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Detection of Bacterial Contamination in Dental Unit Water Lines and Testing the Effectiveness of Disinfectant Against these Contaminants

Sham Lal[a]*, Javed Ahmed Ujan[b], Sapna[b], Om Parkash[a], Waheed Ahmed Ghumro[a], Ameer Ahmed Mirbahar[c] and Pardeep Kumar[a]

[a]Institute of Microbiology, Shah Abdul Latif University, Khairpur, Sindh, 66020, Pakistan
[b]Department of Zoology, Shah Abdul Latif University, Khairpur, Sindh, 66020, Pakistan
[c]Department of Botany, Shah Abdul Latif University, Khairpur, Sindh, 66020, Pakistan

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Abstract. The quality of water in dental units is of substantial importance since patients and dental staff are regularly exposed to water and aerosols generated during dental procedures. The aim of this study was to detect the bacterial contamination of dental unit water and investigate the effectiveness of the disinfectant. The biofilm of bacterial contaminant in dental unit waterlines was detected using bacterial culturing of heterotrophic bacteria, total coliforms and *Pseudomonas aeruginosa*. In addition, presence of biofilm in dental unit waterlines was visualized using scanning electron microscopy (SEM). Subsequently, dental unit water lines were treated with disinfectant to eradicate bacterial contaminants from dental unit water lines and its effectiveness was tested after three months. This study showed that the bacterial contamination of the water samples ranged from not detected to 2.38×10^6 CFU/mL. Out of 34 DUWLs water samples tested the 30 (88.24%) samples exceeded the CDC/EPA recommended threshold of ≤500 CFU/mL, whereas only 4 (11.76%) samples were able to meet the standards. Contamination by total coliforms and *P. aeruginosa* was detected in 52.94% and 64.7% of samples respectively. SEM displayed a dense biofilm on the lumen of tubing confirming the bacterial contamination. The intervention for disinfection of DUWLs resulted in more than 50% samples with acceptable bacterial count in follow up test performed after three months. The high rate of bacterial contamination of dental unit water highlights the need to disinfect and monitor the quality of DUWLs periodically and use a cleaner source of water.

Keywords: dental unit waterlines, bacterial contamination, biofilm

Introduction

Dental chair unit comprises of channels of narrow flexible plastic tubes (2-3 nm internal diameter) known as dental unit water lines (DUWLs) which is connected with dental instruments including an air-water syringe and high speed dental hand-pieces (drills) and ultrasonic scalers. DUWLs are used to irrigate dental instruments and teeth *via* hydraulic system, while working. DUWLs are highly susceptible to microbial contamination and formation of biofilm due to reduced velocity of water at the periphery of the narrow flexible plastic tube (Ampornaramveth *et al*., 2018; Abdouchakour *et al*., 2015). The bacterial contamination of DUWLs water was first reported by Blake (1963).

Biofilm is an aggregate of same or different micro-organisms living together in self-producing extracellular polymeric substances (Pankhurst and Coulter, 2007). There are several factors responsible for contamination and subsequent formation of biofilm in DUWLs. For example, water stagnation during off-hours, failure of anti-retraction valves (fitted in dental hand devices), contaminated water supply and the presence of water heaters (Szymanska *et al*., 2008; Szymanska, 2003). As soon as the biofilm is formed in DUWLs, it becomes continuous source of bacteria in DUWLs (Alkhulaifi *et al*., 2019; O'Donnell *et al*., 2011).

Majorly DUWLs are responsible for disseminating bacteria although some reports have also revealed the presence of protozoa and fungi (Putnins *et al*., 2001; Meiller *et al*., 2000). The most common contaminants of DUWLs are gram-negative non-pathogenic environmental bacteria, however, they can be harmful to immuno-compromised people. These bacteria include but not limited to *P. aeruginosa*, *Legionella pneumophila* and non tuberculosis *Mycobacterium* that cause respiratory diseases (Ditommaso *et al*., 2016; Fotedar and Ganju, 2015; Pankhurst and Coulter, 2007; Singh and Coogan, 2005; Szymanska, 2003). During dental

*Author for correspondence; E-mail: shamlal@salu.edu.pk

procedures, aerosols are generated which are also responsible for dissemination of bacteria (Szymanska, 2003).

In this field, a very few epidemiological studies have been conducted such as Martin (1987) reported that two cancer patients got infected with *P. aeruginosa* originating from DUWLs. Two other studies have reported increase in antibody titer in dental staff compared to general public (Borella *et al.*, 2008). Gungor *et al.* (2014) and Szymanska (2005) have reported immune system suppression in patients and dentists who were exposed to DUWLs contaminated water aerosols from dental unit. In another study transmission of *L. pneumophila* from a contaminated dental unit to patient has been reported. The patient developed a sudden onset of Legionnaire's disease and died from septic shock (Ricci *et al*., 2012).

Internationally there are no unique guidelines for the acceptable limit of heterotrophic bacteria in DUWLs and application of disinfectant for eliminating the biofilm. However, the American dental association suggests that DUWLs output water must contain ≤200 colony forming units (CFU)/mL of heterotrophic bacteria (ADA, 2004), whereas, Centers for Disease Control (CDC) and Environmental Protection Agency (EPA) recommends ≤500 CFU/mL of heterotrophic bacteria (William *et al*., 2003). CDC also recommends regular disinfection and monitoring of DUWLs. However, a vast number of studies have reported the failure of dental practices to achieve these recommended limits (Leoni *et al*., 2015; Arvand and Hack, 2013; Chate, 2010; Artini *et al*., 2008; Szymanska, 2007). There could be various reasons of disinfection protocol failures, such as bacterial resistance towards disinfectants, staff negligence for proper application of disinfection protocol, old dental units, anti-retraction valves failure and low dose of disinfectant use (Lizzadro *et al*., 2019; Ditommaso *et al*., 2018).

Although DUWLs contamination is a universal problem, the dimension of this problem in our country is less studied and thus patients and dental staff are at risk in acquiring infection from contaminated DUWLs water and aerosols generated during dental procedures. The aim of this study was to analyse the quantitative and qualitative bacterial contamination of DUWLs output water in private dental clinics of district Khairpur, Sindh, Pakistan. The significance of this research is to prevent potential occupational/public health outbreaks.

Materials and Methods

Recruitments of dental surgeries and ethical approval. In this study, 34 different dental practices located in district Khairpur, Sindh, Pakistan were designated for collection of DUWLs water samples. Before laboratory investigation, information sheet of project and consent letter was delivered to each dental practice by hand along with verbal discussion.

Sample collection. In designated dental practices, source of water for their dental units was distilled water or municipal/groundwater stored in storage tanks made up of polyethylene, steel or cement. Thirty four DUWLs water samples were collected from consented dental practices. Before collecting water the air-water syringe (connected with DUWLs) was flushed for 2 min to release the stagnant water. Water samples (50 mL) were collected in sterilized glass bottles (containing 0.1 mL of 10% sodium thiosulfate solution for neutralizing residual chlorine) during the working hours of dental practices, while collecting the water samples, another glass bottle containing sterilized water was exposed (as a control for splashes and aerosols). All the samples were labelled and transported to laboratory in a cool box within two hours for further analysis (Lisboa *et al*., 2014; Turetgen *et al*., 2009).

Determination of total bacterial count (TBC). All water samples (N=34) were serially diluted in sterilized physiological saline (0.85%) followed by inoculation (0.1 mL) on 90 mm R2A agar (oxoid) plates in triplicates by using aseptic techniques. All the samples were incubated at 22 °C for seven days (Morris *et al*., 2010). Plates displaying bacterial colonies within the range of 30-300 were used to calculate the final number of CFU/mL by adjusting the dilution factor.

Qualitative assessment of water samples. This study focused on the detection of total coliforms (TC) and *P. aeruginosa* in DUWLs water samples.

(a) Detection of TC. Water sample (0.1 mL) was inoculated in lactose broth (oxoid) tube containing Durham tube and phenol red (a pH indicator). Subsequently, the test tubes were incubated at 37 °C for 48 h (Fotedar and Ganju, 2015; Park *et al*., 2006).

(b) Detection of P. aeruginosa. DUWLs water samples (0.1 mL) were inoculated on cetrimide agar (oxoid) plates (Ditommaso *et al*., 2019; Al-Hiyasat *et al*., 2007; Tambekar *et al*., 2007) and incubated at 37 °C for 48 h. Green coloured colonies (due to pyocyanin pigment)

were noted. The pure culture was prepared on nutrient agar (oxoid) plates followed by identification on the basis of cultural, Gram staining and conventional biochemical tests (John *et al*., 1993).

Scanning electron microscopy (SEM). DUWLs tube (connected with air-water syringe) was obtained as a gift from a dentist who was planning to change the DUWLs of one of the dental units of his clinic due to blockage in the tube (connected with a high speed dental drill). This dental unit was in use for 7 years. The portion of the tube closest to the air-water syringe was cut (1 cm) with a sterilized scalpel and transferred to the microbiology lab in refrigerated temperature (4 °C) for processing. In lab, the tube was further split lengthwise to expose its lumen and immerse fixed in 2.5% gluteraldehyde (sigma) upto 4 h at 4 °C. Subsequently tube was washed in PBS and then left in new PBS for 8 h at 4 °C. This was followed by fixation in 2% osmium tetroxide solution (sigma) for 2 h and then washed in distilled water. The specimen was then dehydrated in ethanol of various concentrations (70% to absolute). The specimen was placed in glass desiccator for overnight (Lal *et al*., 2017; Dillon *et al*., 2014). Finally, the specimen was sent to University of Punjab for commercial imaging of specimen.

Follow up test for DUWLs water samples. Results of TBC count were reported to the dentists and they were advised to follow CDC guidelines for flushing and disinfection of DUWLs to eradicate biofilm and keep the bacterial count within the accepted limit. After three months, water samples from all the dental surgeries were retested for TBC as per method described earlier in this report.

Statistical analysis. Where necessary, experiments were performed in triplicates. Results were displayed as mean ± standard division. Since data of TBC in first and follow up study was skewed, the nonparametric Mann-Whitney U test was performed to detect statistically significant differences ($P<0.05$).

Results and Discussion

Bacterial contamination of DUWLs water. Although DUWLs contamination was identified more than 55 years ago, this issue still persists as evidenced by a vast number of research articles being published internationally on DUWLs contamination and its control. Before commencing this research, the interviews regarding microbial contamination of dental unit water were undertaken (as a part of under graduate student assignment) from 50 dentists running their private clinics. The response of dentists (unpublished data) revealed that many of them were unaware of microbial contamination of DUWLs and its negative impact on health of dental staff and patients. Therefore, this study was designed to monitor the quality of dental unit water used in private dental clinics. In this study, the bacterial contamination of the DUWLs water samples ranged from not detected to 2.38×10^6 CFU/mL. Out of 34 DUWLs water samples analysed, 30 (88.24%) samples crossed the CDC/EPA recommended value of ≤500 CFU/mL (Fig. 1), whereas only 4 (11.76 %) samples were able to meet the CDC/EPA standards suggesting that majority of DUWLs were contaminated with heterotrophic bacteria (Fig. 1). Sterilized water samples (control) exposed during the collection of DUWLs water samples displayed no growth. The high bacterial count in the majority of DUWLs water samples in this research agrees with already published studies (Ji *et al*., 2016; Fotedar and Ganju, 2015; Szymanska and Sitkowska, 2013; Chate, 2010). Small number of dental practices water samples (N=4) showed acceptable limit possibly because their dental units were newly installed (2-5 months old). However, statistical analysis showed a negative correlation (-0.027) between contamination level and age of dental chair (Table 1). These surprising statistical results could be due to low sample size.

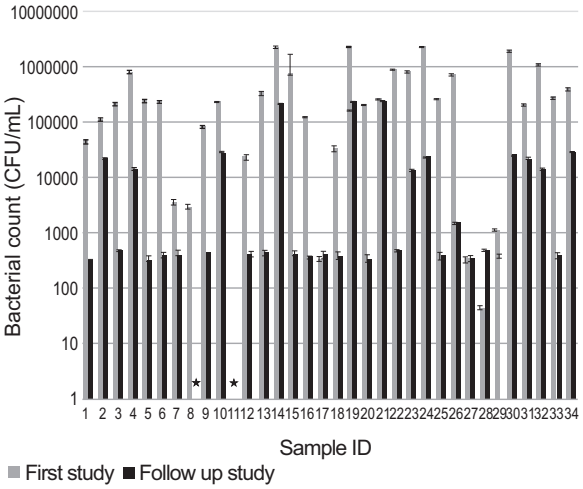

Fig. 1. Total bacterial count from dental unit water samples [Black line within graph indicates acceptable limit as suggested by CDC/EPA (≤500 CFU/mL)].
★=no growth

Table 1. Contamination level of water samples and age of dental chairs, (Correlation coefficient=-0.027)

Sample ID	Average log CFU/mL	Dental chair age since installation (months)	Sample ID	Dental chair age since installation (months)	Average log CFU/mL
DUWL1	4.5×10^4	13	DUWL 2	60	1.1×10^5
DUWL 3	2.2×10^5	85	DUWL 4	35	8.2×10^5
DUWL 5	2.5×10^5	50	DUWL 6	55	2.3×10^5
DUWL 7	3.6×10^3	48	DUWL 8	59	3.0×10^3
DUWL 9	8.4×10^4	11	DUWL 10	61	2.3×10^5
DUWL 11	0	5	DUWL 12	84	2.4×10^4
DUWL 13	3.4×10^5	26	DUWL 14	36	2.3×10^6
DUWL 15	7.5×10^5	73	DUWL 16	65	1.3×10^5
DUWL 17	3.4×10^2	2	DUWL 18	108	3.4×10^4
DUWL 19	2.9×10^6	80	DUWL 20	81	2.1×10^5
DUWL 21	2.6×10^5	25	DUWL 22	36	8.8×10^5
DUWL 23	8.7×10^5	8	DUWL 24	21	2.3×10^6
DUWL 25	2.6×10^5	12	DUWL26	15	7.3×10^5
DUWL 27	3.7×10^2	3	DUWL 28	3	4.4 x 10
DUWL 29	1.1×10^3	40	DUWL30	20	1.9×10^6
DUWL 31	2.0×10^5	49	DUWL 32	40	1.1×10^6
DUWL 33	2.8×10^5	36	DUWL 34	49	4.0×10^5

Therefore, in future more studies should be carried out with a large sample size to authenticate statistical analysis.

In this study, TBC was evaluated on R2A plates since inoculation of water samples on low-nutrient medium (such as R2A) and incubation for longer time is suggested for enumerating bacterial cells from water sources in which disinfectants have been used such as municipal water. R2A was introduced by Reasoner and Geldreich (Reasoner and Geldreich, 1985) and since then, number of authors have used R2A for counting heterotrophic bacteria in water (Watanabe *et al*., 2016; Morris *et al*., 2010; Nikaeen *et al*., 2009; Uzel *et al*., 2008; Sungur *et al*., 2008; Zhang *et al*., 2007). In this study one sample showed no growth that indicates presence of less than 100 CFU/mL, bacteria were not detected due to inoculation of 0.1 mL water sample on the R2A plates. However, membrane filtrate method could be used to detect the less number (<100 CFU/mL) of bacteria.

Qualitative assessment of water samples. Since *P. aeruginosa* has notable ability to form biofilms in many environments including DUWLs (Bjarnsholt *et al*., 2013; Hoiby *et al*., 2010) and the presence of coliform bacteria in water is considered as an indicator of unsafe drinking water (O'Donnell *et al*., 2011; Coleman *et al*., 2009), this study also investigated the presence of these bacteria.

Out of 34 water samples tested, 18 (52.94%) samples were positive for TC as confirmed by acid (yellow coloration) and gas production (in Durham tube), whereas 22 (64.7%) samples were positive for *P. aeruginosa*. The results indicated that 4 samples which were negative for TC displayed the presence of *P.*

Table 2. Microscopic, biochemical and sugar fermentation profile for confirmation of *P. Aeruginosa*

	Characteristics	Result
Cultural characteristics	Growth on Cetrimide agar	Positive (Yellow-green colonies)
Microscopy	Gram's staining	Gram negative, rods
	Motility	Motile
	Flagella staining	Polar flagella
	Capsule staining	Non-capsulated
	Spore staining	Non- sporing
Biochemical tests	Catalase	Positive
	Oxidase	Positive
	Indole	Negative
	Methyl red	Negative
	Voges Proskauer	Negative
	Citrate	Positive
	Urease	Negative
	Nitrate reduction	Positive
Sugar fermentation tests	Glucose	Negative
	Maltose	Negative
	Lactose	Negative
	Sucrose	Negative

aeruginosa. The macroscopic, microscopic, biochemical and sugar fermentation profile for identification of *P. aeruginosa* is shown in Table 2.

The dental surgeries whether they were using distilled water or overhead tank water showed contamination by TC and *P. aeruginosa*. In contrast with these results, the research performed by Aprea *et al*. (2010) and Watanabe *et al*. (2008) showed absence of TC, whereas, in similar to these results, multiple studies have revealed the presence of *P. aeruginosa* in DUWLs water samples (Lizzadro *et al*., 2019; D'Ovidio *et al*., 2011; Barben *et al*., 2009). High prevalence of *P. aeruginosa* in water samples is alarming.

There is a drawback of determining TC in this study since presence of TC does not confirm the presence of fecal coliforms or *Escherichia coli*. Detection of *P. aeruginosa* in 4 samples that were negative for coliforms was surprising. It suggests that traditional indicators of drinking water quality may not be sufficient for regulatory monitoring of drinking water samples. The distilled water already contaminated, addition of distilled water in residual water, improper cleaning of the storage tank may have contributed for TC and *P. aeruginosa* contamination. However, only the use of water with an initial low contamination level cannot prevent the high number of bacteria in high speed and the air-water syringe, if the efforts are not taken for reducing or eliminating biofilm in DUWLs.

SEM visualisation of biofilm on DUWLs. SEM image of tubing showed a dense biofilm matrix on the lumen of tubing (Fig. 2). Bacteria especially rod-shaped was observed in matrix. The cracks seen in the matrix could be due to the stress on the sample during SEM preparation. However, these cracks reveal the thickness of biofilm and confirm the maturity of biofilm. In this research, a thick biofilm on DUWLs lumen agrees with the high planktonic bacterial count found in DUWLs water samples. However, SEM imaging shows both live and dead bacteria. Alternatively, live/dead assay by using propidium iodide (Lal *et al*., 2017) followed by confocal microscopy can be a good method to observe both live and dead cells. Live/dead assay can be more useful while evaluating disinfection strategies to eradicate biofilm.

Follow up test of DUWLs water samples. When the results of TBC were reported to the dental staff, they were also suggested to follow CDC guidelines for flushing and disinfection. After intervention of researchers of this study, although all previously tested samples (N=34) were re-tested but out of 30 dental units that were previously contaminated with high bacterial count (above CDC/EPA standards), 17 (56.67%) samples revealed acceptable bacterial count (≤500 CFU/mL) and 13 (43.33%) revealed low bacterial count than previous test although above the standard threshold (Fig. 1). Four dental units that showed acceptable limit in 1st test revealed the acceptable limit once again. Mann-Whitney U test indicated that there was a statistically significant difference ($P<0.05$) in contamination level during 1st study and follow up study. This study revealed that due to the intervention of researchers, more than 50% dental units met the CDC recommended standards for DUWLs water quality. Additional measures and repeated involvement were needed to achieve acceptable levels in all other dental units. This follow up study highlights the importance of using biocides to clean DUWLs. In literature, the use of various biocides such as hypochlorous acid, Alpron, Sterilox, Bio 2000, Dentosept, oxygenal and sodium hypochlorite have been suggested to disinfect DUWLs (Shajahan *et al*., 2016; Schel *et al*., 2006; Martin and Gallagher, 2005; Walker *et al*., 2004). However, to meet the CDC/EPA recommended standards (≤500 CFU/mL), dentists should follow the specified protocols for using biocides as suggested by manufacturers of biocides/dental unit.

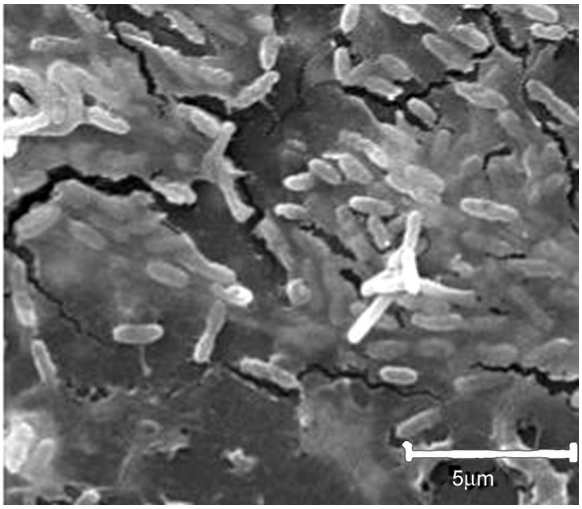

Fig. 2. SEM Image of biofilm produced on DUWL tube lumen (x7000).

This study highlights the importance of routine monitoring the DUWLs water quality by using a

microbiological test that provides valid results. Till now this can be done by testing DUWLs water samples by using conventional microbiological techniques, which involves the culturing on R2A or similar media plates. However, for dental practitioners it can be laborious and expensive to send the DUWLs water samples to microbiology laboratory for conventional microbiological testing. To overcome this problem, various authors have tested in-office tests such as Petrifilm™ test, Heterotrophic Plate Count Sampler™ (Momeni *et al*., 2012; Morris *et al*., 2010); Aquasafe™ water test (Momeni *et al*., 2012), Dip slide™ (Lal *et al*., 2014) for monitoring the quality of DUWLs water samples. According to their suggestions, although these in office tests are not very sensitive, their specificity values are very high and show gross bacterial contamination level of water samples. This will help the dentists to inspect the failure of disinfection protocols.

The present investigation indicates that the DUWLs water can be an important source of cross infection in dental practices. Therefore, for maintaining the sterility of DUWLs, dentists should follow good operating procedures.

Conclusion

For the first time, DUWLs have been evaluated for bacterial contamination in district Khairpur, Sindh, Pakistan. High level of bacterial contamination and presence of TC and *P. aeruginosa* in DUWLs water samples highlights the need for effective strategies to disinfect DUWLs and regular by monitoring the bacterial quality of DUWLs output water. Further, research is needed to investigate the risk of bacterial transmission to patients and dental staff. Local and National Health department should take measures to provide guidelines to dental staff for using disinfectants for the eradication of biofilm in DUWLs and routinely monitoring the quality of water. Subsequently, the health department should ensure the compliances with guidelines by the dental practices. Manufacturers of the dental units should also take efforts for developing biofilm resistant DUWLs.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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