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

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

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

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 deaths [1]. 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 hotels[4-7]. In Bangladesh also, *Legionella pneumophila* was detected in one tap water sample collected from a residential hotel in 2016[8]. 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

Corresponding author
Dr. Sangita Ahmed, Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh. E-mail: sangita@du.ac.bd

after the water had drained for 15–30 s in a sterile 2.5-litre plastic bottle containing 2.5 mL of 0.1 N sodium thiosulfate to neutralize residual free chlorine. The collection of samples was performed by trained staff according to standard methods [9].

Detection of Legionella by the standard culture method

Each water sample was filtered and concentrated by pouring the sample into a sterile 47 mm funnel assembly containing 0.45 µm polycarbonate filters. The filter was placed aseptically into 5 ml of sterile water, and the tube was then vortexed for one minute to loosen the bacteria into the water. The diluted and non-diluted water was surface plated on Buffered Charcoal Yeast Extract agar medium (Difco, Germany) and the plates were then incubated at 35°C in a 2.5% CO_2 incubator for seven days before being counted. Presumptive *Legionella* colonies were identified based on colony morphology, and colonies that appeared round, glistening, and convex with frosted glass appearance were subjected to a latex agglutination test by *Legionella* Latex Test Kit (LK04-Hi, Himedia, India) as per the manufacturer's instructions. The presumptive colonies of *Legionella* contamination determined are tabulated as per Technical Guidelines for the Investigation, Control, and Prevention of Travel Associated Legionnaires' Disease[10] presented in Table 1. Then, the proportions of positive samples were confirmed by molecular detection as described below.

Molecular Detection

Isolation of total DNA

One liter of water was filtered with a 0.45-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. Following incubation at 100°C for 5-10 minutes in a water bath, the tube was immediately chilled on ice for 30 minutes The suspension was centrifuged for 5 minutes 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[11].

Legionella pneumophila-specific 16SrRNA PCR

Metagenomic DNA of water samples was amplified by *Legionella* gene fragment 16SrRNA following the method described by Jonas[12]. The primers used for PCR are LPFP: 5'–AGGGTTGATAGGTTAAGAGC-3' and LPRP:5'–CCAACAGCTAGTTGACATCG-3'. PCR reaction was performed in a final reaction volume of 25 ìl in a PCR tube containing 12.5 ìl of master mix (OneTaq quick load 2x Master mix, New England Biolabs), 0.5 ìl of each forward and reversed primer, 2 ìl of metagenomic DNA and 9.5 ìl of nuclease-free water. Amplification was carried out in Veriti Thermal Cycler (Applied Biosystems, USA) and comprised initial denaturation at 95°C for 5 minutes followed by 40 cycles consisting of 94°C for 1.5 min, 57°C for 1.5 min, and 72°C for 1 min and a final extension at 72°C for 10 min. Then, the product was held at 4°C. After amplification product was processed for gel documentation or kept at - 20°C till tested. The amplified DNA fragment of 386 bp was identified by electrophoresis in 1.5% agarose gel with ethidium bromide. With strict adherence to the manufacturer's instructions, a PCR cleaning kit (Favorgen, Taiwan) was used to clean the amplified PCR products, and about 0.5-100µg of the purified product was sent to Macrogen (Korea) for sequencing. Sequence data generated by 16S rRNA gene amplification were submitted to the NCBI GenBank nucleotide sequence database.

Statistical analysis

Data analysis was performed by two-way analysis of variance (ANOVA). Tests were two-tailed, with á = 0.05. All analyses were performed using Microsoft Excel 13 software.

Table 1: *Definition of levels of contamination with Legionella in potable water*[13]

Level of *Legionella* contamination	*Legionella* spp. concentration (CFU/100 mL)
No or low level of concentration	<1 and ≥100
Medium level of concentration	>100 and ≥1,000
High level of contamination	>1,000 and ≥10,000
Extremely high level of concentration	>10,000

Results and Discussion

Bangladesh is one of the most densely populated countries. Its subtropical monsoon climate, seasonal rainfall, high temperature, and humidity provide an excellent environment for microbial growth. This environment is also quite suitable for Legionnaires' disease. In support of this view, this pathogen has been identified from respiratory tract and urine samples of patients in Bangladesh as well as water samples from patient vicinity and pond water[14]. Recently, water systems of several food industries and hospitals in Bangladesh have been found to harbor this bacterium in Bangladesh[11]. However, to our understanding, no one attempted to see the extent of this organism in the hotel water system in Bangladesh.

Chemical parameters of water systems were evaluated to study risk factors for colonization of *Legionella* species[15]. The pH of all water samples was within the standard level; however, cascade water and chilled water were found alkaline at 8.0 and 8.1(Table 2). The *Legionella* spp. can survive moderately acidic and alkaline conditions (pH levels of 5.5 to 9.2)[16]. Naturally occurring *L. pneumophila* multiplied at a temperature between 25 and 37 degrees C, and at concentrations of 6.0 to 6.7 mg/liter of dissolved oxygen. The TDS, salinity, and conductivity were higher in swimming pool water, while oxidation-reduction potential was higher in cascade water. The bacterium can survive in various environmental conditions and has been isolated from environmental sources with a pH ranging from 2.7 to 8.3. Temperatures between 20 and 45°C are ideal for the proliferation of the organism, with an optimal temperature range of 35–45°C. As the temperature falls, reproductive rates decrease, and there is little or no increase in bacteria numbers when the temperature is below 20°C. *Legionella* can survive for long periods at a low temperature and then proliferate when the temperature increases. At the temperature of 66°C *Legionella* dies within two minutes, and at temperatures above 70°C they are destroyed instantly[17]. Stagnating warm water and a water temperature between 20 and 45°C provide an ideal habitat for the massive growth of this bacterium[18]. Previous studies showed high isolation rates of *Legionella* spp. in building and accommodations sites worldwide, with 43.6% in Kuwait[19], 32.7% in Germany[13], 31.5% in Iran[20], 8.3% in South Korea[21], 84.1% in Italy[22], 74.77% in Poland[23], and 34.1% in Japan[24]. Several studies in European countries have reported a high prevalence of *Legionella* spp. in hot water systems in hotels, hospitals, and domestic buildings[25]. These studies demonstrate the importance of survey, monitoring, and control of *Legionella* colonization in hot water systems, while little is known about the occurrence of *Legionella* and contamination of hot water systems in Bangladeshi hotels. In this study, we conducted environmental monitoring of a luxury hotel in Dhaka to determine *Legionella* prevalence in the hotel's water distribution systems and investigate risk factors associated with contamination that are suspected to be related to the presence and/or growth of *Legionella*. However, Physio-chemical parameters did not influence the presence of *Legionella* spp., and survival in cooling tower water was reported earlier by Ahmadrajabi 2016 [26].

The average aerobic bacterial count in the water samples collected from the hotel was recorded as 4.20 log CFU/ml (Figure 1). The maximum load was observed as 6.68 log CFU/ml, and the minimum was 1.60 log CFU/ml, as shown by direct culture plating. On the other hand, the average *Legionella* load was recorded as 3.90 Log CFU/ml. The maximum load was 5.77 log CFU/ml in hot water tank samples, and the minimum was 2.0 log CFU/ml in chilled water (Fig 1). The *Legionella* spp. contamination at specific water samples in the water distribution systems of the investigated hotel is presented in Table 4. Although an extremely high level of *Legionella* spp. contamination was found in 33.3% of cooling towers and hot water tanks, and a medium level of *Legionella* spp. contamination was recorded in 100% of cascade water, 83.3% of AC water, and domestic water outlets, and 66.6% of cooling tower water, hot water tank, and swimming pool water; and 33.3% of chilled water, in culture detection method, however, molecular

Table 2: *Physio-chemical parameters of hotel water samples*

	Physio-chemical parameters of water samples				
	pH	TDS	salinity	Conductivity	ORP
		ppm	ppm	µS	mv
Cooling Tower Water	7.6	570	3.98	6.41	110
Hot Water Tank Water	6.7	221	169	305	97
Swimming pool water	7.1	318	245	449	103
Cascade water	8.0	253	194	352	119
Chiller room water	8.1	210	121	230	101
AC water	6.6	150	130	222	103
Domestic water	7.5	215	162	296	110

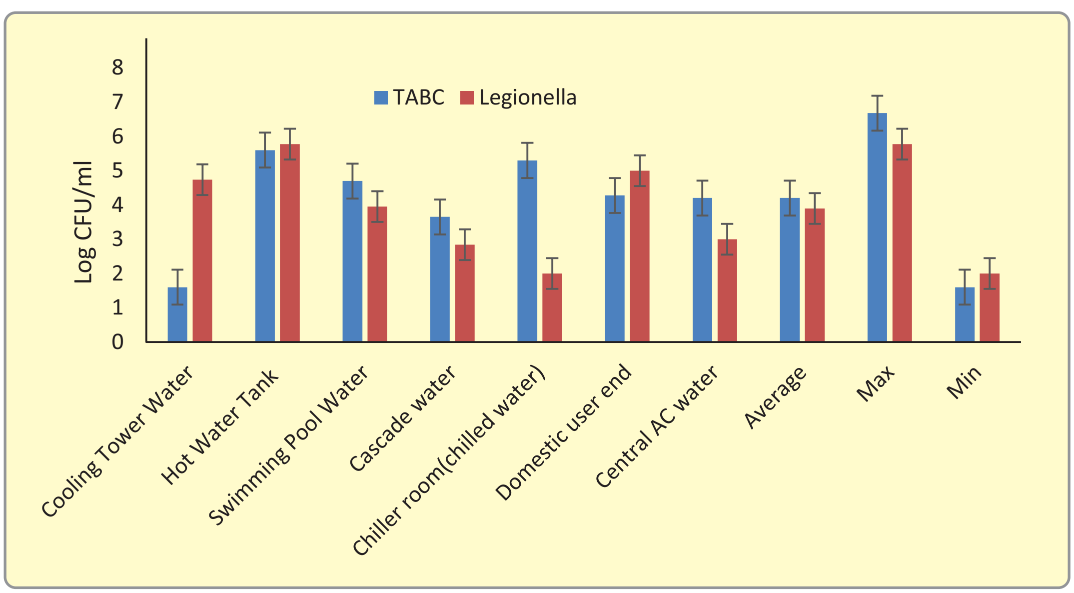

Fig 1: *Distribution of TABC and Legionella spp. in the water supply systems of the investigated hotel.*

identification confirms only cooling tower water and cascade water (Table 4). Cascade is a decorative fountain that produces aerosols, thus not intended for use by persons. However, the investigated hotel has a bar where 50 seats are available to serve drinks to guests. Therefore, the guests are at increased risk of Legionnaires' disease depending on the period and frequency of exposure. In the cooling towers, more than 10^4 CFU/ml *Legionella* spp. was detected indicating the action required to reduce the risk. The Action to be taken following the microbial monitoring for cooling towers as per European Technical Guidelines 2017 to minimize the risk of *Legionella* infections in building water systems is presented in the following Table 3.

The domestic use water such as showers of the investigated hotel, were manual mixers, from which 100% were supplied with hot water from the electric tank and instantaneous heater, and a total of 8 hot water tank was kept on 14F of the hotel building. Cold water for all showers was supplied from the main reservoir located in the basement of the hotel building. The mean age of the building was 3.5 years, but some of the water pipes had been partially renovated over the years. According to the data collected through the questionnaire interviews, 35% of the construction was more than 15 years old, 25% were from 10 to 15 years old, and 40% had been constructed in the last 3.5 years. The hotels had an independent water heating system, and there was no recent mention of water leakage or water supply disruption. Based on questionnaire results, seven plumbing materials were commonly used in water distribution systems as follows: polypropylene random copolymer (PPR), galvanized steel, polyvinyl chloride (PVC), cross-linked polyethylene (PEX-c), chlorinated polyvinyl chloride (CPVC) stainless steel and copper (stainless steel and copper are used partly in the pipes of the hotel).

This study finds that the most implicated risk factor of *Legionella* contamination was the water temperature fluctuations in circulation. In addition, a regular flushing program is needed to prevent water stagnation in less frequently used outlets. Water treatment strategies, such as chlorination, to control bacterial growth. Cleaning and disinfecting water storage tanks, cooling systems, and other water-related equipment. Awareness of hotel staff about the risks of *Legionella* spp. and the importance of proper maintenance and hygiene. The deviation of items from the standard value of the risk factors analyzed is presented in Table 5. The highest score was reported as 2.33 out of 4 for staff skill and water control measures. The examination of poor flow/ stagnancy, scale, sediments, corrosion, and so on, as well as monitoring and evaluation, yielded the lowest score (1.00), suggesting that action was needed to improve the condition.

Table 3: *Action level following microbial monitoring for cooling towers (Adapted from EU Technical Guidelines, 2017)*

Aerobic count (cfu/ml) at 30°C (mini.48 hours incubation)	*Legionella* (cfu/L)	Interpretation/action required
10,000 or less	Not detected	Acceptable
	1000 or less	Check all parameters (e.g., pH, biocide levels, etc.) regularly and keep within standard limits
More than 10,000 and up to 100,000	More than 1000 and up to 10,000	A review of the control measures and risk assessment should be carried out to identify any remedial actions
More than 100,000	more than 10,000	The system should immediately be stopped and corrective action should be done.

Table 4: *Level of L. pneumophila contamination in the hot water system of the investigated hotels (n=42)*

Parameters	Numbers of water samples (x = *Legionella* colony count CFU/L)					
	Negative (x<10)	10 d” x <10^2	10^2 d” x <10^3	10^3d” x <10^5	Total positive	Molecular Identification (GENBANK accession number)
Cooling Tower Water	0 (0.0%)	4(66.6%)	0(0.0%)	2(33.3%)	6 (100%)	ON924464
Hot Water Tank Water	0(0.0%)	4(66.6%)	0(0.0%)	2(33.3%)	6 (100%)	-
Swimming pool water	1(16.6%)	4(66.6%)	1(16.6%)	0(0.0%)	6 (100%)	-
Cascade water	0(0.0%)	6 (100%)	0(0.0%)	0(0.0%)	6 (100%)	ON924468
Chiller room water	0(0.0%)	2(33.3%)	3 (50.0%)	1(16.6%)	6 (100%)	-
AC water	0(0.0%)	5(83.3%)	1(16.6%)	0(0.0%)	6 (100%)	-
Domestic water outlet	0(0.0%)	5(83.3%)	1(16.6%)	0(0.0%)	6 (100%)	-

Table 5. *Findings from the completion of the checklists of the hotel investigated. Only the items for which a deviation from the standard value was recorded are presented herein.*

Scoring Items		Score (%)
1.	**Assessment of the ability of premises personnel to control risk**	
	Is there a responsible person appointed for *Legionella* control?	3
	Is this person(s), and other relevant staff, properly trained to control *Legionella*	2
	Is the person competent, to understand the system(s) risk factors and control measures?	2
	Average score	2.33
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?	4
	Is a private water supply (e.g., a well, spring, or bunkered water) used as a source of water?	4
	Is there evidence that the hot water temperatures in the entire hot water system are always kept between 50^{0}C and 60^{0}C?	1
	Is there evidence that the cold-water temperatures in the cold-water system are less than 25^{0}C?	1
	Are there any additional protective measures in place (such as chlorine, chlorine dioxide, copper/silver ions, and so on)?	3
	Is there evidence that proper monitoring and effective biocide levels are maintained throughout the circuits and the outlets?	1
	Average score	2.33
3.	**Assessment of other elements that may favor *Legionella* growth (poor flow/stagnancy, scale, sediments, corrosion, etc.)**	
	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)?	1
	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.)	1
	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.)?	1
	Is there any visible or considerable deposit of silt, biofilm/slime, filth, corrosion, or scale in any component of the water network?	1
	Average score	1.00
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?	3
	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?	1
	Is the complete water network cleansed at the start of each season when a building is not used throughout the year?	2
	Is it necessary to disinfect water filters (sand filters, multimedia filters, etc.) and softeners regularly, at least every three months?	2
	Are carbon filters (if used) replaced following the manufacturer's recommendations and before the start of each season?	2
	Average score	2.00
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)?	3
	Is a Legionella control program in place for each system that may represent a risk?	0
	Is this program appropriate and sufficient for all systems on the premises that could offer a risk of Legionnaires' disease?	0
	Are frequent records (logbooks, for example) of essential monitoring activities (temperatures, chlorine levels, etc.) kept on site?	2
	Are these records reviewed by the person(s) in charge?	0
	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)?	1
	Is the risk assessment as well as the operation of the control measures evaluated regularly by an independent and competent person?	1
	Average score	1.00
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?	2
	Are there any records of biocide treatment and spa water pH monitoring?	2
	Is there a skilled and competent individual in charge of cooling tower operation and monitoring?	3
	Is a microbiological monitoring system in place, as well as a chemical monitoring system (e.g., chlorine or bromine treatment);	2
	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)?;	2
Average score		2.2

The rating scale was developed based on the following criteria;

0= Element non-existing;

1= Components and structures of elements in place but not operational;

2=Components and structures of elements in place but limited operational,

3 =Components and structures of elements in full operation, and

4=Components and structures of elements fully operational and demonstrated signs of sustainability and evolution (dynamic nature)

Our study indicates that the hotel water system investigated in Dhaka represents a possible source of risk for Legionnaires' disease (LD) and confirms the sensitivity of the molecular method. We observed that several samples were presumptively identified as *Legionella* spp., using morphological, cultural, biochemical, and latex agglutination tests. However, when these culturally presumptive samples were analyzed by using a specific LP16SrRNA PCR primer, among the 42 water samples, *Legionella pneumophila* was detected only in 2 (4.7%) water samples, collected from the cooling tower and cascade water. The poor sensitivity of the culture method might be due to the lack of some particular nutrients necessary for the growth of *Legionella pneumophila*, which is yet to be known [11].

In hotel buildings, potable water systems begin when the water enters the building and end where the pipe links to a faucet, showerhead, or another distribution outlet. All pipes, water heaters, storage tanks, faucets, nozzles, and other distribution outlets are included in the systems. Chlorination concentration, temperature, plumbing system design, and plumbing materials are all factors that may influence *Legionella* proliferation in the plumbing system[27]. The risk factors investigation of the hotel showed that > 50 ppm chlorine is used for routine water disinfection. However, *Legionella* was detected in this investigation, meaning that this chlorination concentration was not enough to inactivate *Legionella* spp.

Hotels utilize hot and cold-water tanks to operate their hydroelectric energy, and if left unregulated, these water tanks can be a source of fatal *Legionella* bacteria, as well as corrosion and formation of biofilm[28]. Rust is another potential indicator of a suitable environment for growing *Legionella* spp. An aging water boiler, for example, may no longer be able to provide the approved storage temperature minimum of 60^0C due to age, resulting in deterioration of water quality and an increased risk of *Legionella* bacteria growth[29].

Occupancy is also a consideration, as no hotel is ever fully booked, and thus, unused rooms water will potentially sit undisturbed within unused outlets, increasing the likelihood of a perfect environment for *Legionella* bacteria. Furthermore, if parts of the hotel are closed for renovations, the water in the closed sections' pipes is at risk of developing similar favorable conditions due to them being temporarily closed, rerouted, or wholly cut and dead-ended. In any situation where water is not flowing through a clean exit but instead sitting and growing old in one place, germs such as *Legionella* can emerge. When water is non-flowing, also known as stagnant, it can risk fostering and developing multiple species of bacteria inside its waters or on its surface[30,31].

At a surface level, the bacteria will clump together in a microfilm, which can float along the waterways until it enters a building through any watercourse. Within this biofilm of bacteria runs the risk of being *Legionella* bacteria, amongst other possible harmful or non-harmful bacteria. This is not the only way *Legionella* can enter a hotel or domicile, however, as multiple different scenarios within and without the walls could play a risk of the growth of these harmful bacteria and risk people's safety.

Conclusion

The laboratory analysis demonstrated the presence of *Legionella* species including *L. pneumophilla* in cooling water and cascade water systems, and environmental site assessment results also showed poor monitoring and maintenance of water distribution to be the reason for *Legionella* contamination. The findings underscore the need for proactive measures, including proper maintenance, temperature control, a regular flushing program to prevent water stagnation, and water treatment strategies to minimize the risk of *Legionella* spp., proliferation in the water distribution systems of the hotel. The limitation of our study is selecting only one hotel. With current observation, we consider public health should investigate both luxuries as well as ordinary hotels to avoid future outbreaks of LD. In addition, training and awareness for hotel staff are necessary to educate them about the dangers of *Legionella* spp.

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