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"ORAL AND DIALYSIS EFFLUENT PROTOZOA COLONIZATION IN CHRONIC KIDNEY DISEASE PATIENTS UNDERGOING PERITONEAL DIALYSIS."

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Oral and dialysis effluent protozoa colonization in chronic kidney disease patients undergoing peritoneal dialysis

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ABSTRACT

The prevalence of chronic kidney disease (CKD) is increasing worldwide. Peritoneal dialysis (PD) is a home-based and widely used therapy of renal replacement for patients with end-stage renal disease (ESRD). Despite the improvements in this renal replacement therapy, peritonitis is still one of the most important causes of technique failure in peritoneal dialysis. Still nowadays, culture-negative peritoneal inflammation accounts for between 5 and 20% of cases of peritonitis in PD patients. In patients with persistent culture-negative peritonitis, consideration should be given to unusual microorganisms, such as protozoa. So, the aim of the present study was to evaluate the presence of protozoa in saliva and PD effluent from PD patients. Also, the prevalence of PD patients' oral protozoa colonization was compared with a healthy population. For that purpose, clinical and demographic information was collected from 41 PD patients and 18 healthy controls (non-CKD family members of PD patients) were included. A non-invasive intra-oral examination was performed in order to evaluate the following parameters: decayed, missing, and filled teeth (DMF) as well as oral hygiene indexes. Saliva was collected before oral examination for pH and flow rate evaluation as well as protozoa analysis. Samples from PD effluent were also collected to PD patients. Saliva and PD effluent were examined by direct microscopy for protozoa identification using Giemsa staining, Lugol's solution and modified Ziehl-Neelsen's technique. It was found protozoa in the samples of dialysis effluent collected from five different PD patients. In two PD patients it was found *Blastocystis hominis* and in the other three PD patients it was found *Entamoeba*, *Giardia lamblia* or *Endolimax nana*. In addition, no oral protozoa colonization was found in PD patients and their healthy familiar controls. In conclusion, PD effluents from PD patients were susceptible to asymptomatic protozoa colonization, highlighting the need for a more systematic screening of protozoa in PD population. The clinical impact of these sub-clinical infections should be investigated. In addition, the fact that oral protozoa colonization was absent in PD patients and their healthy familiar controls, suggests that in Portuguese population the oral protozoa colonization may be low.

Keywords: Peritoneal Dialysis, Chronic Kidney Disease, Protozoa, Oral colonization, Peritoneal dialysis effluent, sub-clinical infection, saliva

RESUMO

A prevalência da doença renal crónica (DRC) tem vindo a aumentar em todo o mundo. A diálise peritoneal (DP) é uma terapia domiciliar e amplamente utilizada como terapia de substituição renal em doentes no estadio final da DRC. Apesar dos últimos avanços nesta terapia, a peritonite contínua a ser uma das mais importantes causas para a falha técnica da DP, levando ao insucesso da mesma. Ainda hoje em dia, em 5 a 20% das peritonites decorrentes da DP, o resultado final de diagnóstico é negativo ou apresenta uma incorreta identificação dos agentes infeciosos. Assim, neste grupo de doentes, deve ser tido em conta microrganismos menos usuais, como os protozoários. Assim, o objetivo do presente estudo foi avaliar a presença de protozoários na saliva e no fluído peritoneal de um grupo de doentes renais crónicos a realizarem DP. Adicionalmente, foi também estudada a prevalência de protozoários na saliva de familiares saudáveis. Para tal, informação demográfica e clínica foi colhida de 41 doentes em DP e 18 controlos saudáveis (familiares saudáveis dos doentes em DP). Foi realizado um exame intraoral não invasivo de forma a avaliar o número de dentes cariados, perdidos e obturados (índice CPO) bem como o índice de higiene oral. Recolheu-se uma amostra de saliva antes do exame intraoral para determinação do fluxo e pH assim como análise de protozoários. Foram recolhidas também amostras de efluente peritoneal dos doentes em DP. As amostras de saliva e efluente foram analisadas por microscopia para a deteção de protozoários recorrendo às colorações com Lugol, Giemsa e Ziehl-Neelsen modificado. Foram encontrados 5 protozoários em amostras de fluído de efluente peritoneal em 5 doentes diferentes. Em dois doentes foi encontrado *Blastocystis hominis* e nos restantes três foi encontrado *Entamoeba, Giardia lamblia* e *Endolimax nana.* Adicionalmente, não se observou colonização oral de protozoários em doentes em DP e controlos saudáveis. Em conclusão, os efluentes peritoneais de doentes em DP revelaram-se suscetíveis à colonização assintomática de protozoários, realçando a necessidade de um rastreio mais sistemático da presença de protozoários nesta população em PD. O impacto clínico destas infeções subclínicas devem ser investigadas. Adicionalmente, a ausência de colonização oral de protozoários em doentes em DP e controlos saudáveis, sugere que a colonização oral deste microorganismos na população portuguesa poderá ser reduzida.

Palavras chave: Diálise peritoneal, Doença renal crónica, Protozoários, Efluente peritoneal, colonização oral, saliva, infeção subclínica

INTRODUCTION

The prevalence of chronic kidney disease (CKD) is increasing every year worldwide. In Portugal, more than 9000 individuals receive renal replacement therapy, and the number of patients with kidney failure that require renal replacement therapy grows 10 to 15% every year (1). CDK is characterized by a number of systemic complications in consequence of a severe hidroelectrolytic, metabolic and immunological imbalance (2, 3). When the function of the kidney is severely damaged, the patients require renal replacement therapy, whether in the form of peritoneal dialysis, hemodialysis or renal transplantation (2). Despite all the improvements in patients care and success of the renal replacement therapies in replacing the major functions of the kidney (4), the impact of CDK on patient's morbidity and mortality is extremely high (3).

Peritoneal dialysis (PD) is a home-based widely used therapy of renal replacement comparable to hemodialysis in terms of risks and expenses. In PD, the peritoneal membrane is used as an artificial kidney. Sterile dialysis fluid is introduced through a catheter into the abdominal cavity, drained and refreshed several times during the day or throughout the night (4).

Despite de improvements in health care 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 (5, 6), as well as catheter loss; transfer to hemodialysis and prolonged hospitalization (7). Therefore, prevention of infection is crucial to the success of PD therapy (8). The factors that influence the PD-related infection occurrence are still not entirely understood. It is assumed that contamination at the time of the dialysis effluent exchange remains a major cause of peritonitis (9, 10), occurring mainly by an external route (8). For this reason and to overcome this issue, high standard hygiene procedures during the PD exchange are promoted in PD patients.

PD related peritonitis occur mainly in the context of bacterial infection, being the most common agents *Staphylococcus* and *Streptococcus* species. Although they are known colonizers of the skin and nasal/oral cavities (11), there is effectively low correlation between peritoneal catheter exit-site infections and peritonitis (12). Interestingly, some authors have highlighted the importance of the oral cavity infection as a starting point for dissemination of pathogenic organisms to different distant body sites, mainly through bacteremia (13). The proximity of the oral microorganisms with the blood stream highlights the possible occurrence of the transfer of oral microorganisms to blood a common but transient event, that may result form routine daily activities or from invasive dental procedures (13, 14). Despite the possible association between oral cavity diseases and an adverse survival of renal diseases patients, the scientific evidence linking this route of infection to peritonitis is still rare or inexistent (15).

On the other hand, culture-negative peritoneal inflammation accounts for between 5 (16) and 20% (17) of cases of peritonitis in PD patients. Unfortunately, in some cases (16), patients die before the cause of peritonitis is determined, after several weeks of persistent culture-negative peritonitis. In these patients with persistent culture-negative peritonitis, consideration should be given to unusual microorganisms, such as protozoa (16). Tilak et *al*. (18) reported a case of *Acanthamoeba* peritonitis in a patient on peritoneal dialysis where culture of PD effluent was negative for bacteria and fungi. It is interesting to notice that the exit-site and tunnel of the peritoneal dialysis catheter were healthy and both blood and urine cultures were culture-negative (18). Also, a severe peritonitis due to *Balantidium coli* acquired in France by an alcoholic pork-butcher was reported in 2004 (19). Furthermore, Chung-YoYeum et *al.*,(20) reported in a patient with no clinical evidence of peritonitis an incidental detection of an *Anikasis* larva in peritoneal dialysis effluent, a parasite present in raw or undercooked fish (20).

Compared to other groups of microorganisms, few parasites colonize the oral cavity, although several recent studies have revealed that the protozoa are more frequent than previously thought (21, 22). Notwithstanding, its prevalence may vary significantly with the worldwide geographic distribution, ranging from 4% to 53% (22). Within oral parasites, the protozoan *Entamoeba gingivalis* and *Trichomonas tenax* are the most frequent and are normally non-pathogenic commensal microorganisms. Although their oral colonization is associated with poor oral hygiene and a low socioeconomic status, these protozoa can also be found in caries-free children and adolescents (21, 23, 24). The protozoa's rate of colonization increases with age, being much more prevalent in adults than in children, particularly in those with periodontal disease (22).

It is of notice that parasitic disease continues to cause significant morbidity and mortality throughout the world (25). It is estimated that there are approximately 340 parasite species capable of infecting humans, with the majority of the 3 billion people currently infected residing in developing regions of the world (26). Despite the fact that the prevalence of parasites decreased in the developed countries with the widespread of use of modern plumbing, footwear and better hygiene measures (27), between 1992 and 1997, the Centers of Disease Control and Prevention (CDC) estimated that more than 2.5 million cases of giardiasis occur annually (28). In agreement, the World Health Organization (WHO) refers that, probably, every water hearth surface are contaminated with Giardia (28,(29). Also, it is known that more than 30% of children worldwide are infected by *Enterobius vermicularis* (30).

The risk factors for the acquisition of parasites are the same in both immunocompetent and immunosupressed individuals (25). However, through local and systemic responses, the immune system plays an integral part in modifying the establishment of infection, controlling disease once it is established, limiting the severity and dissemination of the disease, and assisting in clearance or control of the parasite (25). Thus, immunosupressed hosts are more likely to acquire infection after exposure, have more severe disease once the infection is established, have disseminated infection rather than localized infection, and have more difficulty in clearing parasites, becoming chronic carriers (25). These all lead to, and account for, the greater morbidity and mortality in immunosupressed patients (25).

Being the immunosuppression one of the main characteristics of the chronic kidney disease patients, parasites can be one of the causes of the peritonitis episodes, as previously described (18). Besides the immunosuppression, contact with infected children, living in poor conditions, consumption of non-bottled water, eating unpeeled fruits, eating raw food or vegetables, and traveling, may constitute other risk factors for parasites infections (27).

Given the above stated, the aim of the present project was to evaluate PD patients' protozoa colonization, specifically in saliva and in PD effluent. Also, the prevalence of oral protozoa colonization in a CKD population undergoing PD was compared with a healthy population.

MATERIAL AND METHODS

Study participants

A group of CKD patients engaging the PD program from August of 2011 and March of 2012 and followed up in the outpatient clinic of the Nephrology Department of Hospital S. João were invited to participate in the present study. Also, non-CKD family members living in the same house, closest in age, were invited to participate as control group. To all participants, the study was explained orally and was asked their written informed consent. The informed consent and the study protocols were approved by the Ethic's Committee of "Hospital de S. João". The exclusion criteria were: inability to give informed consent, pregnancy and severe acute illness. The study sample included: 41 PD patients and 18 non-CKD family members.

Clinical patient information was gathered including: age, gender, ethnicity, smoking habits, education level, blood pressure, etiology of renal disease, residual renal function, time on renal replacement therapy (RRT), infectious complications during RRT, past and present peritonitis episodes and agents. Also, information such as water source, eating habits and environmental conditions was obtained.

Sample collection and analysis

A non-invasive intra-oral examination was performed to DP patients and controls in order to evaluate the following parameters: decayed, missing, and filled teeth (DMF) as well as oral hygiene indexes.

Saliva was collected from PD patients and from non-CKD family members before oral examination for the protozoa analysis as well as pH and flow rate evaluation. Patients were instructed not to eat, drink or perform the normal mouth hygiene at least two hours before the procedure. Samples of non-stimulated and stimulated saliva were collected by the spiting of whole-mouth saliva under resting conditions of 5 min and during chewing paraffin pellets (Ivoclar Vivadent, NY, USA) over 5 min, respectively. The volume was quantified gravimetrically and the salivary flow rate was determined (ml/min). Saliva pH was determined immediately after collection using pH strips (5.0-8.0, Duotest, Germany). Also, preparation of

six smears with 50µl of stimulated saliva from each patient and control was performed for subsequent microscopic observation.

Samples of peritoneal dialysis effluent were also collected in an aseptic environment. A total of 50 ml was centrifuged at 1500 rpm for 10 minutes. Afterwards, six smears with 50µl of the pellet were done for later staining.

Identification of Protozoa was done by standard parasitology methods, namely: (a) direct microscopic smear observation with Lugol's iodine solution and; (b) staining techniques, specifically, Giemsa stain and Modified Kinyoun acid - fast. With Lugol's iodine solution, nuclear structures and fibrils of the flagellates stains yellowish-brown color and become visible; also, iodophilic vacuoles or masses stain intensely brown. With Giemsa, flagella, cilia and nuclei stain red and cytoplasm stains blue, being the ideal method to search for *Trichomonas.* Modified Kinyoun technique was used to search specifically coccidian protozoa, in particular, *Cryptosporidium* sp. oocysts, since these latency cells present alcohol acid-fast resistances, turning red/ pink and distinguishable on the blue background.

Data analysis

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

RESULTS

Clinical and demographic data

The final study population was composed by 41 PD patients and 18 non-CKD family members controls. The clinical and demographic data from PD patients and controls are shown in Table I. Non-CKD family members controls were significantly younger than PD patients. Although more females than males constituted the control group, no significant differences were observed regarding gender between PD patients and control group. In general, the education level was low in both control and study groups and not significantly different between groups.

Table I: Clinical and demographical data from PD patients and control group: age and education level as well as aetiology of CKD, time on peritoneal dialysis, renal function, blood pressure, and consumption water' sources.

	PD patients	**Controls**	***p* value**
Age (years)	45.4±14.6	37.5±16.8	0.800*
Gender			0.082#
Male	51.2%	27.2%	
Female	48.8%	72.2%	
Education Level			0.064#
Illiterate	4.9%	0%	
Elementary School	63.4%	38.9%	
High school	9.8%	33.3%	
University	9.8%	16.7%	
Etiology of CDK			
Diabetic nephropathy	14.6%		
IgA nephropathy	14.6%		
Polycystic kidney disease	7.3%		
Chronic glomerulonephritis	4.9%		
Others	22.0%		
Undetermined	26.8%		
Time on PD (months)	12.7±15.9		
Creatinine clearance (ml/min)	10.4±5.8		
Blood pressure			
Systolic	131±23		
Diastolic	79±13		
Consumption water' source			
Urban water supply network	68.3%		
Private water-well or water-borehole	19.5%		
Unknown	12.2%		

CDK= Chronic kidney disease. Results are shown in prevalence (%) or mean±SD. *Student`s t-test and #Chi-square independence test.

Regarding PD patients, the most prevalent aetiologies of CKD were diabetic nephropathy and IgA nephropathy (Table I). These patients presented a mean time on PD program of 12.7 months, but ranged from 0 months and 71 months. The median residual renal function and blood pressure values were in the normal range, considering the typology of patients.

In PD patients, it is also a concern their water consumption' source. In our study population, most participants drink treated water from urban water supply network. Although approximately 20% still drank water from private water-well or water-borehole. Is important to notice that, in 12% of our PD patients, it was not possible to obtained information about their domestic water supply system.

Oral health and oral protozoa colonization

Smoking habits did not differ in the past and present time between PD patients and healthy controls. However, only a small percentage of individuals smoke at the present time (Table II).

Table II: Clinical patient information and intra-oral examination parameters.

	PD patients	**Controls**	***p* value**
Smoking Habits			
Smoked in the past	37.5%	12.5%	0.083#
Smoke in present	8.3%	6.2%	0.806#
Oral Hygiene			0.135#
Bad	52.6%	23.1%	
Regular	42.1%	76.9%	
Good	5.3%	0	
DMFT index	13.58±6.62	13.20±6.64	0.892
Decayed	3.46±3.13	4.5±6.66	0.509*
Missing	7.04±5.58	5.44±3.65	0.317*
Filled	3.04±3.92	3.31±4.10	0.834*
Saliva Rate Flow			
Non-stimulated saliva	0.39±0.30	0.40±0.28	0.927*
Stimulated saliva	0.82±0.56	1.01±0.63	0.338*
Saliva pH			
Non-stimulated saliva	7.7±0.52	7.06±0.377	0.000*
Stimulated saliva	7.84±0.367	7.59±0.344	0.034*

Results are prevalence (%) or mean±SD. *Student`s t test and # Chi-square independence test.

Regarding oral hygiene index, it was only possible to assess the oral health of 27 of the 41 PD patients. Most of PD patients presented bad global oral hygiene, whereas most of the family

members constituting the control group presented regular oral hygiene (Table II). The prevalence of participants with good oral hygiene was found to be very low. Both groups had a very high DMFT index (Table II). Although the DMF index was very similar in both groups, prevalence of decayed teeth tended to be lower in PD patients, but with no statistical significance.

Although no differences were observed regarding salivary flow rate between PD patients and controls (Table II), PD patients presented stimulated saliva flow rate values below the normal range (1.0 to 3.0mL/min). Interestingly, pH value of non-stimulated saliva and stimulated saliva was higher in PD patients when compared with control group (Table II).

No protozoa were found in smears of stimulated saliva collected from both PD patients and healthy controls (Table III).

Table III– Protozoa prevalence and identification in PD effluent from the 41 PD patients.

Sample	Prevalence	Identification
Saliva	0%	-
Dialysis effluent	12.2%	*Entamoeba* sp
		Blastocystis hominis
		Endolimax nana
		Giardia lamblia

PD-related infections and PD effluent protozoa colonization

Taking in to account the previous peritonitis, 34.1% PD patients had previous reports and 4.9% patients had 2 previous peritonitis onsets (Table IV). None of the patients had a peritonitis caused by a fungus. Gram-positive cocci were responsible for 75% of the total number of episodes. The most common genus was *Staphylococcus*, being *S. epidermidis* the most prevalent species. Other Gram-negative bacilli were also found as etiological peritonitis agents such as *Pseudomonas aeruginosa*, *Burkholderia* spp. and other species from *Enterobacteriaceae* family (Table IV).

Taking in to account the previous exit-site infections, 51.2% PD patients had previous reports and 34.1% patients had more than one episode (Table IV). Gram-positive cocci were responsible for 68.6% of the episodes. The most common was *Staphylococcus aureus*, responsible for 55.6% of the episodes, and the second most common cause was *Corynebacterium*, responsible for 30.6%. Gram-negative bacteria were also responsible for exit-

site infections, being the most prevalent *Pseudomonas*, causing 19.6% of the episodes. Bacteria from *Enterobacteriaceae* family, such as *Serratia marcescens. E. coli* and *Klebsiella*, were also found. Fungus, namely *Candida parapsilosis*, were responsible for 4.9% of exit-site infections.

Table IV: Peritonitis and exit-site infection data in PD patients.

	Patients	Episodes	Gram-positive cocci	Gram-negative bacilli	Fungi
Peritonitis	34.1%	16	75%	25%	0
Exit-site infection	51.2%	51	68.6%	33.3%	4.9%

Results are prevalence (%).

Table III shows the prevalence of PD patients with protozoa in dialysis effluent. It was found protozoa in the samples of dialysis effluent collected from five different PD patients, although only 1 to 5 protozoa were observed per smear (50µl), from each positive PD patient. In two PD patients it was observed *Blastocystis hominis* (Figure 1A) and in the other three PD patients it was found *Entamoeba* sp (Figure 1B), *Giardia lamblia* (Figure 1C) and *Endolimax nana* (Figure 1D).

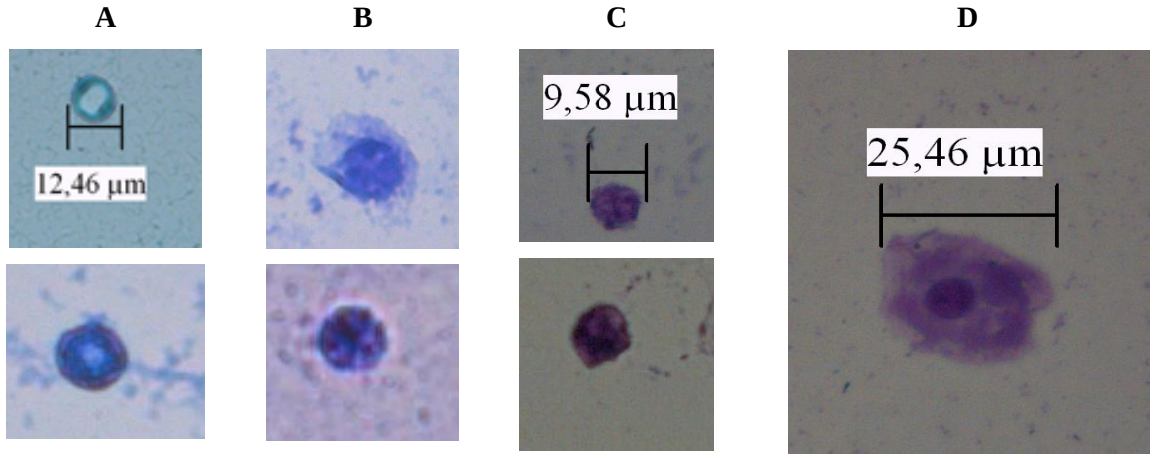


Figure 1: Pictures of protozoa found in PD effluents samples from PD patients, after Giemsa staining method, namely: (A) cysts of *Blastocystis hominis* and (B) *Endolimax nana*; (C) trofozoits of *Giardia lamblia* and (D) ameba of *Entamoeba* sp. Light microscopy, 40x.

PD patients presenting protozoa in PD effluent were further characterized regarding age, gender, educational level, profession, CKD etiology, diabetes and environment/social condition such as children cohabiting, domestic poultry, consumption water source, vegetable (especially lettuce) consumption frequency and frequent summing in rivers or lakes (Table V). Two of these

patients died during the study, one due to complications during a bacterial peritonitis and the second of heart attack.

Table V– PD patients' characterization regarding age, gender, educational level, profession, CKD etiology, diabetes and environment/social conditions

Protozoa ID	***E. nana***	***B. hominis***	***B. hominis***	***Entamoeba sp.***	***G. lamblia***
Patient ID	**1**	**2**	**3**	**4**	**5**
Age (years)	46	27	42	47	57
Gender	Female	Female	Male	Male	Male
Educational level	Elementary school	Elementary school	Elementary school	Elementary school	Elementary school
Profession	Merchant	Cash operator	Locksmith	Managing partner	Unemployed
CDK etiology	Chronic glomerulonephritis	Alport Syndrome	Unknown	Diabetic nephropathy	Undetermined
Time on DP (months)	5,00	0,00	4,00	31,00	3,00
Diabetes	No	No	No	DM type 1	No
Children cohabiting	No	No	No	No	b)
Domestic poultry	Chicken	Chicken and rabbit	a)	No	b)
Lettuce consumption	Yes	Yes	a)	Yes	b)
Swimming (rivers or lakes)	No	No	a)	No	b)
Water source	Water well	Water well	Urban water supply network	Urban water supply network	b)

a) Patients 3 and 5, died during the study, so, some information was not possible to be gathered.

All the PD patients with protozoa colonization have low education level, different CDK etiologies and were not diabetics. Interestingly, 40% were female and having contact with domestic poultry; also, obtained water for from their private well and normally consume lettuce. Although similar information were obtained from male and females, no correlations could be found between specific protozoa microorganism and environmental/ social conditions.

DISCUSSION

The main objective of this study was to evaluate the presence of protozoa in saliva from PD patients and health controls, as well as PD effluent from PD patients. Although this study was performed at Hospital São João (Porto, Portugal), the main hospital located on the North of Portugal, only 41 patients with PD from the Nephrology Service participated. Five PD patients presented positive PD effluents, suggesting asymptomatic protozoa colonization.

In this study, oral health and oral protozoa colonization was assessed from a stimulated saliva sample obtained from each PD patients, which was compared with control group. Stimulated saliva was used instead of non-stimulated due to the low salivary rates of many patients. Because oral microbial colonization is strongly correlated with oral hygiene, dietary habits and familiar pre-disposition, as also oral protozoa is strongly related with living environment conditions as well as alimentary habits, the recommended control group should be as we employed, non-CKD family members of PD patients, rather than unrelated healthy individuals (31, 32). In addition, oral protozoa colonization evaluation is relevant in PD patients' family members since they may represent important vehicles for opportunistic microorganisms' transmission and may be a potential source of infection. In agreement, Bistrup (1997) (33) supports the value of the adoption of special measures of hygiene by the family members of PD patients. Water in a relevant source of protozoa as previously mentioned (27, 34, 35), and both study and control groups are exposed to the same water source. Nevertheless, no oral protozoa was found on our PD patients or their healthy familiar controls, suggesting low oral protozoa colonization among these study population from the north of Portugal. A Portuguese study (36) performed on the water basins and rivers from the North of Portugal, showed the low infection risk for population to be contaminated with *G. lamblia* and *Cryptosporidium parvum* with surface raw and drinking water samples (36). However, this low risk is conditioned by the access of the whole population to the drinking water public network system. According to our national statistics, only 84% of the Portuguese (36) population has access to public drinking water system and, a significant segment of population is still provided by water obtained in wells and other origins. Our study population fits well in these statistics, given that around 20% obtained their drinking water in private wells or boreholes, being water-wells more referred preferentially on the inquiry. In comparison to borehole, water wells are more prone to contamination given their open access to the open-air. Interestingly, 50% of our positive protozoa PD patients drink water from water-wells, suggesting that in these cases water source could represent the protozoa contamination vehicle. Other common transmissions routes are person-to-person (through direct

or indirect contact) and animal-to-animal, animal-to-human, food-borne and recreational water (37-40). Also, It is well documented that raw vegetables, especially lettuce, could be a source of microbes contamination since these vegetables are the natural soil filter; so, if in their irrigation is performed with contaminated water or soil fertilization is due with organic compounds, these vegetables became contaminated and could be an infection vehicle for humans (34, 41-43). In our population, 80% have domestic poultry and consume lettuce, being also others possible contamination vehicles for protozoa acquisition.

Although previous studies pointed out changes in the oral microbiota of patients on hemodialysis when compared to healthy controls, it remains unclear the factors that have a severe impact on the presence of oral pathogens in people with systemic diseases and the role of these microorganisms on the risk for complications (44). Recently, it was reported that factors as age, specific diet, poor dental condition, gingival pathology, limited mobility and difficulties in maintenance good oral hygiene are consequences of systemic illness that favor the occurrence of oral parasites, bacteria and fungi (44). Also, the serious metabolic instabilities, insulin therapy and immunosuppressive drugs change the general health status and the oral microbiota. So, these altered circumstances may enhance the development of protozoa as well as the other numerous opportunistic microorganisms (44).

In this study, although the family member participating as control group should be the member closest in age, most of the PD patients were accompanied by their child's due to the lack of availability of other family members. So, our control group was younger than PD patients and, this age discrepancy could have some interference in oral health and protozoa colonization. Our results revealed a slightly better oral hygiene of the family member although, for both groups, results were not so good. Accordingly to a prior study, where the authors showed a direct correlation between education level and oral hygiene status, probably this results were a consequence of the low education level of most study participants (45). Interestingly, our studied sample was mainly composed by non-smokers, which reveals greater wary with their own health, avoiding exposure to potential and inevitable aggressors but, unfortunately, this concern with general health was not so remarkable with their oral hygiene.

In most studies, no differences were found in oral protozoa colonization regarding the sex of participants, (46, 47) but not all (48). The prevalence of oral protozoa colonization is more prevalent in adults, (48) apparently more common between 41 to 51 years of age (44). Although no oral protozoa colonization was found in our population, four from the five PD patients positive colonized in PD effluent were within this age range.

Saliva flow rate of PD patients was lower than controls, although not attaining statistical significance, which can be supported by the fact that the PD patients have fluid intake restriction, drug therapy side effects, possible salivary gland alteration and oral breathing, consequence of lung perfusion problems (1, 49-51). Moreover, PD patients presented higher saliva pH, due to increased ammonia concentration as a result of urea hydrolysis. Furthermore, several studies reported that PD patients have higher plaque indexes than healthy population (1). This could be related to physical and psychology stress, where often oral hygiene may be neglected (1, 45). Likewise, it was also previously reported that CKD patients visits the dentist less frequently than general population (1). Even though, it is particularly important for these patients to diminish all the infection's focus, including the oral cavity, the idea that oral microorganisms may trigger an infection elsewhere in the body is still not clear to general population (1, 45) playing the dentist an important role in explaining the importance of oral health to maintain systemic health. Dental health status, determined by DMFT index as recommended by the WHO, is calculated by the sum of the number of decayed (D), missing (M) and filled (F) teeth (45, 50). In this study, the DMFT index found for PD patients as well as for controls was considerably high, since the average result for both groups corresponds to approximately half of teeth present in the normal dentition. When comparing the individual values of decayed, missing and filled teeth it can be observed that PD patients have greater number of missing teeth but reduced number of decayed and filled teeth in comparison to the control group, although with no statistical difference. Effectively, in literature is described that PD patients show an index of decay teeth relatively lower than general population, possibly associated with a higher urea concentration in saliva. This fact has a protective effect that inhibits bacterial growth, neutralizes bacterial plaque acids and contributes to enamel remineralization (45, 50, 52). To our knowledge, no study was performed in Portugal evaluating oral protozoa colonization and, no oral protozoa colonization was observed in PD patients' saliva, as well as healthy controls.

Other studies demonstrated that infection rate of *Entamoeba gingivalis* and *Trichomonas tenax* was associated to the presence of calculus and the progression of periodontal disease (22, 48). Notwithstanding, other study found no conclusive evidence to demonstrate the correlation between *Entamoeba gingivalis* and periodontal disease (47). Unfortunately, the periodontal health was not evaluated in the studied population.

The low oral protozoa colonization could be a geographic characteristic, given that it is known that oral protozoa prevalence may vary significantly with the worldwide geographic distribution, ranging from 4 to 53% (22). Also, the limited number of patients analysed as well

as the applied methodology could conditioned the obtained results. It is well documented that protozoa detection is highly dependent on the methods used to collect and analyse samples, as also for protozoa concentration. Also, conventional methods with microscopic observation of stained biological fluids and water samples, are too time consuming and dependent on the technician's skill and expertise, showing rather low sensitivity and specificity (53, 54). A survey (46) of the incidence of oral protozoa performed in 1958 (46) in U.S.A., revealed that 41% of 300 patients presented *Entamoeba gingivalis*, 22% *Trichomonas tenax* and 18% both protozoa (46). It is interesting to notice that 6% of the samples collected from the debris in carious cavities and gingival crevice, diagnosed as negative by wet smear examination were found positive on culturing (46). These finding suggest that, if we add employed another methods to detect protozoa (e.g. immunofluorescence and enzyme immunoassays, culture methods, PCR and flow cytometry) whith a higher sensitivity and specificity than microscopy, we probably could found more protozoa either in saliva or PD effluent. However, even more specific, those methods present also limitations since they are very precise for each protozoa species, even belonging at the same genus. For example, we need to know the: (a) specific antigen-antibody correlation (for immunoassays and flow cytometry methods); (b) gene sequence (for PCR methods) or; (c) culture media compounds and quantities for each protozoa species (55-58).

Interestingly, other oral protozoa were also found in samples from nose, mouth and pharynx swabs of Mexican females, such as *E. histolytica, Acanthamoeba castelanni, Naegleria fowleri* and *Balantidium coli*, suggesting that healthy patients may be healthy carriers of protozoa cysts (59). Taking into account that, since 1978, free-living amoebae have been collected form tap water, water tanks and bottled mineral water in Mexico, these findings could be explained, highlighting the water as an very important vehicle of these protozoa (59). More recently, hemodialyzed patients, diabetic and nondiabetic, were clinically assessed for their pretreatment oral cavity condition and samples from the periodontium and dental plaques were collected for light microscopic studies with wet slides and Giemsa or Trichrome stained slides (44). Motile protozoa as well as *E. gingivalis* and *T. tenax* were found. Curiously, the average prevalence of protozoa in control group was higher (20%) than in the other two groups: diabetic and kidney allograft recipients/hemodialyzed patients (4%) and non-diabetic recipients and hemodialyzed patients (8%) (44). This result highlights the importance of the control group that in our study has a limited number of cases. In diabetic and non-diabetic hemodialyzed patients, multi-organ disturbances influence both the episode of secondary clinical oral manifestations as well as prevalence and species of mouth microorganisms harbouring (44). Unfortunately, no data on

oral protozoa colonization was found for CKD patients undergoing dialysis, highlighting the importance of oral screening of this specific population.

Oral protozoa are normally considered as non-pathogenic commensal microorganisms. However, the presence of protozoa in other body sites, such as PD effluent, intestine or even in blood, can represent serious healthy complications. Patients in dialysis are susceptible to opportunistic infections, as a result of immunologic impairment (60-67). Peritoneal infection is still an important cause of mortality in peritoneal dialysis patients, (68, 69) being bacteria and fungi the most common infection agents. However, parasites were also reported as peritonitis agents. This fact is in agreement with the view that opportunistic parasites are often responsible for serious and recurrent infections in immunocompromised patients, but are self-limited in normal and healthy populations (70).

The first case referred in the literature about finding a protozoon in dialysis fluid dates 1993, (71) and was found a free-living *Acanthamoeba*. A case-report of *B. hominis* infection during *Pseudomonas* sp peritonitis in an Italian male patient on continuous ambulatory peritoneal dialysis (CAPD) was reported in 1996 (72). The protozoa was found in both fresh-observation of faeces and peritoneal fluid and it was the first case referred in the literature of *B. hominis* infection in CAPD patients (72). In this case, the patient was treated with only specific antibiotic therapy for *Pseudomonas* sp infection, raising the question of the role of this specific protozoa: symbion or pathogen (72). Also a 65-year-old Japanese[17] woman on hemodialysis therapy admitted on an hospital for abdominal pain and diarrhea was infected by *B. hominis*, indicating that this microorganism could be overlooked as a cause of diarrhea, especially in immunosupressed patients[17]. Infection with *B. hominis* occurs worldwide, with prevalence ranging from 0.5% to 10% in developed countries and from 30% to 50% in developing countries (73, 74). In our study, even with a small PD patients group, we reported a 5% prevalence for this protozoa, being the normal average found on the developed countries. The role of *Blastocystis* sp as a cause of disease is far from completely understood (72). Pathogenicity of *B. hominis* is in debated given that some studies show resolution of diarrhoea without treatment and others refer the need of specific treatment, also, its presence in stools in asymptomatic individuals is frequently recognized (75).

In a cross sectional study, (76) in 2011, direct stool smear analyzed by trichrome staining of 155 HD Iranian patients and 294 controls, 43.9% of HD patients and 43.1% of control group were infected by intestinal parasites. As in our study, *B. hominis* was the most common cause of parasite infection (8%), followed by *E. coli* (5.6%) and *E. nana* (4.2%). This study highlights the

importance of stool exam for an early parasites diagnosis, especially in patients who suffer from diarrhea, a common symptom of CKD. As CKD is a known cause of diarrhea per se, (77) the early diagnose of intestinal protozoa infection may be difficult, and normally other causes such as bacteria, fungi and virus have to be first eliminated (78). Also, the investigations of the role of factors like water source, personal hygiene as well as alimentary and living habits is crucial to understand the transmission routes of these microorganisms.

As expected for CDK pathology, the PD patients presented, on average, low creatinine clearance values corresponding to 10 to 25% of glomerular filtration, according to the classification of renal function (50), and revealing a compromised renal function. Cerveró and co-workers (2008) (50) discloses that only 2 to 3% of all patients with chronic renal failure have polycystic kidney disease, being the most prevalent disease onset diabetes *mellitus* (40-60%), followed by hypertension and glomerulonephritis (30% and 10%, respectively) (50). However, in this studied population, the most prevalent aetiologies of CKD were diabetic nephropathy and IgA nephropathy followed by polycystic kidney disease and chronic glomerulonephritis. This difference may be due to the reduce number of patients included in the present study.

CONCLUSIONS

No oral protozoa colonization was found in PD patients and their healthy family members controls, suggesting that in Portuguese population the oral protozoa colonization may be low. However, 12% of PD patients presented asymptomatic colonization of dialysis effluent with *Blastocystis hominis*, *Entamoeba* sp, *Giardia lamblia* or *Endolimax nana*, highlighting the need for a more systematic screening of protozoa in PD population. The clinical impact of these sub-clinical infections should be further investigated.

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ANEXOS