Comparing Efficiency between Conventional and Molecular Methods for Detecting *Legionella pneumophilia* in Dental Unit Waterline Systems

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Abstract

The aim of our study was to detect the prevalence of *Legionella pneumophilia (L. pneumophilia)* in $DUWL_S$ using standard culture technique (SCT) and the real-time polymerase chain reaction (PCR) method in order to assess the risk of *L. pneumophilia* contamination within a dental setting. A total of 65 water samples were collected from 16 dental units and one cold water supply system from all clinical departments. *L. pneumophilia* could not be detected in any of the water samples using the standard SCT (0%), whereas *L. pneumophilia* was detected using real time PCR in three (4.6%) water samples collected from the tap system. Following the detection of *L. pneumophilia*, the tap systems were disinfected with surface disinfectant and water samples were recollected. The recollected water samples following disinfection were negative for *L. pneumophilia* once analyzed using culture and real time PCR technique. Although the culture method using BCYE media is the 'gold standard' for the detection of *L. pneumophilia;* Real Time PCR analysis may also be a quick, useful, and sensitive method for the detection of *L. pneumophilia* in order to control and prevent possible infections that may arise in the dental setting.

Keywords: *Legionella pneumophila*, real-time polymerase chain reaction, culture, dental unit, waterline

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Introduction

Legionella pneumophilia (L. pneumophilia) is a pathogenic bacteria that spreads via waterways and is mostly isolated from such aquatic environments as air-conditioning systems, water tanks of hotels, ornamental pools, shower heads, cooling towers, spas, whirlpools, and water distribution systems in hospitals, and in medical equipment that include respirators, dialyzers, inhalers, humidifiers, and balneo therapy. In addition to these environments, dental unit waterline's (DUWLs) narrow-plastic tubing structures provide an opportunity for *Legionella* and other bacteria for colonization and biofilm formation. These tubing systems carry water at high-speed to hand pieces and air/water sprays of dental chairs and present an optimum environment for microbial colonization [1-2].

During dental treatment, dentists use high-speed handpieces, air/water syringes, or ultrasonic devices that can lead to the expansion and inhalation of *Legionella* aerosols [3]. Aerosols produced during dental treatments are the most important pathway for the migration of *Legionella* via dental practices. DUWL$_S$ that are contaminated with *Legionella* may contribute to respiratory diseases among dentists, dental nurses, and patients [4]. The high prevalence of *Legionella* sero-positivity in dental personnel supports the notion that dental healthcare staff are at a high level of risk infection [5].

The routine method used for environmental inspection of *Legionella* is the standard culture technique (SCT), a complex technique that includes the concentration of microorganisms from water sources by filtration and/or centrifugation followed by heat and acid treatments prior to inoculation of the selective medium GVPC (BCYE, or buffered charcoal yeast extract agar, which contains additional supplements and antibiotics such as glycine, vancomycin, polymixin, and cycloheximide). Other methods have been developed for the detection of *Legionella* in aquatic environments using molecular methods in order to overcome the disadvantages associated with culture methods [6-7].

The aim of the current study was to detect the prevalence of *L. pneumophilia* in DUWLs, to compare the efficiency of SCT and real time PCR in detecting *L. pneumophilia,* and to conduct a risk assessment of *L. pneumophilia* contamination in a dental setting.

Material and Methods

Collection of Water Samples

A total of 65 water samples were collected from 16 dental units (two dental units from each department and one from each operating room) and one cold-water supply system (input and output taps) from clinical departments (e.g., periodontology, pedodontics, orthodontics, operative dentistry, endodontics, oral surgery, prosthodontics, and two operating rooms) between October and December 2015. The dental units were selected randomly. Water samples were collected in 1 L sterile glass bottles. The faculty building is supplied with a single cold-water supply system and the age of all dental units is eight years. The water samples from dental units were taken from the following four sites: outlets of air/water syringes, outlets of high-speed hand pieces, water cup fillers, and the taps of each selected unit.

Processing of Water Samples

Water samples of 1,000 mL were filtered through 0.45 μm pore cellulose filters (Advanced Microdevices Pvt. Ltd, India) using a membrane filtration system. Filters were washed for 5 min in an acid buffer (0.2 mol/l HCl), then rinsed in a Page saline solution (NaCl, MgSO$_4$. 7H$_2$O, CaCl$_2$.2H$_2$O, Na$_2$HPO$_4$, KH$_2$PO$_4$) and divided into two parts with a sterile lancet for culturing of the bacteria and DNA extraction.

Isolation and Identification of *Legionella* strains

Filters were placed onto BCYE containing supplements (Liofilchem, Italy). The plates were incubated at 37ºC for 6-14 days with a daily check for growth. Suspected colonies of *Legionella* spp. were Gram-stained and subcultured onto selective BCYE agar (Liofilchem, Italy) and blood agar. The suspected colonies were identified to the species level and serogrouped with a *Legionella* Latex Kit (Microgen Legionella, UK) that supports the identification of *L. pneumophila* by serogroups.

Real-time PCR

All the samples were analyzed with a real-time PCR instrument using the DNA extracted from the cellulose filters using Ribospin Vrd (GeneAll Biotechnology Co., LTD) according to the manufacturer's instructions. The Real-Time PCR system (CFX 96-Real Time System, ThermalCycler C1000, Bio-Rad) was used for detection and quantification of *L. pneumophila*.

Results and Discussion

Results

65 water samples were tested in our study (Table 1). *L. pneumophilia* was not detected in any of the water samples by the SCT (0%). Only three water samples (4.6%) collected from the tap system were positive for *L. pneumophilia* using real-time PCR. According to manufacturer's instructions the following scoring system can be used to determine whether a sample is positive for *L. pneumophilia*: (+) if the value is under 10^2cfu/mL, (++) if the value is between 10^2-10^5cfu/mL, and (+++) if the value is greater than 10^5cfu/mL. The three samples that tested

Table 1. Locations and numbers of selected dental units.

Location	Division	Number of sample
-1 Floor	Cold Water storage tank	2
Ground Floor	Periodontology	8
	Operating Room	4
First Floor	Pedodontics	8
	Orthodontics	8
Second Floor	Operative Dentistry	8
	Endodontics	8
Third Floor	Oral Surgery	8
	Operating Room	3
Fourth Floor	Prosthodontics	8
Total 7 (65 water samples)	Department and -1 Floor	16 dental units and 1 cold water supply system

*Water samples were collected from outlets of air/water syringes, outlets of high-speed hand pieces, water cup fillers, and taps of each selected dental unit (63 water samples)
*Input and output water samples were collected from cold water supply system (two water samples)
*In total, 65 water samples were collected from seven departments and one cold-water supply system

positive for *L. pneumophilia* were collected from the tap system of the periodontology and oral surgery departments, and the oral surgery operating room (Table 2).

After identifying the contaminated water samples, the tap systems were disinfected with a surface disinfectant (35% ethyl alcohol, 5% propan-2-ol, didecyl-dimethyl ammonium chloride, deionized water) and then water samples were recollected and analyzed using both SCT and real time PCR. The recollected water samples from the tap systems were found to be negative for *L. pneumophilia* by both methods.

Discussion of Results

High levels of respiratory diseases among dentists and dental staff compared to the general public have been

Table 2. Evaluation and comparison of *Legionella pneumophila* in water samples using standard culture and real-time PCR techniques.

Legionella pneumophila Evaluation		
Type of Water Sample	Culture	Real-Time PCR
Tap water	(-)	3 (+)*
DUWL$_S$	(-)	(-)
Total	% 0	4.6%

*≤ 10^{2}cfu/mL

reported by a number of studies [1, 8]. Composition of aerosols by air/water syringes and high-speed hand pieces during dental treatments were reported to be the cause of the spread of *Legionella* to dental staff and patients [9)]. In this study, we determined the risk of *L. pneumophilia* infection by monitoring the presence of *Legionella* in water supplied from the tap system from three dental units.

In 2015 the guidelines from the U.S. Centers for Disease Control (CDC) and the U.S. Environmental Protection Agency (EPA) for dental health care procedures set the microorganism quantity for safe drinking water at ≤500 cfu/mL. According to Italian standards for safe drinking water, the acceptable microorganism concentration varies; heterotrophic plate count at 22ºC≤100 cfu/mL, heterotrophic plate count 36ºC≤20 cfu/mL (The European Council Directive) [1, 5]. *Legionella* concentrations that are detected at levels of more than 10^4-10^5cfu per liter of water constitute a risk to human health, and epidemiologic data suggests that *Legionellosis* outbreaks arise from these values [10]. In our study the *L. pneumophilia* concentrations from the three positive samples were evaluated at under 10^2cfu/mL.

Even though the SCT allows for the isolation and the detection of *L. pneumophilia* from environmental aquatic sources, there are some associated limitations with the method: 1) Selective media is required for the culturing of *L. pneumophilia* and requires an extended incubation period (up to 14 days), 2) bacterial loss may occur during the concentration phase preceding pretreatments with acid and heat, and 3) the possible overgrowth with other microorganisms and the case of viable but non-cultivable (VBNC) *L. pneumophilia* [11-12].

The advantages of molecular methods, particularly real time PCR, are as follows: 1) isolation of VBNC, 2) detection of *L. pneumophilia* at very low concentrations, 3) rapid and sensitive identification of *L. pneumophilia,* and 4) better manipulation of large amounts of samples [13-14]. Real Time PCR can also determine the presence of non-cultivable *Legionella* spp., especially VBNC types [15]. Our results have shown that real-time PCR was more sensitive and less time consuming to detect the *L. pneumophilia* in water samples. On the other hand, real-time PCR cannot discriminate between live and dead bacterial cells. Ditommaso S. et al. reported that the *L. pneumophilia* detection rate was at 67% with real-time PCR compared with 46% with the culture method. Furthermore, 21% of samples tested positive by real time PCR were negative for SCT [16]. In our study, *L. pneumophilia* was not detected in any of the water samples by the SCT (0%), whereas 4.6% was found to be positive with real-time PCR. Kramer A. et al. determined that contamination with *L. Pneumophilia* was low in dental units and *L. Pneumophilia* was detected in only four out of 130 (3.07%) water samples [17]. In the current study, three out of 65 (4.6%) water samples tested positive for the presence of *L. pneumophilia*. Therefore, our results are comparable to that of other studies. Ditommaso et al. reported that the load of *L. pneumophilia* DNA identified

by real-time PCR was larger than the concentration of *L. pneumophilia* determined using SCT. The *L. pneumophilia* load analyzed using real-time PCR was 28-fold higher than that detected by SCT [18]. In our study, the positive signal obtained from real-time PCR was estimated to be under 10^2 cfu/mL.

Recently, Leoni E. et al. investigated and outlined a risk management plan for *L. pneumophilia*-contaminated DUWLs. They reported the age, functional features, several disinfection protocols, and locations of operating rooms associated with *L. pneumophilia* contamination of DUWLs [1]. Previous studies reported that chemicals such as ethylene diamine tetra acetic acid, sodium hypochlorite, chlorhexidine gluconate, povidine iodine, ethanol, peroxide, phenol, and glutaraldehyde were commonly used for chemical treatments of the DUWLs [19]. In the current study, following the detection of *L. pneumophilia* the tap systems were disinfected with a surface disinfectant containing 35% ethyl alcohol, 5% propan-2-ol, didecyl-dimethyl ammonium chloride, and deionized water for 30 seconds. Following disinfection the recollected water samples from the tap system tested negative using both SCT and real-time PCR.

There have only been a few similar studies conducted in Turkey. The first of these was investigated and reported by Bodrumlu et al. in Ankara, Turkey [20]. Although they could not detect any *Legionella* spp. in any DUWLs, they were able to detect *L. pneumophilia* in one of the tap water samples using the SCT. In another study regarding bacterial load of dental units conducted by Güngör et al. in İstanbul, Turkey [21], *Legionella* was not detected in any water and aerosol samples, but *Pseudomonas aeruginosa* was isolated in three DUWLs. Furthermore, *Pseudomonas* and *Staphylococcus* also were found in water and aerosol samples.

Conclusions

In this study, the accumulation of polluted water on the ground floor, the rare utilization of tap water, and a deficiency of periodic disinfection protocols are considered to be the most likely causes of *L. pneumophilia* contamination in tap water. Our study reveals the importance and benefits of microbial detection in DUWLs and tap water. Furthermore, our results highlight the importance of changing and disinfecting tap filters routinely and flushing tap water systems.

Based on our findings, precautions should be taken in dental care environments with regards to possible *L. pneumophilia* contamination in the water system. Furthermore, real-time PCR may be a useful method to apply during routine controls of DUWLs and tap water systems.

Acknowledgements

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