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Original Article

The Prevalence of *Entamoeba gingivalis* and *Trichomonas tenax* in Relation to Periodontal Disease among Patients Attending Dental Clinics in Urmia, Iran: A Cross-Sectional Study

Yahya Azadeh [1], *Shahram Khademvatan [1,2], Zahra Goharfar [3], Shokoufeh Shahrabi-Farahani [4], Rasool Jafari [1]

1. *Department of Medical Parasitology and Mycology, Cellular and Molecular Research Center, Cellular and Molecular Medicine Research Institute, Urmia University of Medical Sciences, Urmia, Iran*
2. *Department of Veterinary Biosciences, Melbourne Veterinary School, Faculty of Science, The University of Melbourne, Parkville, Victoria, Australia*
3. *Department of Periodontics, School of Dentistry, Urmia University of Medical Sciences, Urmia, Iran*
4. *Department of Diagnostic Sciences, College of Dentistry, University of Tennessee, Knoxville, Tennessee, USA*

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***Correspondence Emails:**
khademvatan.s@unimelb.edu.au

Abstract

Background: We aimed to determine the prevalence of *Entamoeba gingivalis* and *Trichomonas tenax* in relation to periodontitis in Urmia, Northwestern Iran, in 2022.

***Methods*:** Gingival crevicular fluid (GCF) samples were collected from133 patients and analyzed using microscopic and molecular techniques to identify parasites. Data were analyzed using SPSS software and Chi-square and Fisher's exact test.

***Results*:** 42.1% of patients were infected with *E. gingivalis* and 11.3% with *T. tenax*. In comparison, microscopic diagnosis identified only 1.5% and 0.8% of patients infected, respectively. This highlights the increased sensitivity of molecular methods for parasite detection. A statistically significant relationship was observed between the presence of these parasites (identified by the molecular method) and several factors. Male patients had a significantly higher prevalence of *E. gingivalis* infection (P=0.037). Additionally, a history of diabetes (P=0.011) and cardiovascular diseases (P=0.021) was associated with a higher prevalence of *E. gingivalis*. The highest prevalence of *E. gingivalis* was observed in the 41–50-year age group (15 cases). Importantly, both *E. gingivalis* and *T. tenax* were significantly more prevalent in patients with severe periodontitis (P=0.003, P<0.001).

***Conclusion*:** This study demonstrates a higher prevalence of *E. gingivalis* compared to *T. tenax* in the oral cavity, suggesting its potentially greater role in periodontal disease, particularly among individuals with specific risk factors. Further research is warranted to elucidate the precise contribution of these parasites to periodontitis, identify additional risk factors, and develop improved diagnostic and treatment approaches.

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Introduction

Periodontal disease (PD), which includes gingivitis (inflammation of the gums) and periodontitis (gum infection), is an important health issue that is highly prevalent in adults, and the severity of the disease increases with age (1). Epidemiological studies have shown that up to 56.8% of the population to some degrees are suffering from PD (2). Periodontitis is a chronic inflammatory disease that is caused by the presence of a group of microbes called dental plaque, which causes damage to the ligaments and bones around the teeth (3). Alveolar bone loss is one of the main consequences of periodontitis (4). Gingivitis in general as a specific local inflammation due to conditions caused by the accumulation of dental biofilm and Periodontal attachment loss is considered with redness and swelling of the gums and the absence of periodontal attachment loss (5). Important risk factors in increasing the probability of PD include smoking habit, type 1 diabetes, economic level of living, age, sex, level of oral and dental hygiene, level of dental care, systemic diseases including cardiovascular diseases, respiratory diseases and Blood pressure, obesity, osteoporosis, stress, history of antibiotic use, and acquired immune system deficiency (AIDS) have been mentioned (6, 7).

The microbial flora of the mouth not only plays an important role in maintaining the health of the oral cavity, but also plays a role in the pathophysiology of its diseases (8). The loss of balance between the host's immune system and the pathogenicity of the microbial flora is considered as the initiator of periodontal diseases (9). A wide variety of microorganisms including bacteria, parasites, fungi, viruses and Archaebacteria inhabit the human oral cavity (10). Therefore, another risk factor for oral and dental diseases is infectious diseases such as fungi, parasites, viruses and bacteria (10). Microscopic protozoan parasites are protista that can live free or parasitic (11). Protozoa like as *Entamoeba gingivalis* and *Trichomonas tenax* present in the oral cavity and can cause PD (12, 13). Studies conducted over decades show that *E. gingivalis* and *T. tenax* protozoa induce inflammation in periodontal tissue by increasing the expression of pro-inflammatory molecules and neutrophil recruitment, which contribute to the process of PD (14).

E. gingivalis is found only in the trophozoite form, which varies in size from 5-35 µm (15). The global prevalence of *E. gingivalis* is estimated to be 37% with a 95% confidence interval until August 2020, according to different countries, the highest and lowest prevalence of *E. gingivalis* is Jordan with 87% and Portugal with 3% respectively (16). Another protozoan that is usually seen in samples obtained from patients with periodontitis and gingivitis with *E. gingivalis* is *T. tenax*, also called *Trichomonas buccalis*, and like *E. gingivalis*, it is generally found in the upper part of the digestive system in the spaces of infected gums and tissue grooves of Tonsils are found (17, 18). The global prevalence of *T. tenax* 17%, the highest prevalence in Chile is 56%, the lowest prevalence is 3% in Kenya respectively (19).

Several studies conducted in different regions of Iran, including Tehran, Shiraz, and Ahvaz, have reported the presence of *E. gingivalis* and *T. tenax* in patients with periodontal disease (20-22).

Considering the importance of oral and dental hygiene and investigating the etiology of oral and dental diseases Since no comprehensive research on this topic has been previously conducted in Urmia, this study will provide valuable insights into the local epidemiology of these parasites and their potential contribution to periodontal disease in the region. The findings will inform future research efforts and potentially contribute to the development of more targeted preventive and therapeutic strategies for periodontal health management in Urmia.

Materials and Methods

Study population and sampling

This descriptive study was conducted on 133 patients with moderate to severe chronic periodontitis who referred to Urmia Dental Faculty Clinic in 2022-2023.

The protocol was approved by the Committee on the Ethics of the Urmia University of Medical Sciences (IR.UMSU.REC.1401.254).

After a brief explanation about the goals and methods used in the study, the patients who agreed to participate in the study signed the consent form and completed a questionnaire on oral and dental hygiene, smoking systemic diseases and eating habits. It was examined by a dentist specializing in oral and dental diseases, and the type and severity of the disease was determined (Community Periodontal index-CPI) (23). Moreover, in order to comply with ethical standards, written consent was obtained from the participants. Samples were prepared by absorbent paper point by collecting Gingival Crevicular Fluid (GCF) from periodontal pockets and placing it in a microtube containing 0.5^{cc} of normal saline, the microtubes were labeled and transported to the laboratory of the pathology department of Dental Clinic and were subjected to microscopic examination for identification of oral parasites. Direct microscopic and molecular methods were selected for rapid detection of oral protozoa. After the initial direct study, the samples were stored at -20 °C until subsequent molecular PCR analysis.

Direct microscopy and Geimsa's staining

The prepared samples were immediately subjected to microscopic study as wet slides with 10X and 40X lens, then the slides were fixed with methanol after drying in air and stained with Giemsa dye (with 1/10 dilution for 30 to 60 sec) and subjected to microscopic study with A 100X lens was placed and the results were reported.

E. gingivalis, when present, is distinguished by size (10 20μ), presence of prominent pseudopodia, and slow movement. Another oral protozoan, *T. tenax* is seen as a pear-shaped flagellated trophozoite, about 5-13μ in length, with circular movement (15).

DNA extraction

GCF samples were prepared from the periodontal pockets of periodontal patients by paper point absorption and placed in a microtube containing 0.5 ^{cc} of normal saline and placed in a freezer at -20$^{°C}$ until the time of DNA extraction. The frozen samples were removed from the freezer and after thawing, they were subjected to DNA extraction using the sedimentation method DNA extraction kit of Karmania Pars Gene, (Iran) (CN:KPG-MDNABt).

PCR conditions

The primers used in this study were synthesized by CinaClon, Tehran, Iran. The primers for *E. gingivalis* were chosen GEg3F (5'-GTAATTCCACCTCCAATAGTRT-3') and GEg3R (5'AACTAAGAAC-GGCCATGCAC-3') (24). All amplifications were preceded by an initial cycle of 2 min at 94 °C, amplification consisting of 35 cycles at 94 °C for 30 sec (denaturation), 57 °C for 30 sec (annealing), and 72 °C for 30 sec (extraction) and ended with a 3-min cycle at 72 °C (final extraction). These primers gave amplicons of around 700 bp for GEg3F-GEg3R. TRICHO-F and TRICHO-R (5-CGGTAGGTGAAC-CTGC CGTT-3) and(5-TGCTTCAG-TTCAGCGGGTCT-3) primers were used to identify the 5.8 SrRNA gene of *Trichomonas tenax* and internal transcription regions (ITS1 and ITS2) (25). respectively, amplification consisted of: 1 cycle of 94 °C for 2 min, followed by 30 cycles each consisting of denaturation for 20 sec at 94 °C, 10 sec of annealing at 60 °C, and 40 sec of extension at 72 °C. A final extension step at 72 °C for 3 min was included (26). These primers gave amplicons of around 368-bp for TRICHO-F and TRICHO-R(accession no. U86615) (26).

Statistical analysis

To determine the prevalence of microorganisms and whether the observed differences are significant according to the characteristics of gender and age and different risk factors, we performed statistical analysis (χ^2 or Fisher's exact test) using the SPSS ver. 16 (Chicago, IL, USA) with a significant level of $P<0.05$.

Results

By sampling the gums of 133 patients with periodontitis, the prevalence of *E. gingivalis* and *T. tenax* was investigated by two direct (Wet slide and Giemsa staining) and molecular method Out of 133 participants, (41.4%) were men and (58.6%) were women. The highest number of patients (34, representing 25.2%) were in the 41-50 age group, 96.2% were urban dwellers and 3.8% were rural dwellers, 21.8% did not brush their teeth at all, 12% had the habit of smoking and 97.7% used tap water (Table 1).

Table 1: Frequency distribution of demographic variables and risk factors of patients included in the study

Variable		Count	%
Gender	Men	55	41.4
	Women	78	58.6
Age(yr)	10-20	7	5.2
	21-30	28	20.7
	31-40	24	17.8
	41-50	34	25.2
	51-60	25	18.5
	>60	15	11.1
Location	City	128	96.2
	Village	5	3.8
Brushing	Yes	104	78.2
	No	29	21.8
Smoking	Yes	117	86.67
	No	16	13.33
Drinking water source	tap water	130	97.7
	Household purified water	3	2.3

12.8% (17 people) were suffering from diabetes, 12% had a history of heart diseases, 2.3% had a history of lung diseases, 51% had a history of corona, 0.8% had a history of asthma, 52.6% had taken antibiotics in the 6 months leading up to sampling and 62.5% had mild periodontitis, 27.8% moderate and 9.8% Intense (Table 2).

Moreover, out of 133 patients participating in the study, 56 (42.1%) were positive for *E. gingivalis* and 15 (11.3%) were positive for *T. tenax* using molecular diagnosis methods. In addition, in microscopic examination, 2 (1.5%) cases were positive for *E. gingivalis* and 1 (0.8%) for *T. tenax* (Table 3).

Table 2: Frequency distribution of History of disease of patients included in the study

Variable		Count	%
Diabetes	Yes	17	12.8
	No	116	87.2
Cardiovascular disease	Yes	16	12
	No	117	88
Pulmonary disease	Yes	3	2.3
	No	130	97.7
History of corona infection	Yes	68	51.1
	No	65	48.9
History of asthma	Yes	1	0.8
	No	132	99.2
History of antibiotic use in the last 6 months	Yes	70	52.6
	No	63	47.4
Severity of periodontitis	Mild	83	62.4
	Moderate	37	27.8
	Intense	13	9.8

Features of smoking, brushing teeth, history of antibiotic use in the last 6 months, place of residence, and source of drinking water did not significantly affect *E. gingivalis* infection. However, gender, diabetes, history of cardiovascular diseases, and severity of periodontitis were significant factors related to the frequency of *E. gingivalis* in patients with periodontitis (Table 4).

The variables of smoking, brushing teeth, history of antibiotic use in the last 6 months, place of residence, gender, diabetes, history of cardiovascular diseases and source of drinking water did not significantly affect *T. tenax* infection. However, severity of periodontitis was a significant factor related to the prevalence of *T. tenax* in patients with periodontitis (Table 5).

Table 3: Frequency distribution of *E. gingivalis and T. tenax* positive cases in study patients

Molecular method			
Variable		**Frequency**	**%**
Entamoeba gingivalis	Positive	56	42.1
	Negative	77	57.9
Trichomonas tenax	Positive	15	11.3
	Negative	118	88.7
Microscopic method			
Entamoeba gingivalis	Positive	2	1.5
	Negative	131	98.5
Trichomonas tenax	Positive	1	0.8
	Negative	132	99.2

Table 4: Distribution of demographic variables and risk factors and history of diseases as a result of infection with *Entamoeba gingivalis* parasite

Variable		*Entamoeba gingivalis*					
		Positive		**Negative**		**Odds Retio**	***P*-value**
		Count	%	Count	%		
Gender	Men	29	52.7	26	57.1	2.1	0.037
	Women	27	36.6	51	71.4	1	
Age(yr)	10-20	3	42.9	4	57.1	1	0.709
	21-30	8	28.6	20	71.4	1.33	
	31-40	12	50	12	50	2.5	
	41-50	15	44.1	19	55,9	1.001	
	51-60	11	44	14	56	1.267	
	>60	7	46.7	8	53.3	1.273	
Location	City	55	43	73	57	3	0.297
	Village	1	20	4	80	1	
Brushing	Yes	41	39.42	63	60.58	0.607	0.235
	No	15	51.7	14	48.3	1	
Smoking	Yes	11	68.75	5	31.25	3.52	0.22
	No	45	38.5	72	61.5	1	
Drinking water source	tap water	56	43.1	74	56.9	-	0.263
	Household purified water	0	0	3	100	-	
diabetes	Yes	12	70.6	5	29.4	3.93	0.011
	No	44	37.9	72	62.1	1	
Cardiovascular disease	Yes	11	68.8	5	32.2	3.59	0.021
	No	45	38.5	72	61.5	1	
Pulmonary disease	Yes	2	66.7	1	33.3	2.81	0.38
	No	54	41.5	76	58.5	1	
History of corona infection	Yes	30	44.1	30	55.9	1.18	0.38
	No	26	40	26	60	1	
History of asthma	Yes	1	100	0	0	-	0.42
	No	55	41.7	77	58.3	-	
History of antibiotic use in the last 6 months	Yes	30	42.9	40	57.1	1.06	0.45
	No	26	41.3	37	58.7	1	
Severity of periodontitis	Mild	24	28.9	59	71.1	1	0.003
	Moderate	23	62.2	14	38.8	0.878	
	severe	9	69.2	14	37.8	1.58	

Table 5: Distribution of demographic variables and risk factors and history of diseases as a result of infection with *T. tenax* parasite

Variable		*T. tenax*					
		Positive		Negative		Odds Retio	*P*-Value
		count	%	count	%		
Gender	Men	3	5.5	52	94.5	2.1	0.037
	Women	12	15.4	66	84.6	1	
Age(yr)	10-20	0	0	7	100	-	0.562
	21-30	3	10.7	25	89.3	2.059	
	31-40	4	16.7	20	83.3	3.293	
	41-50	5	14.7	29	85.3	2.797	
	51-60	3	12	22	88	2.333	
	>60	0	0	15	100	-	
Location	City	15	11.7	113	88.3	-	0.544
	Village	0	0	5	100	-	
Brushing	Yes	12	11.54	63	10.3	1.13	0.214
	No	3	88.46	14	89.7	1	
Smoking	Yes	0	0	16	100	-	0.214
	No	15	12.8	102	102	-	
Drinking water source	tap water	14	10.8	116	89.2	0.241	0.222
	Household purified water	1	33.3	2	66.7	1	
diabetes	Yes	3	17.6	14	82.4	1.85	0.295
	No	12	10.3	104	89.7	1	
Cardiovascular disease	Yes	2	12.5	14	87.5	1.14	0.567
	No	13	11.1	104	88.9	1	
Pulmonary disease	Yes	0	0	3	100	1.13	0.696
	No	15	11.5	115	88.5	1	
History of corona infection	Yes	8	11.8	60	88.2	1.1	0.537
	No	7	10.8	58	89.2	1	
History of asthma	Yes	0	0	1	100	-	0.887
	No	15	11.4	117	88.6	-	
History of antibiotic use in the last 6 months	Yes	7	10	63	90	0.764	0.544
	No	8	12.7	55	87.3	1	
Severity of periodontitis	Mild	11	13.3	72	86.7	1	<0.001
	Moderate	2	5.4	35	94.6	0.374	
	severe	2	15.4	11	84.6	1.19	

Discussion

In this study, the prevalence of *E. gingivalis* (42.1%) and *T. tenax* (11.3%) aligns with findings from previous investigations, including in Shiraz, Southern Iran) (21), Jordan (7), Iraq (27), Nigeria (28), further highlighting the common occurrence of these parasites in individuals with periodontal disease. An analysis of global data reveals the prevalence of periodontal disease, oral cancer, and dental caries increased

significantly by 45.6% during the first decade of the 21st century (29).

Several factors can contribute to infection with these parasites, including: Gender, age, underlying diseases (diabetes, cardiovascular and respiratory diseases, etc.), smoking, oral and dental hygiene measures, severity of periodontal disease, etc. (27, 30).

Hyperglycemia in diabetic individuals exacerbates susceptibility to periodontal disease and impedes wound healing. Consequently, managing chronic infections and modulating host immune responses are promising therapeutic targets for this comorbid population (31, 32). In the present study, a significant association was found between the prevalence of *E. gingivalis* in periodontitis patients and diabetes (Table 3). However, the relatively small number of diabetic patients should be considered when interpreting these findings (Table 3). However, statistical analysis did not reveal a significant association between the prevalence of *T. tenax* in periodontitis patients and diabetes (Table 5). The findings of the present study are in line with the results of previous studies regarding *Entamoeba gingivalis*, but not with *Trichomonas tenax*. In Spain, the odds ratio for diabetic patients developing periodontitis was reported as 1.22 (33). According to Nazir et al., individuals with diabetes who have severe periodontitis are 3.2 times more likely to experience mortality compared to those with mild or no periodontitis (34). A significant association was reported between the prevalence of single-celled parasites and diabetes in periodontitis patients, with an odds ratio (OD) of 4.5 (7).

Cardiovascular diseases are also associated with an increased risk of developing periodontitis. Patients with periodontitis are at an increased risk of cardiovascular events, including heart attack, peripheral artery disease, stroke, and heart failure (3). In the present study, a significant association was found between the prevalence of *E. gingivalis* and cardiovascular diseases (OD=3.59, *P*=0.021). However, the limited number of cardiovascular patients in the present study may affect the statistical interpretation of this association. However, no significant association was observed between the prevalence of *T. tenax* and cardiovascular diseases. A significant association was reported between cardiovascular diseases and the prevalence of *E. gingivalis* and *T. tenax* (*P*=0.012) (27). Furthermore, a meta-analysis by Bahekar et al. involving 86,092 patients demonstrated that individuals with periodontal disease (gum disease) are 1.14 times more likely to develop coronary heart disease compared to healthy individuals (While this study does not specifically address parasitic infection, it highlights the potential link between poor oral health and the risk of other infections) (35). These results are consistent with the findings of the present study.

Gender is a factor that can influence oral health. Scientific studies on the prevalence of periodontal diseases based on gender have reported conflicting results. Aggressive periodontitis appears to be more common in men than in women (7, 27, 36, 37).

In the present study, the prevalence of *E. gingivalis* in men and women was 52.7% and 36.6%, respectively (*P*=0.037). The prevalence of *T. tenax* in men and women was 5.5% and 15.4%, respectively (*P*=0.063). These results were significant for *E. gingivalis*, but not for *T. tenax*. In contrast to the findings of the present study, the study by Ella Yassin et al. did not show a significant relationship between the prevalence of parasites and the gender of the participants (7).

In the present study the prevalence of *E. gingivalis* and *T. tenax* was higher in patients with severe periodontitis compared to individuals with mild forms (OR=1.58 and OR=1.19). This finding is consistent with the study in Iran, that reported a significant effect of severe gingivitis on the prevalence of *E. gingivalis* (OR=50, *P*<0.001) and showed that severe periodontitis is associated with a 50-fold increase in the risk of *E. gingivalis* infection in adolescents (38).

This study had some limitations, including: The sample size of this study was relatively small, have limited the statistical power of the

study to detect significant associations between *E. gingivalis* and *T. tenax* prevalence and the risk factors examined. This study used a cross-sectional design, which cannot establish causal relationships between *E. gingivalis* and *T. tenax* prevalence and the risk factors examined and Information on other potential risk factors for *E. gingivalis* and *T. tenax*, such as immune status and diet, was not collected in this study.

Conclusion

This study highlights a higher prevalence of *E. gingivalis* compared to *T. tenax* in the oral cavity, suggesting its potentially greater role in periodontal disease, particularly among individuals with specific risk factors such as diabetes and cardiovascular diseases. Men exhibit an increased susceptibility to *E. gingivalis* infection, and the prevalence of these parasites directly correlates with the severity of periodontitis. Understanding these parasites and their associated risk factors can empower individuals to take preventive measures against oral infections and maintain oral health.

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Conflict of interest

The authors declare that there is no conflict of interests.

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