



SHORT COMMUNICATION

***Legionella* surveillance in stagnant water systems during COVID-19 lockdown in Istanbul**

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ABSTRACT

Aim: The aim of this study was to monitor the restricted water systems during the period of COVID-19 lockdown for the waterborne pathogen *Legionella pneumophila*. Selected water systems were monitored to evaluate the effect of stagnation in terms of *Legionella* colonization.

Methodology and results: A total of 160 water samples were taken from buildings which were temporarily shut down or had restricted usage between April–December 2020. None of the sampling points had *Legionella* positive history in the last three years. All water samples were tested using classic microbiological culture as the gold standard and rapid Vermicon ScanVIT Legionella test kit combined with epifluorescence microscopy. Sixteen (10%) out of 160 water samples were recorded as *Legionella*-positive by both methods, where none of them was positive before the COVID-19 lockdown. All positives were tested as *L. pneumophila* serogroup 1 with latex agglutination kit.

Conclusion, significance and impact of study: Colonization of *Legionella* in stagnant water systems occurs rapidly. Before reopening, routine monitoring in buildings is extremely important especially under restricted use or closure is in case. Flushing, disinfecting and testing are crucial for minimizing the health risks for the public health.

Keywords: *Legionella*, COVID-19, stagnant water, ScanVIT, monitoring

INTRODUCTION

Due to COVID-19 pandemic in 2020, business premises, care facilities, shopping malls and hotels have been temporarily shut down to maximize safety and reduce the risk of COVID-19 spread. A plan by the Turkish government to relax measures has been approved as of May 2021. However, unprecedented restriction causes stagnant water in distribution systems and that prolonged lockdown have led to the growth of waterborne pathogens like *Legionella pneumophila* in man-made water systems (Dey and Ashbolt, 2020; Proctor *et al.*, 2020). As a result of lockdown, pathogenic bacteria and waterborne protozoa could multiply up to harmful levels and this might pose a serious public health risk (Seenivasan *et al.*, 2005; De Giglio *et al.*, 2020).

The opportunistic pathogen *L. pneumophila* is a common member of freshwater environments. Whereas their natural habitats are rivers, ponds, lakes, even soil, *Legionella* proliferate rapidly in man-made water systems (Turetgen *et al.*, 2005; Berjeaud *et al.*, 2016). *Legionella pneumophila* is the etiologic agent of Legionnaires' disease and commonly found in high numbers in whirling

pools, spas, wet cooling towers and decorative fountains (Turetgen, 2004; Lu *et al.*, 2015; LeChevallier, 2019; Lytle *et al.*, 2020). Stagnant water in unused networks could be a good reservoir which *L. pneumophila* could settle in. It was reported that *L. pneumophila* is the significant cause of sporadic and epidemic community-acquired pneumonia (Lu *et al.*, 2015). The disease can be fatal and is caused by the inhalation of aerosols containing *Legionella*, disseminated from man-made water systems (fountains, cooling towers, showerheads etc.). Inhalation of *Legionella* contaminated aerosols might cause a severe form of pneumonia called Legionnaires' disease (Principe *et al.*, 2017). The incidence of *L. pneumophila* took the first place in the list of waterborne pathogens. Graham *et al.* (2020) analyzed that about 13% of the healthy population was seropositive for *L. pneumophila*. The maximum antibody percentage was estimated at 45%. This data suggests that the distribution and presence of *L. pneumophila* is ubiquitous. The majority of legionellosis cases are sporadic and community-acquired.

If nutrients are available in network water, temperature is suitable for growth and the routine monitoring/disinfection procedures are neglected, an

outbreak is quite possible due to rapid proliferation ability of *L. pneumophila* in man-made water systems (LeChevallier, 2019). It is anticipated that outbreaks will increase after reopening of buildings where favorable conditions (e.g., stagnant water, nutrient accumulation) are present. Stagnant water is an ideal environment for biofilm settlement on pipe and storage tank walls which can also harbor amoebae to serve as a host for family Legionellaceae (Turetgen, 2004). It is known that short-term stagnation also results in loss of disinfectant residual, but no recent study was found about the impacts regarding long-term water stagnation (Proctor *et al.*, 2020).

The objective of this study was to monitor the restricted water systems during the period of closure to evaluate the effect of stagnation in these systems regarding *Legionella* colonization. The stagnant water system of buildings possesses a risk of proliferation. It is known that routine monitoring is the key to keep the systems clean as regulated by the law and mandatory surveillance requirements have therefore been put in place in many countries. It is clear that technical staff of the buildings must manage the risks in water systems and take actions to prevent possible outbreaks after the relaxation of lockdown conditions.

MATERIALS AND METHODS

Sample collection

Water samples were collected from man-made water systems in Istanbul city center which were shut down or have restricted access during COVID-19 lockdown between April–December 2020. In total 160 samples were taken from business premises (60 sampling points), shopping malls (30 sampling points) and hotels (70 sampling points). In this study, 44 samples were collected from wet cooling tower basins, 82 from hot water taps and 34 from showerheads. Those buildings were asked for valid *Legionella* test reports at first and chosen for the study when they have no *Legionella*-positive test history within the last three years. Detection of *Legionella* was done by using two methods – classic culture on buffered charcoal yeast extract agar (Oxoid™, UK) and rapid ScanVIT Legionella test kit (Vermicon™, Germany). All samples were taken into single use, pre-sterilized dark plastic containers with 1000 mL volume, containing 1 mL 10% sodium thiosulfate as a neutralizer against chlorine-based disinfectants. The samples were transferred to the laboratory without any delay and analyzed within 24 h upon arrival.

Legionella microbiological culture

For microbiological culture, water samples (1000 mL) were concentrated using nylon membrane filter with 0.22 µm pore size (Sartorius, Germany). A volume of 2 mL of concentrated sample was incubated at 50 °C for 30 min and another 10 mL of sample was treated with 0.2 M 1:1 HCl–KCl (pH 2.2) to eliminate non-target microbes

(Turetgen *et al.*, 2005). A volume of 0.1 mL of each of these samples were spread plated onto Buffered Charcoal Yeast Extract (BCYE) agar (Oxoid, UK). All plates were incubated at 37 °C for 10–14 days in a humidified incubator. *Legionella* forms typically greyish-white colonies with a cut-glass appearance on the BCYE agar (Figure 1). Definitive identification of the resulting isolates was performed by latex agglutination (Oxoid™, UK). Agglutination positive colonies were counted as colony forming unit per liter. Latex particles with specific rabbit antibodies allow a separate identification of *L. pneumophila* serogroup 1 and serogroups 2–14. A loopful colony was emulsified in a drop of manufacturer's buffer on a cardboard. A drop of reagent was added on it. A result recorded as positive if "agglutination" of the black polystyrene latex particles occurs within 1 min.



Figure 1: *Legionella pneumophila* colonies on BCYE agar plate.

ScanVIT Legionella test

Manufacturer's instructions were followed for the ScanVIT Legionella test (Invitrogen, Germany). Water sample (50 mL) was treated with 10 mL of acid solution (0.2 M HCl/KCl solution, pH 2.2) for 5 min and then filtered through the ScanVIT membrane. The membrane filter was rinsed with 10 mL of distilled water and incubated at 37 °C for 72 h. The membrane filter was then transferred to an empty Petri dish using sterile tweezers and three drops of solution B3 were added on it and left to dry for 30 min. After drying, two drops of PreVIT solution were added onto the filter and left on it for 30 min. The nutrient pad was placed in the middle of the ScanVIT reactor and wetted with six drops of PreVIT solution. The dried membrane filter was placed on the wet nutrient pad and two drops of VIT M solution dropped onto it and incubated at 46 °C for 90 min. The washing solution was prepared with 0.2 mL D1B solution + 1.8 mL distilled water and incubated at 46 °C for 30 min. The washing solution was transferred to the reactor incubated at 46 °C for 15 min. The washing solution in the reactor was removed and 2 mL of distilled water was added for rinsing. The membrane filter was placed in a Petri dish and three

drops of Finisher S were added. The filter was examined using an epifluorescence microscope (Nikon 80i, Japan). The test was carried out with positive and negative controls as supplied by the manufacturer.

RESULTS AND DISCUSSION

Sixteen *L. pneumophila*-positive results were recorded in a total of 160 environmental water samples using the culture method. The ScanVIT test kit also detected the same number of positive results (16 positives) in comparison to the classical culture method for the same water samples (Table 1). *Legionella pneumophila* colonies appear red in color (Figure 2) on ScanVIT membrane, whereas those of other *Legionella* spp. would be in green color (Gruas *et al.*, 2013). The detection of *Legionella* takes place on a cultivated filter brought into contact with the gene probes marked with a dye. During the ScanVIT analysis, the marked gene probes enter the bacteria and bind to the matching signatures within the cells. In total of 16 *L. pneumophila* positive results, six samples were from cooling towers, five were from hot water showerheads and another five were from hot water taps (Table 2). The minimum *Legionella* bacteria count among the positive samples was 402 CFU/L from a hotel showerhead, whereas the maximum detected number was 19750 CFU/L from a shopping mall cooling tower water sample (Table 1). Sampling was done between April–December 2020 which coincides mostly to hot summer season in Turkey, which might facilitate bacterial colonization in water networks. The results showed that both methods gave identical results for all positive and all negative control samples.

This study revealed that *Legionella* can rapidly colonize in water systems when closure or restricted use was implemented by the authorities. The first positive result was recorded in the second week of restriction, which could be interpreted as stagnation makes the water systems more vulnerable in terms of bacterial colonization. Depending on the accredited laboratory tests, the chosen buildings were free of *Legionella* (within



Figure 2: Two red light emitting *Legionella pneumophila* microcolonies on ScanVIT membrane, visualized under epifluorescent light (magnification 100x).

the last three years) before lockdown. Positive results were therefore highly associated with inactivity in the buildings surveyed. Stagnant water also supports biofilm growth on surfaces which have contact with the water and after reopening biofilm detachment might contaminate the distal sites of the network. Eight out of 16 positive samples contained *Legionella* above 1000 CFU/L, which requires action for remediation, depending on national laws and regulations pertinent to *Legionella* (National Academies of Sciences, Engineering and Medicine, 2019). All positive samples were tested as serogroup 1 by latex agglutination, which was the most infectious serogroup of the genus *Legionella* (Zhang *et al.*, 2014).

De Giglio *et al.* (2020) monitored the microbiological quality of the distributed water in a COVID-19 hospital and found that it was severely colonized by *L. pneumophila*, where stagnant water favored bacterial growth in pipe rigs and storage tanks. Palazzolo *et al.* (2020) reported a legionellosis case from a restaurant in Italy, right after the end of the first COVID-19 lockdown.

Table 1: *Legionella pneumophila* test results taken from selected water systems.

Total sample number (n)	<i>Legionella</i> -positive samples (culture)	<i>Legionella</i> -positive results ScanVIT	Percentage of <i>Legionella</i> positive	Maximum number of <i>Legionella</i> (CFU/L)	Minimum number of <i>Legionella</i> (CFU/L)
160	16	16	10%	19750	402

Table 2: *Legionella* colony forming unit (CFU/L) data from positive sampling points.

Sampling point	Sample codes and CFU/L counts					
Cooling tower (6 positive)	#12 1031 CFU/L	#23 798 CFU/L	#39 1225 CFU/L	#84 19750 CFU/L	#101 7041 CFU/L	#151 9608 CFU/L
Hot water tap (5 positive)	#21 731 CFU/L	#77 910 CFU/L	#91 1009 CFU/L	#103 708 CFU/L	#155 1020 CFU/L	
Hot water showerhead (5 positive)	#47 777 CFU/L	#49 402 CFU/L	#52 1007 CFU/L	#81 960 CFU/L	#144 558 CFU/L	

The restaurant was closed during the lockdown. This situation shows the importance of regular controls and disinfection in public places and facilities after reopening. A different case arose from Egypt. Arashiro *et al.* (2020) reported a coronavirus and *Legionella* co-infection. An 80-year-old Japanese male developed legionellosis symptoms returning to his home country in March 2020. Fattorini *et al.* (2020) and Lai *et al.* (2020) emphasized the possibility of co-infection with coronaviruses and other respiratory pathogens like *Legionella*.

The ScanVIT *Legionella* test was shown to be rapid and sensitive for *Legionella* detection in water samples (Bargellini *et al.*, 2010; Gruas *et al.*, 2013; Di Maio *et al.*, 2020). Therefore, it was chosen to support the culture method as a complementary test in the current study. The disadvantage of the ScanVIT *Legionella* test kit was the inability to recover and subculture the growing colonies on the ScanVIT membranes. Isolated strains have critical importance for further investigation and diagnosis regarding epidemiological actions. Ditomasso *et al.* (2010) reported the ScanVIT *Legionella* could be recommended for investigating the presence of *Legionella*. They also concluded that the culture method and ScanVIT *Legionella* test kit were equally adequate for the detection of *Legionella* from environmental samples. The primary advantage of the ScanVIT *Legionella* test kit is giving results after 72 h, whereas the culture method needs up to 10 days for colony observation. Rapid detection of *Legionella* contamination is critical to minimize the risk of legionellosis cases (Bargellini *et al.*, 2010). The rapid test kit also allows the differentiation between *L. pneumophila* and other *Legionella* spp. Despite its low incidence, *L. pneumophila* is a significant causative agent of community- and hospital-acquired pneumonia.

The current study showed that even *Legionella*-free water systems are prone to colonization during lockdown or restricted use. There is an increased risk of possible outbreaks because *Legionella* can rapidly proliferate in stagnant water systems. One of the potential limitations in this study was to find *Legionella* free premises and buildings, because generally man-made water systems were colonized by *Legionella*. Hozalski *et al.* (2020) reported that flushing stagnant network water systems after the COVID-19 shutdown can reduce *Legionella* infection risk. The same study suggested that flushing could rapidly improve water quality in restricted buildings, but the improvement may only last a few days. Therefore, routine control and disinfection regime in premises should be implemented regularly. Water quality may not have priority in COVID-19 pandemic, but surveillance for waterborne pathogens should have been implemented for the post-pandemic period.

CONCLUSION

To conclude, this study showed that long time *Legionella*-free water systems could favor bacterial growth in a short time, not months but weeks. The first positive isolate recorded right after the start of the stagnation, where the

sampling point was not associated with *Legionella* before lockdown. When buildings reopen after lockdown, it is critical that water systems are not put back into use without considering the risk of *L. pneumophila* colonization. Environmental surveillance is still a valuable tool for risk management and control. In this study, *Legionella* colonization in positive buildings are not associated with Legionnaires' disease cases. However, further studies are needed for disease surveillance and outbreak detection after the COVID-19 lockdown.

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