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Isolation and identification of potentially pathogenic free-living amoeba in dental-unit water samples

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ABSTRACT

Although the presence of free-living amoebae (FLAs) in various water sources has been reported, few studies have been surveyed on their abundance in medical-unit and dental-unit water samples. The current study aimed to identify morphological and molecular characteristics of FLA isolates in the water samples of the dental unit in Iran. A total of 232 water samples were collected from 17 dental units. Then, filtration and cultivation were conducted on a non-nutrient agar (NNA) medium. Also, polymerase chain reaction (PCR) assay and sequencing were performed by using the genus/species-specific primers plus a common primer set on positive samples. One hundred and sixty-six samples were positive for FLA by the microscopic method, whereas 114 samples were positive by the molecular method with a common primer set. Considering the PCR assay with genus/species-specific primers, 23.27% (54/232) samples were identified as *Acanthamoeba* spp. (belonging to T4 genotype), 36.63% (85/232) as *Vermamoeba vermiformis*, and 1.72% (4/232) as Vahlkampfiidae family (*Naegleria lovaniensis*). These results highlight a need to improve filtration systems in dental units and periodic screenings for FLA in dental-unit water.

Key words: dental units, free-living amoeba, Iran, water

HIGHLIGHTS

- Among 232 water samples of the dental unit, 166 samples were positive for FLA by the microscopic method, whereas 114 samples were positive by the molecular method with a common primer set.
- Considering the PCR, 23.27% (54/232) samples were identified as *Acanthamoeba* spp. (belonging to T4 genotype), 36.63% (85/232) as *Vermamoeba vermiformis*, and 1.72% (4/232) as Vahlkampfiidae family (*Naegleria lovaniensis*).

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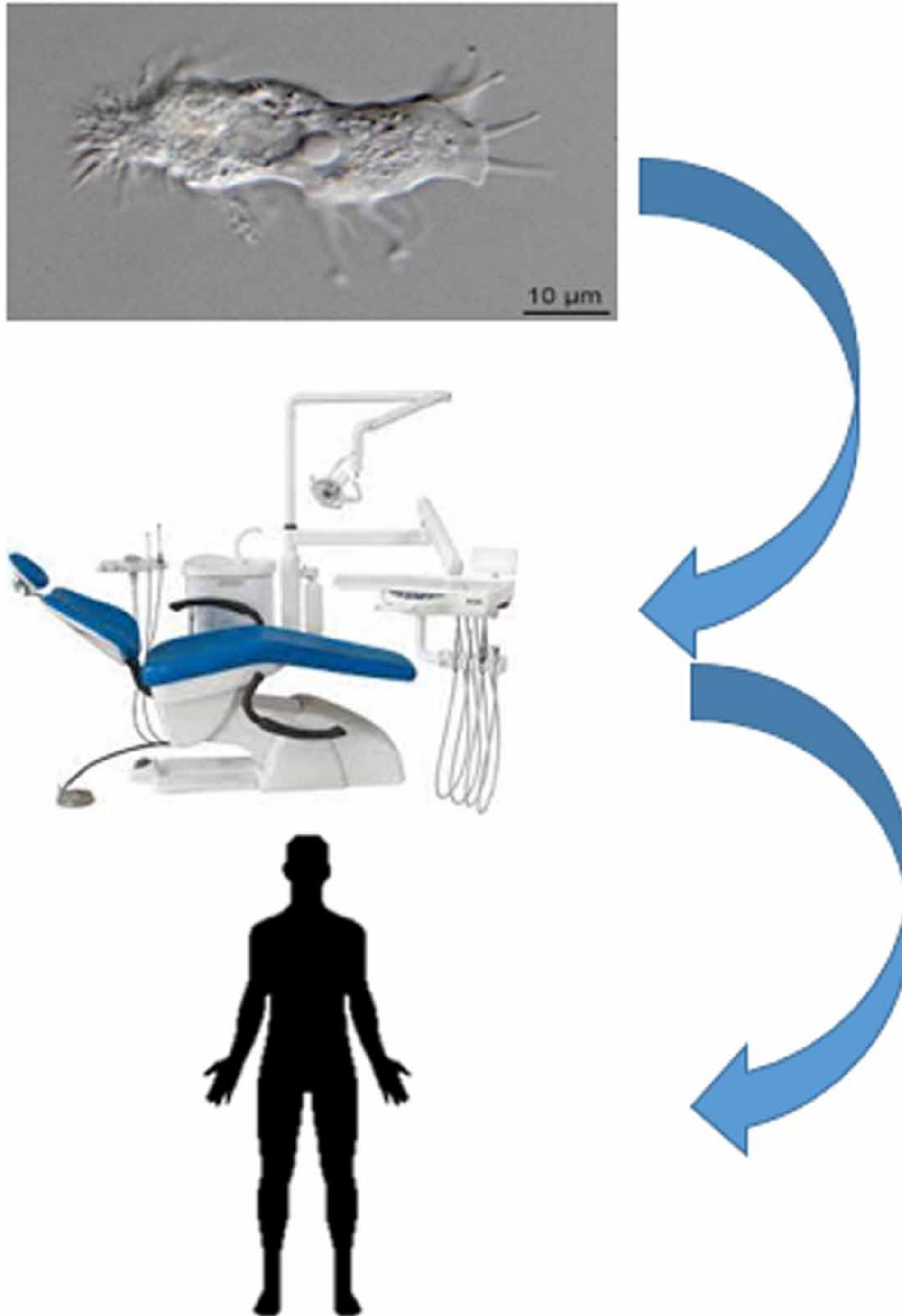
GRAPHICAL ABSTRACT



1. INTRODUCTION

Free-living amoebae (FLAs) are ubiquitous protozoans which could be detected in diverse environmental niches such as water, soil, and air (Badirzadeh *et al.* 2011; Mahmoudi *et al.* 2021). FLA has an amphizoic life cycle that exhibits two different forms: trophozoite as the vegetative form and cyst as the resistant form (Padzik *et al.* 2018). In this regard, cysts are able to withstand adverse environmental conditions such as ultraviolet (UV) radiation, the presence of toxins and disinfectant

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solutions, and very high or low changes in temperature, osmolarity, and pH (Aksozek *et al.* 2002; Dudley *et al.* 2005; Solgi *et al.* 2012a). The most medically important FLA genera include *Acanthamoeba*, *Naegleria*, *Balamuthia*, and *Vermamoeba* (Hoffmann & Michel 2001; Niyyati *et al.* 2016). These organisms are involved in causing neurological and ocular complications by attacking the central nervous system (CNS) and cornea (da Rocha-Azevedo *et al.* 2009). *Acanthamoeba* is the most common FLA, which is classified into 23 genotypes (T1–T23) based on the small subunit of 18 s rRNA gene (Chelkha *et al.* 2020; Putaporntip *et al.* 2021). Considering the *Acanthamoeba*-related diseases, T3, T4, T5, and T11 genotypes have been isolated from many clinical specimens, including *Acanthamoeba* keratitis (AK), and granulomatous amoebic encephalitis (GAE) (Khan 2009; Mirjalali *et al.* 2013). Among the various *Naegleria* species (*Vahlkampfiidae* family), *Naegleria fowleri* is the only pathogenic species in humans that can cause the lethal disease primary amoebic encephalitis (PAM) (Maciver *et al.* 2020; Moseman 2020). *Balamuthia mandrillaris* is considered an infrequently caused CNS disorder known as GAE with a high fatality rate (>98%) (Matin *et al.* 2008). Another FLA, *Vermamoeba vermiformis* (formerly named *Hartmannella vermiformis*) is less commonly reported in clinical specimens than in environmental specimens (Delafont *et al.* 2018; Scheid 2019; Siddiqui *et al.* 2021).

Many studies have shown that FLAs are able to form biofilm in water supply systems by adhering to each other on a surface (Barbeau & Buhler 2001; Pinto *et al.* 2021). In this regard, dental-unit hydraulic systems contain a high number of long and narrow pipes made of nylon or polyvinyl chloride (Göksay *et al.* 2008). Therefore, long-term flow of water in these pipes may provide suitable conditions for the formation of microbial biofilms (Barbeau & Buhler 2001). In addition, most dental units are directly connected to municipal water, which may be contaminated with a broad range of microorganisms (Göksay *et al.* 2008). However, few studies have surveyed the status of FLA in the water system of dental units (Trabelsi *et al.* 2010; Hassan *et al.* 2012). Hence, the purpose of this research was to investigate morphological and molecular characteristics of FLA isolates in dental-unit waterlines.

2. MATERIALS AND METHODS

2.1. Sampling, culture, and microscopy detection

The present cross-sectional study was performed between April and September 2017 on the water samples of hydraulic systems in 17 dental offices from Jahrom City (a city in southern Iran, Fars Province). In this study, water samples from dental units used for mouthwash were examined. In total, 232 water samples were collected into sterile bottles after disinfection of the water outlet handpiece head. After mixing the contents completely, 500 mL of each water sample was filtered through filter papers of Whatman Company with 4–7 μm pore filter size. Then, the filters were transported onto 1.2% non-nutrient agar (NNA) medium covered with heat-killed *Escherichia coli* K12 (HB101), as a source of nutrition (Eftekhari-Kenzerki *et al.* 2021; Biglarnia *et al.* 2022). All plates were sealed with parafilm, and the three plates per sample were incubated at 30, 37, or 42 °C for up to 30 days under a light microscope (Solgi *et al.* 2012b; Eftekhari-Kenzerki *et al.* 2021). Next, all the FLA-grown plates were cloned in order to isolate FLA, this process was performed as described by Lorenzo-Morales *et al.* (2006). In this regard, FLAs were sub-cultured into fresh NNA plates in order to reduce fungal and bacterial contaminations. Considering Pussard & Pons (1977) and Page (Page 1988) keys, isolated FLAs were specified to the genus level using morphological characteristics such as cyst structure (size and cyst wall shape), vacuoles, acanthopodia (spine-like structures), and nucleus criteria. Also, *Naegleria* species and the *Vahlkampfiids* were described by the presence of rounded endo and ectocysts, or worm-like shaped trophozoites.

2.2. DNA extraction

For this purpose, all trophozoites and cysts were collected from plates, then washed with sterile phosphate-buffered saline (PBS, pH 7.4). Afterwards, DNA extraction was performed by the Nucleic Acid extraction *kit* (*Yekta Tajhiz Azma*, Iran), as described by the manufacturer's protocol.

2.3. Polymerase chain reaction amplification

As shown in Table 1, genus/species-specific primers were applied for *Acanthamoeba*, *Naegleria* species, and *V. vermiformis* plus a set of common primers for the all FLAs in molecular detection. For polymerase chain reaction (PCR) amplification, 20 μL of PCR mixture contained 4 μL DNA, 10 μL Taq Master Mix (Cinnagen, Iran), 2 μL of primers, and 4 μL distilled water. To evaluate run validity, each PCR run included a negative control without DNA and a positive control comprising DNA extracted from FLA registered in the GenBank.

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Table 1 | Primer set used in the present study (5′ → 3′)

Species	Forward primer	Reverse primer	Reference
Common for FLA	**FLA1:** CGGGGTAATTCCAGCTCCAATAGC	**FLA2:** CAGGTTAAGGTCTCGTTGTTCGTTAAC	Tsvetkova *et al.* (2004)
Acanthamoeba spp.	**JDP1:** GGCCCAGATCGTTTACCGTGAA	**JDP2:** TCTCACAAGCTGCTAGGGGAGTCA	Schroeder *et al.* (2001)
Vermamoeba vermiformis	**HV1:** TTACGAGGTCAGGACACTGT	**HV2:** GACCATCCGGACTTCTCG	Kuiper *et al.* (2006)
Naegleria species	**ITS1:** GAACCTGCGTAGGGATCATTT	**ITS2:** TTTCTTTTCCTCCCCTTATTA	De Jonckheere (2007)

2.3.1. The thermal cycling conditions for FLA detection

The thermal cycling was performed according to the following protocol: 30 cycles of denaturation at 94 °C for 1 min, followed by annealing at 64 °C for 1 min, and 72 °C for 3 min, and final extension at 72 °C for 5 min. These FLA species can be identified based on band size, fragments of 800-bp (*V. vermiformis*), 900-bp (*Naegleria* species), 1,080-bp (*A. polyphaga, A. castellani, A. lenticulata, and A. hatcheti*), 1,350-bp (*A. comandoni*), and 1,700-bp (*Acanthamoeba* spp.) (Tsvetkova *et al.* 2004).

2.3.2. The thermal cycling conditions for *Acanthamoeba* spp. detection

The PCR amplification conditions were conducted in 40 cycles with denaturation (95 °C, 1 min), annealing (61 °C, 1 min), and primer extension (72 °C, 2 min). Final extension was continued for 5 min at 72 °C. All *Acanthamoeba* species can be specified by band size, fragments of 420-bp (*A. lenticulata*), 450-bp (*A. castellani, A. polyphaga, A. hatchetti,* and *A. culbertsoni*), and 500-bp (*A. comandoni, A. griffini*) (Schroeder *et al.* 2001).

2.3.3. The thermal cycling conditions for *V. vermiformis* detection

The thermal cycling profile was followed in 30 cycles of denaturation at 95 °C for 20 s, the annealing step at 62 °C for 40 s, and 72 °C for 35 s. The final extension was performed at 72 °C for 5 min. *V. vermiformis* can be amplified in a 502-bp-specific fragment (Kuiper *et al.* 2006).

2.3.4. The thermal cycling conditions for *Naegleria* species detection

The PCR amplification conditions consisted of 35 cycles with denaturation (94 °C, 30 s), annealing (55 °C, 40 s), and primer extension (72 °C, 40 s). The final extension was sustained for 5 min at 72 °C. *Naegleria* species can be identified by band size (450-, 500-, and 550-bp fragments) (De Jonckheere 2007).

Finally, all DNA amplification products were electrophoresed by 1.5% agarose gel stained with a safe stain and visualized under UV light. All samples were compared with 50- and 100-bp DNA ladders.

2.4. Sequencing

In this stage, sequencing of PCR products were performed by a sequencer machine (Applied Biosystems® 3130X Automatic Genetic Analyzer). To specify its genus, species or genotypes, the obtained sequences were compared with the available sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program. Then, the new sequences data were submitted to the GenBank database using BankIt tool.

3. RESULTS

3.1. Morphological characteristics

FLAs were detected in 166 (71.5%) out of 232 water samples in the dental unit. Regarding the morphological characteristics of FLA (Table 2 and Supplementary Figures 1 and 2), 19.82% (46/232) identified as *Acanthamoeba* spp., 38.36% (89/232) samples as *Vermamoeba*, and 13.36% (31/232) samples as *Naegleria* species. It is important to mention that after conducting morphological assay, *B. mandrillaris* remained undetected in all the water samples. Morphological distribution of FLA in dental-unit water using Page keys (Page 1988) are presented in Table 2.

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Table 2 | Frequency of FLA by morphological characteristics based on Page keys

FLA species (cyst shape)	Grouping by Page keys	Total sample, $n = 232$
Acanthamoeba spp. (with wrinkled outer wall, and inner wall in star, polygonal or triangular shapes, cyst size <18 μm), $n =$ positive (%)	Group II (*A. mauritaniensis, A. quina, A. dinionensis, A. triangularis, A. lugdunensis, A. castellani, A. polyphaga, A. griffni, A. rhysodes, A. paradivionensis, A. hatchetti*)	4 (1.72%)
Acanthamoeba spp. (with smooth and thin outer wall, and circular inner wall, cyst size <18 μm), $n =$ positive (%)	Group III (*A. palestiensis, A. royreba, A. culbertsoni, A. pustulosa, A. lenticulata*)	42 (18.10%)
Vermamoeba (smooth and spherical double-walled cysts), $n =$ positive (%)	*Vermamoeba* cyst	89 (38.36%)
Naegleria species (spherical cyst with two completely distinct walls), $n =$ positive (%)	*Naegleria* cyst	31 (13.36%)
Total of FLA, $n =$ positive (%)	–	166 (71.5%)

3.2. Molecular detection

All positive microscopic samples were surveyed using the PCR assay. In the first stage, we used general primer pairs to identify FLA species for all 166 positive samples. Despite several attempts on the 52 isolates, they were not amplified. Thereby, 114 FLA isolates were amplified using the FLA1 and FLA2 primer set (Figure 1). Considering a specific primer (JDP1 and JDP2), 54 *Acanthamoeba* spp.-positive samples were amplified and showed band sizes of 450-bp ($n = 37$), 500-bp ($n = 12$), and 550-bp ($n = 5$) (Figure 2). As shown in Table 3, the different species of *Acanthamoeba* are categorized based on the band size. In the case of *Vermamoeba*, 85 samples were amplified with HV1 and two specific primers, and the 502-bp band size confirmed the presence of *Vermamoeba* (Figure 3). In *Naegleria* species, only four samples were amplified with band sizes of 450-bp with a specific primer (ITS1 and ITS2) (Figure 4).

3.3. Sequencing

For this purpose, five samples of *Acanthamoeba* spp., two *Vermamoeba*, and one *Naegleria* were sequenced. All the sequencing results for *Acanthamoeba* spp. revealed the presence of genotype T4 (accession numbers: MG581703, MG581704, MG581707, MG581709, and MG581710). Both *V. vermiformis* samples were also confirmed (accession numbers: MG581706 and MG581708). About *Naegleria*, *N. lovaniensis* (accession number: MG581705) was confirmed.

4. DISCUSSION

This study reports the occurrence of waterborne FLA belonging to the *Acanthamoeba* T4 genotype, *N. lovaniensis* and *V. verformis* in water samples of the dental unit. In the present study, the frequency of FLA by the microscopic method was higher than the molecular method. Also, the frequency of FLA using common and specific primers was different. There are two possible reasons for these differences: (1) high bacterial and fungal contaminations as a distorting and interfering factor and (2) the sensitivity and specificity of each diagnostic method (da Rocha-Azevedo *et al.* 2009; Goh *et al.* 2018). In microscopic detection, there is a possibility of misdiagnosis of FLA with other microorganisms or artifacts (Attariani *et al.* 2020). Thereby, misdiagnosis may be one of the possible reasons for the high frequency by this method. Regarding the molecular method, the sensitivity and specificity of each test is highly dependent on the input sample (Paknejad *et al.* 2020). Hence, some samples were highly contaminated with bacterial and fungal, so their amplification was unsuccessful. Moreover, some studies have shown that specific primers are more sensitive than common primers, leading to differences in frequency rates (Schroeder *et al.* 2001; Tsvetkova *et al.* 2004).

The previous studies have reported that *Acanthamoeba* has a higher frequency than other FLA in both clinical and environmental samples (Hajialilo *et al.* 2015; Mahmoudi *et al.* 2015; Saburi *et al.* 2017). *Acanthamoeba's* high adaptability to different environmental conditions is one of the most important reasons for its widespread distribution (Aksozek *et al.* 2002). With regards to *Acanthamoeba* diseases, the most reported cases of AK are related to people who wear soft contact lenses, which occur due to inappropriate maintenance of their lenses (by cleaning of contact lenses with distilled water or non-sterile water) (Lindsay *et al.* 2007). T4 genotype isolated in this study is considered as one of the important genotypes reported in patients with AK (Ledee *et al.* 2009; Omaña-Molina *et al.* 2016). Also, there have been reports of isolation of

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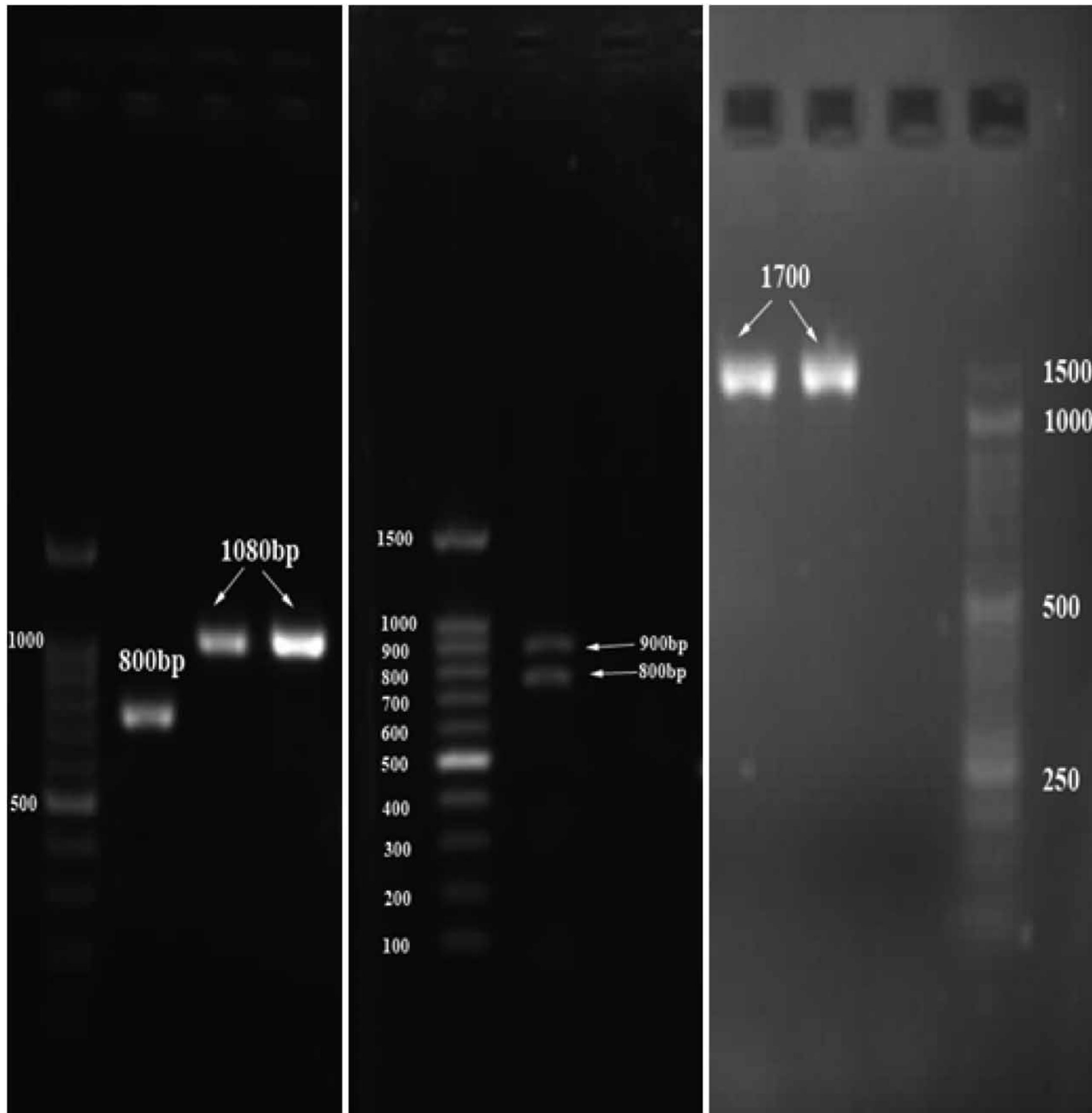

Figure 1 | The PCR product of FLA species based on the band size by the FLA1 and FLA2 primer pairs.

pathogenic *Acanthamoeba* T4 genotype from the mucous tissue of immunocompromised individuals, which may predispose them to ocular and cerebral involvement (Memari *et al.* 2015, 2017; Niyyati *et al.* 2017; Tananuvat *et al.* 2019). In this regard, some studies have reported *Acanthamoeba* pathogen genotypes (T1, T3–T5, T11) from the oral and nasopharyngeal cavity of patients with immunodeficiency diseases (Memari *et al.* 2016; Niyyati *et al.* 2017; Arab-Mazar *et al.* 2019). As a consequence, due to the fact that the water supply system of dental units is often supplied from municipal water, immunocompromised individuals and people wearing contact lenses need to be fully aware of the transmission, pathogenesis, and ways to prevent this microorganism. Moreover, it is well known that dental-unit water is a suitable place for the growth of a wide range of microorganisms. Based on the published literature, FLA (especially *Acanthamoeba*) are able to proliferate attached to bacterial biofilms in waterlines (Barbeau & Buhler 2001; Pinto *et al.* 2021). In this regard, they provide a favorable microhabitat for the survival, growth and multiplication of bacterial agents residing in them (Muchesa *et al.* 2017). In the other studies, Hassan *et al.* (2012), Retana-Moreira *et al.* (2015), and Trabelsi *et al.* (2010) reported the presence of *Acanthamoeba* spp. in 45, 14, and 13.3% samples collected from the dental-unit water from Egypt, Costa Rica, and Tunisia, respectively. Therefore, the infection of immunocompromised patients with *Acanthamoeba* spp. may pose a possible risk factor for serious and opportunistic infections due to the immunocompromised status of the patients.

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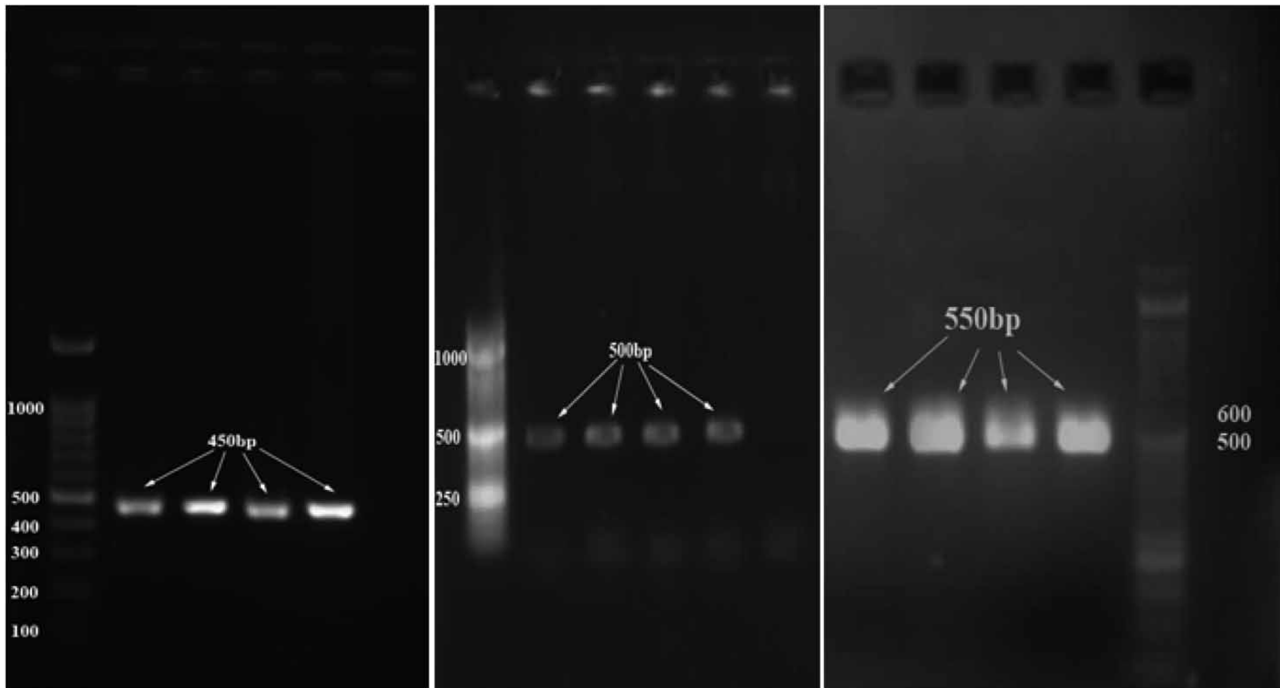

Figure 2 | The PCR product of *Acanthamoeba* spp. based on the band size by the JDP1 and JDP2 primer pairs.

Table 3 | Frequency of DNA fragments based on *Acanthamoeba*-specific primer (JDP1 and JDP2) in the present study

Band size	450-bp	500-bp	550-bp
Frequency, n (%)	37 (68.52%)	12 (22.22%)	5 (9.26%)
Acanthamoeba species	*A. castellani, A. polyphaga, A. hatchetti, A. culbertsoni*	*A. comandoni, A. griffni*	*A. astronyxis*

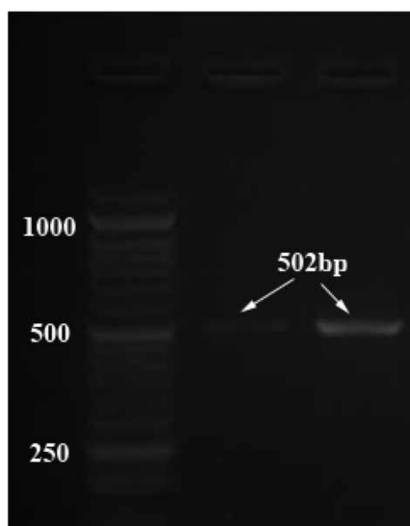

Figure 3 | The PCR product of *Vermamoeba vermiformis* based on the band size by the HV1 and HV2 primer pairs.

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Figure 4 | The PCR product of *Naegleria* species based on the band size by the ITS1 and ITS2 primer pairs.

Among the species of *Naegleria*, we detected only the *N. lovaniensis*. It is suggested that flagellation and temperature tolerance tests with PCR be used in future studies to differentiate *Naegleria* spp. from Vahlkampfia and other FLA.

In this study, one of the most abundant FLA identified was *V. vermiformis*. Although the pathogenicity of *V. vermiformis* has not been fully established, some case reports have suggested that this amoeba is a factor in patients with keratitis (Lorenzo-Morales *et al.* 2007; Abedkhojasteh *et al.* 2013). For example, a mixed infection of *Acanthamoeba* and *Hartmannella* has been reported in an AK patient (Lorenzo-Morales *et al.* 2007). Hence, it is suggested that more studies need to be conducted to gain a deeper understanding of the status and pathogenesis mechanism of this FLA.

Although the occurrence of *B. mandrillaris* has been reported in different sources such as hospital water distribution system, hot springs, and dust (Niyyati *et al.* 2009; Muchesa *et al.* 2015; Latifi *et al.* 2016), this FLA was not isolated in water samples of the dental unit of the present study. Therefore, more epidemiological studies are required to assess the exact niches of *B. mandrillaris* in different sources.

5. CONCLUSION

Given that one of the main routes of transmitting these FLAs is the eye, it is suggested that patients wear safety glasses during dental treatments to prevent possible water droplets from falling into the eye. Also, treatment of patients with oral ulcers is not recommended until their ulcers have healed. Moreover, every morning before starting work as well as after each patient, the handpiece should be washed and disinfected with sterile water or a sterile saline solution. These results reveal an essential need to improve filtration systems (pore lower than 0.2 μm) in dental units and periodic screenings for FLA in dental-unit water.

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AUTHORS' CONTRIBUTION

All authors contributed to study design. KS and HK contributed to all parts of the study. HR and HK contributed to study implementation. BA and KS collaborated in the analysis and interpretation of data. AT, AA and KS collaborated in the manuscript writing and revision. All the authors commented on the drafts of the manuscript and approved the final version of the article.

ETHICAL APPROVAL

This study was approved by Jahrom University of Medical Sciences Ethics Committee (ethical approval ID: IR.JUMS.-REC.1396. 098).

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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