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Low prevalence of oral protozoa colonization in Portuguese healthy and end-stage renal disease patients undergoing peritoneal dialysis

Baja prevalencia de la colonización oral de protozoos en Portugueses saludables y o pacientes renales del último estadio de enfermedad y que necesitan de diálisis del peritoneo

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SUMMARY

Infections are a major complication in end-stage renal disease (ESRD) patients undergoing peritoneal dialysis (PD). Because the oral cavity may act as a source of systemic pathogens, some authors advocated specific measures when these patients are submitted to oral interventions, such as the administration of prophylactic antibiotics. Oral protozoa colonization may vary significantly with geographic distribution and to our knowledge no studies were performed in Portugal. The aim of the present study was to evaluate protozoa colonization in the saliva of ESRD patients undergoing PD and of their family members, living in the north of Portugal. Saliva was collected from 39 PD patients with a mean time on PD therapy of 12.7 - 15.9 months, and from 18 healthy volunteers (ESRD family members) for microscopic evaluation of protozoa by Lugols' direct smear and specific staining techniques (Giemsa, Trichrome and Kinyoun). After the analysis of 456 smears obtained from 57 participants, only one PD patient (2.6%) presented an amoeba trophozoite in saliva. In conclusion, very low oral protozoa colonization was found, both on PD patients and family controls, suggesting that the oral protozoa colonization of Portuguese population is low and not significantly modified by the presence of end-stage chronic kidney disease. Further studies are required to address this issue.

Key words: Peritoneal Dialysis, Chronic Kidney Disease, Protozoa, Oral microorganisms, Saliva.

RESUMEN

Las infecciones son la principal complicación en pacientes renales del último estadio (ESRD) y que necesitan de diálisis del peritoneo (PD). Como la cavidad oral puede funcionar como una fuente de patógenos sistémicos, algunos autores indican medidas específicas cuando esos pacientes son sometidos a intervenciones orales, como la administración de antibióticos profilácticos. La colonización oral puede variar significativamente con la distribución geográfica. Según nuestros conocimientos, no han sido realizados estudios similares en Portugal. El principal objetivo fue evaluar la colonización de protozoos en saliva de pacientes ESRD del Norte de Portugal que hacían PD y, también, de sus familiares. Muestras de saliva fueron recogidas de 39 pacientes PD, con tiempo medio de terapia de PD de 12,7-15,9 meses y, también de 18 voluntarios saludables (familiares de ESRD). Las mismas utilizadas para evaluación microscópica de protozoos en laminas con lugol y tinciones especificas (Giemsa, Trichrome and Kinyoun). Después del análisis de 456 laminas, obtenidas de los 57 participantes, solamente en un paciente PD (2.6%) se observó un trofozoíto del ameba. En conclusión, se encontró una baja prevalencia de colonización oral de protozoos en el grupo estudiado. Así, la colonización oral de la población Portuguesa por protozoos es baja y no se cambia con la evolución de la enfermedad. Para mejor analizar esta situación, futuros estudios son necesarios.

Palabras clave: *Diálisis del peritoneo, Insuficiencia Renal Crónica, Protozoos, Microorganismos del cavidad oral, Saliva.*

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INTRODUCTION

Compared to other groups of microorganisms, few parasites colonize the oral cavity. Theprotozoan flagellate Leishmaniasp.can rarely, but severely, affect the human oral cavity and it does so indirectly by causing granulomatous growth disfigurations involving the mouth and nose (Bergquist, 2009). Other parasites can cause severe systemic disease after entering through oral cavity, such as Naegleria fowleri and Acanthamoeba encephalitis that may reach nasal cavity, penetrating the olfactory epithelium and, eventually, invading the brain (Cervantes-Sandoval, Serrano-Luna Jde, Garcia-Latorre, Tsutsumi, & Shibayama, 2008; Kaushal *et al.*, 2008). However, the most frequent human oral protozoa are usually non-pathogenic commensals such as *Entamoeba gingivalis*,followed by *Trichomonas tenax* (Wantland & Lauer, 1970). The oral colonization by these protozoa is associated with poor oral hygiene and with the severity of periodontal disease (Bergquist, 2009; Feki, Molet, Haag, & Kremer, 1981; Ghabanchi, Zibaei, Afkar, & Sarbazie, 2010; Kurnatowska, Dudko, & Kurnatowski, 2004; Pomes *et al.*, 2000; Wantland & Lauer, 1970).

Although literature reports are scarce, recent studies have revealed that oral protozoa colonization is more frequent than previously thought, and that its prevalence may vary significantly with the worldwide geographic distribution (Bergquist, 2009; Ghabanchi, *et al.*, 2010). In central Europe two studies from the 80s report an oral protozoa prevalence between 28% and 85% (Cambon *et al.*, 1979; Feki, *et al.*, 1981) whereas in eastern Europe two more recent studies showed an oral protozoa prevalence of 18% and 37% in Slovakia and Poland, respectively (Kurnatowska, *et al.*, 2004; Vrablic, Tomova, Catar, Randova, & Suttova, 1991).In Iraq and Iran,the prevalence varies between 4% and 46% (Ghabanchi, *et al.*, 2010; Madhi & Saeed, 1993) and, in Borneo, Indonesia, an oral protozoa prevalence of 18% was reported (Palmieri, Halverson, Sudjadi, Purnomo, & Masbar, 1984). In 1970,Wantland reported a prevalence of up to 55% in north of America (Wantland & Lauer, 1970) whereas in central and south America, studies from the 90s,described a prevalence of 21% and 62% in Guatemala and Brazil, respectively (Favoreto Junior & Machado, 1995; Pomes, *et al.*, 2000). To our knowledge no studies were performed so far in Portugal.

Parasitic disease continues to cause significant morbidity and mortality throughout the world (Stark *et al.*, 2009). Specifically protozoa are one of the most important pathogens that affect immune compromised patients (Ferreira, 2000). Individuals with impaired cell immunity such as carriers of the human immunodeficiency virus, patients with haematologic neoplasias, patients submitted to transplant of solid organs or under high-dose corticosteroid therapy are among the most infected by this pathogens group.

Immunosuppressed hosts are more susceptible to acquire infection after microorganisms exposure, and, although not commonly valorised, the oral cavity may act as a source of systemic pathogens (Bahrani-Mougeot *et al.*, 2008; Kshirsagar *et al.*, 2009; Li, Kolltveit, Tronstad, & Olsen, 2000). Once the infection is established,disease becomes more severe and disseminated, since the patients' immune response has more difficulty in eliminating the parasites, becoming chronic carriers (Stark, *et al.*, 2009). Being the immune system dysfunction a characteristic of end-stage renal disease (ESRD) patients due to uraemia(Kato *et al.*, 2008), parasite colonization can represent a risk factor in ESRD patients. In accordance, the major causes of death in ESRD patients are cardiovascular disease and infections, together accounting for up to 70% of all deaths in this patient population (Kato, *et al.*, 2008). In this population several reports demonstrate the risk of intestinal protozoa colonization (Barazesh, Fouladvand, Tahmasebi, Heydari, & Fallahi, 2015; Gil *et al.*, 2013; Visvesvara *et al.*, 2013). In Brazil, Gil and colleagues reported a prevalence of 51.6% of intestinal parasites in hemodialysis patients, but although the prevalence of colonization did not differ from control patients, ESRD patients were more easily symptomatic than controls (Gil, *et al.*, 2013). In Iran, Barazesh and colleagues reported a28.4%of hemodialysis patientsinfected with one or more intestinal parasites (Barazesh, *et al.*, 2015). Blastocystishominis, *Entamoeba coli* and *Cryptosporidium* were the most common parasitic infections in ESRD patients (Barazesh, *et al.*, 2015; Gil, *et al.*, 2013).

Peritoneal dialysis (PD) is a home-based widely used renal replacement therapy, and represents an alternative to hemodialysis (HD), with comparable survival, lower cost, and improved

quality of life (Chaudhary, Sangha, & Khanna, 2011). In PD, the patients peritoneal membrane is used as a dialysis membrane. Sterile dialysis fluid is introduced through a catheter into the abdominal cavity, drained and refreshed several times during the dayand throughout the night. Despite the improvements in the last decades, infection-related morbidity is still a significant complication in PD patients, accounting for 16 to 18% of the deaths in this population, as well as catheter loss, transfer to HD or prolonged hospitalization(Bloembergen & Port, 1996; Pereira, Sayegh, & Blake, 2005; Szeto, Wong, Chow, Leung, & Li, 2003). Therefore, prevention of infection is crucial for the success of PD therapy (Barraclough, Hawley, Playford, & Johnson, 2009).

Taking into account that there were no studies evaluating oral protozoa colonization in Portugal and that in ESRD patients undergoing PD the focus of infection should be a major concern, the aim of this study was to evaluate protozoa colonization in saliva of Portuguese healthy individuals and ESRD patients undergoing PD therapy at the Hospital S. João, the main hospital from the North of Portugal.

MATERIAL AND METHODS

Study participants

A group of ESRD patients undergoing PD therapy at the outpatient clinic of the Nephrology Department of "Centro Hospitalar de S. João" (Porto, Portugal)was invited to participate in the present study. Also, non-ESRD family members living at the same houseand closest in age were invited to participate as controlgroup.The study was explained orally and a written informed consent was obtained from all participants. The written informed consent as well as the study protocols werein accordance with the Helsinki Declaration of 1975, as revised in 1983,and were approved by the Ethic's Committee of "Centro Hospitalar de S. João". Exclusion criteria were applied, namely: (a) inability to give informed consent, (b) pregnancy, and (c) severe acute illness. The study sample included 39 PD patients and 18 familyvolunteers.

Clinical patient information was gathered, including: (a) age, (b) gender, (c) ethnicity, (d) education level, (e) aetiology of renal disease, (f) time on renal replacement therapy (RRT), (g) past and present peritonitis episodes, and (h) microbial agents responsible for peritonitis episodes. Also, information regarding the water source consumption was obtained.

Sample collection

Saliva was collected from all participants for protozoa analysis. Patients were instructed not to eat, drink or perform the normal oral hygiene at least two hours before the procedure. Samples of stimulated saliva were collected by the spiting of whole-mouth saliva during chewing paraffin pellets for 5 minutes (IvoclarVivadent, NY, USA).

Protozoa evaluation

Stimulated saliva was collected from each patient and placed ineightsmears (50µl each). Microscopic evaluation was performed by direct mounts with Lugol's iodine solution and after stainingwith Giemsa, Trichrome and Kinyoun techniques, in duplicate. Microscopic observation and photographs were performed using a Leica DM4000B microscope (Wetzlar, Germany) connected to a camera Leica DFC320 with 400x and 1000x amplification. The images were processed using Leica Application Suite software (version 2.3.1 R1).

Data analysis

Statistical analyses were performed by using IBM® SPSS® version 21.0 (Statistical Package for Social Sciences). The categorical variables were described through relative frequencies (%) and analysed by Chi-square independence test. Continuous variables were described using mean ± standard deviation (SD) and analysed by student`s t-test. A $P<0.05$ was assumed to denote a significant difference.

RESULTS

Demographic data from PD patients and controls are shown in Table I. Control family members were slightly younger and females were more prevalent in controls than in PD patients, although no significant differences were observed regarding age and gender. In general, education level was low in both control and study groups,with no significant differences between groups.

Table I. Demographical data from ESRD patients undergoing peritoneal dialysis (PD) and healthy family members controls.

	PD patients	Controls	Pvalue
Age (years)	45.4±14.6	37.5±16.8	0.800a
Gender			0.082b
Male	51.2%	27.2%	
Female	48.8%	72.2%	
Education level			0.064b
Illiterate	4.9%	0%	
Elementary School	63.4%	38.9%	
High school	9.8%	33.3%	
University	9.8%	16.7%	

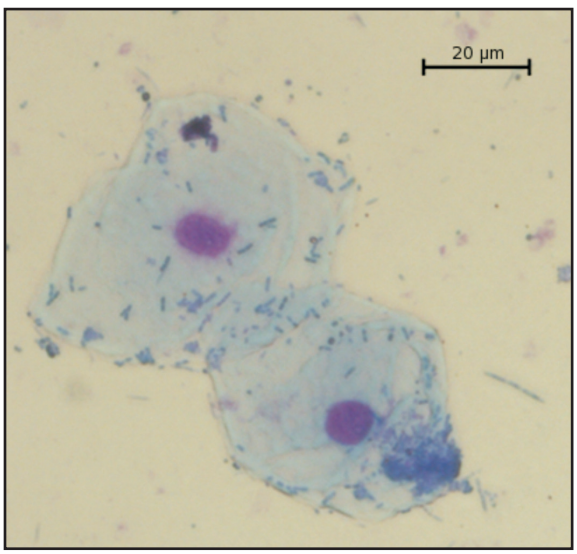
Results are shown in prevalence (%) or mean±SD. [a]Student's t-test and [b]Chi-square independence test.

In PD patients, the most prevalent aetiologies of chronic kidney disease were chronic glomerulonephritis (19.5%, with a high prevalence of IgA nephropathy) and diabetic nephropathy (14.6%). The mean time on PD program was 12.7 months. Before the study, 25.6% of the PD patients had previous peritonitis episodes and gram-positive cocciwere the most prevalent isolated microorganisms. None of the patients had reported peritonitis caused by fungi or protozoa.

Most participants drank treated water from urban water supply network (68.3%), although 19.5% still used water from private water-well or water-borehole.We were not able to obtain information regarding the water source from 12.2% of PD patients.

In this study, oral protozoa colonization was assessed in stimulated saliva due to the low salivary rates observed in many PD patients. Concerning the microscopic observation of saliva, normal epithelial cells and oral microbiota (cocci, bacilli and, in some cases, yeasts) (Fig. 1) were observed in the wet mounts of all samples. Despite the use of specific staining methods for *E. gingivalis*, *T. tenax*, *Giardia* sp and *Cryptosporidium* sp, an amoeba was observed in only one PD patient (2.6%) (Fig. 2).The respective family member control, as well as all otherparticipantsof the study, did not present oral protozoa colonization.

Fig. 1. Microscopic view of saliva samples after giemsa staining, showing an assortment of epithelial cells and bacteria (400x, Leica DFC320).

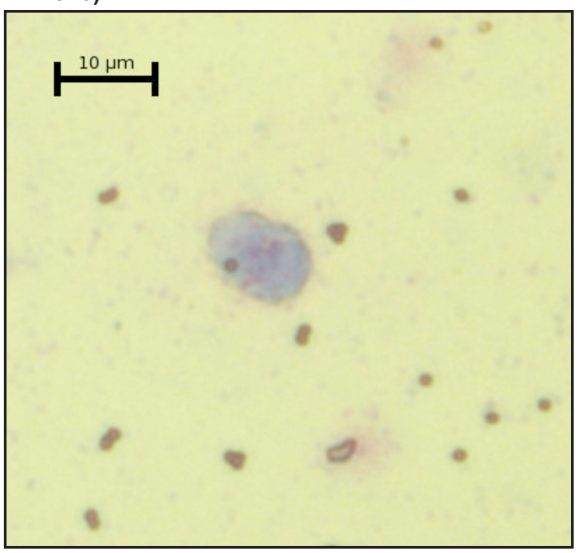


DISCUSSION

This is the first study to evaluate oral protozoa colonization in a Portuguese population of ESRD patients undergoing PD and respective family controls, as no other study was found in the literature.

It is well known that oral microbial colonization is strongly correlated with oral hygiene, dietary habits and familiar pre disposition; additionally, oral protozoa prevalence is associated with living environment conditions as well as alimentary habits (Almeida *et al.*, 2010; Smith, Caccio, Cook, Nichols, & Tait, 2007). Thus, for this type of studies, the recommended control group should be family members of the patients, as here employed, rather than unrelated healthy individuals(C. Areias *et*

Fig. 2. Microscopic view of saliva sample after trichrome staining, showing a trophozoite of an amoeba with a centrally karyosome, peripheral chromatin and a granular cytoplasm (400x, Leica DFC320).



al., 2012; C. M. Areias, Sampaio-Maia, Guimaraes, Melo, & Andrade, 2011). In addition, evaluation of oral protozoa colonization in PD patients' family members is crucial since they could represent potential source of infection and act as vehicles for opportunistic microorganism transmission.

In this study only one PD patient presented oral protozoa colonization, probably by *Entamoeba gingivalis* due to the fact that is the most frequent amoeba in the oral cavity. Interestingly, protozoa colonization was not observed in his corresponding control. Despite the very low oral protozoa colonization among the studied PD population, no protozoa were observed in the control group, probably suggesting a higher susceptibility of PD patients to protozoa colonization in comparison to non-ESRD individuals.

In particular, the patient presenting an amoeba in saliva was suggested to be immune compromised, given that *Aspergillus fumigatus* and *Acinetobacter baumannii* were identified in his bronchial secretions before his death,6 months after saliva collection for protozoa isolation. Despite the fact that no peritonitis episodes were described in this patient before the study, two peritonitis occurred one and five months after saliva collection, caused by *Streptococcus* sp. and *Escherichia coli* respectively, this last in the context of an intestinal perforation that resulted in the patient's death. Future studies with a larger sample group are required to confirm this difference between health and CKD patients.

It has been previously documented that oral protozoa prevalence may vary significantly with the geographic distribution (Wantland & Lauer, 1970). Despite the fact that no data are available for the Portuguese population this study, performed at the main reference hospital of the North of Portugal, could suggest a low colonization among the northern Portuguese population. In accordance to this, a Portuguese study performed on the water basins and rivers from the north of Portugal reportedthe existence of a low infection risk for protozoa with surface raw and drinking water samples (Almeida, *et al.*, 2010). However, it is well established that drinking water from private wells or boreholes may represent a significant risk for infection transmission. In our study, 20% of the individuals obtained their drinking water from private wells or boreholes; so, the low rate of protozoa colonization may be explained by the generalized access of our population to water from the public network system. Despite this, the water source of the PD patient colonized by protozoa in saliva was the public network system, what may suggest that others transmission routes may have a significant impact in the oral colonization by these infectious agents.

The low rate of oral protozoa in our PD patients, namely 2.6%, and its absence in their healthy family controls may also be conditioned by the limited number of patients analysed, as well as by the methodology applied. The direct visualization of cells (cysts or trophozoites) in the samples presents lower sensitivity and specificity than other methods, such as immunofluorescence and enzyme immunoassays, culture methods, PCR as well as flow cytometry (J. Barbosa, Costa-de-Oliveira, Rodrigues, & Pina-Vaz, 2008; J. M. Barbosa *et al.*, 2008).

In conclusion, very low oral protozoa colonization was found in this study,suggesting that this may reflect the oral protozoa colonization of Portuguese population. Future studies are required to evaluate the possible higher susceptibility to oral protozoa colonization of ESRD patients undergoing PD.

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Conflict of interest

The authors declare that they have no conflict of interest.

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