracing. The *pilE* gene encodes the type IV pilin protein, a key structural element of the bacterial type IV pili, which facilitates *Legionella* attachment to protozoa, macrophages, and biofilm matrices in water systems (Stone & Abu Kwaik, 1999). In *L. pneumophila*, the *asd* gene encodes aspartate β -semialdehyde dehydrogenase, an essential

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housekeeping enzyme involved in synthesizing amino acids such as lysine, methionine, and threonine (Harb & Kwaik, 1998). The *mompS* gene encodes the major outer membrane protein (MOMP), critical for bacterial physiology and molecular typing (Gaia et al., 2003). Additionally, the *proA* gene in *L. pneumophila* encodes proline aminopeptidase, an enzyme that cleaves N-terminal proline residues from peptides (Black et al., 1990).

1.6 *Legionella* spp. with coinfection

Individuals with compromised immune systems, such as those undergoing stem cell transplantation (Palmore et al., 2009) or receiving anti-tumour necrosis factor-alpha therapy (Lanternier et al., 2017) are at heightened risk of *L. pneumophila* infection. Furthermore, patients suffering from chronic lung diseases or those with a smoking history (Edelstein & Lück, 2015) face an increased likelihood of contracting Legionnaires' disease. In hospital environments, the case fatality rate for Legionnaires' disease can be alarmingly high, making it a significant concern for healthcare providers. This is especially true for patients with AIDS (Head et al., 2019) or those who have recently undergone organ transplants (Moemen et al., 2019), as their weakened immunity places them in even greater jeopardy.

1.7 Epidemiology/Incidence

Legionellosis is a significant global disease with a rising incidence in many countries. Outbreaks have been reported worldwide in both developed and developing nations. *L. pneumophila*, the main cause, accounts for 1-3% of community-acquired pneumonia cases and is involved in up to 26% of atypical pneumonia cases (Pedro-Botet & Sabrià, 2005; Diederer et al., 2008; Mathys et al., 2008). According to the Yu et al. (2024) the incidence of Legionnaires' disease has surged nearly ninefold from the year 2000 to 2018.

Table 1.1 List of Legionnaires' disease outbreaks worldwide
(retrieved from wiki accessed on 14.08.2025)

Year	City	Cases	Death	Venue	Fatality rate
2022	Argentina	22	6	Health Clinic	45%
2020	USA	5	1	Brookdale Senior Living	20%
2018	USA	27	1	Cooling towers (sugar building)	3.7%
2017	USA	22	1	Cooling towers (Disneyland)	4.5%

Legionnaires' disease (LD) is recognized as the leading cause of drinking water disease outbreaks in the United States (Barskey et al., 2022). From 2000 to 2014, reported cases of legionellosis per 100,000 population increased by 300% in the USA (Alarcon Falconi et al., 2018). Over the last five years, several outbreaks have been reported across the United States, with a startling fatality rate of 4.5% recorded in 2017, which shockingly escalated to five times that figure by 2020. Table 1.1 displays some notable outbreaks from the USA. Records indicate that the most significant incidents are associated with increased exposure to potential sources of infection, particularly cooling towers. A severe outbreak in Argentina in 2022 witnessed an alarming fatality rate of 45%, underscoring the troubling rise of infections caused by *L. pneumophila*.

Legionnaires' disease in the United States, 2000-2022

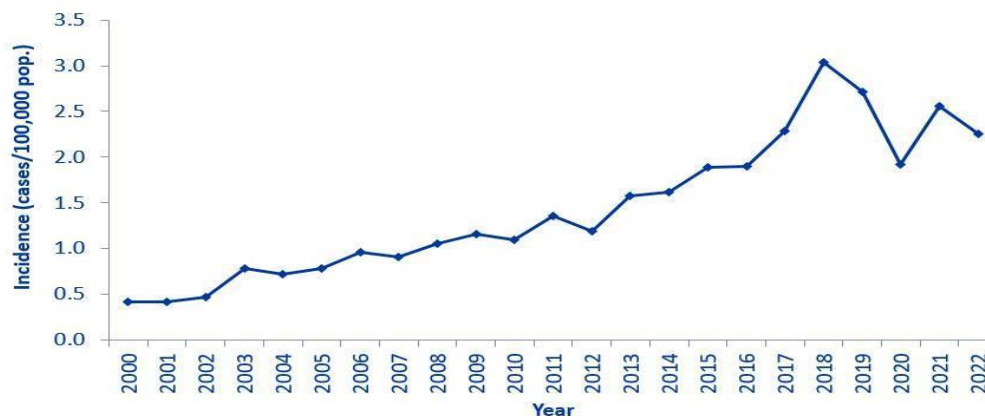


Figure 1.2 Legionnaires' disease incidence has been increasing since 2000-2022 (CDC, 2025)

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In general, reported cases of Legionnaires' disease have been increasing since the early 2000s, with a peak in 2018 (Figure 1.2). While reported cases dropped during the first year of the COVID-19 pandemic, they rebounded in 2021 (CDC, 2025).

According to the latest reports from the European Centre for Disease Prevention and Control (Legionnaires' Disease, ECDC, 2021), the highest annual notification rate of Legionnaires' disease in Europe was seen in 2021, with an incidence rate of 2.4 cases per 100,000 population. A staggering 75% of all reported cases originated from just four countries: Italy, France, Spain, and Germany. Investigations revealed that many of these infection cases were linked to water features in hospitals and hotels, such as decorative fountains and cooling towers (Lam et al., 2011). High isolation rates of *Legionella* spp. were also evident in cooling towers within hospital water systems in Spain, reaching an alarming 23.8% (Rivera et al., 2007). In 2018, a major Legionnaires' disease outbreak caused by *L. pneumophila* serogroup 1 (Lp1) occurred in Bresso, Italy, where researchers traced the outbreak back to a contaminated fountain, establishing it as the primary source responsible for the epidemic (Faccini et al., 2020). Countries such as Japan, China, and Australia have reported a notable uptick in cases of legionellosis. In Japan, national surveillance data collected since the 2000s indicate a steady rise in annual cases, surpassing 1,000 occurrences each year (Fukushima et al., 2021).

The increasing trend of legionellosis throughout the world can be attributed to the growing utilization of complex water systems in healthcare facilities, hotels, and public buildings. Several factors are considered to influence this increase, including enhanced surveillance efforts, modifications in testing and reporting protocols, an ageing population, evolving travel patterns, infrastructural developments, as well as variations in climate and weather conditions (ECDC, 2020; Rubin, 2018; Sakamoto, 2015). Factors identified to be associated with a significant risk of widespread epidemic diseases include inadequate hygienic design within the air conditioning and refrigeration systems, compounded by poor cleaning and disinfection protocols, affecting employees, visitors, and consumers of food products contaminated by *L. pneumophila*. The rising use of various water products and devices, mainly those conducive to the proliferation of *Legionella* bacteria, coupled with exposure to bioaerosols from sources like cooling towers, decorative fountains, hot tubs, and potable water systems across multiple

facilities, has led to an uptick in Legionnaires' disease outbreaks (Bentham & Broadbent, 1993; Cunha et al., 2016).

1.8 Detection methods

Identifying *L. pneumophila* poses significant challenges primarily due to the lack of reliable microbial indicators or model organisms that can correlate effectively with its presence. As a result, direct culture analysis is paramount for accurately assessing the risk of Legionellosis. Two pivotal techniques employed for monitoring disease incidence and detecting *L. pneumophila* are conventional culture and polymerase chain reaction (PCR) and other nucleic acid amplification tests, such as nested PCR, Real Time PCR (qPCR), multiplex PCR and RT-PCR (Reverse Transcription PCR), which have been recognized for their effectiveness in environmental assessments (Whiley et al., 2016).

1.8.1 Culture-based method for the detection of *L. spp.*

Diagnosing Legionnaires' disease predominantly involves detecting *L. pneumophila*, with culture analysis considered the gold standard method due to its capacity to identify a wide range of *Legionella* species (ISO 11731:2017 - Water Quality Enumeration of *Legionella*). Unlike many bacteria, it does not thrive on standard culture media, making its isolation a unique challenge.

Optimal conditions for *Legionella* include temperatures surpassing 20°C, the presence of iron, and L-cysteine, an amino acid that acts as a crucial nutrient. Based on these growth requirements, different media have been formulated for the isolation of *Legionella* from clinical and environmental samples. Scientists often utilize buffered charcoal and yeast extract agar to isolate this bacterium effectively, which creates an environment conducive to its growth. The most widely utilized medium for the isolation of *Legionella* species is Buffered Charcoal Yeast Extract (BCYE) Agar, which provides essential nutrients and a buffering system that supports the growth of these fastidious bacteria. For the effective isolation of *Legionella* from both environmental and clinical samples, the use of specialized media GVPC is recommended. GVPC Medium (Glycine Vancomycin Polymyxin Cycloheximide) is particularly useful for suppressing the growth of competing microflora while allowing for the selective isolation of *Legionella*. Additionally, the Modified Wadowsky Yee (MWY) Medium serves as an alternative that enhances the

recovery of *Legionella* from varied sources, further facilitating the identification and study of these pathogens in both laboratory settings and environmental assessments (Kusnetsov et al., 1994).

However, the process of culture analysis to detect *Legionella* is labor-intensive and can take up to ten days to yield results. Moreover, the effectiveness of traditional culture methods is significantly influenced by the physiological state of the bacterial cells, leading to variability in detection and introducing additional challenges. Another notable limitation of culture-based methodologies is their inability to detect viable but non-culturable (VBNC) bacteria (Gilpin & Gilpin, 2014) or those residing within protozoan hosts, which raises the risk of failing to identify these pathogens in water samples. Culture methods also encounter other issues, such as potential interference from competing microbiota, and concerns regarding low recovery rates and sensitivity (Collins et al., 2017). This scenario raises alarm about the possibility of undetected *Legionella* exposure, highlighting an urgent need to develop rapid and accurate testing methods.

1.8.2 Rapid Detection Methods for the *Legionella* spp.

Urine Antigen Test

Various rapid testing methodologies have been developed to address the challenges encountered in culture-based methods. This includes urinary antigen assays (Sathapatayavongs et al., 1982; Kazandjian et al., 1997; Shimada et al., 2009). While this rapid test provides a valuable tool in the diagnostic process, it often does not achieve perfect sensitivity and falls below the 100% threshold (Hayden et al., 2001). Many healthcare facilities predominantly depend on the *Legionella* urinary antigen test to diagnose cases of *L. pneumophila* (Jomehzadeh et al., 2019). This test offers substantial advantages by delivering results within hours, in stark contrast to the five days generally required for culture tests. Furthermore, obtaining a urine sample is less invasive and more straightforward than collecting a sputum sample. Nevertheless, this urinary antigen test is limited to detecting only the antigen associated with *L. pneumophila* serogroup 1 (LP1) (Kawasaki et al., 2022), which means it cannot identify infections caused by non-LP1 strains or other species of *Legionella*. Additionally, the urinary antigen test does not yield bacterial isolates, which can hinder public health officials during investigations of outbreaks of Legionnaires' disease. This limitation underscores the necessity for

complementary diagnostic approaches to enhance public health responses to potential outbreaks (Kim et al., 2022).

Latex Agglutination Test

The identification of *Legionella* species predominantly relies on serologic methods, which are widely regarded as the standard technique in this field. Among these methods, the rapid identification of *L. pneumophila* serogroup 1, as well as other serogroups, can be performed directly from culture isolates or clinical specimens. This approach has proven effective for the detection of the majority of *Legionella* strains through slide agglutination tests, as documented by researchers such as Tacker et al. (1985) and Benson et al. (1998).

However, it is essential to acknowledge the limitations associated with the Latex Agglutination test. This method necessitates the prior isolation of bacterial colonies, rendering it unsuitable for use with direct samples obtained from patients. Additionally, there is a risk of cross-reactivity with other pathogens, which underscores the need for further confirmatory testing to ensure accurate identification of the *Legionella* species (Benson et al., 1987).

Direct fluorescent antibody test

Direct fluorescent antibody (DFA) staining for *Legionella* spp. is a rapid test for respiratory samples and tissue, providing results in 2-4 hours. It uses a highly specific monoclonal antibody, though cross-reactions with *Pseudomonas* are rare, and false positives may occur in tularemia cases. The sensitivity is relatively low, between 33-70% (La Scola & Maltezou, 2004), influenced by specimen quality, organism quantity, and technician experience. A negative DFA result does not exclude a *Legionella* infection. DFA testing for *Legionella* sp. is insensitive, and clinical microbiology labs should prioritize more sensitive tests like PCR (She et al., 2007).

1.8.3 Nucleic acid amplification assays

Nucleic acid amplification assays represent a promising alternative, improving the detection of *Legionella* (Koide & Saito, 1995; Rantakokko-Jalava & Jalava, 2001; Wellinghausen et al., 2001). The genes encoding the 5S (Hayden et al., 2001) and 16S

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ribosomal subunits have distinctive signature sequences that aid in identifying *L. pneumophila* (Cloud et al., 2000). Various qPCR techniques explicitly developed for *Legionella* detection are available, focusing on either genus-specific detection based on the 16S rRNA gene or species-specific detection via the *mip* gene (Ratcliff et al., 1998; Wellinghausen et al., 2001; Benitez & Winchell, 2013; Whiley & Taylor, 2016). Unlike traditional culture methods, polymerase chain reaction (PCR) is faster and more efficient, avoiding the inherent drawbacks of culture-based techniques. This advanced detection method has effectively identified *Legionella* in lower respiratory tract samples (Bencini et al., 2007; Murdoch et al., 2013). Recent studies show that nucleic acid-based detection methods, such as *Legionella*-specific PCR, successfully identify all known species with commendable specificity and sensitivity (Diederens et al., 2008).

A PCR-based method targeting a conserved region of the 16S rRNA gene specific to *L. pneumophila* (LP) is widely used in clinical, environmental, and research settings (Jonas et al., 1995; Liu et al., 2003). This gene is highly conserved across *L. pneumophila*, but certain regions allow for species-level discrimination, especially when combined with species-specific primers (Stølhaug & Bergh, 2006). This method is sensitive than traditional culture-based methods, likely due to its ability to detect both culturable and viable but non-culturable (VBNC) bacteria (Rafiee et al., 2014). Because of its sensitivity, specificity, and speed compared to traditional culture methods (Janczarek & Palusińska-Szys, 2016). The LP16SrRNA PCR method has proven to be an efficient and accurate tool for identifying pathogenic *L. pneumophila*, making a valuable contribution to microbiological diagnostics.

16SrRNA PCR has several limitations. Firstly, it detects both living and dead bacteria, which complicates the assessment of real health risks (Yin et al., 2022). Additionally, PCR inhibitors found in complex water systems, such as cooling towers, can result in false-negative results (Yaradou et al., 2007; Morio et al., 2008).

Nested PCR has emerged as a powerful technique for the detection of *Legionella* species in both environmental and clinical samples, significantly enhancing sensitivity and specificity (Bernander et al., 1997; Catalan et al., 1997). This method is particularly advantageous in scenarios where samples are characterized by low bacterial load or the presence of inhibitory substances that may interfere with conventional PCR.

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The nested PCR process involves two distinct amplification rounds. In the initial amplification round, a broad region of the 16S ribosomal RNA (rRNA) gene is targeted, effectively capturing a wide array of bacterial sequences. This step serves to enrich the sample by increasing the overall quantity of genetic material present. Following this, the second round of amplification targets a more specific sequence associated with *L. pneumophila*, allowing for precise detection of the organism. This two-step approach not only enhances the ability to detect *Legionella* in challenging samples but also significantly improves both sensitivity and specificity compared to traditional PCR methods (Fiume et al., 2005).

Ultimately, the refined nature of nested PCR makes it an invaluable tool in microbiological diagnostics, particularly in the identification of pathogenic bacteria like *Legionella*, facilitating timely and accurate responses to potential outbreaks and infections. Despite these advancements, the absence of diagnostic tests for species other than *L. pneumophila* remains concerning and may lead to underreporting and overlooked cases of legionellosis. The global burden of disease linked to non-serogroup 1 *L. pneumophila* and other species is largely unknown but is believed to be significant (Gobin et al., 2009). Developing a more inclusive detection method that enhances identification and characterization capabilities is essential to address these challenges.

1.9 Impact of meteorological factors on the growth of *Legionella* spp.

Recent research underscores a significant relationship between climatic factors, specifically enhanced precipitation, elevated temperatures, and increased relative humidity, and Legionnaires' disease (LD) incidence (Fasano et al., 2019). Studies have shown that conditions such as higher wind speeds, atmospheric pressure, visibility, ultraviolet (UV) radiation, and prolonged sunshine duration appear to be inversely correlated with LD rates (Pampaka et al., 2022). Moreover, LD exhibits a distinct seasonal pattern, with a marked increase during the warmer months, particularly in the summer and autumn seasons (Ng et al., 2008; Karagiannis et al., 2009; Garcia-Vidal et al., 2013). Studies demonstrate that rising temperatures are closely associated with a heightened incidence of LD (Conza et al., 2013; Dunn et al., 2013; Brandsema et al., 2014). Interestingly, Karagiannis et al. (2009) found that the peak incidence of LD often coincides with humid and wet conditions, even when temperatures remain near average. This observation aligns with findings from (Fisman et al., 2005), who noted that the

environment's dampness, rather than extreme heat, significantly elevates the risk of legionellosis. In addition, elevated environmental temperatures, combined with the intermittent use of plumbing systems, create environments rich in warm, stagnant water. These conditions are particularly conducive to the growth of opportunistic aquatic pathogens, such as *Legionella* spp. (Ji et al., 2017). These environmental variables play a crucial role in shaping conditions that facilitate the proliferation of *Legionella* species and the subsequent exposure to bioaerosols, potentially explaining the rising incidence of LD observed in recent years (Beauté et al., 2020). Findings shed light on the intricate and multifaceted relationship between environmental conditions and the prevalence of Legionnaires' disease, highlighting the importance of ongoing research and monitoring as climate patterns evolve.

1.10 Prevention and Control of *Legionella* spp.

Legionella species are commonly found in various environments, particularly in freshwater bodies and artificial water systems, where they thrive under specific conditions, especially within a temperature range that supports their growth. Preventing and controlling *Legionella* therefore involves limiting its growth and transmission in man-made water systems, which are the primary sources of human infection. Effective strategies include maintaining proper water temperatures, ensuring adequate disinfection, and preventing water stagnation to inhibit the formation of biofilms where *Legionella* can thrive. Despite the efforts to improve preventive approaches, *Legionella* spp. control remains one of the most challenging issues in the water treatment industry. Legionellosis incidence is on the rise and is expected to keep increasing as global challenges become a reality (Pereira et al., 2021). Regular monitoring, risk assessments, and maintenance of cooling towers, hot water systems, and other potential reservoirs are essential components of *Legionella* spp. control programs (Mouchtouri et al., 2010)

1.10.1 Temperature Control

Water systems must implement strict guidelines for temperature and disinfectant levels to effectively manage and curb the proliferation of *Legionella* spp. Ideally, water temperatures should be kept below 20°C for cold systems and maintained above 50 °C for hot systems. In comparison, temperatures between 25°C and 45°C should be avoided to thwart *Legionella* colonies' development effectively. In contemporary water management,

strategies to prevent *Legionella* spp. dissemination are paramount, with various chemical disinfection methods extensively researched and proposed (Bonetta et al., 2018; Coniglio et al., 2018).

1.10.2 Chemical Disinfection

Chemical disinfectants, particularly oxidizing agents like chlorine, chlorine dioxide (Liu et al., 2009), monochloramine (Flannery et al., 2006) and ozone, are extensively employed for controlling *Legionella* spp. Chlorine derivatives are recognized as the most common biocides for mitigating waterborne pathogens (Walker et al., 1995; Schwartz et al., 1998; Cervero-Aragó et al., 2015). Regular water testing must complement the disinfection practices to verify that residual disinfectant levels are maintained throughout the water system, ensuring optimal efficacy against bacterial invasion.

1.10.3 Alternative Controls

Utilization of metal ions like copper or silver has been shown to exert biocidal effects when dissolved in water. Copper-silver (Cu-Ag) ionization has gained traction as an effective method for controlling *Legionella* spp. in building water systems due to its relative affordability and low maintenance requirements (Kim et al., 2002). Recent studies indicate that hydrogen peroxide is one of the most effective disinfection methods (Stavrou et al., 2020). While ultraviolet (UV) light may not achieve direct microbial lethality, it effectively impairs the DNA and proteins of microorganisms, inhibiting their ability to replicate and cause infections.

Implementing chemical and physical control measures while setting clear limits within the water systems is essential to effectively mitigate the risk of *Legionella* spp. growth. Continuous water quality monitoring is imperative to identify any shifts that may promote *Legionella* spp. growth, such as reductions in chlorine levels. Furthermore, maintaining water heaters at adequate temperature settings is critical for preventing the establishment of *Legionella* spp. colonies.

Regularly maintaining decorative fountains is crucial for minimizing debris and visible biofilm accumulation. In parallel, monitoring and maintaining adequate disinfectant levels in cooling towers and hot tubs is necessary to safeguard against microbial growth.

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Any surfaces exhibiting visible biofilm should be thoroughly cleaned to eliminate potential microorganisms. Control measures for *Legionella* spp. in food factories, as articulated by Moens (2002) emphasize the elimination of the organism, environmental manipulation to thwart colonization, and growth limitation through measures such as nutrient control and temperature regulation, as well as preventing low flow and stagnation within the system.

Moreover, limiting the quantity and types of nutrients, particularly organic materials, available within the water systems is a critical control measure. As Crespi & Ferra (1997) noted, applying chemical additives to manage scaling, corrosion, and microbial growth at well-defined and effective concentrations is essential. Adhering to these comprehensive control measures and maintaining rigorous monitoring practices can significantly minimize the risks associated with *Legionella* growth and water system contamination.

1.11 Treatment of Legionnaires' Disease

Mild Legionnaires' disease, a form of pneumonia, can be effectively treated using a single oral antibiotic regimen. Recommended antibiotics include fluoroquinolones (Giglio et al., 2015) such as levofloxacin and moxifloxacin, both known for their broad-spectrum activity against various bacterial pathogens, including those responsible for this disease. Additionally, macrolides like azithromycin and clarithromycin are practical options, offering good tissue penetration and a favorable side effect profile.

In cases where patients may not tolerate these antibiotics or when an alternative therapy is required, tetracyclines such as doxycycline serve as a viable substitute. Doxycycline, in particular, has been recognized for its efficacy in treating atypical pneumonia and can provide a robust response against the *Legionella* species. Tailoring antibiotic therapy based on the patient's health status, potential drug interactions, and local resistance patterns is essential to ensure the most effective treatment outcome.

1.12 Research perspective with current hypotheses

Infections caused by *Legionella* spp. specifically, *L. pneumophila* is increasingly recognized as a significant public health concern, and addressing it effectively is crucial due to its high rates of mortality and morbidity when left untreated. This is a growing

Introduction

concern in many countries, including underdeveloped ones like Bangladesh. With the rapid economic growth in Bangladesh, which has led to the establishment of numerous food industries, upscale hotels, modern hospitals, and high-rise buildings, there is a genuine concern about the presence of *Legionella* spp. in this artificial environment. Unfortunately, there are only a few reports on the detection of *Legionella* spp. in Bangladesh (Jahan et al., 2015; Haque et al., 2016; Mahmud et al., 2025), and no data is available on pneumonia infections caused by *L. pneumophila*, which could potentially lead to an outbreak in unhygienic conditions.

The prevalence of Legionnaires' Disease (LD) remains unknown in Bangladesh due to the lack of knowledge about the infective dose and a casual approach to the causative agents. It is crucial to regularly monitor the presence of *Legionella* spp. in processed water systems within the food industry, hospitals, and hotels. Furthermore, studying the impact of water's physicochemical parameters on their prevalence is essential. Reported cases of legionellosis increased over threefold from 2001 to 2012 in the United States, highlighting the need for improved public health research and policy development regarding legionellosis. Strengthening guidance for its prevention is essential due to its rising impact on morbidity and mortality (Parr et al., 2015). This study, therefore, focuses on various water system sites to understand the prevalence of *L. pneumophila*, with the ultimate goal of establishing a national guideline for managing the presence of *Legionella* spp. in processed water systems.

The main objective of this study is to assess the current distribution of *L. pneumophila* across environmental water sources in public facilities such as hotels, hospitals, food industries and natural surface water throughout Bangladesh, with a major focus on the capital, Dhaka. We aim to detect the prevalence of *Legionella* spp. in different potential sites in Bangladesh to prevent the outbreak of LD, as we are not adequately prepared to handle such a critical situation as a developing country. Due to the lack of resources and facilities, we want to detect *Legionella* spp. in the easiest yet specific and sensitive way possible.

1.13 Objectives

The specific objectives of the study are as follows:

- A) To detect the prevalence of *Legionella* spp. in natural and processed water systems of the food industry, hospitals, and hotel in Bangladesh
- B) To compare the conventional culture method, serological test, and PCR technique to evaluate their effectiveness in determining the prevalence of *Legionella* spp. and to identify the most suitable detection method.
- C) To assess the prevalence of the virulence factor associated with the mip gene of *Legionella pneumophila* in the collected samples.

Chapter 02:

Materials and Methods

2. Materials and Methods

Study location

The study was conducted in the Department of Microbiology, University of Dhaka.

2.1 Sample collection

A total of one hundred twenty-eight water samples were collected from two hospitals, a single hotel, and five different food industries in Dhaka City between 2019 and 2020. The main source of this water supply is the Dhaka WASA (Dhaka Water Supply and Sewerage Authority). From hospitals, food industries, and the hotel, processed water samples were collected from 13, 8, and 7 different sites, respectively, based on the permission of the respective authorities (Table 2.1, Figure 2.1).

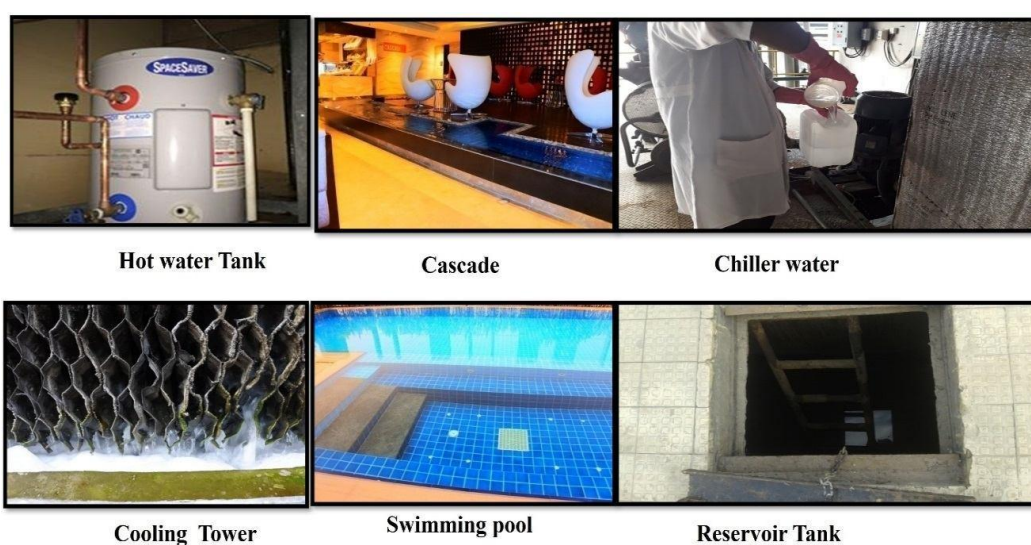


Figure 2.1 Different collection sites of water samples from the food industry (hot water tank), hospital (chiller water, reservoir tank) and hotel (cascade, cooling tower, swimming pool)

Additionally, 43 natural surface water samples were collected from different parts of Bangladesh, including Dhaka city (section 3.6.4). Water samples were collected in a sterile, non-transparent plastic bottle, maintained at an average temperature, and immediately transported to the laboratory within 2 hours.

Tracheal aspirate samples were collected from patients admitted to the intensive care unit of a tertiary care hospital in Dhaka and processed as described in section 2.7.

Table 2.1 Collection of water samples from the Hospital, Food industry and Hotel water systems in Dhaka city

Sites of water samples				
Sl. Nos.	Hospital	Food industry	Hotel	
1	ICU Tap water (2)	Reservoir water / Holding Tank (10)	Cooling Tower Water (2)	
2	Reservoir water / Holding Tank (4)	Cooling Tower water (35)	Hot Water Tank Water (2)	
3	Reverse Osmosis Dialysis water (3)	Softener water (10)	Swimming Pool Water (2)	
4	Oxygen Flowmeter water (2)	Potable water/ Drinking water (5)	Cascade water (2)	
5	Sterilize water for utensils (2)	Bottle washing water (5)	Chiller room (2)	
6	Fan Coil unit water (1)	Hot Water Tank water/Preheated water (10)	Central AC water (2)	
7	ICU Potable water/ drinking water (3)	Chiller water (5)	Potable water (2)	
8	Cooling Tower water (1)	Air conditioning water (10)		
9	Softener water (1)			
10	Inlet water (1)			
11	Air Handling water (1)			
12	Hot Water Tank water / Preheated water (1)			
13	Chiller water (2)			

Each sampling site at the hotel, hospital and food industry was inspected visually to identify the potable water source, holding tank, primary disinfection procedures, building plumbing characteristics (such as plumbing materials, construction age, and water circulation temperature), and hot water production processes (including heating systems,

tanks, and their volume). Additionally, the investigation covered cascade (decorative fountain) water, cooling towers, air-conditioning, domestic water usage, and the hospital ICU.

A detailed general survey questionnaire was developed to collect all this data.

The checklists of the water system investigation.

1. Assessment of the ability of premises personnel to control risk

Is there a responsible person appointed for *Legionella* control?

Are this person(s) and other relevant staff properly trained to control *Legionella*

Is the person competent to understand the system (s) risk factors and control measures?

2. Control measures evaluation (domestic cold and hot water temperatures, as well as biocide levels)

Is a reliable source of potable water provided by a public utility?

Is a private water supply (e.g., a well, spring, or bunkered water) used as a source of water?

Is there evidence that the hot water temperatures in the entire hot water system are always kept between 50°C and 60°C?

Is there evidence that the cold-water temperatures in the cold-water system are less than 25°C?

Are there any additional protective measures in place (such as chlorine, chlorine dioxide, copper/silver ions, and so on)?

Is there evidence that proper monitoring and effective biocide levels are maintained throughout the circuits and the outlets?

3. Assessment of other elements that may favor *Legionella* growth (poor flow/stagnancy, scale, sediments, corrosion, etc.)

Materials and Methods

Is there evidence that all taps, showers, and other water-use locations in all buildings are flushed for several minutes once a week (enough to remove any stagnant water)?

Is it required, as advised in the risk assessment, to clean, descale, and disinfect showerheads, hoses and tap filters, aerators, etc., TMVs, and sieves regularly? (The frequency varies depending on scale deposition and usage.)

Is there any pipework in the water network that has intermittent or no water flow (bypasses, dead legs, blind ends, sites that are not used or are used infrequently, etc.)?

Is there any visible or considerable deposit of silt, biofilm/slime, filth, corrosion, or scale in any component of the water network?

4. Assessment of the cleaning and disinfecting practices

Are the calorifiers inspected, drained, cleaned, and disinfected at least once a year, and when a building is not in use for an extended period, before the start of each season, and after any maintenance?

Are cold water tanks inspected, cleaned, and disinfected on an annual basis, and at the start of each season when a facility is not used throughout the year?

Is the complete water network cleansed at the start of each season when a building is not used throughout the year?

Is it necessary to disinfect water filters (sand filters, multimedia filters, etc.) and softeners regularly, at least every three months?

Are carbon filters (if used) replaced following the manufacturer's recommendations and before the start of each season?

5. Assessment of the surveillance and monitoring practices and associated documents

Is there a defined protocol in place for cleaning and disinfecting the water systems (for example, 50 ppm chlorine for an hour)?

Materials and Methods

Is a *Legionella* control program in place for each system that may represent a risk?

Is this program appropriate and sufficient for all systems on the premises that could offer a risk of Legionnaires' disease?

Are frequent records (logbooks, for example) of essential monitoring activities (temperatures, chlorine levels, etc.) kept on site?

Are these records reviewed by the person(s) in charge?

Is a risk assessment evaluation of the premises' water systems undertaken on a regular (at least every two years) basis or whenever there are changes that may affect the risk assessment (e.g., change in frequency and type of use, key personnel)?

Is the risk assessment as well as the operation of the control measures evaluated regularly by an independent and competent person?

6. Evaluation of specific water systems (spa pools, wet cooling towers) on the sites

Are there papers proving that the spa pool is checked regularly by responsible individuals?

Are there any records of biocide treatment and spa water pH monitoring?

Is there a skilled and competent individual in charge of cooling tower operation and monitoring?

Is a microbiological monitoring system in place, as well as a chemical monitoring system (e.g., chlorine or bromine treatment)?

Are the entire cooling tower and associated pipes cleaned and disinfected at least twice a year (and at the start of each season when a building is not utilized all year)?

Materials and Methods

A total of ninety water samples were meticulously collected from eight distinct sampling sites across five food industries located in Dhaka city. Each site was selected to ensure a comprehensive representation to assess potential *Legionella* spp. contamination. Information regarding the collecting water sampling sites is provided in Table 2.2. The water samples were also analyzed to evaluate various parameters within the region.

Table 2.2 Name of water samples collected from the Food industry water systems in Dhaka city

Sl. Nos.	Water Sampling Sites	No. of samples	Food Industry A	Food Industry B	Food Industry C	Food Industry D	Food Industry E
1.	Reservoir water / Holding Tank	10	4	3	3	-	-
2.	Cooling Tower water	35	3	2	3	10	17
3.	Softener water	10	-	2	8	-	-
4.	Potable water/ Drinking water	5	2	1	2	-	-
5.	Bottle washing water	5	-	-	5	-	-
6.	Hot Water Tank/ Preheated water	10	4	-	3	-	3
7.	Chiller water	5	-	-	1	1	3
8.	Air conditioning water	10	4	2	1	3	-
	Total water samples:	90	17	10	25	15	23

2.2 Treatment of samples

Many of the water samples collected from different sources, including hotels, hospitals, and the food industry, underwent treatment with disinfectants. To effectively neutralize

these disinfectants, particularly in specific contexts such as cooling tower, potable, cascade, swimming pool, softener, reservoir, and bottle washing water, each 1.0-liter sample was treated with 0.5 milliliters of a 0.1N sodium thiosulfate solution. This procedure was implemented to ensure that any residual disinfectant would be rendered inactive, thereby facilitating accurate analysis and assessment of the water quality in these critical environments (ISO 11731:2017).

2.3 Determination of the physicochemical parameters of the water sample

Physicochemical parameters of each water sample, such as pH, TDS (Total dissolved solids), salinity, and conductivity, were examined according to the standard methods for investigating water and wastewater (Standard Methods for the Examination of Water Wastewater, APHA, 24th edition (American Public Health Association)).

2.3.1 Measurement of pH

The pH of the water samples was determined using a pH meter (model: SD 50, Lovibond, Germany) according to the method outlined in Standard Methods for the Examination of Water and Wastewater (APHA). A pH meter measures the electrical potential of a pH-sensitive electrode immersed in the water sample. This electric potential is a function of the hydrogen ion activity in the sample. The pH meter (model: SD 50) device was held without a protective cap in the sample water. The device was immersed in the sample water no higher than the seal ring, and the measurement was displayed.

2.3.2 Measurement of Conductivity, Salinity, and Total Dissolved Solids (TDS)

Conductivity, Salinity, and TDS of the water samples were measured with a portable conductivity meter (model: SD 70, Lovibond, Germany), a salinity meter (model: SD 90, Lovibond, Germany), and a TDS meter (model: SD 80, Lovibond, Germany). The conductivity meter consists of a probe and a meter. Voltage is applied between two electrodes in a probe immersed in sample water. The voltage drop caused by the resistance of the water is used to calculate the conductivity per centimeter. The meter converts the probe measurement to micro-Siemens and displays the results. Salinity and TDS were measured according to the manufacturer's instructions (describe in detail). The conductivity meter (model: SD 70) device was held without a protective cap in the sample

water. The device was immersed in the sample water no higher than the seal ring, and the measurement was displayed. The salinity meter (model: SD 90) device was held without a protective cap in the sample water. The device was immersed in the sample water no higher than the seal ring, and the measurement was displayed. The meter was briefly pressed to freeze/release the measurement. It was pressed and held for 3 sec. to save the measurement. The TDS meter (model: SD 80) functioned similarly. Both Salinity and TDS concentration were expressed in mg/L as ppm (parts per million).

2.4 Bacteriological analyses of the water samples

Each water sample was also analyzed to assess the bacteriological quality. In particular, the presence of total aerobic bacterial count (TABC), total coliform count, *E. coli*, and *Salmonella sp.* was evaluated, and TABC data were analyzed by statistical software Mann-Whitney U test (Fay and Proschan, 2010).

2.4.1 Determination of Total aerobic bacterial count (TABC)

Each water sample was diluted with normal saline to perform a serial dilution, reaching concentrations up to 10^{-6} . This process was carried out to assess both the total aerobic bacterial count (TABC). For measuring the TABC, the spread plate technique was employed, where 100 μ l aliquots from the dilutions of 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} were applied in triplicate onto plate count agar plates. The chemical composition of the media and reagents used is given in Appendix -1. Following an overnight incubation period at 37°C, observations of the results were recorded.

2.4.2 Determination of coliform count

To determine the total coliform count and the presence of *E. coli*, a standardized technique was used. For measuring, the spread plate technique was employed, where 100 μ l aliquots from the dilutions of 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} were applied in triplicate onto MacConkey agar for coliform count and onto mFc agar for fecal coliform count. The plates were kept at 37°C for coliforms and at 44°C for *E. coli*. The results were then assessed to evaluate microbial presence.

2.4.3 Determination of *Salmonella* count

To detect and quantify *Salmonella*, Bismuth sulfite agar, a selective medium, was used. The liquid sample was mixed with buffer peptone water in a 1:10 dilution to create a pre-enriched sample, which was then incubated for 18 to 24 hours at 37°C. After this incubation, 100 µl of the pre-enriched culture was transferred into the selective enrichment broth, Muller-Kauffmann tetrathionate (MKTT) broth. Following an overnight incubation at 37°C, the selective enrichment broth was streaked onto selective agar plates. The plates were then incubated at 37°C for 24 to 48 hours, and the results were recorded.

2.5 Detection of *Legionella* spp. in the water samples

Following a thorough examination of the physicochemical parameters of the collected water samples, including factors such as pH levels, TDS, salinity, and conductivity, the samples were then subjected to a comprehensive detection method for *Legionella* spp. specifically *Legionella pneumophila* and detection of coliform, *E. coli*, and *Salmonella* present in the water, ensuring a detailed analysis of its overall quality and safety for public health and environmental impact. The overall methodology is summarized in the flow chart (Figure 2.2).

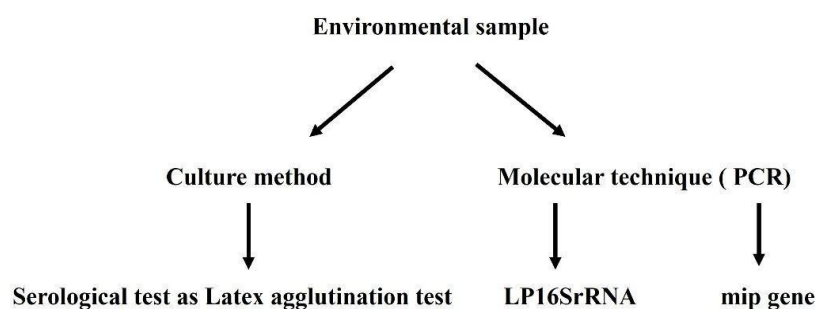


Figure 2.2 Overview of the method used to detect *Legionella pneumophila*

2.5.1 Detection of *Legionella pneumophila* using the standard culture-based method

To detect the presence of *Legionella pneumophila*, each water sample was cultured following the detection method described in Water Quality-Enumeration of *Legionella* ISO 11731:2017 (Ditommaso et al., 2022). Due to the complex nature of different sample

matrices, it was necessary to determine the appropriate method for each sample type. The choice of the method used for the enumeration of *Legionella* species depends on the origin/characteristics of the sample and the reason for sampling or investigation (Figure 2.3). An assumption was made about the expected concentration of interfering microorganisms based on the origin of the sample, and a membrane filtration concentration technique was applied for the detection of *L. pneumophila* from water samples. For highly contaminated samples and to reduce non-target bacteria that may interfere with *L. pneumophila* recovery, dilution and direct plating were conducted after the pre-treatment (heat and/or acid treatments) of the water sample. Hot water systems, such as taps and showers, can have high levels of background bacteria, including heat-adapted strains of *L. pneumophila*. Cooling tower water is often heavily contaminated with various microorganisms. In hospitals and hotels, water systems are designed to enhance specificity and prevent the overgrowth of harmful bacteria. Biofilm suspensions contain many competing organisms, making them complex environments. Industrial process water may also contain diverse microbial communities. Storage tanks and distribution systems carry the risk of stagnant water, which can lead to the development of complex microbial populations. Heat treatment was applied primarily to environmental water samples, especially those expected to contain high levels of non-*Legionella* bacteria (background flora) that may interfere with culture detection. It selectively reduces competing microorganisms while preserving viable *L. pneumophila*, which are more heat-resistant. Acid treatment was applied for selective inhibition of non-*Legionella* bacteria.

Each 1-liter 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 placed aseptically into 5 ml of sterile water, and the tube was then vortexed for one minute to loosen the bacteria into the water. This concentrate (5 ml) 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. Initially, a precise aliquot, typically encompassing 0.1 ml of the concentrated sample, was meticulously plated onto a selective medium, often enriched with cysteine to promote the growth of specific microorganisms. These plates were then carefully incubated at a controlled temperature of 35°C within a humidified chamber, maintaining a carbon dioxide atmosphere of 2.5% for a period of 7 to 10 days.

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In instances where concentrated samples exhibited an exceptionally high concentration of interfering microorganisms, a dilution process was employed, typically at ratios of 1:10 and 1:100. Before dilution, a thorough pre-treatment involving heat and acid was conducted. For the acid treatment, one volume of the water sample was combined with nine volumes of a KCl–HCl acid solution, adjusted to a pH of 2.2. This mixture was then subjected to meticulous shaking and left to incubate at room temperature for a duration of 5 min, following the methodology established by (Bopp et al., 1981).

Subsequently, for the heat treatment, the entirety of the filtered concentrated water sample was transferred into a sterile container and immersed in a water bath set at 50 °C for 30 min. To ensure accurate temperature control, smaller volumes not exceeding 5 ml were utilized, with the timing for the treatment commencing only when the desired temperature was attained. The caps of the tubes were securely tightened to prevent any evaporation of the precious water sample, as emphasized by (Maiwald et al., 1998).

In cases where a combined treatment was necessary, the heat treatment was performed first, followed by the acid treatment. It was crucial to allow the heat-treated sample to cool to room temperature before initiating the acid treatment. After these rigorous preparatory steps, after a vortex of filtrated, acid-treated, and heat-enriched water samples, 0.1 ml of each suspension was surface plated onto the same selective media used in the primary isolation phase. The plates were held at 35°C within a 2.5% CO₂ incubator and were inspected daily for seven days for signs of *Legionella* growth. Following the procedures outlined in the standard methodology, only those colonies that proliferated on BCYE agar were deemed as potential *Legionella* strains.

Presumptive *Legionella* colonies were identified based on colony morphology. Colonies that appeared round, glistening, and convex with a frosted glass appearance (CDC ,2005) was presumptively identified as *Legionella* spp. At least five suspected colonies from each plate were sub-cultured on BCYE with and without cysteine. Growth solely on cysteine-supplemented media indicates the presence of *L. pneumophila*.

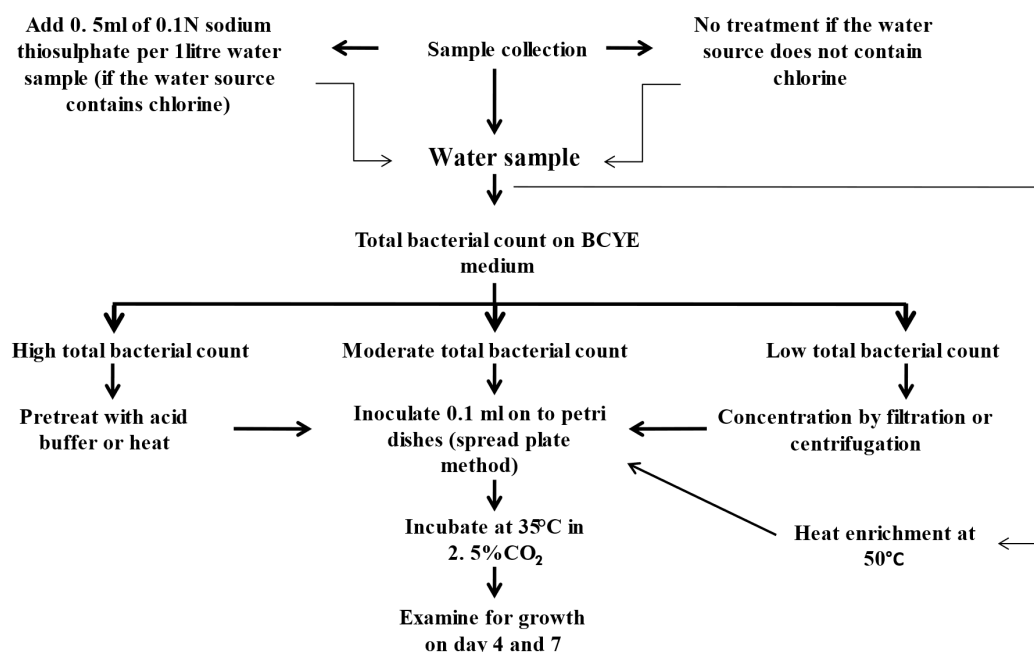


Figure 2.3 Diagram of the Overview of the procedure for the detection of *Legionella* spp. in water sample

2.5.2 Identification of *Legionella pneumophila* by the latex agglutination test

The colonies from BCYE agar that produced *Legionella*-like colonies were subjected to a latex agglutination test using the *Legionella* Latex Test Kit (LK04-Hi, Himedia) as per the manufacturer's instructions. Hi*Legionella* Latex Test Kit is a latex agglutination test designed for the confirmatory identification of *L. pneumophila* grown on selective media. The test is suitable for organisms derived from environmental sources and allows for the separate identification of *L. pneumophila* serogroup 1 and serogroups 2-15.

According to the manufacturer's instructions, each suspected *Legionella* colony was evaluated using a final agglutination test. First, 20 µl of isotonic saline (LK04d) was added to each of two wells on the agglutination slide. Then, *Legionella*-like colonies from the BCYE agar plate were added to one of the wells and mixed with the saline to create a smooth, thick, even smear. If the suspension appeared stringy or granular (often the result of old and/or mucoid cultures) on the slide, then representative colonies from the BCYE agar plate were suspended in 0.5 ml of 0.85% isotonic saline, boiled for 5 min, followed by cooling to room temperature to prepare a homogeneous turbid suspension.

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The prepared smooth suspension or the boiled suspension was placed onto each of the two wells of the agglutination slide. Subsequently, 20 µl of the *Legionella* 1 Latex Reagent (LK04a) was added to one bacterial suspension, and 20 µl of the *Legionella* serotype 2-15 Latex Reagent (LK04b) was added to the other suspension. The reagents and suspensions were mixed using clean mixing sticks for each combination, ensuring that the liquid spread evenly over the entire area of each well. The slide was gently rocked for 2 min and then observed for agglutination.

If the sample contains *L. pneumophila* antigens, an immunochemical reaction would occur, causing the latex particles to agglutinate into visible aggregates. The latex reagent coated with serogroup 1 antibodies would only agglutinate in the presence of serogroup 1 antigens. Similarly, the latex reagent coated with serogroup 2-15 antibodies would agglutinate only in the presence of antigens from any of the serogroups 2-15.

For a positive result, 20 µl of a positive control (LK04c) was added to one well of the agglutination slide, followed by 20 µl of the *Legionella* 1 Latex Reagent (LK04a). This mixture was gently rocked for 2 min and then observed for agglutination. A positive result was indicated by agglutination for *L. pneumophila* serogroup 1. The process was repeated using the *Legionella* serotype 2-15 Latex Reagent (LK04b) to check for agglutination for *L. pneumophila* serogroups 2-15. After use, the mixing sticks and slides were discarded into an appropriate disinfectant.

2.6 Detection of *Legionella pneumophila* by PCR

A widely accepted PCR-based method has been described by Jonas et al. (1995) for the detection of *L. pneumophila* from metagenomic DNA. This method amplifies a *L. pneumophila*-specific 16SrRNA gene fragment. The primers used for PCR are LPFP: (5'-AGGGTTGATAGGTTAAGAGC-3'); and LPRP: (5'-CCAACAG CTAGTTG ACATCG-3').

2.6.1 Isolation of total DNA

One liter of water was filtered with a 0.22-micron membrane filter, and the filter was washed with 10 ml of sterile deionized water. The filtered wash-off water was centrifuged at 13000 rpm for 5 min, and the pellet was resuspended in 0.1 ml of sterile deionized

water. The suspension was centrifuged for 5 min at 12000 x 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. Following incubation at 100°C for 5-10 min in a water bath, the tube was immediately chilled on ice for 30 min (Naher et al., 2022).

2.6.2 PCR reaction

PCR was performed in a final reaction volume of 25µl in a PCR tube consisting of 12.5µl of master mix (OneTaq quick load 2x Master mix, New England Biolabs), 0.5µl of each forward and reverse primer, 2µl of metagenomic DNA, and 9.5µl of nuclease-free water. PCR conditions are described in Table 2.3. Amplification was carried out in a Veriti Thermal Cycler (Applied Biosystems, USA).

Table 2.3 LP 16S PCR assay

Initial Denaturation	95°C for 5 min
Denaturation	94°C for 1.5 min (40 cycles)
Annealing	57°C for 1.5 min
Extension	72°C for 1 min
Final extension	72°C for 10 min

The amplified DNA fragments of 386bp were identified by electrophoresis in a 1.5% agarose gel with ethidium bromide.

2.6.3 Agarose gel electrophoresis

Amplification was ascertained by horizontal electrophoresis in a 1.5% agarose slab gel (based on the expected PCR product size) in Tris-acetate EDTA (TAE) buffer following the PCR process. To achieve the desired agarose gel concentration, agarose was dissolved in 1x Tris-acetate EDTA buffer and heated for approximately one minute in a microwave oven. And when the temperature came down to around 50°C, Ethidium bromide (EtBr) was added to stain the gel (4µl in 60ml TAE). Next, it was poured onto the tray that had been previously prepared with the comb and left to harden. Each gel well was filled with a 3µl aliquot of the PCR result. Based on the expected band pattern, 100 bp (Genedirex, USA) was used to ensure amplification of the desired gene with the exact product size.

The UV transilluminator (Infinity Vilber Lourmat, France) was used to see the EtBr-stained DNA bands. Photographs were taken using VisionCapt software.

2.6.4 Purification of PCR product and sequencing

With strict adherence to the manufacturer's instructions, a PCR cleaning kit was used to clean the amplified PCR products. The QIAquick PCR Purification Kit was utilized following the manufacturer's protocol to achieve a high yield of concentrated DNA suitable for downstream applications. This purification procedure effectively removes unwanted components such as primers, unincorporated nucleotides, enzymes, mineral oil, salts, and various impurities that may interfere with subsequent analyses.

5 volumes of Buffer PB were added to 1 volume of the PCR sample and mixed thoroughly. A pH indicator was incorporated into Buffer PB to check the color of the mixture; it should appear yellow. If the colour of the mixture was orange or violet, 10 µl of 3 M sodium acetate was added to make the pH 5.0, and mixed until the mixture turned yellow. The QIAquick spin column was placed in the provided 2 ml collection tube. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 30–60 s. Discarded the flow-through. That column was placed back into the same tube. For washing, 0.75 ml Buffer PE was added to the QIAquick column and centrifuged for 30–60 s. Discarded the flow-through and placed the column back in the same tube. The column was centrifuged for an additional 1 min. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of the QIAquick membrane, and the column was centrifuged for 1 min. Alternatively, for increased DNA concentration, 30 µl elution buffer was added to the centre of the QIAquick membrane, let the column stand for 1 min, and then centrifuged.

To assess the purity and concentration of the purified DNA, we employed the Nanodrop-2000 spectrometer, which allows for precise measurements of nucleic acid concentrations and provides an evaluation of sample purity through A260/A280 and A260/A230 ratios. The purified DNA was then used as a template for our nested PCR reaction, ensuring optimal efficiency and reliability in amplification.

About 0.5-100µl of the purified product was sent to MacroGen (Korea) for sequencing.

2.6.5 Analysis of sequence data

Sequence data generated by 16S rRNA gene amplification were submitted to the NCBI GenBank nucleotide sequence database for identification. Sequencing and analysis of the data were performed using the NCBI database with *L. pneumophila*. Sequences that displayed a percentage identity between 98% and 100% and had 95% to 100% query coverage were considered a perfect match.

2.7 Prevalence of *Legionella pneumophila* from tracheal aspirates

To assess the prevalence of *L. pneumophila* in pneumonia patients admitted to the Intensive Care Units (ICU), a total of fifty tracheal aspirate (TA) samples were meticulously collected from individuals receiving treatment at a tertiary care hospital in Dhaka, Bangladesh. This study was subject to a thorough ethical review process, which was granted approval by the Faculty of Biological Sciences at the University of Dhaka (Ref. No. 305/Biol.Scs). The collection of samples aimed to enhance our understanding of the role of *L. pneumophila* in respiratory infections within this vulnerable patient population, potentially providing insights that could inform clinical practices and improve patient outcomes in the future.

2.7.1 Sample Collection

A tracheal aspirate sample was collected through a tracheostomy by a trained nurse in the hospital. The procedure involved using a sterile catheter connected to a Lukens trap, which was carefully inserted into the trachea via the tracheostomy site. Suction was applied to aspirate a sample of approximately 0.5 ml to 1.0 ml into the Lukens trap. The collected sample was then placed in a sterile container containing normal saline for testing and analysis, and was transported to the laboratory immediately.

2.7.2 Processing of the sample

Each tracheal aspirate sample was diluted in Phosphate Buffered Saline (PBS) at a 1:10 ratio to ensure proper homogenization. The mixture was vortexed vigorously for a specified duration to achieve a uniform consistency, and then subjected to centrifugation

at 3000g for 10 min to separate the cellular components from the supernatant effectively. Following centrifugation, the supernatant was carefully discarded using a sterile pipette to avoid contamination, and the concentrated sediment was resuspended in PBS to facilitate further analysis. Metagenomic DNA isolation from each sample was performed following section 2.6.

2.8 Construction of phylogenetic tree

Analyzing the set of 16S rRNA sequences, the first step involved proper alignment of the sequences, which is crucial prior to constructing a phylogenetic tree. The process of aligning the 16S rRNA gene sequences was carried out using the MEGA software version 11, and then a phylogenetic tree was constructed using the maximum likelihood method. The maximum likelihood (ML) approach for the phylogenetic analysis utilized 1,000 bootstrap replicates and considered 24 nucleotide substitution models (Tamura & Nei, 1993). All evolutionary analyses were conducted within the MEGA11 software environment, ensuring robust and accurate results (Tamura et al., 2021). For the alignment of the sequences, we used the Clustal W algorithm, which allows for accurate multiple sequence alignment.

2.9 Detection of virulence gene *mip* in *Legionella pneumophila*

Macrophage Infectivity Potentiator (MIP) protein is an essential virulence factor involved in the invasion process of *L. pneumophila* into host cells (Lindsay et al., 1994). To detect the virulence potential of the detected *L. pneumophila*, the water and the TA samples that displayed the presence of these bacteria were screened for the presence of the *mip* gene (FP 5'-AAAGGCATGCAAGACGCTAT-3' and RP 5'-ACGTTGCTGGCTTAGCAGTT-3') by PCR (Ahmadrajabi et al., 2016). The primers and reaction mixture used for PCR were described in Table 2.4.

Table 2.4 Components of PCR reaction (for 25µl reaction volume)

Master Mix	12.5µl
Forward Primer	0.5µl
Reverse Primer	0.5µl
Metagenomic DNA	3µl

Nuclease-free Water	8.5µl
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The polymerase chain reaction (PCR) assay of mip gene is detailed in Table 2.5, which outlines the specific conditions and components used in the reaction. The resulting amplification product from this assay is characterized by a size of 340 base pairs (bp), indicating the successful targeting of the intended DNA sequence. This precise product size suggests a high level of specificity in the amplification process.

Table 2.5 mip gene PCR assay

Initial Denaturation	95°C for 2 min
Denaturation	94°C for 30 S (35 cycles)
Annealing	53°C for 30 S
Extension	72°C for 30 S
Final extension	72°C for 5 min

2.10 PCR product purification

With strict adherence to the manufacturer's instructions, a PCR cleaning kit was used to clean the amplified PCR products, following section 2.6.4.

Chapter 03:

Results

3. Results

3.1 Physicochemical parameters of processed water samples from the different Food industries

The physicochemical properties of processed industrial water, including pH, TDS, salinity, and conductivity, were assessed because *Legionella* bacteria can thrive in a variety of water conditions, with optimal growth not being strictly tied to any single physicochemical property like pH, TDS, salinity, or conductivity, but rather a combination of factors including sufficient temperature (often within the range of 25-45°C) and the presence of organic matter and biofilms, which are facilitated by these properties but are not directly measured by them. While extreme values for some properties can influence microbial growth, *Legionella* spp. are commonly found in warm, stagnant water systems, such as cooling towers, and their presence is more a symptom of poor maintenance and biofilm formation than a direct consequence of specific pH, TDS, salinity, or conductivity levels. *Legionella* spp. can survive a wide range of pH levels, but it tends to favour slightly acidic to neutral conditions, often between 5.5 and 7.5; however, it can also grow in alkaline water. High TDS levels can indicate a rich nutrient source for bacteria, including *Legionella* spp. The salts and minerals present in high-TDS water provide essential elements for their growth and the formation of protective biofilms. *Legionella* spp. are typically found in freshwater environments, but they can tolerate some level of salinity, especially in industrial settings where they might be present in mixed water sources. Electrical Conductivity (EC) is a measure of dissolved ions, which are the building blocks of TDS. Higher conductivity can indicate higher levels of dissolved salts and minerals, contributing to a more nutrient-rich environment where *Legionella* spp. can proliferate.

3.1.1 Physicochemical parameters of processed water from the Food Industry A

Seventeen processed water samples were collected from seven locations within the food industry A. The pH levels of these water samples ranged from 6.6 to 8.3 (Table 3.1). The acceptable pH range for drinking water is between 6.4 and 7.4, similar to the reservoir and potable water pH of 6.7 (Table 3.1). The hot water tank and AC water showed higher pH values, ranging from 8.0 to 8.3, indicating slightly alkaline conditions. The total dissolved solids (TDS) concentrations varied significantly among the samples, from 8

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ppm in domestic AC water to 541 ppm in storage water. The salinity levels in our samples varied widely, ranging from 10 ppm to 551 ppm, with the hot water tank samples consistently exhibiting the highest salinity and conductivity values.

The conductivity measurements for various water sources indicate notable differences that could inform management practices. The cooling tower water conductivity ranges from 409 to 470 μS , while the hot water tank water shows higher levels, between 954 and 994 μS . The reservoir water conductivity ranges from 680 to 747 μS , indicating moderate water quality. Domestic AC water has a low conductivity range of 11 to 12 μS . Clean room AC water exhibits conductivity between 193 and 225 μS , and potable water conductivity is recorded between 289 and 304 μS .

Table 3.1 Results of physicochemical parameters of processed water from the
Food Industry A

Sl. Nos.	Food Industry A	pH	TDS	Salinity	Conductivity
	Sampling sites		ppm	ppm	μS
1	Cooling Tower Water 1	7.8	351	254	409
2	Cooling Tower Water 2	7.7	324	250	470
3	Cooling Tower Water 3	7.4	316	238	433
4	Hot Water Tank 1	8.2	127	545	994
5	Hot Water Tank 2	8.0	115	509	954
6	Hot Water Tank 3	8.1	131	523	979
7	Hot Water Tank 4	8.2	120	551	989
8	Reservoir water 1	6.7	516	407	747
9	Reservoir water 2	6.8	450	420	680
10	Reservoir water 3	6.7	541	447	730
11	Reservoir water 4	6.6	530	398	716
12	Domestic AC water 1	7.3	8	10	12
13	Domestic AC water 2	7.3	16	11	11
14	Clean Room AC water 1	8.3	162	120	225
15	Clean Room AC water 2	8.1	150	109	193
16	Potable water 1	6.7	510	167	304
17	Potable water 2	6.6	510	170	289

3.1.2 Physicochemical parameters of processed water from the Food Industry B

In the food industry B, potable and reservoir water pH was 7.3 and 7.1, respectively, but other water samples were alkaline, 7.8 -8.5 (Table 3.2). Higher TDS ranging from (1097-1166 ppm) were observed in the reservoir tank and softener water, indicating harmful contaminants and metals. The reservoir tank and softener water were found to be of fair quality. However, the electrical conductivity ranged from 1498-1540 $\mu\text{S}/\text{cm}$ in reservoir tanks and softener water. Higher EC values indicate a higher concentration of ions, suggesting greater salinity or dissolved solids.

Table 3.2 Result of physicochemical parameters of processed water from the Food Industry B

Sl. Nos.	Food Industry B	pH	TDS	Salinity	Conductivity
	Sampling sites		ppm	ppm	μS
1	Cooling Tower Water 1	8.5	366	271	450
2	Cooling Tower Water 2	8.3	313	234	446
3	Softener Water 1	7.2	1166	820	1537
4	Softener Water 2	7.2	1109	762	1498
5	Reservoir Tank water 1	7.1	1146	814	1540
6	Reservoir Tank water 2	7.1	1065	230	420
7	Reservoir Tank water 3	7.2	1097	246	437
8	Domestic AC water	7.8	78	57	102
9	Central Packing AC water	8	64	72	96
10	Potable water	7.3	650	482	880

3.1.3 Physicochemical parameters of processed water from the Food Industry C

Processed water samples were collected from 9 different sites in the food industry C, and the highest pH levels were observed in softener water (Table 3.3). The pH of the drinking water was within the range of the Bangladesh Standards for Packaged Drinking Water. The pH of the 8 softener waters ranged between 8 and 8.3. The majority of the water samples had pH values ranging from 6.5 to 7.7. The TDS values varied widely across the samples, and the TDS levels were found below 100 in potable, AC, hot water, and bottle washing water. However, TDS levels ranged from 428 to 568 in the rest of the water samples. The salinity level was low in bottle washing and hot water tank water, where the

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highest value ranged from 344 ppm to 437 ppm. The highest salinity and conductivity values were observed in all cooling tower water samples.

Table 3.3 Results of physicochemical parameters of processed water from the Food Industry C

Sl. Nos.	Food Industry C	pH	TDS	Salinity	Conductivity
	Sampling sites		ppm	ppm	μS
1	Cooling Tower water 1	7.7	568	437	794
2	Cooling Tower water 2	7.5	549	449	781
3	Cooling Tower water 3	7.7	559	428	799
4	Softener water 1	8	457	349	652
5	Softener water 2	8.1	449	360	667
6	Softener water 3	8.3	471	352	673
7	Softener water 4	8	455	347	659
8	Softener water 5	8.2	449	358	665
9	Softener water 6	8.1	428	365	660
10	Softener water 7	8	460	351	653
11	Softener water 8	8.1	450	366	369
12	Preheater water	8.1	27	23	33
13	Holding Tank water 1	7.3	458	344	641
14	Holding Tank water 2	7.2	440	353	652
15	Holding Tank water 3	7.4	465	362	667
16	Air Conditioning water	6.9	23	87	181
17	Potable water 1	6.7	58	190	220
18	Potable water 2	6.6	69	182	200
19	Chiller water	7.1	442	346	641
20	Hot Water Tank water	6.8	72	52	90
	1				
21	Hot Water Tank water	6.5	85	68	81
	2				
22	Bottle washing water 1	6.6	95	75	134

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Sl. Nos.	Food Industry C	pH	TDS	Salinity	Conductivity
23	Sampling sites		ppm	ppm	μS
24	Bottle washing water 2	6.7	81	63	126
25	Bottle washing water 3	6.5	90	69	140
26	Bottle washing water 4	6.6	81	83	122
27	Bottle washing water 5	6.7	97	87	149

3.1.4 Physicochemical parameters of processed water from the Food Industry D

Ten processed water samples were collected from different cooling towers in the food industry D. These samples showed an alkaline pH level, high salinity, and conductivity (Table 3.4). In contrast, air-conditioning water samples have lower pH levels (from 6.4 to 6.6), making them more acidic than cooling tower water. The TDS values of cooling tower samples vary widely (ranging from 390 ppm to 1131 ppm), indicating diverse impurities or dissolved substances. Conversely, TDS levels for air conditioning water are low, typically around 105 ppm to 118 ppm. The salinity levels in cooling tower water also differ, with the highest measured at 813 μS, suggesting variations in water source quality or contamination. Air conditioning water salinity is much lower, ranging from 109 μS to 124 μS. Conductivity values for cooling tower samples vary from 219 μS to 1495 μS, illustrating the ionic content and overall water quality. In contrast, air conditioning water displays relatively low conductivity, between 157 μS to 169 μS. Among all cooling towers, the total dissolved solids (1119, 1131 ppm), salinity (800, 813 ppm), and conductivity (1487, 1495 μS) levels were highest in cooling tower DL. The differences observed in TDS, salinity, and conductivity among the cooling towers indicate varying operational or maintenance conditions.

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Table 3.4: Results of Physicochemical parameters of processed water from the Food Industry D

Sl. Nos.	Food Industry D	pH	TDS	Salinity	Conductivity
	Sampling sites		ppm	ppm	μS
1	Cooling Tower water 01 PCL	8.7	721	509	964
2	Cooling Tower water 02 PCL	8.8	735	515	873
3	Cooling Tower water 03 IM	8.7	816	625	1129
4	Cooling Tower water 04 IM	8.8	819	623	1113
5	Cooling Tower water 05 IM	8.8	390	154	219
6	Cooling Tower water 06 IM	8.5	805	611	1126
7	Cooling Tower water 02 DL	8.9	1119	813	1487
8	Cooling Tower water 03 DL	8.8	1131	800	1495
9	Cooling Tower water 12 Bakery	8.7	169	140	233
10	Cooling Tower water 14 Bakery	8.9	157	133	152
11	Air Conditioning water 1	6.6	105	109	157
12	Air Conditioning water 2	6.4	118	124	169
13	Air Conditioning water 3	6.6	110	116	163
14	Husky Chiller water	7.5	210	186	327

3.1.5 Physicochemical parameters of processed water from the Food Industry E

Twenty-three processed water samples from the food industry were collected. This included cooling tower water from five different locations, as well as chiller and hot water tank samples. The pH level of the cooling tower water was notably high at the Generator cooling tower water, 9.6, in contrast to the other samples (Table 3.5). The hot water tank pH varied between 6.6 and 6.8, while the chiller water ranged from 7.1 to 7.3. Although TDS and salinity levels were average overall, the cooling tower water displayed a wide range of conductivity, from 12.37 to 759. The hot water tanks exhibited the lowest TDS, salinity, and conductivity, with slightly acidic pH compared to chiller water. Meanwhile, chiller cooling towers had moderate to high TDS, salinity, and conductivity levels, with pH mostly neutral to slightly basic.

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Table 3.5 Results of physicochemical parameters of processed water from the Food Industry E

Sl. Nos.	Food Industry E	pH	TDS	salinity	Conductivity
	Sampling sites		ppm	ppm	μS
1	Generator Cooling Tower 1	9.5	940	7.11	12.41
2	Generator Cooling Tower 2	9.6	1020	7.97	12.37
3	Generator Cooling Tower 3	9.6	1180	8.03	12.5
4	Chips Cooling Tower 1	8	561	420	750
5	Chips Cooling Tower 2	8.2	553	411	759
6	Chips Cooling Tower 3	8.1	540	415	755
7	Hot Water Tank 1	6.8	71	53	92
8	Hot Water Tank 2	6.6	79	61	86
9	Hot Water Tank 3	6.8	85	59	96
10	Chiller water 1	7.1	441	345	643
11	Chiller water 2	7.3	431	352	649
12	Chiller water 3	7.1	447	339	632
13	Air High Compressor Cooling Tower 1	6.3	57	30	55
14	Air High Compressor Cooling Tower 2	6.1	38	27	51
15	Air High Compressor Cooling Tower 3	6.8	143	120	200
16	Air High Compressor Cooling Tower 4	6.9	130	117	214
17	Air High Compressor Cooling Tower 5	7.2	159	124	226
18	Chiller Cooling Tower 1	6.9	459	323	568
19	Chiller Cooling Tower 2	7	536	394	750
20	Chiller Cooling Tower 3	6.7	509	378	679
21	Chiller Cooling Tower 4	7.0	550	401	701
22	Chiller Cooling Tower 5	6.8	497	375	643
23	Chiller Cooling Tower 6	7.7	374	270	455

3.1.6 Comparative analysis of physicochemical parameters of processed water from different food industries

An extensive analysis was conducted on the physicochemical characteristics of various processed water samples collected from different food industries to evaluate the overall quality of the water used in these settings. Upon examination, all samples demonstrated pH values ranging between 6 and 7.5 (Figure 3.1), typically considered acceptable for most food processing operations. However, there were instances where specific samples recorded elevated pH levels.

Notably, cooling tower water from the food industry E had the highest pH level of all samples, nearing 9.6. This higher pH could be attributed to specific chemical treatments used in the cooling tower or changes in the water source. The cooling tower averages for industries A, C, and D were slightly lower, with pH readings of 7.5 and 7.6, respectively. This puts them in a more typical range, but it emphasizes the importance of monitoring to ensure they do not trend higher in alkalinity.

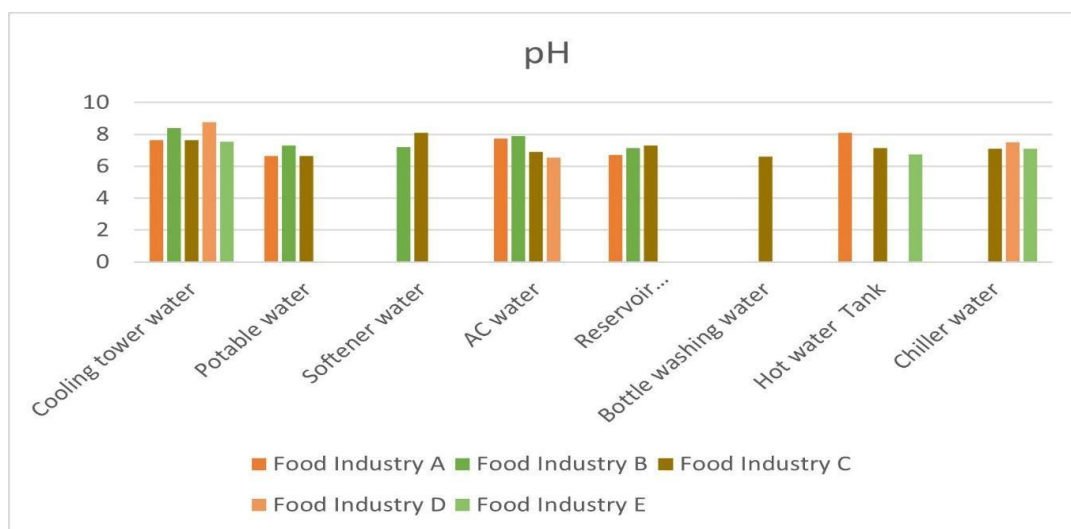


Figure 3.1 pH of different processed water samples from five food industries

In contrast, industries B and D had higher pH levels in their cooling tower systems, measuring 8.4 and 8.7, respectively. These figures indicate a potential need for more inquiry into these companies' operational practices, as elevated pH can affect scaling and corrosion processes.

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Furthermore, the average pH of chiller water used in industries C, D, and E was 7.1 and 7.5, indicating a similar consistency throughout these facilities, albeit slightly lower than their cooling tower equivalents. Overall, the water quality assessment across diverse food industries emphasizes the significance of monitoring and managing water chemistry to preserve operational efficiency and product safety.

The assessment of total dissolved solids (TDS) levels within various water samples used across different operational contexts revealed a broad range, specifically from 23 to 150 parts per million (ppm). Among these samples, the water derived from the softener and reservoir linked with food industry B had the highest TDS concentrations (Figure 3.2), indicating probable water quality management difficulties in that sector.

When TDS levels in cooling tower water were examined, it was discovered that both food companies A and B produced equivalent findings, implying that their water quality management methods are either equally successful or challenged. In contrast, a significant difference developed in the average TDS levels of chiller water from food industries C and E, which were startlingly similar (442 ppm and 447 ppm, respectively). Meanwhile, food business D reported a substantially lower average TDS level of only 210 ppm, which was roughly half the value recorded in industries C and E.

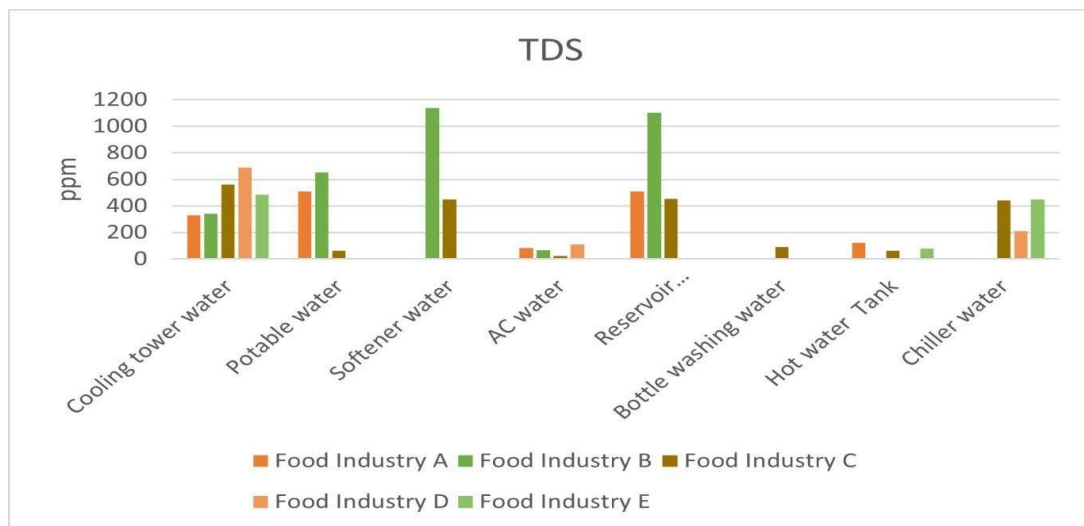


Figure 3.2 TDS of different processed water samples from five food industries

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Significant findings were observed in an analysis of salinity levels across five distinct food industries, particularly concerning the water used in softeners and hot water tanks. Industry B exhibited the highest salinity levels in its softener and hot water tank water, while Industry A also recorded elevated salinity in its hot water tank (Figure 3.3). This heightened salinity in Industry B raises concerns about the efficiency of its water treatment processes, particularly in the softening phase.

In contrast, water samples from other sources within the same industries, including air conditioning (AC) systems, bottle washing facilities, and hot water tanks, exhibited markedly low salinity levels. Additionally, salinity measurements of reservoir water remained consistent across the three industries analyzed, indicating a uniform quality in this source.

When evaluating the average salinity levels among Industries A, B, and E, it was noted that they were relatively similar, suggesting a potential standardization in water treatment processes across these locations. Furthermore, Industries B and D also demonstrated comparable salinity values. The consistency in reservoir water salinity across the various food industries implies that they may draw from the same or similar water sources or employ uniform treatment methodologies.

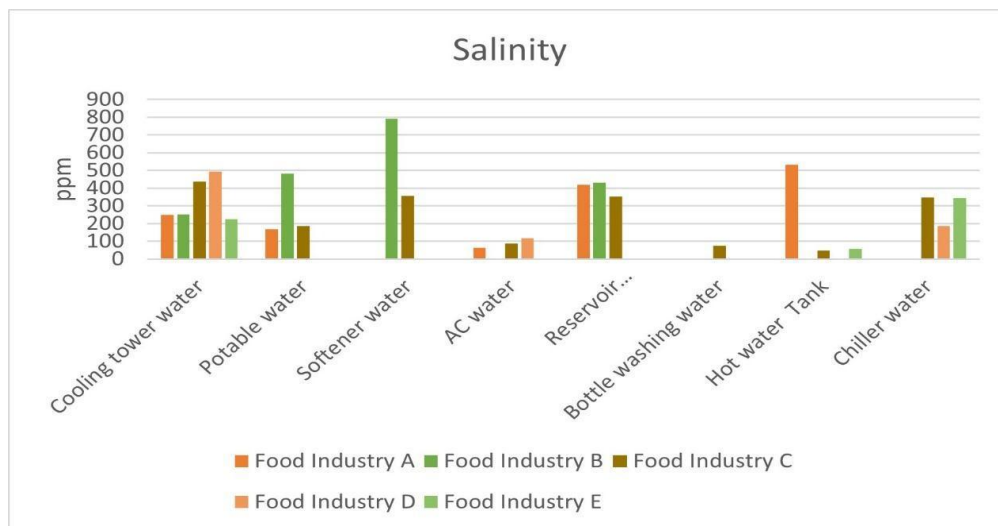


Figure 3.3 Salinity of different processed water samples from five food industries

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In the food industry B, the water softener was found to have the highest conductivity, with an impressive average reading approaching 1,500 micro-Siemens (Figure 3.4). This indicates a significant concentration of dissolved salts and minerals in the water, which could impact operations. In contrast, the cooling towers across food industries A, B, and E displayed nearly identical conductivity levels, suggesting a uniformity in water quality or treatment processes among these facilities. Meanwhile, food industries C and D exhibited comparable but somewhat lower conductivity readings, indicating potential differences in water treatment or usage practices.

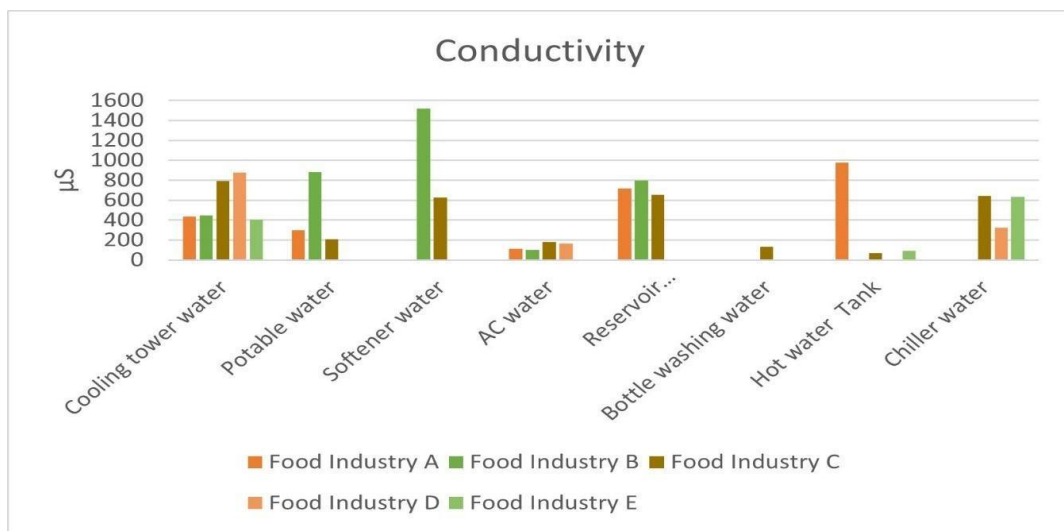


Figure 3.4 Conductivity of different processed water samples from five food industries

When examining the conductivity of reservoir tank water, food industries A and B showed similar measurements, which may reflect consistent water sourcing or treatment methods. In contrast, food industry C reported slightly lower conductivity, possibly hinting at variations in water composition or treatment efficacy.

Regarding chiller water, the results for pH, total dissolved solids (TDS), and salinity were notably consistent across all food industries. Specifically, food industries C and E recorded the same levels for these parameters, indicating a shared approach or similar environmental conditions affecting their cooling systems. However, the food industry D's measurements were significantly lower, showing only half the values of its counterparts. This may suggest unique treatment processes or operational adjustments that could significantly affect the water chemistry.

3.2 Microbiological analysis and identification of processed water from food industries

3.2.1 TABC (Total Aerobic Bacterial Count) of processed water from the Food Industry A

Industrial facilities, with their large, complex water systems, are especially sensitive to microbial growth. These systems often provide the ideal conditions for opportunistic pathogens to flourish warm temperatures, stagnant water, and the presence of biofilms. Examining conventional fecal indicators is the most common practice in evaluating potable water quality in engineered water systems. However, fecal indicators indicate only the presence of enteric pathogens and/or fecal contaminants and cannot infer the presence and concentrations of water-based opportunistic pathogens. This study collected and evaluated five industrial facilities' water for microbial contamination, including *Legionella* spp. an opportunistic pathogen. In total, 17 water samples were collected from six locations, including cooling tower water, hot water tank water, reservoir tank water, domestic AC outflow water, clean room AC outflow water, and potable water of food industry A. All these waters were distributed through pipelines to the point necessary from the reservoir tank, and the microbiological quality and opportunistic pathogens of these waters were evaluated. The results of industry A showed that all of the water exceeded the maximum allowable Total Aerobic Bacterial Count (TABC), with TABC values ranging from 3.2 to 4.8 log CFU/ml (Table 3.6). The highest TABC count was found in AC outflow water (4.4-4.8 log CFU/ml), while the lowest was found in the hot water tank (3.2-3.5 log CFU/ml). This could be due to the temperature, severely injuring the bacterium, or the duration, which eventually inactivates the bacteria.

The TABC count in the reservoir tank ranged between 3.9 and 4.2 log CFU/ml; however, higher levels of TABC were detected in the cooling tower, AC outflow water, and potable water, which might be attributed to the existence of biofilms within the pipeline, which serve as nutrients for bacteria to thrive. However, no coliform or *E. coli* were found in any of the 17 water samples analyzed.

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Table 3.6 TABC of processed water from the Food Industry A

Food Industry A	TABC	TCC	<i>E. coli</i>	<i>Salmonella</i> sp.
Sampling Sites	Log CFU/ml			
Cooling Tower Water 1	4.30	ND*	ND	ND
Cooling Tower Water 2	4.47	ND	ND	ND
Cooling Tower Water 3	4.54	ND	ND	ND
Hot Water Tank 1	3.17	ND	ND	ND
Hot Water Tank 2	3.30	ND	ND	ND
Hot Water Tank 3	3.47	ND	ND	ND
Hot Water Tank 4	3.39	ND	ND	ND
Reservoir water 1	3.92	ND	ND	ND
Reservoir water 2	4	ND	ND	ND
Reservoir water 3	4.17	ND	ND	ND
Reservoir water 4	4.11	ND	ND	ND
Domestic AC water 1	4.60	ND	ND	ND
Domestic AC water 2	4.77	ND	ND	ND
Clean Room AC water 1	4.41	ND	ND	ND
Clean Room AC water 2	4.60	ND	ND	ND
Potable water 1	4.39	ND	ND	ND
Potable water 2	4.54	ND	ND	ND

ND*: Not Detected

3.2.2 TABC of processed water from the Food Industry B

In the context of the food industry B, a comprehensive analysis of water samples revealed significant variations in Total Aerobic Bacterial Count (TABC count) across different water sources (Table 3.7). The reservoir tank water demonstrated the highest levels, with TABC counts fluctuating between 5.60 and 5.77 log CFU/ml. This indicates a considerable bacterial presence, highlighting potential concerns for water quality in this area.

Pathogenic enteric bacteria were tested among the cooling tower water samples, and *Escherichia coli* (*E. coli*) was identified in only one sample. The TAB count for this

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specific sample ranged from 4.84 to 4.90 log CFU/ml, suggesting isolated contamination that could pose health risks if not addressed.

Further inspection of two air conditioning (AC) water systems yielded TABC counts measured at 3.0 log CFU/ml each. This level suggests a moderate bacterial presence, warranting monitoring to ensure that it remains within safe limits. In contrast, the potable water supply indicated a TABC count of 3.6 log CFU/ml, which is relatively acceptable for drinking water standards but still highlights the need for regular checks on water hygiene.

The softener water exhibited the lowest TABC count among the samples analyzed, with values ranging from 1.90 to 2.07 log CFU/ml. This suggests that the softening process may be effective in reducing bacterial presence in this type of water. Importantly, throughout the testing process, no total coliforms, *E. coli*, or *Salmonella* were detected in any of the water samples analyzed, which is a positive outcome and suggests that, despite the elevated TABC counts in certain samples, there were no indications of harmful pathogens that could affect health. This information underscores the need for ongoing monitoring to ensure the safety and quality of water used within the food industry.

Table 3.7 TABC of processed water from the Food Industry B

Food Industry B	TABC	TCC	<i>E. coli</i>	<i>Salmonella</i> sp.
Sampling Sites	Log CFU/ml			
Cooling Tower Water 1	4.84	ND*	1	ND
Cooling Tower Water 2	4.90	ND	ND	ND
Softener Water 1	1.90	ND	ND	ND
Softener Water 2	2.07	ND	ND	ND
Reservoir Tank water 1	5.77	ND	ND	ND
Reservoir Tank water 2	5.60	ND	ND	ND
Reservoir Tank water 3	5.69	ND	ND	ND
Domestic AC water	3.00	ND	ND	ND
Central Packing AC water	3.00	ND	ND	ND
Potable water	3.60	ND	ND	ND

ND*: Not Detected

3.2.3 TABC of processed water from the Food Industry C

In assessing water quality within the food industry C, the chiller water sample stood out as the most problematic, exhibiting alarmingly high levels of microbial contamination. The Total Aerobic Bacterial Count (TABC) for the other water samples varied, with values ranging from 1.30 to 3.95 log CFU/ml, indicating noticeable microbial presence (Table 3.8). In contrast, the bottle-washing water samples demonstrated remarkably low contamination, remaining below 10 CFU/ml.

The softener water samples also reflected moderate microbial levels, with TABC readings fluctuating between 1.3 and 2.39 log CFU/ml. Consistency was observed in the three cooling tower samples, which yielded TABCs that hovered between 1.9 and 2.11 log CFU/ml, indicating a stable level of microbial activity. Additionally, the TABC levels for reservoir, potable, and AC water samples were notably higher, ranging from 3.0 to 3.95 log CFU/ml, highlighting potential concerns regarding water safety and hygiene in these areas.

Table 3.8 TABC of processed water from the Food Industry C

Food Industry C	TABC	TCC	<i>E. coli</i>	<i>Salmonella</i> sp.
Sampling Sites	Log CFU/ml			
Cooling Tower water 1	2	1.47	ND*	ND
Cooling Tower water 2	1.90	ND	ND	ND
Cooling Tower water 3	2.11	ND	ND	ND
Softener water 1	1.30	ND	ND	ND
Softener water 2	1.90	ND	ND	ND
Softener water 3	2	ND	ND	ND
Softener water 4	1.69	ND	ND	ND
Softener water 5	2.30	ND	ND	ND
Softener water 6	1.77	ND	ND	ND
Softener water 7	2.17	ND	ND	ND
Softener water 8	2.39	ND	ND	ND
Preheater water	2.95	ND	ND	ND
Reservoir Tank water 1	3.25	ND	ND	ND
Reservoir Tank water 2	3.47	ND	ND	ND

Results

Food Industry C	TABC	TCC	<i>E. coli</i>	<i>Salmonella</i> sp.
Sampling Sites	Log CFU/ml			
Reservoir Tank water 3	3.07	ND	ND	ND
Air Conditioning water	3	ND	ND	ND
Potable water 1	3.95	ND	ND	ND
Potable water 2	3.90	ND	ND	ND
Chiller water	4.30	ND	ND	ND
Hot Water Tank 1	3.04	ND	ND	ND
Hot Water Tank 2	2.90	ND	ND	ND
Bottle washing water 1	<10	ND	ND	ND
Bottle washing water 2	<10	ND	ND	ND
Bottle washing water 3	<10	ND	ND	ND
Bottle washing water 4	<10	ND	ND	ND
Bottle washing water 5	<10	ND	ND	ND

ND*: Not Detected

3.2.4 TABC of processed water from the Food Industry D

Among the five food sectors analyzed, water samples from the food industry demonstrated the highest total aerobic bacterial counts (TABC) levels. In particular, the cooling tower associated with Food Industry D exhibited the most significant elevation in TABC levels. Fourteen water samples were systematically collected from three different sites, revealing that ten cooling towers recorded TABC values ranging from 3.60 log CFU/ml to 5.17 log CFU/ml (Table 3.9). Notably, in the samples collected from cooling tower 03 DL, the total aerobic count was categorized as too numerous to count (TNTC), indicating an extremely high bacterial presence.

Additionally, the TABC measured in the water sample from the air conditioning unit surpassed that of all other food industry samples, with recorded values between 5.07 and 5.32 log CFU/ml. This finding underscores the concerning levels of microbial contamination in the food industry's cooling and air conditioning systems, highlighting potential implications for food safety and sanitation practices.

Results

Table 3.9 TABC of processed water from the Food Industry D

Food Industry D	TABC	TCC	<i>E. coli</i>	<i>Salmonella sp.</i>
Sampling sites	Log CFU/ml			
Cooling Tower water 01 PCL	5.17	ND*	ND	ND
Cooling Tower water 02 PCL	4.77	ND	ND	ND
Cooling Tower water 03 IM	3.77	ND	ND	ND
Cooling Tower water 04 IM	3.60	ND	ND	ND
Cooling Tower water 05 IM	4	ND	ND	ND
Cooling Tower water 06 IM	3.69	ND	ND	ND
Cooling Tower water 02 DL	4.07	ND	ND	ND
Cooling Tower water 03 DL	TNTC	ND	ND	ND
Cooling Tower water 12 Bakery	4.17	ND	ND	ND
Cooling Tower water 14 Bakery	4.30	ND	ND	ND
Air Conditioning water 1	5.14	ND	ND	ND
Air Conditioning water 2	5.32	ND	ND	ND
Air Conditioning water 3	5.07	ND	ND	ND
Husky Chiller water	3.47	ND	ND	ND

ND*: Not Detected

3.2.5 TABC of processed water from the Food Industry E

In the food industry E, the microbial count in the chip cooling tower water peaked at 4.90 log CFU/ml (Table 3.10). Interestingly, AHC CT2, AHC CT3, and AHC CT5 had no microbial presence, while AHC CT1 and AHC CT4 displayed TABC. Overall, AHC samples tended to show lower counts than chips and chiller samples. The lowest TABC load in all water samples was recorded at 2.0 log CFU/ml, highlighting the variability of TABC levels among the different cooling tower water samples. Chiller CT3 was classified as TNTC (Too Numerous to Count), indicating an extremely high microbial concentration that surpasses the counting limit. Moreover, the TABC in chiller water ranged from 3.43 to 3.69 log CFU/ml, while TABC levels in three hot water tanks were between 2.9 and 3.11 log CFU/ml. All TCC, *E. coli*, and *Salmonella sp.* results were reported as ND (Not Detected) for the tested samples.

Results

Table 3.10 TABC of processed water from the Food Industry E

Food Industry E	TABC	TCC	<i>E. coli</i>	<i>Salmonella sp.</i>
Sampling Sites	Log CFU/ml			
Generator Cooling Tower 1	4.60	ND*	ND	ND
Generator Cooling Tower 2	4.30	ND	ND	ND
Generator Cooling Tower 3	4.47	ND	ND	ND
Chiller Cooling Tower 1	4	ND	ND	ND
Chiller Cooling Tower 2	3.90	ND	ND	ND
Chiller Cooling Tower 3	TNTC	ND	ND	ND
Chiller Cooling Tower 4	3.69	ND	ND	ND
Chiller Cooling Tower 5	3.95	ND	ND	ND
Chiller Cooling Tower 6	3.84	ND	ND	ND
Air High Compressor Cooling Tower 1	2.47	ND	ND	ND
Air High Compressor Cooling Tower 2	<10	ND	ND	ND
Air High Compressor Cooling Tower 3	<10	ND	ND	ND
Air High Compressor Cooling Tower 4	2	ND	ND	ND
Air High Compressor Cooling Tower 5	<10	ND	ND	ND
Chips Cooling Tower 1	4.84	ND	ND	ND
Chips Cooling Tower 2	4.77	ND	ND	ND
Chips Cooling Tower 3	4.90	ND	ND	ND
Hot Water Tank 1	2.90	ND	ND	ND
Hot Water Tank 2	3.11	ND	ND	ND
Hot Water Tank 3	3	ND	ND	ND
Chiller water 1	3.69	ND	ND	ND
Chiller water 2	3.43	ND	ND	ND
Chiller water 3	3.51	ND	ND	ND

ND*: Not Detected

3.2.6 Comparative analysis of TABC of processed water from different Food Industries

Cooling tower water serves a vital function in maintaining optimal temperature control across various operations within the food industry. Among the five food industries examined, Food Industry B had the predominant TABC in cooling tower water, having 4.87 log CFU/ml. In contrast, Food Industry C represented the lowest TABC, with 2 log CFU/ml (Figure 3.5).

Moreover, potable water, critical for human consumption and various operational needs, is universally essential across all food sectors. For instance, Food Industry A recorded a significant TABC 4.47 log CFU/ml level in its potable water supply. In contrast, Food Industry B showed a slightly lower contamination at 3.6 log CFU/ml.

Water softeners are integral in treating hard water to mitigate scale buildup. TABC was particularly prevalent in Food Industries C and D. The Total Aerobic Bacteria Count (TABC) for Industry C was measured at 1.99 log CFU/ml. In comparison, Industry D registered a marginally lower count of 1.94 log CFU/ml, indicating effective management practices in water treatment.

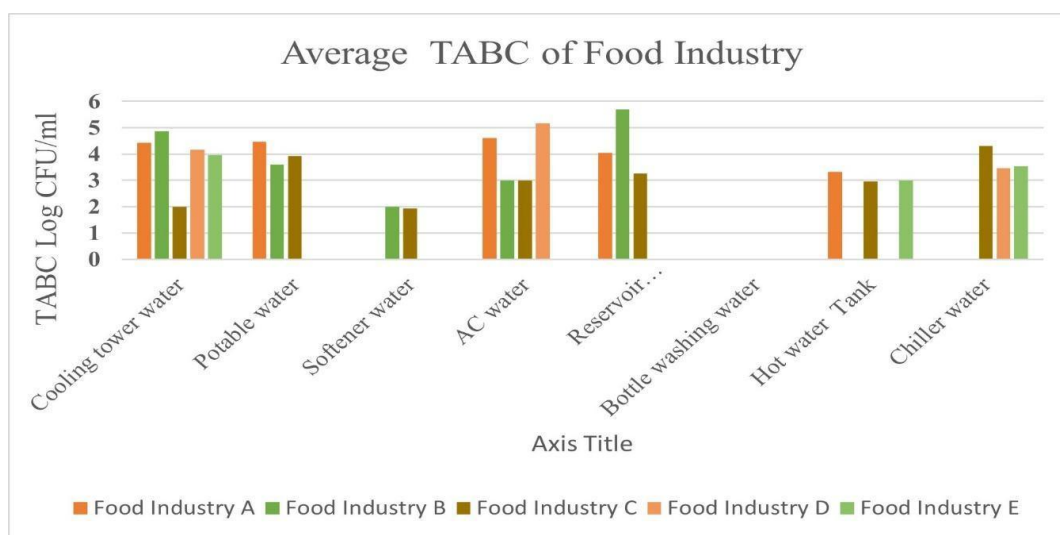


Figure 3.5 Overview of the total aerobic bacterial count of the processed water samples of different food industries

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The importance of air conditioning (AC) water also cannot be overstated, especially in more extensive industrial facilities or warmer climates. Food Industry A reported a Total Aerobic Bacteria Count of 4.6 log CFU/ml for its AC water, whereas Industry D recorded a notably higher level at 5.17 log CFU/ml.

There is considerable variation across the industries when examining storage water, which encompasses water held in reservoir tanks. Industry A reported a count of 4.05 log CFU/ml, which is significantly lower than the peak measurement of 5.69 log CFU/ml found in Food Industry B's reservoir tank. This highlights discrepancies in water quality standards and storage practices among the industries.

On average, the Total Aerobic Bacteria Count across these food industries falls between 3 and 4.5 log CFU/ml, with Food Industry B exhibiting the highest TABC measurement in its reservoir tanks. Furthermore, other types of water, such as those used in hot water tanks and chillers, reveal a more significant degree of variability, indicating the complex water management needs within the food industry sector.

3.3 Physicochemical and microbiological analysis of processed water samples from the Hospital

A total of sixteen processed water samples were collected from Hospital 1 in 2019 and 2020, and 8 from Hospital 2 (2019), to compare water quality and determine whether there is an association between water quality and *Legionella pneumophila* and other pathogens.

3.3.1 Physicochemical parameters of processed water from Hospital 1

The physicochemical parameters of hospital-processed water samples remained broadly consistent, with slight variations. In Hospital 1, the pH of the chiller room, flow meter, holding tank, and RO dialysis water increased in 2020 (Table 3.11) compared to 2019. The pH of ICU tap water and drinking water was within the limit of the Bangladesh standards 1240:2021 for packaged drinking water. In total dissolved solids (TDS), variation was observed for RO dialysis water, flow meter water, and ICU drinking water values between 2019 and 2020, while the rest did not display any significant variation. Compared to 2019, salinity results showed a significant decrease in ICU tap water,

Results

reservoir water, and RO dialysis water in 2020, while an increase in ICU drinking water in 2020 was evident; however, the rest of the samples did not exhibit any remarkable variation. The conductivity of ICU tap water, reservoir water, RO dialysis water, and ICU drinking water decreased in 2020. In particular, RO dialysis water had significant variations in all tested parameters. Conductivity also showed considerable variability.

Table 3.11 Results of physicochemical parameters of processed water from Hospital 1 (2019 and 2020)

Hospital 1	pH		TDS		Salinity		Conductivity	
Sampling Sites			ppm		ppm		μS	
	2019	2020	2019	2020	2019	2020	2019	2020
Chiller Room	6.7	7.7	194	182	143	141	257	254
ICU Tap water	7.4	6.9	231	205	290	165	417	288
Reservoir water	7.0	7.8	197	183	250	149	455	252
Holding Tank	7.5	6.8	204	203	156	156	287	280
water								
RO Dialysis	5.2	7.2	36	11	50	18	82	12
water								
Flow meter water	8.8	9.3	418	78	ND	45	ND	163
Sterilize water	8.7	8.6	155	150	124	122	226	209
ICU Drinking	7.4	7.0	100	213	82	161	142	300
water								

3.3.2 Physicochemical parameters of processed water from Hospital 2

In the analysis conducted on the processed water samples from Hospital Water 2, the pH level of the air-handling water was significantly elevated compared to the other water samples tested, as detailed in Table 3.12. Notably, the pH values for the potable water were within the acceptable standard limits established by the Bangladesh Standards and Testing Institution (BSTI) for packaged drinking water.

Further examination revealed notable differences in other water quality parameters. The total dissolved solids (TDS), salinity, and conductivity levels were markedly higher in the

Results

cooling tower water, indicating potential issues with water quality that may need addressing. In contrast, air-handling and reverse osmosis (RO) water demonstrated considerably lower levels for these parameters, suggesting higher purity.

It's important to note that, due to the limited number of water samples collected from the Fan Coil Unit (FCU), comprehensive TDS, salinity, and conductivity measurements could not be performed for that specific water type. This limitation highlights the need for increased sampling to ensure a more thorough assessment of water quality across all sources within the hospital.

Table 3.12 Results of physicochemical parameters of processed water from Hospital 2 (2019)

Hospital 2	pH	TDS	Salinity	Conductivity
Sampling Sites		ppm	ppm	μS
Cooling Tower water	8.3	693	598	1046
Softener water	7.3	227	177	319
Inlet water	7	217	169	306
Air Handling water	9.1	25	28	38
RO water	6.2	36	32	48
Potable water	7.1	280	181	330
FCU water	7.3	ND	ND	ND
Hot Water Tank water	7.1	242	192	349

3.4 Microbiological analysis and identification of processed water from Hospital 1 and Hospital 2

A comprehensive study conducted in 2019 and 2020 systematically collected sixteen processed water samples to assess the total aerobic bacterial count (TABC) across various water sources within the hospital premises.

In 2019, Hospital 1 recorded a significantly higher total aerobic bacterial count (TABC) in all the water samples tested compared to 2020, with some values higher in 2019, while others were higher in 2020.

Results

In Hospital 1, compared to 2019, three specific categories of water: tap water from the Intensive Care Unit (ICU), Reverse Osmosis (RO) dialysis water, and water from the flow meters displayed an increase in TABC levels in the year 2020 (Table 3.13). Specifically, the TABC measured in the Intensive Care Unit (ICU) tap water was 3.0 log CFU/ml. However, in 2020, the TABC levels had escalated to the point that they were classified as too numerous to count (TNTC), suggesting a concerning increase in microbial presence. On a positive note, the TABC levels observed in the chiller water and holding tank were lower in 2020, indicating some improvement in those areas.

The most concerning finding among these samples was the extraordinarily high TABC load detected in the oxygen flow meter water used in the ICU during 2019, where 2020 also showed TNTC. Furthermore, significant fluctuations in TABC levels were recorded in several other water sources, including those from the chiller room, holding tank, and the drinking water provided in the ICU. In contrast, the reservoir and sterilizer water exhibited minimal variations in their TABC levels, suggesting a more stable microbiological quality in those sources. Water samples collected from Hospital 1 lacked detectable total coliform counts, indicating a favorable microbial profile. Additionally, there were no indications of pathogenic organisms such as *E. coli* or *Salmonella* in the samples, reassuring for patient safety and health standards. This data highlights the ongoing need for rigorous water quality monitoring in healthcare settings.

Table 3.13 TABC of processed water from Hospital 1 (2019 and 2020)

Hospital 1	TABC		TCC		<i>E. coli</i>		<i>Salmonella sp.</i>	
	Log CFU/ml							
Sampling Sites	2019	2020	2019	2020	2019	2020	2019	2020
Chiller Room	4.77	3.47	ND	ND	ND	ND	ND	ND
ICU Tap water	3.00	TNTC	ND	ND	ND	ND	ND	ND
Reservoir water	3.69	3.84	ND	ND	ND	ND	ND	ND
Holding Tank	5.07	3.34	ND	ND	ND	ND	ND	ND
RO Dialysis water	3.47	TNTC	ND	ND	ND	ND	ND	ND
Flow meter water	5.11	TNTC	ND	ND	ND	ND	ND	ND
Sterilizer water	3.30	3.90	ND	ND	ND	ND	ND	ND
ICU Drinking water	4.17	3.54	ND	ND	ND	ND	ND	ND

Results

The water sample from the hot water tank of Hospital 2 showed a low total aerobic bacterial count (TABC) in (Table 3.14), which may be attributed to the maintenance of high temperatures between 50°C and 60°C. Furthermore, the TABC of the Reverse Osmosis (RO) water, potable water, inlet, and air handling water was less compared to the high count of cooling tower water, softener water, and the Fan Coil Unit (FCU) water. None of the water samples tested positive for total Coliform, *E. coli*, or *Salmonella*. These findings are significant as they indicate potential health risks and the need for further investigation.

Table 3.14 TABC of processed water from the Hospital 2

Hospital 2	TABC	TCC	<i>E. coli</i>	<i>Salmonella</i> sp.
Sampling Sites	Log CFU/ml			
Cooling Tower water	4.30	ND	ND	ND
Softener water	4.60	ND	ND	ND
Inlet water	3.30	ND	ND	ND
Air Handling water	3.17	ND	ND	ND
RO water	3.0	ND	ND	ND
Potable water	3.30	ND	ND	ND
FCU water	4.30	ND	ND	ND
Hot Water Tank water	2.77	ND	ND	ND

3.5 Physicochemical and microbiological analysis of processed water samples from the Hotel

3.5.1 Physicochemical analysis of processed water from the hotel

Fourteen processed water samples were collected in two consecutive years, 2018 and 2019. In 2018, an analysis was conducted on seven water samples collected from different sources: cooling tower water, swimming pool water, cascade water, and chiller room water. The findings revealed that four of these samples exhibited alkaline properties, meaning they had a higher pH level than neutral. Notably, the hot water tank and the potable water samples recorded a pH level that conformed to neutrality, as illustrated in Table 3.15. According to the guidelines set forth by the Bangladesh Standards of Drinking Water, the pH levels observed in potable water fell well within the permissible limits, indicating a level of safety for consumption. Furthermore, the World

Results

Health Organization (WHO) recommends maintaining drinking water's total dissolved solids (TDS) levels at less than 300 ppm. TDS plays a critical role in assessing water quality, as it offers important insights into the overall chemical composition of the water. Elevated TDS levels can serve as a red flag, suggesting the possible presence of harmful contaminants, including heavy metals, which pose severe risks to human health.

To ensure the safety of drinking water, water treatment plants employ a range of techniques to reduce TDS levels. Commonly used methods include reverse osmosis, distillation, and ion exchange. These processes effectively eliminate dissolved solids from the water, resulting in a product that is not only cleaner but also safer for human consumption. A notable trend observed from 2018 to 2019 was increased TDS levels within the potable water samples. Specifically, the TDS value in cooling tower water experienced a significant rise when comparing the two years. The analysis also indicated that the salinity and conductivity levels in cooling tower water had doubled in 2019 compared to those recorded in 2018, emphasizing the need for ongoing monitoring and management of water quality to mitigate potential health risks associated with elevated TDS and its implications.

The TDS, salinity, and conductivity were lower in swimming pool water in 2018. However, these parameters showed no remarkable changes in the cascade, chiller room, and potable water during sampling in 2018 and 2019. The most notable change was observed in cooling towers and AC water, frequently reported as the most common sources of *Legionella pneumophila*. Cooling towers can create ideal conditions for microbial growth, including *Legionella*. In AC water, all physicochemical parameters were almost three times higher in 2019, except in 2018, when pH was increased. Hot water tank and swimming pool water were observed in the other three parameters, and the results did not change significantly during these two collection times. These changes in the physicochemical parameters of the water samples from the Hotel indicate potential health risks and the need for ongoing monitoring and management of water quality.

Results

Table 3.15 Results of physicochemical parameters of processed water from the Hotel 2018 and 2019

Hotel	pH		TDS		salinity		Conductivity	
			ppm		ppm		μS	
Sampling Sites	2018	2019	2018	2019	2018	2019	2018	2019
Cooling Tower Water	8.8	7.6	240	570	1.81	3.98	3.05	6.41
Hot Water Tank Water	7.1	6.7	179	221	144	169	261	305
Swimming Pool Water	8.1	7.1	256	318	218	245	398	449
Cascade water	8.1	8.0	227	253	179	194	330	352
Chiller room water	8.1	8.1	137	210	122	121	210	230
AC water	7.9	6.6	52	150	42	130	72	222
Potable water	7.4	7.5	186	215	143	162	251	296

3.5.2 Microbiological analysis and identification of processed water from the hotel

A total aerobic count was performed to check the microbial concentration in all water samples and the total coliform count for fecal contamination. *E. coli* and *Salmonella*, two of the most prevalent bacteria in municipal water systems in Bangladesh, were also examined (Table 3.16).

The TABC count was consistent in 2018 and 2019 for the cooling tower and swimming pool water. TABC count of potable water showed a significant reduction from 3.29 log CFU/ml in 2018 to <10 in 2019, suggesting improved water quality. However, the TABC result of hot water and chiller water increased from 2019 compared to 2018. In 2018, all samples except the hot water tank and swimming pool water showed a total coliform count ranging from 1.84 log CFU/ml to 3.26 log CFU/ml (cooling tower, cascade, chiller, potable, and AC water). In contrast, in 2019, only swimming pool water had a coliform count. *E. coli* was detected in cascade water (waterfall with aquarium type) in 2018, where fishes were in the water. In 2018, cascade and potable water showed the highest TABC count, whereas in 2019, chiller water. At the same time, TCC was found in the cooling tower, cascade, AC water, and potable water collected in 2018, whereas in 2019, only TCC was present in swimming pool water.

Results

Table 3.16 TABC results of processed water from the Hotel in 2018 and 2019

Hotel	TABC		TCC		<i>E. coli</i>		<i>Salmonella</i> sp.	
Sampling Sites	Log CFU/ml							
	2018	2019	2018	2019	2018	2019	2018	2019
Cooling Tower Water	1.90	1.60	1.84	ND	ND	ND	ND	ND
Hot Water Tank	1.90	3.23	ND	ND	ND	ND	ND	ND
Swimming Pool Water	3.01	3.65	ND	2.77	ND	ND	ND	ND
Cascade water	3.29	2.77	3.26	ND	TFTC	ND	ND	ND
Chiller water	2.74	4.27	2.65	ND	ND	ND	ND	ND
Potable water	3.29	TFTC	3.14	ND	ND	ND	ND	ND
Central AC water	2.69	TFTC	2.25	ND	ND	ND	ND	ND

3.6 Prevalence of *Legionella* spp. in natural and processed water samples using culture-based method

3.6.1 Food Industries

Ninety water samples collected from different food industries were processed as described in Method section 2.2. Each treated and untreated water sample was inoculated on Buffered Charcoal Yeast Extract. Round, glistening, convex, frosted glass colonies, typical of *Legionella* spp., were obtained from different water samples (Figure 3.6).



Figure 3.6 Growth on BCYE agar media *Legionella pneumophila* ATCC 33152 (left), sample with presumptive growth of *Legionella* (middle), sample with no development of *Legionella*-like colony (right)

Results

In the food industry A, a total of 17 distinct processed water samples were meticulously collected from two sampling sites cooling tower 1,4; reservoir water 2,4; and clean room AC water 1, which revealed the presence of presumptive *Legionella* colonies.

In the food industry B, examinations of 10 processed water sources, including potable water, cooling towers, reservoir tanks, and water softener, revealed the presence of presumptive *Legionella* colonies.

Similarly, in the food industry C, laboratory tests detected presumptive *Legionella* colonies in several water systems, including the cooling tower, potable water sources, hot water tanks, softener, chiller and AC water. The highest presumptive colonies were obtained from food industry C water samples.

Furthermore, in the food industry D, chiller, AC and cooling tower water samples were found to contain presumptive *Legionella* colonies cultured on Buffered Charcoal Yeast Extract (BCYE) media.

From the food industry E, only 3 water sampling sites, such as the cooling tower and chiller water, showed a typical colony of *Legionella*. All the sampling sources where presumptive colonies were found on BCYE media are summarized below in Table 3.17.

Table 3.17 Sites of processed water samples from 5 food industries containing presumptive *Legionella* colonies on BCYE media

Water Sampling Sites of Food Industries	
Food Industry A	Food Industry B
Cooling Tower water 1	Potable water
Cooling Tower water 4	Cooling tower water 1
Reservoir water 2	Cooling tower water 2
Reservoir water 4	Reservoir Tank water 1
Clean Room AC water 1	Reservoir Tank water 3
	Softener water 1
Food Industry C	Food Industry D
Cooling Tower water 1	Husky Chiller water
Cooling Tower water 2	Air Conditioning water 1

Results

Cooling Tower water 3	Cooling Tower water 05 IM
Holding Tank water 1	Cooling Tower water 06 IM
Holding Tank water 3	Cooling Tower water 02 DL
Potable water 1	Cooling Tower water 03 DL
Hot Water Tank water 1	Cooling Tower water 14 Bakery
Chiller water	Food Industry E
Softener Water 1	Air High Compressor Cooling Tower water 3
Air Conditioning water	Chiller water 3
	Chiller Cooling Tower water 5

3.6.2 Hospital

Twenty-four processed water samples collected from two hospitals were processed as described in Method section 2.2. Each treated and untreated water sample was inoculated on Buffered Charcoal Yeast Extract. Round, glistening, convex, frosted glass colonies, typical of *Legionella* sp., were obtained from 15 water samples. In this study conducted at hospital 1, a total of 16 water samples were meticulously collected from 8 distinct locations across the facility during two separate time points, specifically in 2019 and 2020. Both the Intensive Care Unit (ICU) drinking water and tap water, from 2019 and 2020, revealed the presence of presumptive *Legionella* colonies cultured on Buffered Charcoal Yeast Extract (BCYE) media. Furthermore, the sterilizer water and reservoir water collected in 2019, as well as the holding tank water sampled in 2020, also showed presumptive *Legionella* colonies.

Simultaneously, hospital 2, wherein all 8 processed water samples taken from various sites throughout the facility yielded positive results for presumptive *Legionella* colonies on BCYE media (Table 3.18).

Table 3.18 Sites of processed water samples from 2 hospitals containing presumptive *Legionella* colonies on BCYE media

Water Sampling Sites	
Hospital 1	Hospital 2
ICU Drinking water (2019)	Cooling Tower water
ICU Tap water (2019)	Softener water

Results

Sterilizer water (2019)	Inlet water
Reservoir water (2019)	Air Handling water
ICU Drinking water (2020)	RO water
ICU Tap water (2020)	Potable water
Holding Tank water (2020)	FCU water
	Hot Water Tank water

3.6.3 Hotel

Fourteen processed water samples collected from the hotel were processed as described in Method section 2.2. Each treated and untreated water sample was inoculated on Buffered Charcoal Yeast Extract. Round. In the water sampling conducted in 2018, four distinct sites were found for the presence of presumptive *Legionella* colonies. These sites included a cooling tower, a hot water tank, a swimming pool, and a cascade of water.

In the following year, 2019, water samples were collected again from similar sources, and colonies were found in the cooling tower and cascade water. Additionally, the air conditioning (AC) water contained the presumptive colonies on selective buffered charcoal yeast extract (BCYE) media.

The results revealed that out of 14 samples, 8 samples demonstrated the presence of these presumptive colonies on the designated selective media (Table 3.19).

Table 3.19 Sites of processed water samples from hotel containing presumptive *Legionella* colonies on BCYE media

Water Sample Sites	
Cooling Tower Water (2018)	AC water (2019)
Hot Water Tank Water (2018)	Cascade water (2019)
Swimming Pool Water (2018)	Cooling Tower Water (2019)
Cascade water (2018)	
Chiller room water (2018)	

Results

3.6.4 Natural surface water

43 natural surface water samples were collected randomly from 17 different sites of 5 districts of Bangladesh (Figure 3.7). Each sample underwent specific treatment methods, including acid treatment, heat treatment, or a combination of both, mixed with untreated water as described in Method section 2.2.

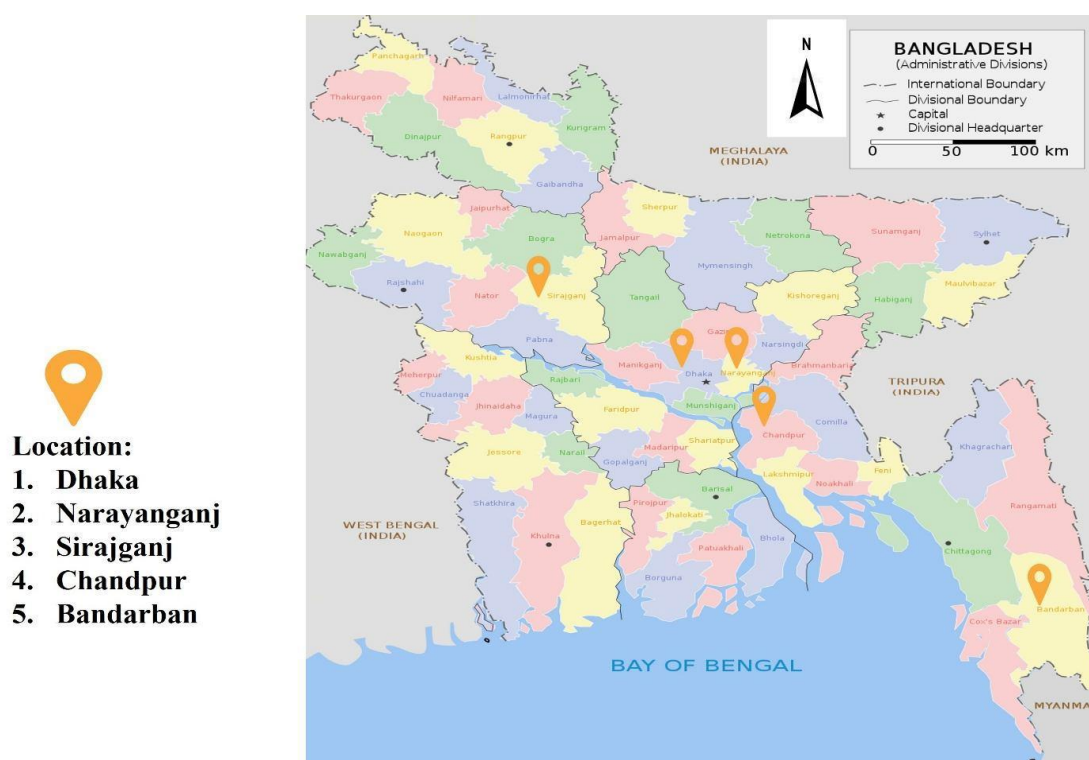


Figure 3.7 Mapping of the locations of collected natural surface water

From these inoculated samples, the appearance of presumptive *Legionella* was successfully isolated from 18 water samples (Table 3.20).

Table 3.20 Sites of natural water samples from the surface containing presumptive *Legionella* colonies on BCYE media

Water Sampling Sites	
Dhaka University	Dhaka
DU swimming pool	Hatirjheel Lake
Shahidullah Hall pond	Dhanmondi Lake

Results

Jagannath Hall pond	IPH pond
Jahirul Haque Hall Pond	Gulshan Lake
Ramna pond	Gulshan Lake (Gulshan 1)
TSC Swimming pool	DBBL Fountain
Bangla Academy Pond	Chandpur
Narayanganj	Chandpur pond 1
Shitalakshya River water	Chandpur pond 2
Sirajganj	Bandarban
Sirajganj pond	Boga lake

Presumptive colonies of *Legionella* spp. were identified on BCYE agar plates sourced from various locations, including food processing industries, hotel, hospitals, and natural water samples. In total, out of 171 water samples, 72 presumptive colonies were obtained. The findings are summarized in Table 3.21 below.

Table 3.21 Presumptive colony from selective media

Sl. Nos.	Water Sampling Sites	No. of samples
1.	Food Industry A	5
2.	Food Industry B	6
3.	Food Industry C	10
4.	Food Industry D	7
5.	Food Industry E	3
6.	Hospital 1	7
7.	Hospital 2	8
8.	Hotel	8
9.	Surface water	18

3.7 Identification of *Legionella* spp. using the Latex agglutination test

The presumptive colonies from BCYE media were subjected to the latex agglutination test. The results of the latex agglutination test revealed that none of the presumptive *Legionella* isolates from any water sample exhibited agglutination, contrasting sharply with the American Type Culture Collection (ATCC) strain, which demonstrated a positive

Results

agglutination response (as illustrated in Figure 3.8). This discrepancy in agglutination behavior strongly suggests that the colonies isolated from the Buffered Charcoal Yeast Extract (BCYE) media, which had been tentatively identified as *Legionella* species, do not belong to the *L. pneumophila* species. The absence of agglutination in the presumptive isolates indicates a likely misclassification, warranting further investigation to confirm their true identity.

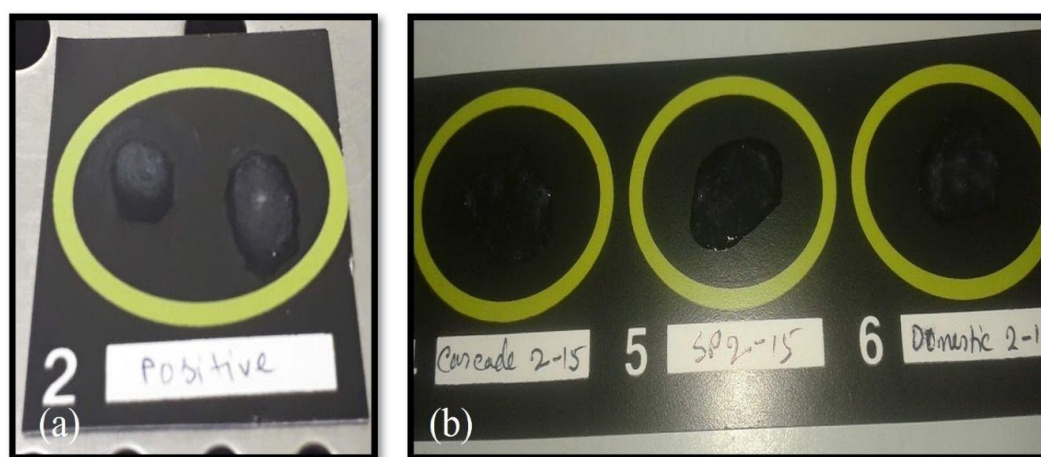


Figure 3.8 Latex agglutination test of presumptive isolates (a) Positive agglutination of *Legionella pneumophila* ATCC culture 33152, (b) no visible agglutination in representative samples

3.8 Detection of *Legionella* spp. in natural and processed water samples using a *Legionella pneumophila*-specific PCR primer

Metagenomic DNA was extracted from water samples collected from various sources, including food industries, hotels, hospitals, and surface water. This metagenomic DNA underwent amplification targeting the 16S rRNA gene fragment specific to *L. pneumophila*.

3.8.1 Food Industry

Among the 31 samples of food industries tested, the *L. pneumophila*-specific PCR product was detected in 4 samples (Figure 3.9).

Results

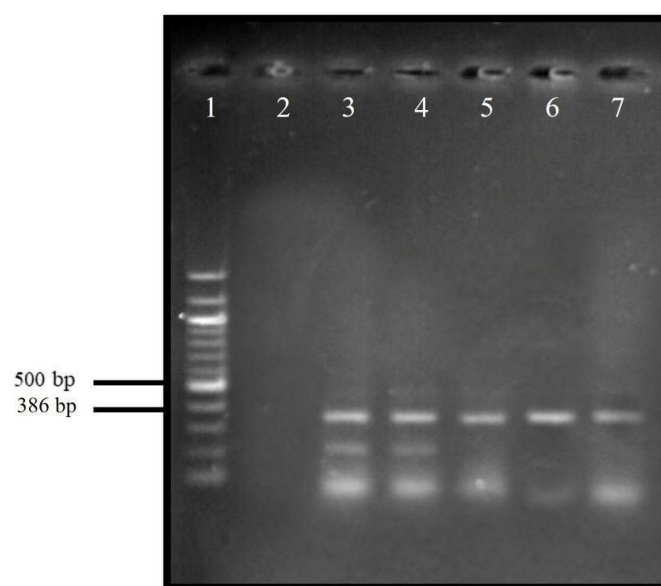


Figure 3.9 *Legionella pneumophila* (LP) specific 16SrRNA gene (386bp) detection in food industry water samples; Lane 1: DNA Ladder (100bp), Lane 2: Negative control, Lane 3-6: Samples, Lane 7: Positive control as *Legionella pneumophila* ATCC 33152

A comparison of the sequence data of these PCR products in the GenBank database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/GenBank>) showed 100% homology of all four isolates with *L. pneumophila*. NCBI GenBank designated the isolates with accession numbers after necessary verifications, which are MZ102257, MZ102258, ON924466, and ON92446 (Table 3.22). These four isolates were obtained from the cooling tower water of food industry A and C, potable water of food industry C, and chiller water of food industry D, respectively.

Table 3.22 GENBANK accession no. of the food industries water sample by NCBI

Water Sampling Sites	No. of positive Water Samples	<i>Legionella pneumophila</i> GENBANK accession no
Cooling tower water 1 (Food Industry A)	1	MZ102257
Cooling tower water 1 (Food Industry C)	1	MZ102258
Potable water 1 (Food Industry C)	1	ON924467
Husky Chiller water	1	ON924466

(Food Industry D)

3.8.2 Hospitals

Among the 24 samples tested collected from hospitals 1 and 2, the *L. pneumophila*-specific PCR product was detected in 5 samples (Figure 3.10).

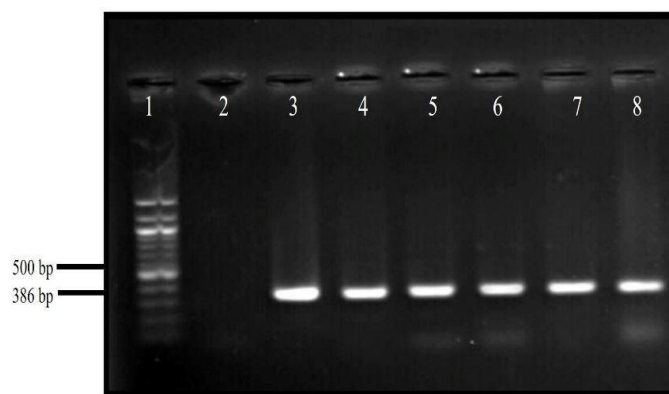


Figure 3.10 Detection of *Legionella pneumophila* (LP) specific 16SrRNA gene (386bp) in hospital water samples; Lane 1: DNA Ladder (100bp), Lane 2: Negative control, Lane 3: Positive control as *Legionella pneumophila* ATCC 33152, Lane 4-8: Samples

A comparison of the sequence data of these PCR products in the GenBank database of the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/GenBank>) revealed 100% homology with all five isolates of *L. pneumophila*. NCBI GenBank designated the isolates with accession numbers after necessary verifications, which are MZ102255, MZ102256, ON924465, PP762241, and PP762242 (Table 3.23). Among these, three were obtained from the ICU tap water, ICU drinking water, and sterilizer water of Hospital 1. In contrast, the remaining two were obtained from the cooling tower water and the fan coil unit water of Hospital 2.

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Table 3.23 GENBANK accession no. of hospital water samples by NCBI

Water Sampling Sites	No. of positive Water Samples	<i>Legionella pneumophila</i> GENBANK accession no
ICU tap water (Hospital 1, 2019)	1	MZ102255
ICU Drinking water (Hospital 1, 2019)	1	PP762241
Sterilizer water (Hospital 1, 2019)	1	PP762242
Cooling tower water (Hospital 2)	1	MZ102256
Fan Coil Unit water (Hospital 2)	1	ON924465

3.8.3 Hotel

Among the 14 samples tested, the *Legionella pneumophila*-specific PCR product was detected in 2 samples (Figure 3.11).

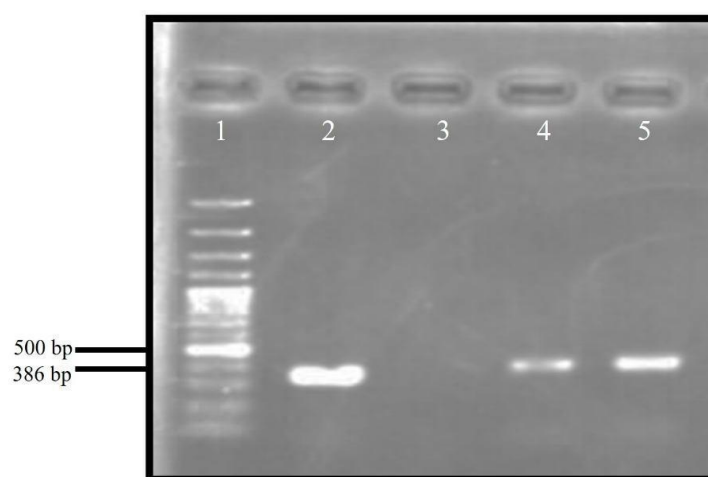


Figure 3.11 Detection of *Legionella pneumophila* (LP) specific 16SrRNA gene (386bp) in hotel water samples; Lane 1: DNA Ladder (100bp), Lane 2: Positive control as *Legionella pneumophila* ATCC 33152, Lane 3: Negative control, Lane 4-5: Samples

Results

A comparison of the sequence data of these PCR products in the GenBank database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/GenBank>) showed 100% homology of two isolates with *L. pneumophila*. NCBI GenBank designated the isolates with accession numbers after necessary verifications, which are ON924464 and ON924468 (Table 3.24). These two isolates were obtained from the cooling tower water and cascade water of the 2019 collection.

Table 3.24 GENBANK accession no. of hotel water samples by NCBI

Water Sampling Sites	No. of Positive Water Samples	<i>Legionella pneumophila</i> GENBANK accession no
Cooling tower water (2018)	1	ON924464
Cascade water (2018)	1	ON924468

3.8.4 Natural surface water

Among the 43 samples tested, the *L. pneumophila*-specific PCR product was detected in 2 samples. A comparison of the sequence data of these PCR products in the GenBank database of the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/GenBank>) showed 100% homology of these two isolates with *L. pneumophila*. NCBI GenBank designated the isolates with accession numbers after necessary verifications, which are ON926906 and ON926907 (Table 3.25). These two isolates were obtained from the surface water of the Dhaka University area.

Table 3.25 GENBANK accession no. of surface water samples by NCBI

Water Sampling Sites	No. of Positive Water Samples	<i>Legionella pneumophila</i> GENBANK accession no
DUSHL	1	ON926906
DUJAGHL	1	ON926907

3.9 Detection of the virulence gene mip of *Legionella pneumophila* in the water samples

The testing for the mip gene, specific to *L. pneumophila*, involved a specialized PCR primer applied to each collected water sample. Out of the total samples tested, only five displayed the presence of the mip gene, indicating a potential risk factor for *L.*

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pneumophila contamination in those water sources. Among these five water samples, one was from the hotel cooling tower (2019), two were from the hospital 1 ICU tap water and the hospital 2 cooling tower, and two were from the cooling tower water of the food industry A and C (Figure 3.12).

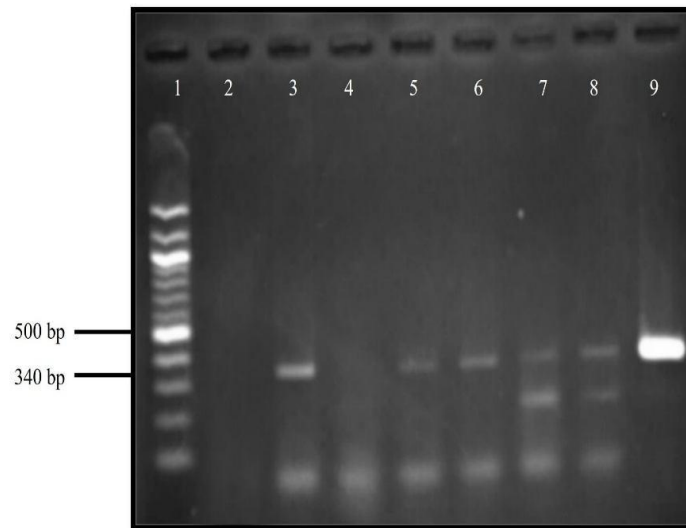


Figure 3.12 Detection of the virulence gene mip of *Legionella pneumophila* in the water samples; Lane 1: DNA Ladder (100bp), Lane 2: Negative control, Lane 3-8: Samples, Lane 9: Positive control as *Legionella pneumophila* ATCC 33152

3.10 Developing a novel Nested PCR method for the detection of *Legionella pneumophila* in environmental (natural and processed water) samples

We encountered challenges in culturing *L. pneumophila* on selective media, which might be due to its viable but nonculturable state as well as its presence in low quantities within the samples. Addressing this, metagenomic DNA from water samples was collected and subjected to LP16SrRNA-specific PCR to detect *L. pneumophila* (section 2.4). However, when employing the LP16SrRNA PCR, it was observed that some samples yielded faint bands on gel electrophoresis. The faint bands suggested that while the DNA was present, it was likely at a lower concentration than optimal, interfering with the identification process. When these PCR products producing faint bands were sequenced, a low percentage of identification was encountered, indicating that the derived sequences were closely related to *L. pneumophila* but did not meet the criteria for definitive identification.

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In response to the challenge of detecting samples with lower PCR yields, we developed an innovative solution, a nested PCR technique.

3.10.1 Primer design

Primers were designed targeting the 386 bp fragment of the *L. pneumophila* LP16SrRNA. The primer design involved obtaining the sequence of the 16SrRNA gene of *L. pneumophila*, sourced from the NCBI database (Figure 3.13), and subjecting it to the NCBI Primer-Blast software.

AACTGAAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTAACA
CATGCAAGTCGAACGGCAGCATTGTCTAGCTTGCTAGACAGATGGCGAGTGG
CGAACGGGTGAGTAACGCGTAGGAATATGCCTTGAAGAGGGGGACAACCTTGG
GGAAACTCAAGCTAATACCGCATAATGTCTGAGGACGAAAGCTGGGGACCTT
CGGGCCTGGCGCTTTAAGATTAGCCTGCGTCCGATTAGCTAGTTGGTGGGGTA
AGGGCCTACCAAGGCGACG CGGTAGCTGGTCTGAGAGGAT GACCAGC CAC
ACTGGAAGTGAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGC
CTGAGGGTTGTAAAGCACTTTCAGTGGGGAGG **AGGGTTGATAGGTTAAGAG**
CTGATTAACTGGACGTTACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAA
AGGGTGCGTAGGTGGTTGATTAAGTTATCTGTGAAATTCCTGGGCTTAACCTG
GGACGGTCAGATAATACTGGTTGACTCGAGTATGGGAGAGGGTAGTGGAATT
TCCGGTGTAGCGGTGAAATGCGTAGAGATCGGAAGGAACACCAGTGGCGAA
GGCGGCTACCTGGCCTAATACTGACACTGAGGCACGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCTGTAAA **CGATGTCAACTAGCTG**
TTGGTTATA

Figure 3.13 The 16SrRNA sequence of *Legionella pneumophila*. The sequence highlighted in cyan represents the PCR product amplified by the primers designed by Jonas et al. (1995). The forward and reverse primers of this PCR are highlighted in yellow

A total of 10 primer pairs were generated by the NCBI Primer-Blast software (Figure 3.14). Based on the GC content and probability of self-complementarity, primer pair 7

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was selected. This primer pair amplifies a 137 bp segment of the 386 bp fragment of the *L. pneumophila* LP16SrRNA and displays the least self-complementarity, with GC content ranging between 50% and 55%.

	Sequence (5'→3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTCCGGTGTAGCGGTGAAAT	Plus	20	228	247	59.68	50.00	4.00	2.00
Reverse primer	TTTCGTGCCTCAGTGTTCAGT	Minus	20	318	299	59.54	50.00	3.00	3.00
Product length	91								
Primer pair 7									
Forward primer	GTAAGGGTGCCTAGGTGGT	Plus	20	120	139	59.68	55.00	2.00	0.00
Reverse primer	TCTCTACGCATTTCACCGCT	Minus	20	256	237	59.47	50.00	2.00	2.00
Product length	137								
Primer pair 8									
Forward primer	ATTTCGGTGTAGCGGTGAA	Plus	20	226	245	59.68	50.00	4.00	2.00
Reverse primer	AATCCTGTTGCTCCCAAG	Minus	20	339	320	60.61	55.00	2.00	2.00
Product length	114								
Primer pair 9									
Forward primer	ACAGAAGAAGCAGCGCTAA	Plus	20	41	60	59.31	50.00	4.00	1.00
Reverse primer	ACCACCTACGACCCCTTAC	Minus	20	139	120	59.68	55.00	2.00	0.00
Product length	99								
Primer pair 10									
Forward primer	GGAATTCGGTGTAGCGGT	Plus	20	223	242	60.39	55.00	7.00	2.00
Reverse primer	TGACATCGTTTACAGCGTGA	Minus	21	374	354	60.00	47.62	3.00	2.00
Product length	152								

Figure 3.14 10 primer pairs were generated by the NCBI Primer-Blast software, where Primer pair 7 (red blocked) was selected as a nested PCR primer

The provided sequence, 386 base pairs (bp) of the 16S ribosomal RNA from *L. pneumophila*, includes highlighted regions in yellow that indicate the binding sites for a set of newly designed nested primers (Figure 3.15). These primers, 137 bp in length, consist of both forward and reverse sequences. The Forward primer is (5'–GTAAGGGTGCCTAGGTGGT–3'), and the reverse primer is (5'–TCTCTACGCATTTCACCGCT–3').

AACTGAAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTAACA
CATGCAAGTCGAACGGCAGCATTGTCTAGCTTGCTAGACAGATGGCGAGTGG
CGAACGGGTGAGTAACGCGTAGGAATATGCCTTGAAGAGGGGGACAACCTTGG

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GGAAACTCAAGCTAATACCGCATAATGTCTGAGGACGAAAGCTGGGGACCTT
CGGGCCTGGCGCTTTAAGATTAGCCTGCGTCCGATTAGCTAGTTGGTGGGGTA
AGGGCCTACCAAGGCGACGATCGGTAGCTGGTCTGAGAGGATGACCAGCCAC
ACTGGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGC
CTGAGGGTTGTAAAGCACTTTCAGTGGGGAGGAGGGTTGATAGGTTAAGAG
CTGATTAAGTGGACGTTACCCACAGAAGAAGCACC GGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGC GTAA
AGGGTGCGTAGGTGGT TGATTAAGTTATCTGTGAAATTCCTGGGCTTAACCTG
GGACGGTCAGATAATACTGGTTGACTCGAGTATGGGAGAGGGTAGTGGAATT
TCCGGTGTAGCGGTGAAATGCGTAGAGATCGGAAGGAACACCAGTGGCGAA
GGCGGCTACCTGGCCTAATACTGACACTGAGGCACGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCTGTAAA CGATGTCAACTAGCTG
TTGGTTATA

Figure 3.15 The sequences highlighted in yellow represent the forward and reverse primers designed in this study for the detection of *Legionella pneumophila* using nested PCR. The sequences in Cyan represent the forward and reverse primer sequences of the LP-16s PCR

3.10.2 Sample preparation for nested PCR

Sample preparation for nested PCR begins with the meticulous process of metagenome extraction from both environmental and clinical samples. For PCR reactions, a mixture of master mix, nuclease-free water, and primers was prepared without any template DNA in a sterile Eppendorf tube. The proper mixture was ensured by a short spin. An aliquot amount of the mixture was taken into PCR tubes from the Eppendorf tube. Template DNA (3µl) was then added to the mixture of the PCR tubes separately. Once the reaction mixture was prepared, all the tubes were gently centrifuged to collect all components at the bottom of the tube. Negative control was kept in which nuclease-free water (3µl) was added instead of the template. The PCR tubes were then placed in a thermal cycler, and the conditions were set correctly for amplification. For each PCR, 35 cycles were applied.

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3.10.3 Optimal conditions for nested PCR reaction

Once the PCR product as a template was prepared, several PCR conditions with different annealing temperatures and extension times were explored to determine the most effective one, facilitating specific binding of both primers to the intended target while minimizing the formation of primer dimers (Table 3.26). For each PCR, 35 cycles were applied. These conditions include precise temperature cycling profiles, reagent concentrations, and recommended thermal profile settings.

Table 3.26 Different PCR Conditions of nested PCR

Primer	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Result
Nested PCR (137 bp)	95°C for 5 min	94°C for 30 s	54°C for 30 s	72°C for 40 s	72°C for 7 min	Non-specified band occurred
	95°C for 2 min	94°C for 30 s	54°C for 1min	72°C for 40 s	72°C for 4 min	Non-specified band occurred
	95°C for 2 min	94°C for 40 s	54°C for 30 s	72°C for 1 min	72°C for 4 min	Non-specified band occurred
	95°C for 5 min	94°C for 30 s	54.5°C for 30 s	72°C for 1 min	72°C for 7 min	Non-specified band occurred
	95°C for 5 min	94°C for 30 s	54.5°C for 1min	72°C for 1 min	72°C for 7 min	Non-specified band occurred
	95°C for 2 min	94°C for 40 s	55°C for 30 s	72°C for 1 min	72°C for 4 min	Specified band of target size with no presence of non-specific band
	95°C for 5 min	94°C for 30 s	55°C for 1 min	72°C for 40 s	72°C for 7 min	Non-specified band occurred
	94°C for 5 min	94°C for 1 min	55.5°C for 30 s	72°C for 1 min 30 s	72°C for 4 min	Primer -dimer
	94°C for 5 min	94°C for 1 min	55.5°C for 1 min	72°C for 1 min 30 s	72°C for 4 min	Primer -dimer
	94°C for 10 min	94°C for 1 min	56°C for 30 s	72°C for 1 min	72°C for 10 min	Primer -dimer

To optimize the process, various annealing temperatures were tested to determine the most effective one, facilitating specific binding of both primers to the intended target while minimizing the formation of primer dimers (Figure 3.16). In this study, the PCR condition consisted of an initial denaturation at 95°C for 2 min, followed by 35 cycles at 94°C for 40s, annealing at 55°C for 30s, extension at 72°C for 1 min and a final extension at 72°C for 4 min.

Results

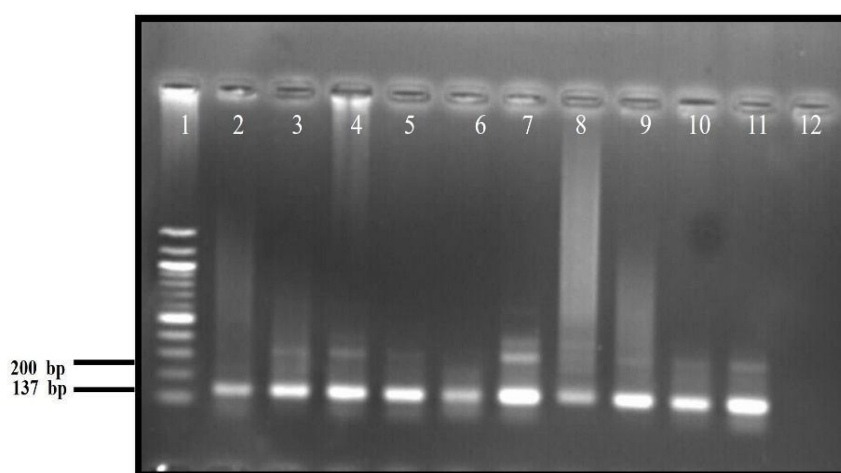


Figure 3.16 Optimisation of the annealing temperature and annealing time for the nested PCR to amplify a 137 bp sequence within the 386 bp *Legionella pneumophila* 16S rRNA sequence; Lane 1: DNA Ladder (100bp), Lane 2: annealing at 55°C for 30s, Lane 3: annealing at 54°C for 30 s, Lane 4: annealing at 54°C for 1min, Lane 5: annealing at 54°C for 30 s, Lane 6: annealing at 54.5°C for 30 s, Lane 7: annealing at 54.5°C for 1 min, Lane 8: annealing at 55°C for 1 min, Lane 9: annealing at 55.5°C for 30s, Lane 10: annealing at 55.5°C for 1 min, Lane 11: annealing at 56°C for 30 s, Lane 12: Negative control.

3.10.4 Detection of *Legionella pneumophila* by the nested PCR method

Once the optimal condition was determined, the nested PCR was employed to detect the presence of *L. pneumophila* on all the samples collected from food industries, hotels, and surface water, which gave faint bands in the LP16s PCR. The novel PCR method successfully identified *L. pneumophila* in processed water of 11 food industries, 10 hotel, and 9 natural water samples (Table 3.27).

Table 3.27 Detection of *Legionella pneumophila* by nested primers in different water samples

Water sampling sites		
Food Industries	Hotel	Surface water
Food Industry A	Hot Water Tank Water (2018)	DU Swimming Pool
Clean Room AC water 1	Swimming Pool Water (2018)	IPH pond
Food Industry B	Chiller room water (2018)	Hatirjheel Lake

Results

Cooling Tower water 2	AC water (2018)	Dhanmondi lake
Softener Water 1	Cooling Tower Water (2019)	Gulshan Lake
Potable water	Hot Water Tank Water (2019)	Gulshan lake (Gulshan 1)
Food Industry C	Swimming Pool Water (2019)	Chandpur pond 1
Hot Water Tank 1	Cascade water (2019)	Chandpur pond 2
Food Industry D	Chiller room water (2019)	Shitalakshya River water
Air Condition water 1	AC water (2019)	
Cooling Tower water 02DL		
Cooling Tower water 03DL		
Cooling Tower water 05 IM		
Cooling Tower water 06 IM		
Cooling Tower water 14		
Bakery		

In the food industry, nested primers detected *L. pneumophila* in common processed water sources, including air conditioning systems, hot water tanks, and cooling towers, as well as in hotel water sources. The nested PCR method effectively detected *L. pneumophila* in the swimming pool of the University and natural waters in other city areas. Additionally, it was found in the natural waters of Chandpur and Narayanganj in Bangladesh (Figure 3.17). This highlights the effectiveness of the nested PCR process even more.

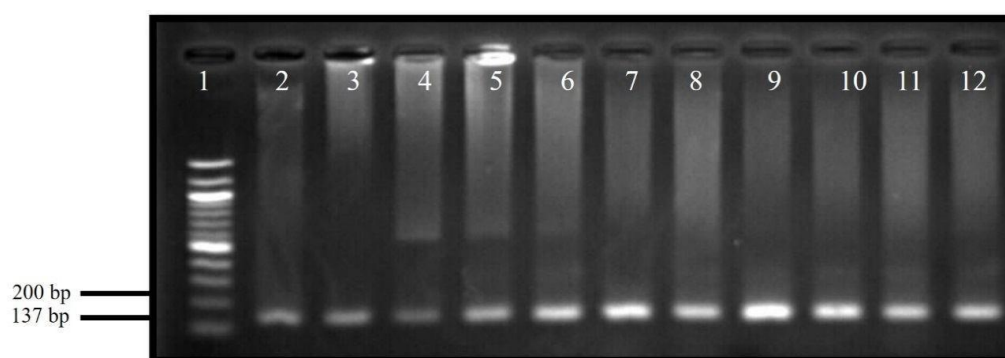


Figure 3.17 Amplification of the 137 bp sequence of *Legionella pneumophila* 16S rRNA sequence by the nested PCR method; Lane 1: DNA ladder 100 bp, Lane 2: Food Industry A Clean room water, Lane 3: Food Industry B cooling tower water 2, Lane 4: Food Industry B potable water, Lane 5: Food Industry C Hot water tank, Lane 6: Food Industry

Results

D Cooling tower water 05IM, Lane7: Hotel AC water (2018), Lane 8: Hotel cooling tower water (2019), Lane 9: Hotel Cascade water (2019), Lane 10: Surface water IPH pond, Lane 11: Surface water Gulshan Lake, Lane 12: Surface water Chandpur pond 2.

3.10.5 Sequencing of nested PCR products

For further confirmation, the nested PCR products were sent for sequencing, and the sequences were compared with available databases at the NCBI site. A comparison of the sequence data of these PCR products in the GenBank database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/GenBank>) showed 98-100% homology with *L. pneumophila* of all the nested PCR products (Figure 3.18).

The image shows a screenshot of the NCBI BLAST results page. It displays two sections of 'Sequences producing significant alignments'. The top section shows four results for *Legionella pneumophila* strains with 100% identity and 100% coverage. The bottom section shows four results for *Legionella pneumophila* strains with 97% identity and 97% coverage. The table includes columns for Description, Scientific Name, Max Score, Total Score, Query Cover, E value, Per. Ident, Acc. Len, and Accession.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Legionella pneumophila strain LEG1116 chromosome, complete genome	Legionella pneu...	147	147	100%	5e-31	100.00%	3455650	CP146990.1
Legionella pneumophila strain LEG1094 chromosome	Legionella pneu...	147	147	100%	5e-31	100.00%	3651777	CP144485.1
Legionella pneumophila strain LEG1091 chromosome	Legionella pneu...	147	147	100%	5e-31	100.00%	3651307	CP144487.1
Legionella pneumophila strain E9_O, complete genome	Legionella pneu...	147	441	100%	5e-31	100.00%	3367205	CP015956.1
Legionella pneumophila strain E9_O, complete genome	Legionella pneu...	141	424	97%	2e-29	100.00%	3367205	CP015956.1
Legionella pneumophila strain E8_O, complete genome	Legionella pneu...	141	424	97%	2e-29	100.00%	3455562	CP015955.1
Legionella pneumophila strain E7_O, complete genome	Legionella pneu...	141	424	97%	2e-29	100.00%	3425999	CP015954.1
Legionella pneumophila strain E6_N, complete genome	Legionella pneu...	141	424	97%	2e-29	100.00%	3405173	CP015953.1

Figure 3.18 NCBI blast result of the amplified 137 bp of *L. pneumophila* LP16SrRNA sequence obtained from environmental samples, showing high-similarity matches

In our study, we identified a total of 30 natural and processed water samples that tested positive for *L. pneumophila* using nested PCR techniques. Out of these samples, nine were selected for sequencing, and the results were found to be identical. It is important to note that a minimum of 150 nucleotide sequences is required to obtain a GENBANK accession number; however, our primers yielded sequences of only 137 base pairs. The detailed findings of the genetic characteristics of the detected strains, such as query cover and percentage identification, are summarized in the table below (Table 3.28).

Results

Table 3.28 Representative sequences producing significant alignments

Sample ID and Sampling Sites	Alignments	Query Cover	Percent Identification
D3 (Dhanmondi Lake)	<i>Legionella pneumophila</i>	100%	99.62%
R (Shitalakshya River)	<i>Legionella pneumophila</i>	97%	100%
P1 (IPH pond)	<i>Legionella pneumophila</i>	100%	99.91%
C2 (Chandpur Pond 2)	<i>Legionella pneumophila</i>	100%	100%
P11 (Hotel Swimming pool)	<i>Legionella pneumophila</i>	99%	98.94
G1 (Hotel cooling Tower water, 2019)	<i>Legionella pneumophila</i>	97%	98.99%
FIB (Potable water, Food Industry B)	<i>Legionella pneumophila</i>	97%	100%
N4 (Cooling Tower 06 IM, Food Industry D)	<i>Legionella pneumophila</i>	99%	98.24%
D1 (Cooling Tower 05 IM, Food Industry D)	<i>Legionella pneumophila</i>	100%	98%

In one of the sequences of nested PCR from the food industry, analyzing potable water, a distance tree was used to illustrate the pairwise comparisons among the selected sequences. This analysis highlighted the genetic similarities and differences, providing valuable insights into the *Legionella pneumophila* diversity present in the water samples. The resulting phylogenetic tree serves as a critical tool for understanding the relationships between various *Legionella pneumophila* strains, which can ultimately inform safety standards and quality control measures in the industry (Figure 3.19).

Results

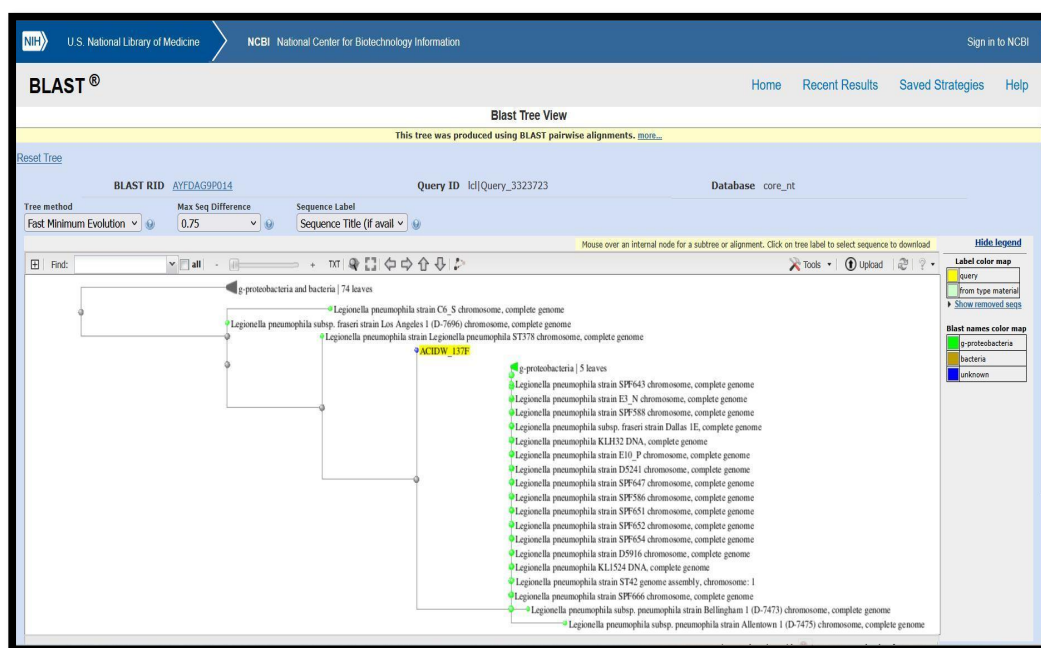


Figure 3.19 BLAST Tree View (phylogenetic tree) generated from nested PCR sequence alignment results, likely for *Legionella pneumophila*. The tree illustrates how our query nested pcr sequence from food industry potable water (highlighted, **ACIDW_13F**) is related to other sequences in the database. Sequences that cluster closely together share higher genetic similarity, suggesting a common evolutionary origin.

3.11 Specificity of nested PCR primer

Assessing the specificity of the nested PCR method developed in this study, PCR reactions were conducted utilizing the nested PCR primers and the total genomic DNA from various bacterial species, excluding *L. pneumophila*, allowing for a comprehensive evaluation of the primers' specificity. The selected bacterial strains included *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, and *Staphylococcus aureus*. The experimental results demonstrated that the primers displayed no binding affinity to the total genomic DNA of any of the tested bacterial species, confirming that they were exclusively reactive to the genetic material of *L. pneumophila* (Figure 3.20).

Results

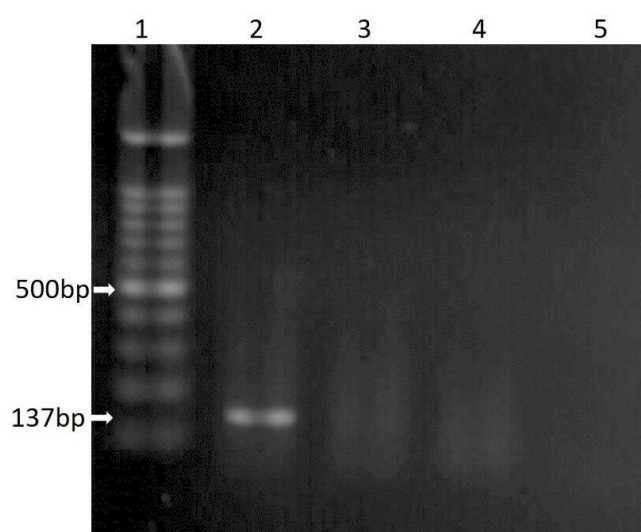


Figure 3.20 Nested PCR with *Legionella pneumophila* and non-*Legionella* control; Lane 1: DNA Ladder (100bp), Lane 2: *Legionella pneumophila* control ATCC 33152, Lane 3: *Pseudomonas aeruginosa*, Lane 4: *Escherichia coli*, Lane 5: *Vibrio cholerae*.

3.12 Sensitivity of nested PCR primer

To assess the sensitivity of the nested PCR method, a series of PCR reactions was conducted using varying concentrations of template DNA, specifically from 1 pg (picogram) to 10 ng (nanogram) of LP16SrRNA PCR products. Faint bands corresponding to LP16SrRNA PCR products were meticulously selected and subsequently purified using a PCR cleanup kit, which included a binding buffer, wash buffer, and elution buffer, along with a spin column and collection tubes (Method 2.10). The purification process was carried out according to the manufacturer's instructions provided with the QIAquick PCR Purification Kit (50).

Following the purification step, the concentration and purity of the DNA were accurately measured using a Nanodrop spectrophotometer, followed by dilution using nuclease-free water to prepare different concentrations of template DNA, ranging from 10 ng to 1picogram/ μ l. Each concentration was again checked by the Nanodrop spectrophotometer and then subjected to the optimized PCR cycle previously established.

Results

The result revealed that the minimal concentration of template DNA that could be consistently detected using polymerase chain reaction (PCR) was one picogram/ μ l (Figure 3.21). This finding underscores the sensitivity of our PCR approach in detecting low concentrations of DNA, which is critical for accurate metagenomic analysis.

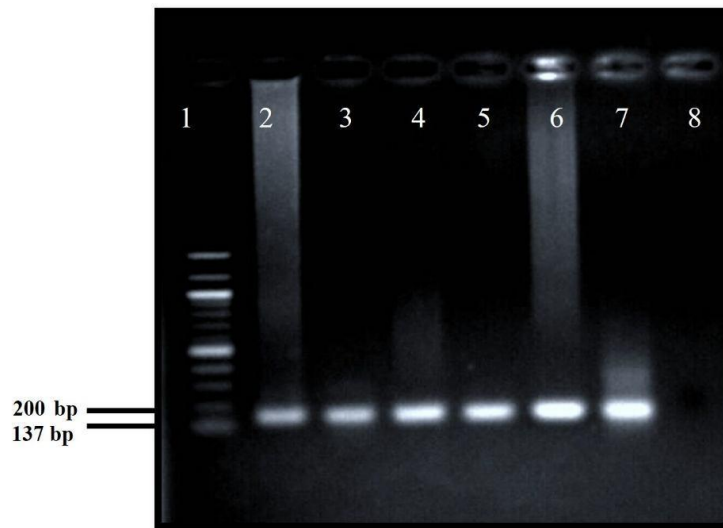


Figure 3.21 Nested PCR at different DNA concentrations; Lane 1: DNA Ladder (100bp), Lane 2: 1 picogram of sample, Lane 3: concentration 1ng of sample, Lane 4: concentration 2ng of sample, Lane 5: concentration 4ng of sample, Lane 6: concentration 6 ng of sample, Lane: concentration 10 ng of sample, Lane 8: Negative control

3.13 Prevalence of *Legionella pneumophila* in clinical tracheal aspirate samples from the ICU of the hospital

Legionnaires' disease primarily affects elderly and immunocompromised individuals, causing life-threatening pneumonia. *L. pneumophila* is responsible for about 90% of cases. It is a significant concern in ICU patients, accounting for up to 30% of pneumonia cases. Although rapid diagnostic techniques have led to increased detection worldwide, in Bangladesh, the detection of *Legionella*-associated pneumonia cases is absent. Moreover, the current study has detected *L. pneumophila* in the hospital water system, including ICU tap and drinking water, suggesting that this pathogen is circulating in the hospital environment of Bangladesh. Therefore, to check whether the patients admitted to the ICU are getting infected by this pathogen, the prevalence of *L. pneumophila* was investigated in the clinically diagnosed pneumonia patients in a tertiary hospital in Dhaka.

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Fifty respiratory tracheal aspirate (TA) samples were collected from patients (Method 2.7.1), followed by inoculation on BCYE media (Method section 2.5.1) and a latex agglutination assay (Method section 2.5.2). Metagenomic DNA was also extracted from each sample (Method section 2.7.2), and PCR was performed using *L. pneumophila* 16S rRNA-specific primers and nested primers (Method sections 2.6.2 and 3.10.4).

3.13.1 Detection of *Legionella* spp. in clinical trachea aspirate samples using culture and latex agglutination method

From each treated and untreated clinical trachea aspirate sample, the appearance of round, glistening, convex, frosted glass colonies, typical of *Legionella* spp., was obtained from 20 clinical sample isolates (Figure 3.22).



Figure 3.22 Growth on BCYE agar media sample with presumptive growth of *Legionella*

Each of the isolates from the culture did not reveal the latex agglutination test, and none of the presumptive *Legionella* isolates from any TA sample exhibited agglutination, contrasting sharply with the American Type Culture Collection (ATCC) strain, which demonstrated a positive agglutination response.

3.13.2 Detection of *Legionella* spp. in clinical trachea aspirate samples using a *Legionella pneumophila*-specific PCR primer

Among the 50 tracheal aspirates, a *L. pneumophila*-specific 16SrRNA PCR product indicative of *L. pneumophila* was successfully detected in 5 of these samples (Figure 3.23).

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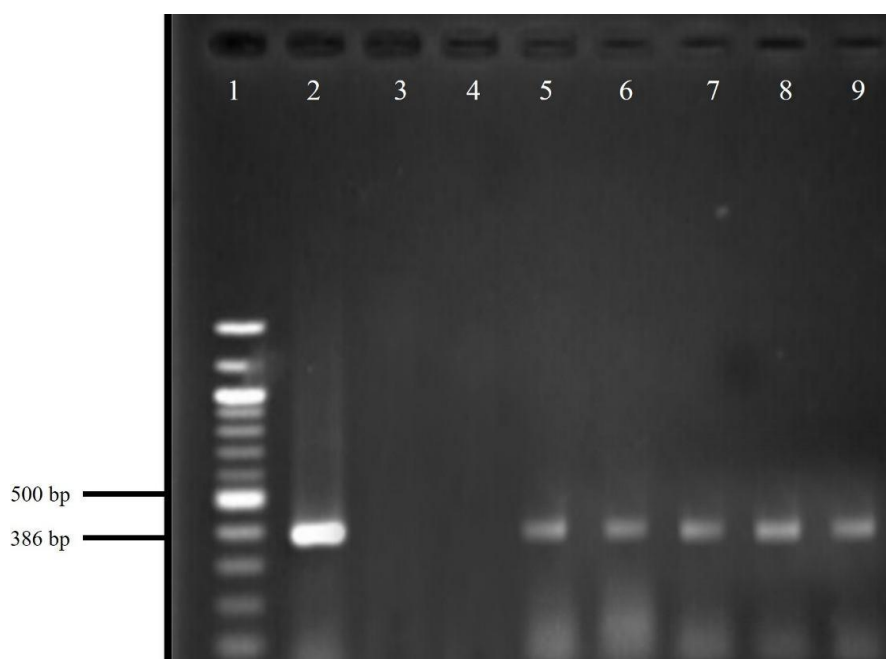


Figure 3.23 Detection of *Legionella pneumophila* (LP) specific 16SrRNA gene (386bp) in Tracheal aspirate samples; Lane 1: DNA Ladder (100bp), Lane 2: Positive control as *Legionella pneumophila* ATCC 33152, Lane 3: Negative control, Lane 5-9: TA Samples

Subsequent analyses included a meticulous comparison of the complete nucleotide sequences obtained from these samples against the extensive genomic data available in the GenBank database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/GenBank>). Notably, one sample exhibited a definitive identification as *L. pneumophila*, as illustrated in Figure 3.24.

The sequencing results demonstrating a perfect match with the reference strain of *L. pneumophila*, resulting in an impressive 100% homology. This level of identity underscores the accuracy of our identification methods and supports the reliability of our findings. Following a rigorous verification process to ensure data integrity and accuracy, the NCBI GenBank assigned the isolate an accession number, OR 378539, confirming its classification within the database. This classification not only facilitates further research but also aids in understanding the epidemiology of *L. pneumophila* in environmental and clinical contexts.

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select all

100 sequences selected

GenBank

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Distance tree of results

MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Legionella pneumophila isolate TA 22 16S ribosomal RNA gene, partial sequence	Legionella pneu...	708	708	100%	0.0	100.00%	383	OR378539.1
Legionella pneumophila strain ERS1305867 chromosome, complete genome	Legionella pneu...	664	1992	93%	0.0	100.00%	3405663	CP048618.1
Legionella pneumophila OIPH 14_48 gene for 16S ribosomal RNA, partial sequence	Legionella pneu...	664	664	93%	0.0	100.00%	616	LC491286.1

Figure 3.24 NCBI blast result of the amplified 386 bp *L. pneumophila* 16SrRNA-specific gene sequence obtained from a tracheal sample (TA) showing high-similarity matches

3.14 Phylogenetic tree construction

To investigate the evolutionary relationship of the *L. pneumophila* sequences, a phylogenetic analysis was performed with relevant sequences of three reference strains of *L. pneumophila* available in the NCBI database. A maximum likelihood tree was constructed based on 16S rRNA sequences from fourteen distinct *L. pneumophila* isolates from various samples, such as water and clinical tracheal aspirate.

In this study, the phylogenetic analysis indicated that the fourteen *L. pneumophila* sequences were closely related to those in the database (Figure 3.25), including the strain held by ATCC 33152.1. Upon review, the sequences displayed a distribution across two distinct lineages, diverging from a common ancestral root. The foundation of this phylogenetic tree is represented by ON926906, which refers to the surface water samples collected from the University area of Dhaka. Furthermore, the sequence PP762241, obtained from an Intensive Care Unit (ICU) drinking water, and the sequence PP762242, obtained from hospital sterilizer water, exhibited homology with the reference sequence NR074231, linked to *L. pneumophila* strain Philadelphia 1. This data indicates the presence of *L. pneumophila*, a pathogenic bacterium, in various environmental settings. Our analysis revealed no specific associations between the sequences and their respective sample collection sites. Nonetheless, a significant finding emerged regarding the

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sequence of OR378539, identified in a clinical trachea aspirate sample. This sequence demonstrated homology to the reference sequence NZLAXV01000040, which was detected in a cooling tower in China, suggesting a potential connection between the two.

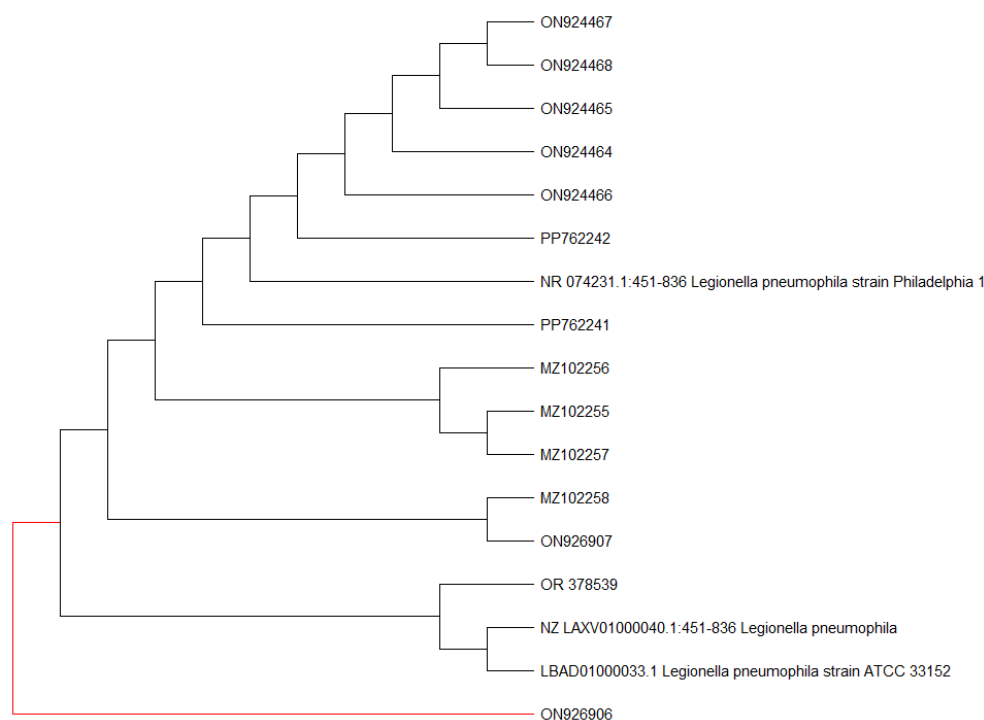


Figure 3.25 Phylogenetic tree of 14 sequences of LP16SrRNA PCR product of *Legionella pneumophila*

3.15 Detection of *Legionella pneumophila* in trachea aspirates (clinical samples) using a novel nested PCR method

L. pneumophila was detected in 11 samples based on faint PCR bands in the 16S rRNA-specific PCR. Analyzing it, a new primer set (137 bp) was designed for a nested PCR that amplified a 386 bp region in the 16SrRNA sequence of *L. pneumophila*, using the *L. pneumophila* 16SrRNA PCR product as a template. The results indicated the presence of *L. pneumophila* in all 11 samples (Figure 3.26).

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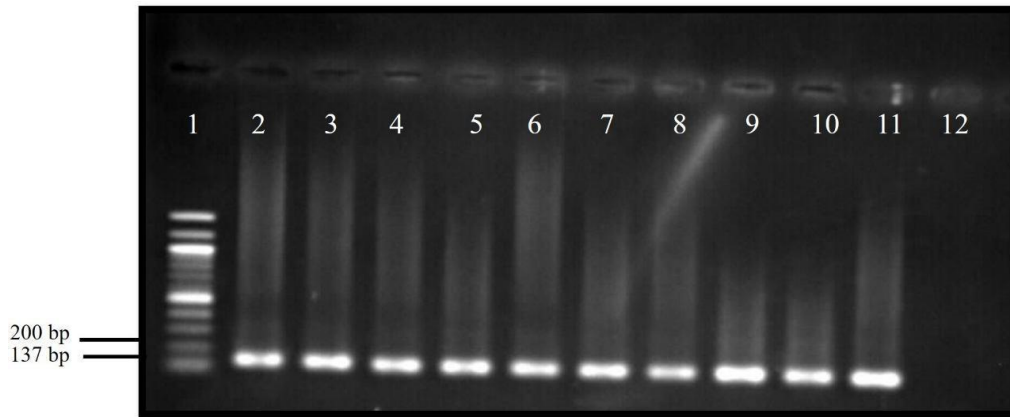


Figure 3.26 Detection of the 137 bp *Legionella pneumophila* 16s rRNA sequence in tracheal aspirate samples by the nested PCR method; Lane 1: DNA Ladder (100bp), Lane 2-11: Samples, Lane 12: Negative control

For further confirmation, the nested PCR products were sent for sequencing, and the sequences were compared with available databases at the NCBI site. A comparison of the sequence data of these PCR products in the GenBank database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/GenBank>) showed identical to *L. pneumophila* of all the nested PCR products. Out of these 11 samples, seven were selected for sequencing, and the results showed 98% homology with *L. pneumophila*. The detailed findings of the genetic characteristics of the detected strains, such as query cover and percentage identification, are summarized in the table below (Table 3.29).

Table 3.29 Representative sequences producing significant alignments

Sample ID	Alignments	Query Cover	Percent Identification
TA 9	<i>Legionella pneumophila</i>	98%	99.11%
TA 11	<i>Legionella pneumophila</i>	95%	98.13%
TA15	<i>Legionella pneumophila</i>	100%	98.83%
TA 17	<i>Legionella pneumophila</i>	100%	99.79%
TA 24	<i>Legionella pneumophila</i>	100%	99.94%
TA 28	<i>Legionella pneumophila</i>	97%	98.87%
TA 36	<i>Legionella pneumophila</i>	100%	98.91%

3.16 Detection of the virulence gene mip of *Legionella pneumophila* in the clinical samples using PCR

In parallel, a thorough screening was conducted on fifty tracheal aspirate samples to assess their virulence potential associated with the mip gene. This investigation revealed that 12 of the tracheal aspirate samples contained the mip gene, providing clear evidence of its role in the pathogenicity of *L. pneumophila*, as illustrated in Figure 3.27.

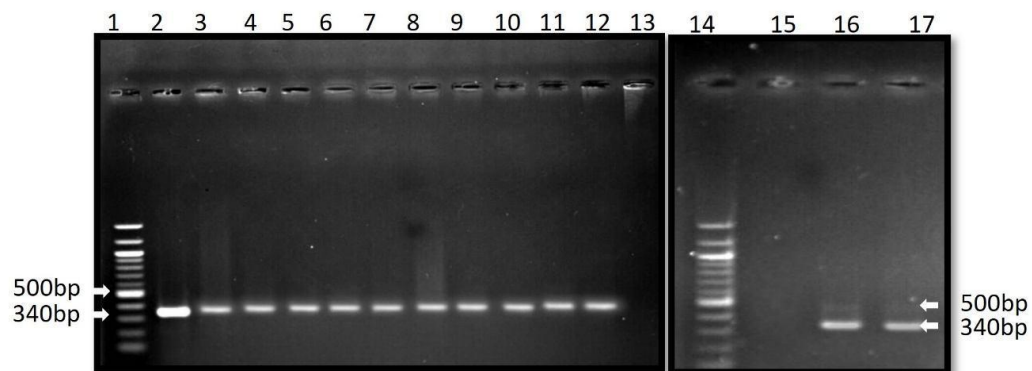


Figure 3.27 PCR detection of the *Legionella pneumophila* virulence gene mip (340 bp) in the tracheal aspirate samples; Lane 1 and 14: DNA Ladder (100bp), Lane 2: Positive control as *Legionella pneumophila* ATCC 33152, Lane 3-12: Samples, Lane 13 and 15: Negative control, Lane 16 and 17: Samples

To verify the identity of the mip gene, NCBI Nucleotide BLAST was employed on six samples derived from the mip gene sequencing data, enabling precise identification and analysis, as depicted in Figure 3.28. This comprehensive approach highlights the importance of the mip gene in understanding the virulence and pathogenic behaviour of *L. pneumophila*.

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[Graphics](#)

	Description ▾	Scientific Name ▾	Max Score ▾	Total Score ▾	Query Cover ▾	E value ▾	Per. Ident ▾	Acc. Len ▾	Accession
☒	macrophage infectivity potentiator [Legionella pneumophila]	Legionella pneumophila	209	209	99%	2e-67	99.05%	128	AR072113.1
☒	macrophage infectivity potentiator [Legionella pneumophila]	Legionella pneumophila	209	209	99%	2e-67	99.05%	124	AR072112.1
☒	macrophage infectivity potentiator [Legionella pneumophila serogroup 11]	Legionella pneumophila	209	209	99%	2e-67	99.05%	134	AEX26089.1

Figure 3.28 NCBI blast result of the amplified 340 bp of the mip gene of *L. pneumophila* sequence obtained from a tracheal sample (TA), showing high-similarity matches

Analysis using LP16SrRNA and nested PCR methods showed that out of fifty tracheal aspirate samples tested, sixteen were positive for *L. pneumophila*. This accounts for 32% of all samples examined (Table 3.30). These results emphasize the presence of this pathogenic bacterium in respiratory specimens and its possible involvement in respiratory infections.

Table 3.30 Overview of the determination of *Legionella pneumophila* from tracheal aspirate samples based on different PCR methods

PCR methods applied	<i>Legionella pneumophila</i> positive
(Total samples 50)	No. of Samples (%)
LP-specific 16Sr RNA	05 (10.0)
LP-specific nested primer	11 (24.44)
mip gene	12 (24.0)
Total	16 (32.0)

3.17 Microbiological analysis of the tracheal aspirate samples

In addition to the analysis of targeting *Legionella* spp., each tracheal aspirate sample was subjected to an extensive and meticulous examination to construct a detailed bacteriological profile. This process began with assessing the total viable count of bacteria, which involved culturing the samples in various conditions to ensure an accurate representation of the microbial load present. Following this, a thorough determination of the coliform count was conducted to identify any potential contamination that could indicate a health concern.

Moreover, the analysis focused on identifying other potential pathogenic organisms that may pose significant health risks to patients. This included testing for various bacteria utilizing advanced microbiological techniques and appropriate culture media. The goal was to comprehensively understand the microbial environment within the tracheal aspirates, thus facilitating targeted treatment strategies and improving patient care.

3.17.1 Determination of total aerobic bacterial count (TABC) and total coliform count (TCC)

Out of the total of 50 samples that were meticulously analyzed, only 10 samples of TABC demonstrated a colony-forming unit (CFU) count falling within the range of 1.71 to 4.93 log CFU/ml. This finding suggests that most samples, precisely 40, exhibited TNTC (Too Numerous to Count) signs of viable microbial growth. The assessment of total coliform count on MacConkey agar from the tested TA samples revealed a wide variability in CFU counts, which ranged from 2 to an alarming 1200 CFU/ml. Among the analyzed samples, 18 were reported as having no detectable colonies and were classified as TFTC (Too Few to Count). Conversely, several remaining samples were categorized as TNTC (Too Numerous to Count), indicating an overwhelming presence of coliform colonies. This data is summarized in detail in Table 3.31.

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Table 3.31 Microbiological analysis of 50 TA samples; Total Aerobic Bacterial Count (TABC) and Total Coliform Count (TCC)

Sl. Nos.	Total Aerobic Bacterial Count (TABC)	Total coliform count (TCC)	Sl. Nos.	Total Aerobic Bacterial Count (TABC)	Total coliform count (TCC)
1.	TNTC	TFTC	26.	TNTC	TFTC
2.	TNTC	2	27.	TNTC	TNTC
3.	TNTC	16	28.	TNTC	30
4.	TNTC	TNTC	29.	TNTC	TNTC
5.	TNTC	TFTC	30.	TNTC	350
6.	400	TFTC	31.	TNTC	TNTC
7.	TNTC	TNTC	32.	TNTC	TNTC
8.	11000	TFTC	33.	TNTC	TNTC
9.	TNTC	TFTC	34.	TNTC	TNTC
10.	TNTC	TFTC	35.	TNTC	TNTC
11.	15000	TFTC	36.	TNTC	120
12.	51	TFTC	37.	TNTC	TFTC
13.	TNTC	24	38.	TNTC	TFTC
14.	TNTC	TFTC	39.	TNTC	TNTC
15.	TNTC	TNTC	40.	TNTC	1200
16.	TFTC	TNTC	41.	TNTC	TNTC
17.	TNTC	300	42.	TNTC	50
18.	TNTC	1200	43.	10000	TFTC
19.	TNTC	TNTC	44.	TNTC	TNTC
20.	70000	34	45.	TNTC	TNTC
21.	10000	TFTC	46.	85000	330
22.	TNTC	600	47.	TNTC	10
23.	60000	TFTC	48.	TNTC	TNTC
24.	TNTC	TNTC	49.	TNTC	TNTC
25.	TNTC	TNTC	50.	80000	TNTC

Chapter 04:

Discussion

4. Discussion

4.1 Prevalence of *Legionella* spp. in processed water samples of the hotel, food industry, and hospital

Legionella spp., especially *Legionella pneumophila*, are opportunistic waterborne pathogens responsible for 70–80% of Legionnaires' disease and Pontiac fever (Spagnolo et al., 2013). *L. pneumophila* is widespread in man-made water systems, particularly where conditions favor biofilm growth and warmth. The majority of studies on *L. pneumophila* distribution focus on man-made aquatic environments, healthcare facilities, air conditioning, cooling towers, and hot tubs, as these are the main sources of human infection (Costa et al., 2005; Doléans-Jordheim et al., 2006; Wullings & Kooij, 2006; Carvalho et al., 2007). Its prevalence varies across different water systems due to factors such as temperature, biofilm formation, water stagnation, and disinfection methods (Kruse et al., 2016). In recent years, incidents of Legionellosis in artificial water distribution systems, such as industrial cooling systems, hospital water distribution systems, have increased (George et al., 2016; Weiss et al., 2017; Lefebvre, 2024; Yao et al., 2024). Therefore, this study was conducted to assess the prevalence of *L. pneumophila* in natural and processed water distribution systems with a major focus on the food industry, hospitals, hotels, and surface water in Bangladesh.

4.1.1 Prevalence of *Legionella* spp. in processed water of the hotel

Hotels can significantly impact tourism promotion and public health protection as they are the main component of the hospitality industry. In the current study, we conducted environmental monitoring of a luxury hotel in Dhaka to determine the prevalence of *Legionella* spp. especially *L. pneumophila* in the hotel's water distribution systems, and investigate risk factors associated with the contamination. Based on the detection of *L. pneumophila* 16S rRNA sequence, we observed the presence of *L. pneumophila* in 85.71% (12 out of 14) of the water samples, including those collected from the cascade, cooling tower, hot water tank, chiller water, air conditioning water, and swimming pool water. This is the first report of *L. pneumophila* in the hotel water systems in Bangladesh. Detecting the same 16S rRNA sequence, a previous study in Bangladesh also detected *L. pneumophila* in tap water from an air-conditioned hotel in Dhaka (Haque et al., 2016), indicating that this pathogen possibly prevails in the hotel water system, but remains

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undetected because of the lack of routine investigations. Recently, Mahmud et al. (2025) revealed a notable prevalence of *Legionella* spp. (28.8%) in environmental water sources in Bangladesh, with *Legionella pneumophila* accounting for the majority (56.1%). Higher detection rates were associated with tap waters from hotel and domestic. Notably, highly virulent strains carrying 19 out of 21 genes were identified in hot and tap water during summer and late autumn. The presence of *L. pneumophila* in the hotel water system also poses a threat to the hotel guests.

Our study demonstrates a notably high prevalence of *L. pneumophila*, with 85.71% (12/14) of water samples testing positive based on 16S rRNA detection. This proportion is substantially higher than most previously reported studies, although careful interpretation is required due to differences in sample size, sampling design, and environmental conditions. Compared to our findings, studies from other regions generally report lower contamination levels. *L. pneumophila* are commonly detected in water samples of hotels across the world. Studies conducted in Greece reported that 62.95% of the sampled hotels had 20.7% of samples contaminated by *Legionella* spp. (Papadakis et al., 2021). Similarly, 57.15% of the sampled hotels in Italy had 25.6% of samples contaminated by *L. pneumophila* (Filippis et al., 2017). Also in the Balearic Islands, Spain, *L. pneumophila* was detected in 15.9% of hot water samples of the 65.4% of the sampled facilities (Domenech-Sanchez et al., 2022). In Israel, 60% of sampled hotels had 17% of samples contaminated by the *L. pneumophila* (Yakunin et al., 2020). Additionally, 25.7% of hotel samples in Bosnia and Herzegovina were contaminated with *L. pneumophila* (Bešić et al., 2021) and in the Netherlands, 85% (Euser et al., 2012). Compared to the above-mentioned studies, the prevalence of *L. pneumophila* in hotel water in our study is higher than in previous studies.

A key factor influencing these differences is sample size and sampling scope. Our study analyzed a relatively small number of samples ($n = 14$), which can amplify the apparent prevalence; each positive sample contributes a large percentage to the total. In contrast, most of the cited studies included much larger sample sizes (often hundreds of samples across numerous hotels), providing more statistically robust and generalizable estimates. Larger datasets tend to smooth out localized spikes in contamination and better represent overall environmental variability.

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Additionally, our sampling included multiple high-risk water sources such as cooling towers, hot water tanks, and air-conditioning systems, which are well-known reservoirs for *L. pneumophila*. This targeted sampling strategy may have increased the likelihood of detection compared to studies that included a broader mix of low- and high-risk water systems.

Methodological differences also play an important role. We used a sensitive 16S rRNA-based detection approach enhanced by nested PCR, which detects low bacterial loads that conventional culture or single-round PCR methods used in earlier studies could miss. This increased sensitivity can contribute to a higher reported prevalence.

However, these studies found *L. pneumophila* in the cold (Donohue et al., 2014), hot (Borella et al., 2005) and cooling tower water (Sun et al., 2011), as well as in decorative fountains (O'Loughlin et al., 2007; Palmore et al., 2009; Haupt et al., 2012; Smith et al., 2015; Papadakis et al., 2018), and swimming pool (Leoni et al., 2001, 2018) in the hotels, many of which align with our sources of *L. pneumophila* detection. Several reasons might be responsible for such a higher prevalence of *L. pneumophila* in the hotel water samples. First of all, the main water supply that provides water to the hotel might be contaminated. In Dhaka, a significant portion (around 78%) of the water supply comes from groundwater, primarily extracted through deep tube wells (Dhaka WASA, annual report 2018–2019), and the remaining portion (around 33%) is sourced from rivers like the Buriganga and the Shitalakshya. Although local authorities treat surface water for public use through various processes such as chemical addition, coagulation, flocculation, sedimentation, filtration, and disinfection, the water often remains contaminated with different pathogenic bacteria (Parveen et al., 2008; Hasan et al., 2019). This issue was similar to that observed in the drinking water supply system in Germany, where abundant levels of *L. pneumophila* were detected (Lesnik et al., 2016). This is possible as although chlorine is regularly added to the water distribution system in Bangladesh, the presence of *L. pneumophila* was documented in water despite after treatment with >5 ppm chlorine (Barbaree et al., 1987; Lin et al., 1998). Additionally, factors such as chlorination concentration, temperature, plumbing system design, and plumbing materials can affect *L. pneumophila* proliferation in the plumbing system (Science et al., 2019). Therefore, the presence of *L. pneumophila* is not unlikely in the supply water. *L. pneumophila* can enter

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a hotel or residence through various pathways inside and outside the premises, posing a risk to people's health and safety (CDC, 2021).

Additionally, hotels use hot and cold-water tanks to generate their hydroelectric energy. However, if left unregulated, these water tanks can become a source of fatal *Legionella* bacteria, corrosion, and biofilm formation (Sciuto et al., 2021). The interactions among *L. pneumophila*, free-living amoebae (FLA), and biofilms are fundamental to the successful survival and spread of this pathogen in aquatic environments. These relationships enable *L. pneumophila* to thrive in challenging conditions, effectively resist disinfectants and antibiotics, and increase its virulence (Declerck, 2010). Amoebae, such as *Acanthamoeba*, *Naegleria*, and *Vermamoeba*, serve as beneficial hosts where *L. pneumophila* can replicate (Greub & Raoult, 2004). Biofilms in water systems act as vital habitats for *Legionella*, where amoebae and bacteria collaborate to form complex ecosystems that facilitate the colonization of *L. pneumophila* (Lau & Ashbolt, 2009). Rust is also a potential indicator of a favorable environment for growing *Legionella* spp. An ageing water boiler may be unable to maintain the approved storage temperature minimum of 60°C due to age, leading to deteriorating water quality and a higher risk of *Legionella* bacteria growth (Tso, 2014). Occupancy is also an essential factor, as hotels are never fully booked. Thus, water in unused rooms may sit unused in outlets, increasing the likelihood of a suitable environment for *Legionella* bacteria to grow. Furthermore, parts of the hotel are sometimes closed for renovations. In that case, the water in the closed sections' pipes is at risk of developing similar favorable conditions due to being temporarily closed, rerouted, or wholly cut and dead-ended. Though the hotel authorities disinfect the municipal water, our analysis suggests that the occurrence of *Legionella* bacteria in the city may be attributed to the inadequate upkeep of hygiene and sanitation.

Unlike reports from some other studies (Murga et al., 2001; Wingender & Flemming, 2011; Garrison et al., 2016; Thom et al., 2022;), the potable water samples taken from hotel water systems did not yield any positive results for the bacterium. It is possible that in the potable water, nutrient levels were minimal due to filtration and purification, making it difficult for *Legionella* to multiply.

Our study provides a partial overview of the presence of *L. pneumophila* in hotel water systems and reveals that the hotel water system in Dhaka, Bangladesh, could be a potential source of Legionnaires' disease (LD). It is crucial to broaden the research to

include more hotels from Dhaka and other parts of the country to evaluate the risk of legionellosis.

4.1.2 Prevalence of *Legionella* spp. in processed water of the food industry

To investigate the prevalence of *L. pneumophila* in the food industry, we explored five different food industries in Dhaka. We prioritized the sampling of cooling towers because it has been reported as the major source of *L. pneumophila*. Additionally, we also collected biofilm swabs from the cooling tower basin to investigate whether *L. pneumophila* is associated with the biofilms. Using the LP-16SrRNA-specific PCR, the 5 samples (20.74%) were found to be positive for *L. pneumophila*, of which four were detected in the potable, chiller, and cooling tower water. Additionally, the nested PCR yielded positive results for 11 additional water samples. Out of the total sources identified, 3(60%) were potable water, 7(20%) were cooling towers, 1(10%) was softener water, 1 (10%) was hot water tanks, 2 (20%) were air conditioning water sources, and 1 (20%) chiller water source.

Globally, there are not many reports on the prevalence of *Legionella* spp. in the food industry. However, our results align with other published works, which suggest that industrial facilities equipped with cooling towers or coolant systems were the most common occupational source of *L. pneumophila* (Brown et al., 1999; Ishimatsu et al., 2001; Li et al., 2015; Abuodeh et al., 2017; Llewellyn et al., 2017). A community-wide outbreak of LD in Paris was linked to industrial cooling towers, ineffective and hazardous control measures, and extensive environmental contamination of an industrial site, resulting in a community-wide outbreak of LD and had significant public health implications for the control and prevention of LD (Nguyen et al., 2006). *Legionella*-contaminated cooling towers as environmental trigger, in particular in the neighborhood of susceptible individuals, can cause severe health problems and even death (Walser et al., 2014). The high prevalence of *L. pneumophila* in cooling towers poses a major threat, as exposure to aerosols contaminated with *Legionella* spp. in industrial facilities equipped with cooling towers or coolant systems is known to spread infection among the workers (Allen et al., 1999; Principe et al., 2017). Cooling towers caused a large outbreak of Legionnaires' disease in a medically vulnerable community in New York City (Hugosson et al., 2007; Ambrose et al., 2014; Fitzhenry et al., 2017). Cooling tower water collected from air-conditioned buildings in Kuwait also displayed a wide prevalence of *L.*

pneumophila in sampling sites (Al-Matawah et al., 2015).

Legionella contaminated distribution systems as a source of occupational legionellosis has been well-established in many industries, including meat processing plants, waste processing plants, dairies, and beverage industries, where the pathogen was detected in water systems, especially in biofilms inside pipes and equipment (Osterholm et al., 1983; Isozumi et al., 2005; Gomez-Valero et al., 2009; Oggioni et al., 2016). Households and public buildings may seem like safe havens, but they're often unwitting hosts to *Legionella* spp., with *L. pneumophila* being the most prevalent culprit. These bacteria can infiltrate our potable water systems, reminding us of the importance of vigilance when it comes to water safety (Maria Stojek & Dutkiewicz, 2006; Sikora et al., 2015; Wang et al., 2012; Kruse et al., 2016). Phosphate-based drinking water softeners are widely used to prevent scale formation in drinking water distribution systems, though, in our study, *L. pneumophila* was detected from food industry of softener water samples, similar to earlier studies (Jereb et al., 2022; Joshi et al., 2023). The current study also revealed a significant prevalence of *Legionella* species in hot water distribution systems, aligning closely with findings reported in similar research (Leoni et al., 2005; Bargellini et al., 2011; Rakić & Štambuk-Giljanović, 2016). This consistency underscores the widespread issue of *L. pneumophila* colonization in such systems, emphasizing the importance of monitoring and implementing effective control measures to mitigate the associated health risks.

4.1.3 Prevalence of *Legionella* spp. in hospital water systems

L. pneumophila is the second most common pathogen causing severe pneumonia in ICU patients (Rello et al., 1991; Grattard et al., 1996). In this study, we analyzed the prevalence of *L. pneumophila* in the water supply systems of two hospitals in Dhaka and results revealed that 20.84% (5 out of 24) of the hospital water samples were positive for *L. pneumophila*, which were found in ICU tap water, ICU drinking water, cooling tower water, sterilizer water, and fan coil unit (FCU) water. Although there is no regular surveillance of hospital water systems for *L. pneumophila* in Bangladesh, a previous study also detected *L. pneumophila* in a hospital water sample (Jahan et al., 2015). The data from the current study further support this previous data and indicate that *L. pneumophila* is circulating in the hospital water system, imposing a great threat to the patients (Tercelj-Zorman et al., 2004).

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Throughout the world, *L. pneumophila* is frequently isolated from hospital water samples (Garcia-Nuñez et al., 2008; Spagnolo et al., 2013). One study found *L. pneumophila* in 48% of potable water samples from hospitals in a European-wide survey (Montagna et al., 2016a). In a study from Taiwan, *Legionella* was isolated from 61.4% of hot water systems in hospitals (Yu et al., 2008). In a tertiary hospital in Japan, 1.3% samples tested positive for *L. pneumophila* from water faucets of patient rooms, toilets, waste rooms, and water sourced from wells (Nakamura et al., 2020). Iran reported a 28.8% prevalence of *L. pneumophila* in hospital water systems, and 60.5% of isolates were *L. pneumophila* (Khaleidi et al., 2018). One previous study in Bangkok found a 20.0% prevalence of *Legionella* spp. contamination, of which 61.3% of *L. pneumophila* contamination was in the cooling tower water of the hospital (Luksamijarulkul et al., 2014). The presence of *L. pneumophila* in hospital water is a great concern, as aerosols generated from various water sources in hospitals are mainly responsible for the spread of this nosocomial infection (Sabria & Yu, 2002; Napoli et al., 2010). CDC received reports of healthcare-associated Legionnaires' disease cases from across the US in 2017, with a 25% case fatality rate (Kyritsi et al., 2018). *L. pneumophila* transmission can occur from cooling towers (Dondero et al., 1980; Sabria et al., 2006; De Olalla et al., 2008), water heaters, and the potable water distribution system of the hospital. Showering is also a risk factor for nosocomial *Legionella* pneumonia (Kessler et al., 2021). The private hospital nursery of Cyprus recorded a 33.3% mortality rate due to aerosol produced by a newly installed cold-mist humidifier filled with contaminated water from *L. pneumophila* (Yiallourous et al., 2013).

Similar to other studies, the cooling tower has been identified as a major source of *L. pneumophila* in our study (Samad et al., 2004; Jalili et al., 2021). In a survey of six cooling towers and multiple potable water sources in the University Hospital Campus, Kuala Lumpur, at least one cooling tower serving a hospital tested positive for *L. pneumophila* serogroup 1 (Wee et al., 2003). In Spain, a survey of 44 hospital cooling towers found *L. pneumophila* in nearly 24% of samples (Yao et al., 2024). A hospital-acquired case of Legionellosis in a 75-year-old patient was traced to a hospital cooling tower in the USA (Engelhart et al., 2008). A 2023 meta-analysis of 23,640 water samples across global hospitals revealed that 41.6% were positive for *L. pneumophila*, with prevalence higher in hot water systems (47.6%) than in cold water (Fang et al., 2023).

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There is very limited published data that specifically examines the prevalence of *L. pneumophila* in fan-coil unit (FCU) water in hospitals. These units are part of HVAC (Heating, Ventilation, and Air Conditioning) systems. In a study spanning four hospitals in Tehran, 116 water samples were collected from sources including hospital chillers and air-conditioning systems (which may include components similar to fan coil units), and 19.8% (23/116) of samples were PCR-positive for *L. pneumophila*, and 6.9% (8/116) were positive by culture (Sakhaee et al., 2022). Kirrage et al. (2007) examined environmental contamination in hospital HVAC systems and water loops of the UK, reporting colonization frequencies in HVAC-related reservoirs, such as cooling coils and humidifiers, typically ranging from 20–40%.

It also appears that there is no published data examining the prevalence of *L. pneumophila* bacteria in sterilizer water (e.g., water used in medical sterilization devices) within hospital settings. To our knowledge, the current study is the first report of the detection of *L. pneumophila* from the sterilizer and Fan coil unit water of a hospital in Bangladesh.

In our study, *L. pneumophila* has also been detected in the ICU tap water, which is used to sponge the bodies of ICU patients and perform other internal accessories work. This is a great concern, as the patients are getting direct contact with *L. pneumophila* contaminated water. A similar presence of *L. pneumophila* in bathing, water taps was reported by Rivera et al. (2007) and Kanatani et al. (2017). The possible source of *L. pneumophila* in tap water and drinking water (Kooij et al., 2013; Mapili et al., 2020) might be from the mains of the water network at concentrations undetectable by routine laboratory methods.

Many reports show that patients with weakened immune systems may acquire *L. pneumophila* infection from hospital water sources through exposure to aerosols from steam-heated towels, cooling towers (Mavridou et al., 2008), decorative fountains, and room air humidifiers (Boer et al., 1998; Brown et al., 2001; García-Fulgueiras et al., 2003; Engelhart et al., 2008). Helms et al. (1983) documented hospital-acquired cases of Legionnaires' disease in immunocompromised patients were directly linked to the presence of *L. pneumophila* in the hospital water outlet and hot water system. Ditommaso et al. (2014) found *L. pneumophila* in hospital water systems linked to hospital-acquired Legionnaires' disease.

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As *L. pneumophila* was detected in the water systems in hospitals and legionellosis is a common cause of hospital-acquired pneumonia, we aimed to investigate whether this pathogen is present in tracheal aspirate samples of the Intensive Care Unit (ICU) patients, who are mostly immunocompromised. Among 50 tracheal aspirate (TA) samples from ICU patients, five samples were initially positive for *L. pneumophila*, using the LP16SrRNAPCR. With an additional 11 positive cases using the nested PCR, taking the total prevalence to 32% (16 out of 50). A previous study also reported the prevalence of *L. pneumophila* in 4.65% clinically diagnosed pneumonia patients in a hospital in Bangladesh (Jahan et al., 2015), indicating that LP has been circulating in the ICU of hospitals in Bangladesh, but remains undetected in the majority of cases.

L. pneumophila is commonly detected in hospitalized patients and is associated with hospital-acquired pneumonia. In India, 2.3% of confirmed pneumonia patients tested positive for *Legionella* spp. (Sreenath et al., 2020). Cillóniz et al. (2012) reported that among severe community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP) cases requiring ICU admission, *L. pneumophila* accounted for 4.7% of microbiologically confirmed cases (Exner et al., 2005). Legionellosis was also found to be present in 5.9% of the patients admitted to the hospitals with a diagnosis of pneumonia (Montagna et al., 2006, 2016, 2018). A French survey found that approximately 15.8% of all reported Legionnaires' disease cases were hospital-acquired (Doleans et al., 2004).

Compared to other studies conducted in Bangladesh and abroad, the current study reports a very high prevalence of *L. pneumophila* in the ICU patients. Most of the studies from around the globe relied on the 16s rRNA-specific PCR for the detection of *L. pneumophila*, and the application of the nested PCR in the current study might have increased the detection rate. Additionally, the hospitals in Bangladesh also use culture-based method for the detection of this pathogen, while this conventional method has been identified as less specific and sensitive in many studies (Allegra et al., 2011; Whiley & Taylor, 2016). The current study also has the same findings as no *L. pneumophila* was detected using the culture-based method, while the use of the PCR-based method detected a higher prevalence. Roig et al. (2002) observed *L. pneumophila* in 3–6% of patients in European ICUs and noted that standard cultures often missed many cases that PCR or urinary antigen tests detected. Moreover, Allegra et al. (2008) demonstrated that under stress, *L. pneumophila* can enter the VBNC state, undetectable by culture but still

potentially infectious. The true prevalence in ICU patients in Bangladesh may also be underestimated due to the atypical symptoms of the disease. ICU protocols should include routine screening for *L. pneumophila* in patients with pneumonia unresponsive to standard antibiotics. The current study, therefore, reflects the importance of the use of current diagnostic methods for the identification of *L. pneumophila* infection, and also emphasizes the regular surveillance of hospital water samples for this pathogen.

4.1.4 Prevalence of *Legionella* spp. in natural water (surface)

L. pneumophila is naturally present in low concentrations in natural freshwater like lakes, rivers, streams, and thermal springs (Fliermans et al., 1981; Bartram, 2007) as shown in numerous scientific studies. Surface waters often harbor *L. pneumophila* bacteria, especially in warm and nutrient-rich conditions.

L. pneumophila in the natural water of Bangladesh has not been documented yet. We studied the prevalence of *L. pneumophila* in the natural surface water of Dhaka city and four regions of Bangladesh for the first time and found that 25.5% of samples tested positive for the bacteria based on the LP 16SrRNA PCR and nested PCR. The surface water from the Dhaka University area was found to contain a significant presence of *L. pneumophila*. Among the four regions studied, the natural water from the Sitalakshya River and the Chandpur Pond exhibited prevalence of *L. pneumophila*. According to Bartram (2007), *L. pneumophila* are widespread in natural aquatic environments and tend to thrive in warm, stagnant, and nutrient-rich waters. Research in Europe and Asia has found *L. pneumophila* bacteria in a significant portion of river and lake water samples, with one study in Northern Italy detecting *L. pneumophila* in 66% of surface water samples using both culture and PCR methods. A study by Wunderlich et al. (2016) also found *L. pneumophila* in surface water, highlighting the public health concern. Zhang et al. (2017) mentioned the presence of *L. pneumophila* in various warm aquatic environments, including natural hot springs. Edagawa et al. (2019) reported the detection of *L. pneumophila* in river water sources used for tap water by qPCR (75.5%). The high prevalence and concentration of *Legionella* spp. and *L. pneumophila* in the surface waters in Taiwan were recorded by (Tung et al., 2013).

Parthuisot et al. (2010) results suggested that *Legionella* spp. may be present at significant concentrations in many more freshwater environments. Although *L.*

pneumophila in surface water rarely causes direct human infections, it can serve as a source of colonization for engineered water systems, leading to Legionnaires' disease outbreaks, as reported in previous studies (Brooks et al., 2004). Although they are part of the natural microbial community, their potential to colonize man-made systems, water that aerosolizes when sprinkled on plants, represents a potential source of Legionellosis, making workers (farmers and gardeners) who were in contact with waters used for irrigation (Napoli, et al., 2019). Therefore, environmental surveillance and risk assessment are essential to prevent outbreaks.

4.2 Impact of physicochemical parameters of water on the survival or growth of *Legionella* spp.

Water is a well-recognized medium for microbial transmission, especially in sensitive settings such as hospitals, hotels, and food processing facilities. These environments must comply with strict national and international water quality standards to ensure safety. Among the various parameters that define water quality, physicochemical factors such as pH, temperature, total dissolved solids (TDS), salinity, and conductivity play a crucial role in determining the microbial safety of water and significantly influence the survival and growth of bacteria, including *Legionella* spp. (Ohno et al., 2003; Zayed et al., 2020; Sciuto et al., 2021;).

Reports have shown that stagnant warm water, particularly between 30°C and 45°C, provides optimal conditions for the proliferation of *Legionella* spp. (Paniagua et al., 2020; WHO, 2022; CDC, 2024). The bacteria prefer a pH range of 6.0 to 8.0, but are capable of tolerating extremes from 5.0 to 9.5 (Ranjdoost & Owrang, 2025). Favorable pH facilitates biofilm development, which protects *Legionella* from heat and disinfectants. Biofilm formation is also facilitated by the high mineral content of water, which is reflected by elevated TDS levels (Declerck, 2010). Even in low-nutrient water, the bacteria may persist within biofilms, especially in complex piping systems. *Legionella* spp. also benefits from metals like iron and manganese, organic matter, and interactions with protozoa, which help shield them from environmental stress and disinfectants.

In Bangladesh, seasonal variation, rainfall, and local environmental factors can lead to significant variation in the physicochemical properties of water (Bhuyan et al., 2019). Given these concerns, this study aimed to assess physicochemical parameters, pH, TDS,

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salinity, and conductivity in water samples collected from hospitals, hotels, and food industries to identify high-risk areas for *Legionella* spp. contamination. Various water systems, such as hot water tanks, potable water, cooling towers, chillers, and pools, are all vulnerable to conditions favoring *Legionella* spp. colonization.

Water quality in the sampled facilities showed wide variation. For example, pH ranged from 6.3 to 9.6, TDS from 8 to 1,180 ppm, Salinity from 10 to 820 ppm, and Conductivity from 12.37 to 1,540 $\mu\text{S}/\text{cm}$. There were wide variations in the physicochemical parameters across the samples collected from different sources. While in hotel water systems, pH ranged from 6.6 to 8.8, and TDS was between 52 and 570 ppm, in the food industry, pH ranged from 6.1 to 9.6, and TDS reached up to 1,180 ppm. Hospital samples also exhibited a slightly different pH range, between 5.2 and 9.3, and TDS from 11 to 693 ppm. Although no direct correlation between any specific parameter with the prevalence of *L. pneumophila* was observed, several sample sources displayed physicochemical features including high TDS, slightly alkaline pH, and warm temperatures, that is considered biofilm-promoting conditions. Research has shown that water quality often declines due to biofilms in pipes, especially in places with complex plumbing like hospitals or water tanks in hotels, which favours bacteria like *L. pneumophila* to survive.

Previous studies have reported inconsistent findings regarding the relationship between *L. pneumophila* and various physicochemical parameters. For example, a study by Ahmadrajabi et al. (2016) found no significant correlation with pH, total dissolved solids (TDS), or salinity. In contrast, Rafiee et al. (2014) revealed that the colonization of *L. pneumophila* appeared to be unaffected by water type, system design, or routine maintenance practices. Although *L. pneumophila* has been detected in multiple samples, further analysis is needed to determine the specific pH, TDS, salinity, and conductivity values associated with these positive samples. Identifying these critical values will be essential for establishing thresholds that support *L. pneumophila* growth and for developing targeted control strategies.

4.3 Impact of background microflora/interferences

L. pneumophila often coexists with other microbial species and protozoa (e.g., amoebae), which act as hosts or reservoirs, helping *Legionella* spp. evade disinfectants (Fields,

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1996; Thomas et al., 2004; Valciņa et al., 2019). Cooling towers can generate biofilms and aerosolize contaminated water, increasing the risk of microbial inhalation. This risk was highlighted by Legionnaires' disease epidemics linked to cooling towers, leading to an extensive study of *L. pneumophila*. While countries have specific standards for acceptable microorganism levels in cooling tower systems, these typically focus only on *L. pneumophila*, despite the presence of various other microorganisms like protozoa, bacteria, and viruses (Pagnier et al., 2009). In the current study, the highest total aerobic bacterial count (TABC) recorded in a cooling tower in the food industry reached a notably elevated log value of 5.17 CFU/ml, while a hospital's flow meter water exhibited a log value of 5.11 CFU/ml. These figures are considerably high and may suggest that the environmental conditions in these systems are conducive to the growth of *L. pneumophila* bacteria; however, these counts alone cannot definitively confirm the presence of *L. pneumophila*. Research by Liu et al. (2009) showed *L. pneumophila* was often present in systems with biofilm and microbial loads exceeding log 3.0 CFU/ml. Thomas et al. (2004) pointed out that microbial loads and biofilm density correlated with *L. pneumophila* presence. Bargellini et al. (2011) reported a positive association between *L. pneumophila* counts with heterotrophic plate count (HPC), indicating a correlation between high bacterial load with the presence of *L. pneumophila* (Temmerman et al., 2006; Valster et al., 2010; Wielen & Kooij, 2013).

Wadowsky & Yee (1983) demonstrated that *L. pneumophila* requires certain heterotrophic bacteria for optimal growth in water systems, showing “satellite” growth facilitated by *Flavobacterium* spp. Fields et al. (2002) also highlighted that heterotrophic bacteria in biofilms provide essential nutrients and shelter for *L. pneumophila*, facilitating their persistence and multiplication. Declerck (2010) explained how dense microbial communities (high TABC) enhance biofilm formation, which directly promotes *L. pneumophila* survival and growth. The report from the current study supports the findings from these works, as the samples where *L. pneumophila* was detected also displayed high TABC (Total Aerobic Bacterial Count). High TABC values typically indicate a rich microbial ecosystem. In our study on culture detection methods, we initially observed colonies on Buffered Charcoal Yeast Extract (BCYE) medium, which suggested the potential presence of *L. pneumophila*. However, this initial observation was not enough to definitively confirm the presence of the bacterium, leading us to treat these colonies as

presumptive identifiers of *L. pneumophila*. Following the latex agglutination testing, we found that none of these colonies met the criteria for confirmation

There is limited information available about the association between TFCC, the presence of *Salmonella*, and *E. coli*, particularly concerning *L. pneumophila*. However, in Bangladesh, fecal contamination of water is very common. High TFCC indicates poor sanitary conditions and possibly biofilm-prone systems, imposing an indirect risk factor for *Legionella* spp. colonization. Therefore, we aimed to investigate whether the water samples had any fecal contamination, which would give an insight into the water quality as well as any association with these pathogens with *L. pneumophila*. The results revealed that no coliform was detected in the water samples tested, except one, indicating a lack of association between fecal coliforms and *L. pneumophila*. Similarly, Whiley & Bentham, (2011) reported that there was no relation of the presence of *L. pneumophila* with fecal contamination; thus, conventional indicator organisms such as coliforms or *E. coli* are not reliable predictors of *L. pneumophila*. Bartram et al. (2007) stated that there was no consistent association between the presence of coliform bacteria and *Legionella* spp. in water systems. Leoni & Legnani (2001) also reported that there was no statistical correlation between fecal coliforms and *L. pneumophila* counts, even in recreational water environments. Fliermans et al. (1981) conducted one of the early landmark studies on the ecology of *L. pneumophila* and found no correlation between the presence of coliform bacteria and *Legionella* spp. in environmental water samples. Water systems can be free of coliforms yet still contain *L. pneumophila* in biofilms or warm stagnant areas. *L. pneumophila* is an environmental organism, not of fecal origin. In the current study, no correlation was observed regarding this issue. Variations in elevated Total Aerobic Bacteria Count (TABC) across samples from food industries, hospitals, and hotels may create favorable conditions for the proliferation of *L. pneumophila*. Therefore, environmental monitoring should not only focus on microbial enumeration but also include routine screening for *Legionella* spp. especially *L. pneumophila* in critical sectors such as food production and healthcare.

4.4 Prevalence of the virulence mip gene in *Legionella pneumophila*

The mip gene (macrophage infectivity potentiator gene) of *L. pneumophila* encodes a protein that promotes the entry, survival, and replication of this pathogen within human macrophages and amoebae, and is crucial for its infectivity. The mip gene appears in all

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strains of *L. pneumophila* but is absent in most other *Legionella* species, making it a dependable target for distinguishing *L. pneumophila* from non-pathogenic or less clinically relevant species (Cianciotto & Fields, 1992). This gene is widely used in diagnostic testing and epidemiological studies as a reliable genetic marker for detecting and identifying *L. pneumophila* through molecular methods like PCR or nested PCR.

In the current study, the *mip* gene was detected in both environmental and tracheal aspirate samples, which is an important finding, as in India (Sreenath et al., 2020) and in the kidney transplants in Tehran (Ebrahimi et al., 2022). These results highlighted a significant exposure to the virulent *L. pneumophila* in the community, and emphasize the need for increased awareness and preventive measures, as echoed by a similar study conducted in China (Zhang et al., 2017; Valciņa et al., 2023). They found virulent genes in environmental water sources, indicating a high virulence potential for *L. pneumophila* and highlighting the need for effective risk management to protect public health. However, we saw a discrepancy in the detection of *L. pneumophila* and the presence of the *mip* gene. Out of 171 water samples, *L. pneumophila* was identified in 43 samples, of which only five samples tested positive for the virulent *mip* gene (11.6%). In the tracheal aspirate samples, 12 (75.0%) tested positive for the *mip* gene, while 16 samples confirmed the presence of *L. pneumophila* 16S rRNA gene. The discrepancy could be due to genetic diversity in the *mip* gene region. The primers targeting the *mip* gene might fail to bind to the target if there are mutations or polymorphisms in the primer-binding sites. Failure to detect the *mip* gene might also be because of low DNA concentration in the sample metagenomic DNA.

4.5 Comparison among applied detection methods

With rapid urbanization and the installation of artificial water systems, Legionnaires' disease is rising throughout the world (Viasus et al., 2022). However, due to lack of a sensitive and accurate diagnostic method, this infection often remains undetected, leading to the suffering of the patients. The current study employed two different methods; culture-based and PCR-based to investigate the detection of *L. pneumophila*, which enabled comparison between the effectiveness of these two methods in the accurate detection of this pathogen from both environmental and clinical samples.

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Culture-based method is the gold standard for the detection and quantification of *L. pneumophila* in environmental and clinical samples. This method involves growing the bacteria on the selective media Buffered Charcoal Yeast Extract (BCYE) agar under specific conditions, with or without selective supplements GVPC (e.g., glycine, vancomycin, polymyxin B, cycloheximide) (ISO 11731:2017; Whiley and Bentham, 2011).

In the current study, it was not possible to detect *L. pneumophila* using the conventional culture-based method (Borges et al., 2012). This could be because of the fact that all the samples that were positive for *L. pneumophila* showed high TABC (Total Aerobic Bacterial Count), which led to a high load of different types of bacteria other than *Legionella* spp. on the BCYE medium, which might have interfered with the growth of *L. pneumophila*. Additionally, the striking similarities between the colonies of the non-*Legionella* bacteria with typical *Legionella* colonies might have led to confusion in diagnosis, and many samples of *L. pneumophila* might have been overlooked or missed. This indicates that cultivation on BCYE medium, considered a selective and gold-standard method, needs further review.

Additionally, this study also explored the use of the latex agglutination test, a rapid diagnostic method commonly used to detect *L. pneumophila*. This test is usually used to confirm colonies grown on selective media like BCYE agar. In the current study, when presumptive *Legionella*-like colonies from BCYE agar were subjected to the latex agglutination assay, it failed to detect any. This is not unexpected, as although this assay has benefits such as speed and simplicity, it does not always provide accurate results and shows low sensitivity. Lee et al. (2011) reported that the latex agglutination test showed low sensitivity in mixed populations, and environmental samples often contain a diverse microbiota, leading to a failure in detection. This assay can also lead to false-negative results due to low concentrations of *L. pneumophila* (Bartram et al., 2007), and also fails to detect non-*pneumophila* species or non-serogroup 1 strains of *L. pneumophila*.

In contrast, the polymerase chain reaction (PCR) is a powerful molecular technique used for the detection of *L. pneumophila*, especially in environmental and clinical samples (Diederer et al., 2006, 2008; Collins et al., 2015; Whiley and Taylor, 2016). It allows

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rapid, sensitive, and specific identification of *L. pneumophila* DNA, often outperforming culture methods in speed and detection rate (Fard et al., 2012). PCR detects *L. pneumophila* DNA within hours, compared to days or weeks required for culture. Additionally, PCR can detect very low levels of *Legionella* spp., including non-culturable or VBNC (viable but non-culturable) cells (Alleron et al., 2008). PCR methods targeting target genes like 16S rRNA, macrophage infectivity potentiator (*mip*), or *dot* (defective in organelle trafficking), *icm* (intracellular multiplication) genes have been employed to detect *L. pneumophila* in water systems such as cooling towers, showers, taps, (Whiley et al., 2014) and clinical respiratory samples, e.g., sputum, bronchoalveolar lavage, tracheal aspirates (Kataoka et al., 2015.)

In the current study, the PCR method targeting the LP16SrRNA sequence also exhibited accuracy and sensitivity in the detection of *L. pneumophila* from environmental and clinical samples. While the traditional culture-based methods failed to identify any *L. pneumophila* (LP) cases, standard PCR screening that targeted the LP16s rRNA sequence detected 18 out of 59 positive samples.

However, the single-step PCR-based method targeting the LP16SrRNA sequence also showed some limitations. In samples with lower metagenomic DNA concentration, this PCR failed to detect *L. pneumophila* or produced a PCR product in too low a concentration to confirm by sequencing. PCR may not reliably detect low levels of *L. pneumophila*, especially in samples with high microbial backgrounds or inhibitors (Lee et al., 2011; Collins et al., 2017). For such samples, the nested PCR method developed in the current study displayed better detection efficiency, which is a significant contribution of this study. Our study presents the development and optimization of a novel nested PCR assay targeting the 16S rRNA gene of *L. pneumophila*, offering significantly enhanced sensitivity and specificity compared to conventional PCR approaches. The implementation of the nested PCR method significantly improved detection rates (30) in water samples, increasing identification from 4.65% to 83.33%. Additionally, there was a notable rise in the detection of clinical tracheal aspirate samples (11), with the rate rising from 10% to 24.45%. This substantial improvement highlights the nested PCR method's potential as a more sensitive and specific tool for diagnosing *L. pneumophila* infections.

We performed multiple iterations of the nested PCR, optimizing the annealing temperature and cycling conditions to enhance primer efficiency. The specificity of these

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primers was confirmed as they did not anneal to the genomic DNA of a range of pathogens, including *Vibrio*, *Salmonella*, *Listeria*, *E. coli*, *Pseudomonas*, and *Klebsiella*. The lack of cross-reactivity with other bacterial DNA emphasizes the robustness and reliability of the nested PCR assay, indicating its potential usefulness for the accurate detection of *L. pneumophila* in various samples without interference from other common bacterial pathogens. Additionally, the primers were highly sensitive and could bind to one picogram of DNA. Nested PCR has emerged as a successful technique for the detection of microbial pathogens in complex environmental and clinical samples (Hassanin et al., 2020). Nested PCR methods targeting the *mip* gene of *L. pneumophila* have been developed to detect this pathogen in clinical and water samples (Bernander et al., 1997; Fiume et al., 2005). Targeting the 16SrRNA sequence in the nested PCR method is expected to be more accurate compared to targeting the *mip* gene, as the possibility of mutation in the *mip* gene, the major virulence factor, is more likely compared to the highly conserved 16SrRNA sequence. Compared with the method described by Devos et al. (2005), which achieved 98% detection in aquatic samples, the present study reports 100% detection and a defined analytical sensitivity. This highlights the improved performance and reliability of the developed assay. Petzold et al. (2025) recommended the integration of quantitative PCR (qPCR) with conventional culture techniques. However, the culture techniques have their limitations, which are already well-established. Our method solely relies on a PCR-based method and does not involve any culture method, increasing the sensitivity. The method designed in the current study is considered novel as it involves a novel set of primers, along with a newly developed PCR method, offering significantly enhanced sensitivity and specificity compared to other reported PCR approaches. This nested PCR method may fill a local or regional gap where commercial kits are unavailable, unaffordable, or poorly validated for environmental surveillance in Bangladesh or similar settings. Such a robust detection method is vital for routine monitoring, early risk assessment, and outbreak response in high-risk settings like healthcare facilities and food-processing environments. However, understanding the conditions that favor *L. pneumophila* in engineered systems, including their growth in biofilms and interactions, will help to develop new detection technologies to minimize human exposure to pathogenic *L. pneumophila* (Buse et al., 2012).

4.6 Conclusion of detection methods

The method for isolating and quantifying cultivable *Legionella* species from environmental and clinical samples has limitations, mainly due to its time-consuming nature, requiring selective media and days of incubation (7 to 10). Although an international standard exists for the detection of *Legionella* in water (ISO 11731:2017), challenges persist, such as overgrowth of other bacteria, presence of viable but non-culturable (VBNC) cells, and loss of viability during sample treatment (Diederer et al., 2007). Our study's inherently low *L. pneumophila* levels may affect data reliability.

Recovering *L. pneumophila* from water samples is challenging due to their low concentrations, necessitating concentration techniques such as centrifugation and filtration for environmental analysis. For clinical samples, it's essential to suppress the growth of competitive flora (Blanco et al., 2008). PCR offers a faster alternative, detecting *L. pneumophila* and non-culturable cells, although culture methods may yield lower detection rates (Wilson et al., 2003; Dusserre et al., 2008).

Traditional culture media often fail to detect *Legionella* spp. while metagenomic procedures successfully identify its DNA. Ongoing research highlights the need for more investigation to support public health efforts, providing insights for assessing risks and preventing the spread of *L. pneumophila*.

Chapter 05:

Conclusion

5. Conclusion

This study provides valuable data on the prevalence of *Legionella* spp. in Bangladesh and presents a robust detection method that can be integrated into public health and environmental monitoring programs. The results emphasize the widespread presence of *Legionella* spp. especially *Legionella pneumophila* in diverse aquatic environments, including rivers, lakes, groundwater, and processed water systems like cooling towers and plumbing systems. The widespread presence of *L. pneumophila* in processed water systems, such as cooling towers and plumbing systems, matches global findings that these environments are ideal for bacterial growth and spread. Warm temperatures, stagnant water, and the formation of biofilm create favourable conditions for *Legionella* spp. colonization. This is especially worrying in Bangladesh, where infrequent maintenance and monitoring of these systems raise the risk of contamination.

Additionally, the detection of *L. pneumophila* in natural water sources, such as rivers and lakes, underscores the importance of vigilance, as these sources are often used for drinking, bathing, and irrigation, potentially exposing numerous people to the pathogen. This highlights the potential public health risk posed by *Legionella* spp., particularly in densely populated countries like Bangladesh, where water quality management remains a significant challenge. The detection of *Legionella* spp. in both natural and processed water systems highlights the importance of comprehensive water quality management practices, including regular monitoring, maintaining water systems, and implementing public awareness campaigns. The findings suggest that interventions should focus not only on processed water systems but also on natural water sources, given their widespread use and potential for contamination. This also highlights the importance of integrating environmental monitoring with public health strategies.

Furthermore, the study provides data on the potential for *Legionella* spp. to adapt to varying environmental conditions in Bangladesh, including high temperatures and humidity. The phylogenetic analysis revealed a notable proximity among the *Legionella* sequences across water samples collected from diverse environments, including the food industry, hospitals, hotels, and clinical settings, highlighting the need for a more extensive genomic study of this pathogen in this region to better understand its pathogenicity and transmission dynamics to elucidate the transmission pathways of *L. pneumophila* in Bangladesh.

Conclusion

This study also validated the use of a novel nested PCR-based approach that offers a rapid, sensitive, and specific detection method of *L. pneumophila* as an alternative to the traditional culture-based method. Data from this study demonstrated the efficacy of the nested PCR-based method in identifying *Legionella* spp. even at low concentrations. This approach is particularly advantageous in resource-limited settings like Bangladesh, where rapid and accurate detection is crucial for timely intervention and prevention of outbreaks. Additionally, the implementation of the developed detection method in routine water quality monitoring programs could enhance the capacity to identify and mitigate *Legionella* spp. contamination in a timely manner.

In conclusion, the findings underscore the need for proactive measures to prevent *L. pneumophila* associated diseases, particularly in settings where water quality management is a persistent challenge. Addressing this issue requires a multidisciplinary approach, combining environmental science, microbiology, and public health strategies to safeguard the population from this emerging waterborne pathogen.

Chapter 06:

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Appendices

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Appendix 1

>MZ102255

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The *Legionella pneumophila* sequences reported in the study have been submitted to the GenBank database under the accession number.

Appendices

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Sequences obtained from the NCBI database

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Appendix 2

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Nested PCR sequences of sample data acquired from the NCBI database.

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Appendix 3**Culture Media Composition**

Composition of Buffered Charcoal Yeast Extract Agar (BCYE agar) Base

Ingredients	Amount (gram per liter)
Yeast Extract	10.00
Ferric Pyrophosphate g	0.25
ACES Buffer	10.00
Charcoal, Activated	2.00
Alpha-Ketoglutarate	1.00
Agar	15.00
pH 6.85 ± 0.1	
Supplement: 4 mL of a 10% solution of L-cysteine HCl	

Composition of Plate Count Agar (PCA)

Ingredients	Amount (gram per liter)
Casein Enzymic Hydrolysate	5.00
Yeast Extract	2.50
Dextrose	1.00
Agar	9.00
pH 7.0 ± 0.2 at 25°C	

Composition of Membrane Fecal Coliform Agar (mFC)

Ingredients	Amount (gram per liter)
Tryptose	10.00
Proteose peptone	5.00
Yeast extract	3.00
Lactose	12.50
Bile salts No. 3	1.50
Sodium chloride	5.00
Agar	12.90
Aniline blue	0.10

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pH 7.4 ± 0.2 at 25°C
Additional: 10 mL of 1% solution of Rosolic Acid in 0.2 N NaOH

Composition of MacConkey Agar

Ingredients	Amount (gram per liter)
Peptone	17.00 g
Proteose peptone	3.00 g
Lactose	10.00 g
Bile salt	1.50 g
Agar	13.50 g
Sodium chloride	5.00 g
Crystal violet	0.001
Neutral red	0.03
pH 7.1 ± 0.2 at 25°C	

Composition of Bismuth Sulphite Agar (BSA)

Ingredients	Amount (gram per liter)
Peptone	10.00
HM Peptone B	5.00
Dextrose (Glucose)	5.00
Disodium phosphate	4.00
Ferrous sulphate	0.30
Bismuth sulphite indicator	8.00
Brilliant green	0.02
Agar	20.00
pH 7.7 ± 0.2 at 25°C	

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Composition of Muller-Kauffmann tetrathionate (MKTT) broth

Ingredients	Amount (gram per liter)
Peptic Digest of animal tissue	4.30
Casein enzymic hydrolysate	8.60
Ox bile	4.75
Sodium chloride	2.60
Calcium carbonate	38.70
Sodium thiosulphate, pentahydrate	47.80
Brilliant green	0.0095
pH 8.2 ± 0.2 at 25°C	

Phosphate Buffer Solution

8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 , and 0.24g KH_2PO_4 were added and dissolved by stirring in 800 ml of distilled water. The pH was adjusted to 7.4 with a few drops of 1.0 M HCl, and the final volume was made up to 1000 ml. The solution was sterilized by autoclaving and stored at room temperature.

Acid Solution (KCl–HCl acid buffer, pH 2.2 ± 0.2)

A 0.2 mol/L solution of hydrochloric acid (HCl) was prepared by adding 17.4 ml concentrated HCl (minimum assay 37%) or 20 ml concentrated HCl (minimum assay 31.5%) to 1 L of water (solution A). A 0.2 mol/L solution of potassium chloride (KCl) was prepared by dissolving 14.9 g of KCl in 1 L of water (solution B). The acid solution was prepared by mixing 3.9 mL of solution A and 25 mL of solution B. pH 2.2 ± 0.2 was adjusted by the addition of a solution of 1 mol/l potassium hydroxide (KOH)

0.1N sodium thiosulfate solution

12.41 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ was dissolved in 500 mL of distilled water

TAE buffer

242g of tris-base, 57.10 ml of glacial acetic acid, and 100 ml of 0.5 M EDTA (pH 8.0) were taken, and distilled water was added to the mixture to make 1 L. 1X concentrated TAE buffer was made by adding 10 ml 50X TAE buffer with 490 ml distilled water and stored at room temperature.

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Ethidium bromide solution

10 μ L of ethidium bromide was dissolved in 100 mL of TAE buffer to make a final concentration of 20 mg/mL and stored at 4 °C in the dark.

QIAquick PCR Purification Kit (50). Catalog No. 28104

Reagents:

QIAquick Spin Columns

Buffer PB (binding buffer)

Buffer PE (wash buffer concentrate) contains 95% ethanol

Buffer EB (elution buffer)

Collection Tubes

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MICROBIAL BIOACTIVES

RESEARCH

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

Nazmun Naher¹, Sangita Ahmed^{1*}, and Md. Latiful Bari²

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*

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Appendices

Bangladesh J Microbiol, Volume 40, Number 1, June 2023, pp 33-39



Original Article

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Risk Assessment and Detection of *Legionella* species in the Water System of A Luxury Hotel in Dhaka city, Bangladesh

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Legionella cause Legionnaires' disease and Pontiac fever, and, more rarely, extrapulmonary infections, collectively referred to as legionellosis. *Legionella pneumophila* causes serious pneumonic infections, and water and Air Conditioning systems are a common source. We performed this study focusing on identifying and assessing the potential sources of *Legionella* spp. in a luxury hotel water system in Dhaka, Bangladesh. As hotels often have complex water distribution systems that can harbor and facilitate the growth of these bacteria, understanding the risks and implementing effective control measures is crucial for public health. This study determines the presence of *Legionella* spp. and potential factors contributing to their proliferation within the hotel water systems. In total 42 water samples and six swabs from 7 different points were collected multiple times between March 2018 and April 2019 and were analyzed. *Legionella* spp. was detected in all samples (100%) tested. The highest colony counts were observed in water samples from the hot water tank and cooling system. The findings highlight the importance of regular monitoring, maintenance, disinfection, and flushing protocols to mitigate the risk of *Legionella* spp., contamination, and subsequent health issues among hotel guests and staff.

Keywords: *Legionella* spp., Hotel water systems, PCR, 16S rRNA

Introduction

Hotel is one of the major hospitality industries and can play an important role in local and international tourism. However, the hotel is also found as one of the sources for certain disease outbreaks such as a source of Legionnaires' disease (LD). Currently, more than 60 *Legionella* species are known, but more than 90% of Legionnaires' disease (LD) cases are caused by *Legionella pneumophila*. LD was first identified in hotels in 1976 when over 180 members of the American Legion staying at the Bellevue-Stratford Hotel in Philadelphia reported unusual symptoms like weariness, chest aches, and fever, which led to 29 deaths¹. An inquiry by the Centers for Disease Control and Prevention identified the causative agent as *Legionella pneumophila*, a bacteria found in the hotel's cooling tanks. Following that, *Legionella* exposure has been linked to different types of premise plumbing systems (e.g., hotels and other buildings with complex water distribution systems)^{2,3}.

It is well known, that this ubiquitous organism is found in underground and surface water, and wet soils, but their main reservoir is man-made aquatic environments, hot scorching water systems in residential buildings, hospitals, nursing homes, hotels, and other private and public buildings, where they can colonize taps, shower heads, cooling towers, spas, and fountains. Of these, hotel water system has been identified as a major source of *Legionella pneumophila*, and many outbreaks of legionellosis from different parts of the world are linked to hotels⁴⁻⁷. In Bangladesh also, *Legionella pneumophila* was detected in one

tap water sample collected from a residential hotel in 2016⁸. However, there has been no further investigation of the hotel water system in Bangladesh for the prevalence of *Legionella pneumophila*, although in recent years the establishment of luxury hotels is on the rise in the country. Therefore, the current study attempted to investigate the presence of *Legionella* spp. in a luxury hotel in Dhaka, Bangladesh.

Methods and Materials

Sampling sites investigation: risk factors investigation

Sampling site investigation includes the visual inspection of potable water source, holding tank, primary disinfection procedure, building plumbing characteristics (plumbing materials, construction age, water circulation temperature), and hot water production procedure (heating systems, tanks, and their volume). Additionally, cascade (decorative fountains) water, cooling towers, air-conditioning, and domestic use water were also investigated. A detailed survey questionnaire was developed to collect all these data.

Water sampling approach

A total of 42 water samples were collected from 7 different points of the hotel from March 2018 to April 2019. The samples were obtained from 7 sites namely, a hot water tank, cooling tower, chiller room, swimming pool, fountain, air cooler water, and domestic use water located furthest from the hot water source, and the investigation of sampling sites of the hotel determined the number of collected samples. The samples were collected

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