FREE-LIVING AMOEBAE (FLA): MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *ACANTHAMOEBA* IN DENTAL UNIT WATER

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Summary:

The aim of our study was to detect free-living Amoebae (FLA) by morphological methods and to identify *Acanthamoeba* spp. by PCR in the dental unit water lines (DUWL). Materials and methods: it was a prospective study dealing with 196 water samples collected from DUWL (94 samples taken in the early morning before materials flush and patient consultations and 102 samples taken after consultations). At the same time, 39 samples from tap water were realized. Results: 135 (69 %) samples were positives by the morphological study with morphotypical diversity. The predominant morphotype was the monopodial (39.2 %). 18 strains of *Acanthamoeba* spp. were detected in DUW (13.3 %) and three strains in tap water (10 %). The amplification of 18S rDNA gene of these strains of *Acanthamoeba* spp. was positive for all samples. Conclusion: the FLA and *Acanthamoeba* were isolated both in tap water and in dental unit. The amoeba pathogenicity has not been demonstrated after oral or dental contamination; but the presence of intracellular and pathogenic bacteria in the amoeba could be a source of microbiological risks for patients in case of deep dental care or immunodepression. The improvement of this dental unit was necessary by putting a filter of 0.2 microns porosity before the arrival of the water in hand-pieces allowing the limitation of FLA passage.

KEY WORDS : *Acanthamoeba* spp., amibe libre, unite dentaire, identification morphologique, PCR, Tunisie.

Résumé : AMIBES LIBRES : IDENTIFICATION MORPHOLOGIQUE ET MOLÉCULAIRE D'*ACANTHAMOEBA* DANS L'EAU DES UNITÉS DENTAIRES

L'objectif de notre étude a été de détecter les amibes libres par des méthodes morphologiques et d'identifier Acanthamoeba *spp. par PCR dans l'eau des unités dentaires. Matériel et méthodes : il s'agit d'une étude prospective portant sur 196 prélèvements d'eau à partir des circuits dentaires (94 échantillons réalisés avant purge et consultation des malades et 102 échantillons après consultation des malades) et 39 échantillons d'eau de robinet. Résultats : l'étude morphologique a permis de détecter les amibes libres dans 69 % des échantillons. Le morphotype prédominant était le monopodial (39,2 %). 18 souches d'*Acanthamoeba *ont été détectées dans l'eau du circuit dentaire (13,3 %) et trois souches dans l'eau de robinet (10 %). L'amplification du gène 18S de l'ADN ribosomal de ces souches d'*Acanthamoeba *spp. a été positive dans 100 % des cas. Conclusion : les amibes libres, dont* Acanthamoeba*, ont été isolées aussi bien dans l'eau de robinet que dans les unités dentaires. Des cas d'infections amibiennes directement attribuables aux soins dentaires n'ont pas été démontrés, cependant, la présence de bactéries intracellulaires au sein des amibes libres doit être considérée comme un risque potentiel surtout chez les patients immunodéprimés. L'assainissement de ces unités s'impose au moins par la mise en place d'un filtre de porosité 0,2 microns avant l'étape d'entrée de l'eau dans les circuits dentaires afin de limiter l'éventuel passage des amibes libres.*

MOTS CLÉS : Acanthamoeba *spp., amibe libre, unite dentaire, identification morphologique, PCR, Tunisie.*

INTRODUCTION

Free-living amoebae (FLA) are opportunistic and ubiquitous protozoa that have a cosmopolitan distribution in the environment. Among the many genera of this FLA, members of only four genera were recognized to cause human disease: *Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris* and *Sappinia diploidea,* which are responsible of opportunistic and non opportunistic infections in humans and other animals (Martinez & Visvesvara, 1997). In dental unit, the quality of water is of considerable importance since patients and dental staffs are regularly exposed to water and aerosols generated from the unit. However, dental water may become heavily contaminated with FLA, bacteria and fungi. Indeed, dental hand pieces were connected to dental unit by a network of small-bore plastic tubing through which water and air were propelled to activate or cool down the instruments. Hydrodynamics shows that the water column inside the small lumen moves in the center of the tubing leaving a thin layer of liquid virtually undisturbed against the walls. Water stagnation associated with this physical state creates biofilms. This phenomenon increases the concentration and favours the proliferation of FLA, which are considered important hosts for *Legionella pneumophila*, *Pseudomonas aeruginosa* and other pathogenic bacteria (Rodrigues *et al.*, 2005). The amoeba pathogenicity has not been demon-

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strated after oral or dental contamination; but the presence of intracellular and pathogenic bacteria in the amoeba such as *Legionella pneumophila, Pseudomonas aeruginosa* and non tuberculous *mycobacteria* is particularly dangerous for patients with decreased immunity (Szymanska, 2004).

The aim of our study was to detect FLA by morphological methods and to identify *Acanthamoeba* spp. by PCR in the dental unit water.

MATERIALS AND METHODS

Sample collect and culture of amoebic isolates

It was a prospective study, during six months (April 2007-September 2007), dealing with 196 water samples collected from dental unit water (DUW) of the regional unit of medical school and university of Sfax (south of Tunisia). Dental unit canalizations were connected directly to municipal distribution systems for potable water and there is two filters 20 µm porosity placed before the arrival of the water in dental hand-pieces. Two different collects were realized one day during the week from tumblers, spittoons and dental instruments (air-water syringes, turbines and porphy-angles). The first collect (94 samples) was taken in the early morning before materials flush and patient consultations. The second collect (102 samples) was taken after consultations. At the same time, 39 samples from tap water were realized.

500 ml of each water sample was filtered through cellulose acetate filter 0.45 µm porosity under a weak vacuum. The filters were suspended in 8 ml of PAS solution 1×. 1 ml of various amoebae suspensions obtained from each filter solution was inoculated on 1.5 % non nutrient agar plates seeded with *E. coli* suspension. The samples were incubated at 25 °C and examined after 3-5 days under a light microscope. A piece of agar plates covered with *Acanthamoeba* was placed in axenic liquid culture medium PYG slightly modified and incubated at 25 °C (Shuster, 2002).

The strain (Linc-AP1) of *Acanthamoeba polyphaga* used was isolated by T.J. Rowbotham, Public Health Laboratory, Leeds, United Kingdom.

Microscopic examination

Amoebae in cultures were observed directly and after stains (Giemsa, trichrome, nuclear read and ethylene bleu) by using a light microscope at 100 ×, 400 × and 1000 × magnification. The genus *Acanthamoeba* was identified from the FLA based on its distinctive feature of trophozoites and cysts, particularly the double-walled cyst shape. For the classification, the Pussard & Pons (1977) and Page (1988) keys were applied.

Extraction of nuclear DNA

The cells collected from liquid culture PYG were centrifuged (1000 g) for 10 min at 4 °C and washed for three times with PBS (phosphate-buffered-saline) pH 7.2. Then, the sediment was resuspended in lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH = 8) and incubated at 56 °C for one hour in association with 10 mg/ml proteinase K. The genomic DNA was extracted using phenol-chloroform method. Until used, the DNA was stored at – 20 °C.

PCR of the SSU rDNA

For molecular identification, the primers P3/P4 were used. P3 (5' CCGAATTCGTCGACAACTGTTGATCCT GCCAGGT 3') and P4 (5' GGATCCAAGCTTGATCCTT CTGCAGGTTCACCTAC 3') (Bhattacharya *et al.*, 1998; Chung *et al.*, 1998). All amplification reactions of PCR were performed in a 50 µl mixture containing 50 ng DNA, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 50 mM of $MgCl_2$, 2 µl SAB (serum albumin bovin), 20 pmol of each primer and 2.5 UI of GoTaq DNA polymerase (Promega, Madisson, USA). PCR consisted of 1 min denaturation at 94 °C, 1 min hybridation at 64 °C and 2 min elongation at 72 °C. After 30 cycles, 10 min of extension time at 72 °C was done. Finally, the PCR products were cheeked by electrophoresis in a 1.5 % agarose gel.

RESULTS

Morphological study

FLA were detected in 135 sample collect (69 %): 69 positive before flush (51.1 %) and 66 positive after flush and consultations (48.8 %) ($p > 0.1$). Six different morphotypes were detected: monopodial (39.2 %), dactylopodial (31.8 %), fan shaped (29.6 %), acanthopodial (26.6 %), eruptive (10.3 %) and rugosa (8.1 %). The morphotype frequency was variable according to the moment of isolation (Fig. 1). Two or more morphotypes were associated in the same culture in 71 %

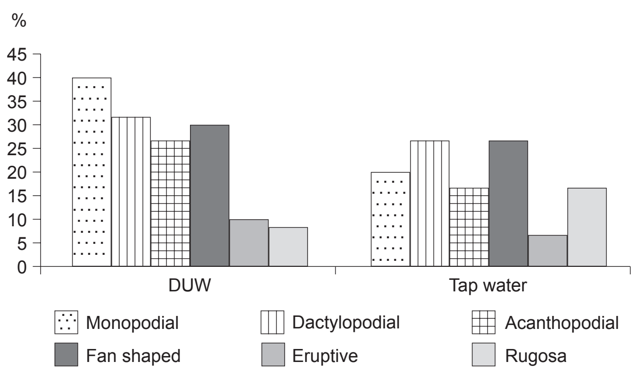

Fig. 1. – Frequency of FLA morphotypes in dental unit and tap water.

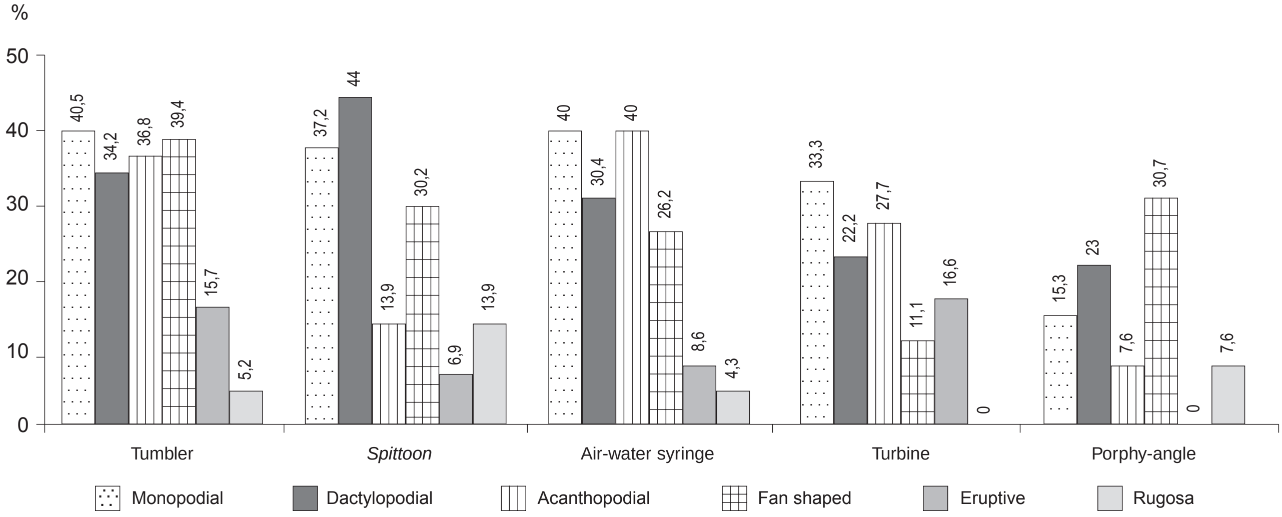

Fig. 2. – Frequency of FLA morphotypes in the different points of collection.

of cases. FLA were detected in spittoon (31.8 %), tumbler (28.1 %), air-water syringe (17 %), turbine (13.3 %) and prophy-angle (9.6 %). In the tap water, 30 samples (77 %) containing different amoebae morphotypes frequency: dactylopodial and fan shaped (26.6 % each), monopodial (20 %), acanthpodial and rugosa (16.6 % each), eruptive (6.6 %) (Fig. 2). There was no significant amoebae frequency variation between DUW and tap water (p > 0.1).

18 strains of *Acanthamoeba* spp. (13.3 %) were detected (ten samples in the first collect and eight in the second collect): five in each of spittoons, bottles and syringes, two in the porphy-angles and one in the turbine. In tap water, *Acanthamoeba* was detected in three samples (10 %).

The *Acanthamoeba* cysts detected in our samples belonged to the morphological group II of Pussard & Pons (1977) and Page (1988).

Molecular study

PCR amplification, with primers P3/P4, of the 21 samples identified as *Acanthamoeba* spp, showed the presence of a band of 2.3 kb in all cases.

DISCUSSION

To the best of our knowledge, this is the first study reporting on the morphological and molecular detection and identification of FLA and *Acanthamoeba* genus in the dental unit waters of Tunisia. A previous study was performed in a dialysis unit (Dendena *et al.*, 2008).

A wide range of microorganisms has been isolated from DUW, including fungi, FLA, opportunistic and human pathogen bacteria (Parrott *et al.*, 1982; Pankhurst *et al.*, 2003), but few studies were published about the diseases related to dental water. Only *Pseudomonas aeruginosa* derived from DUWL has been reported to give rise to infections in two immunocompromised patients (Martin, 1987). Pneumonia, cerebral infections and gastrointestinal disorders caused by waterborne microorganisms, although possible, would be difficult to link to a dental unit (Pankhurst *et al.*, 1998; Barbeau *et al.*, 2000). Eye infection with *Acanthamoeba* after accidental splatter has been reported, but the proofs were absent (Barbeau *et al.*, 2000). There is no evidence of a widespread public health problem from exposure to DUW. Nevertheless, the goal of infection control is to minimize the risk from exposure to potential pathogens and to create a safe working environment in which to treat patients (Pankhurst & Johnson, 1998).

Free amoebae, and particularly *Acanthamoeba* spp., were isolated in both DUW and tap water coming from municipal distribution systems for potable water. Even if chlorinated, this water hosts a diverse micro flora of bacteria, yeasts, fungi, viruses, protozoa, unicellular algae and nematodes. In fact, water is considered as potable if it contained less then one fecal coliforms/100 ml and less then 500 UFC/ml (CDC, 2003).

In our study, FLA have been isolated from 69 % of DUW samples and *Acanthamoeba* accounted for 13.3 % which was similar to the study of Michel & Just, (1984) whose found 12 % of *Acanthamoeba* in DUW. Barbeau *et al.* (2001) found that all DUW samples contained amoebae while *Acanthamoeba* species were detected in 40 % of samples.

Our water samples were collected at different moments but there was no significant amoebae frequency variation between the two collects. This was probably due to an ineffective material flush, water stagnation inside the canalizations and biofilm formation. Therefore, it was necessary, every morning, to flush hand-pieces and dental circuits before the first patient, during 5 at 8 min for reducing at the minimum the microorganism proliferation (Whitehouse *et al.*, 1991; Barbeau *et al.*, 2000). Barbeau *et al.* (2001) suggested that flushing dental unit water for 2 min was indispensable and can reduce the number of amoebae by 66 %. In addition, DUW should be flushed for 30 to 45 seconds between the patients (Barbeau, 2007) for reducing the number of oral microorganisms that may have been retracted into the lines after each patient. In addition, in our dental unit, there was two 20 µm porosity filters before the arrival of the water to hand-pieces. So, a pore size of 0.2 microns is recommended for limiting the passage of different FLA (Pankhurst & Johnson, 1998).

In DUW, all isolates of *Acanthamoeba* belonged to the morphological group II of Pussard & Pons (1977). Kong *et al.* (2002) find the same morphological type in the majority of contact lens storage cases in Korea.

We have completed our investigation by a molecular study of *Acanthamoeba* spp., which is pathogenic. The band size of *A.polyphaga* reference strain was 2.3 kb. This same band has been detected in all our samples. It could be also DNA amplification of other pathogenic species (*A. castellanii, A. culbertsoni, A. hatchetti, A. healyi, A. lugdunensis, A. triangularis, A. palistinensis* or *A. rhysodes*) (Chung *et al.*, 1998; Liu *et al.*, 2005). So, sequence analysis of 18s rDNA is a necessary stage for the identification of different *Acanthamoeba* species. This first study must be completed by the detection of microorganisms whitch can be carried by FLA.

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