Tropical Biomedicine 40(3): 307-312 (2023)
https://doi.org/10.47665/tb.40.3.006

RESEARCH ARTICLE

Molecular detection of oral *Trichomonas tenax* in periodontal disease patients by polymerase chain reaction -based 18S rRNA gene

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ARTICLE HISTORY

Received: 3 April 2023
Revised: 26 June 2023
Accepted: 26 June 2023
Published: 30 September 2023

ABSTRACT

Trichomonas tenax, an oral flagellated protozoon found in humans, potentially associated with the inflammation of periodontal tissues and decreased immunity that causes the tissue damage and tooth loss from chronic infection. Currently, there is a lack of data regarding the prevalence of *T. tenax* infection in Thailand. Therefore, this study aimed to measure prevalence of *T. tenax* in periodontal disease patients by using polymerase chain reaction (PCR) to amplify the 18S ribosomal RNA (18S rRNA) gene and to determine the factors associated with the presence of this protozoan. A cross-sectional descriptive study was conducted among 230 patients with periodontal disease, who visited the oral health center of Suranaree University of Technology Hospital, Thailand from 2021 to 2022. Dental plaque specimens were collected and examined to identify the presence of *T. tenax* using the PCR-based 18S rRNA gene. The occurrence of factors associated with *T. tenax* infection was analyzed by the chi-square test and binary logistic regression. The prevalence of *T. tenax* infection was 13.48% (31/230), in patients, including 96.77% (30/31) and 3.23% (1/31) in periodontitis and gingivitis patients, respectively. The presence of *T. tenax* was associated with periodontal disease (p<0.001) and the Periodontal Screening and Record (PSR) index (p=0.001). The significant risk factors for *T. tenax* infection were periodontitis (OR_{adj}=239.89, 95% CI=23.801-2417.746), no-underlying disease (OR_{adj}=0.31, 95% CI=0.099–0.942), and male sex (OR_{adj}=0.25, 95% CI=0.062-0.981). Dentists should be concerned about this oral protozoan in periodontitis patients. Furthermore, epidemiologic studies of *T. tenax* are still needed to investigate the mechanism of pathogenesis from *T. tenax* infection.

Keywords: Oral protozoa; *Trichomonas tenax;* periodontal disease; polymerase chain reaction (PCR); Thailand.

INTRODUCTION

Trichomonas tenax is a motile-flagellated protozoan is one of the risk factors for inflammation of periodontal tissues. It was first considered to be a commensal protozoan in the oral cavity and nasopharyngeal cavity (Hamadto *et al*., 2014) until the 1940s, when Dobell (1939) and Wenrich (1944) described the oral flagellate with the name *T. tenax* (Honigberg & Lee, 1959). It may be found in the intraoral cavity, such as in dental plaque, calculus, saliva of periodontal disease patients (Ribeiro *et al*., 2015), and in the extraoral cavity, such as in the respiratory tract, lung, maxillary sinus in compromised patients, other organs, and tissue (Mallat *et al*., 2004; Marty *et al*., 2017). For several decades, studies in oral protozoans have been of interest, and some authors have reported that *T. tenax* has a potential pathogenic role and is a coinfection pathogen in various infections (Socransky & Haffajee, 1992; Ribeiro *et al*., 2015; Dybicz *et al*., 2018). The occurrence of *T. tenax* has been observed in the oral cavity of patients with pulmonary disease and rheumatoid arthritis as well as in immunosuppressive patients. The functions of the immune system are impaired due to the main disease (Kikuta *et al*., 1997; Marty *et al*., 2017). Moreover, immunosuppressive patients can develop opportunistic parasitic disease (Dybicz *et al*., 2018). In various studies, *T. tenax* has recently been reported to damage mammalian epithelial cells, and it behaves similarly to and is closely related *Trichomonas vaginalis,* and pathogenic *Trichomonas* species of the genitourinary tract, thus satisfying the requirements to be considered a parasite (Ribeiro *et al*., 2015).

Periodontal disease is a chronic illness in humans that is characterized by inflammation and the loss of both soft and hard tissue supporting the teeth, as shown in Figure 1. In addition, periodontal disease associated to worsening systemic disorders such as diabetes, atherosclerosis, and cardiovascular diseases

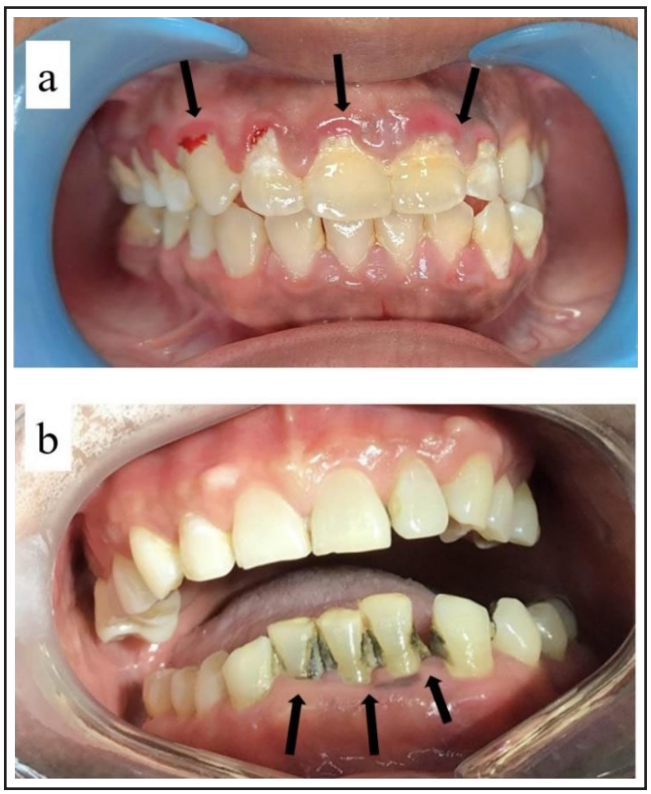

Figure 1. Representative image of periodontal disease. (a) Characteristic image from a gingivitis patient. There is inflammation of the gums, causing bleeding with swelling, and redness. (b) Characteristic image from periodontitis patient, there is loss of alveolar bone, formation of deep gum pockets. The black arrows indicate periodontal lesions.

(Otomo-Corgel *et al*., 2012; Mawardi *et al*., 2015). The destruction due to a local homeostasis disruption caused by the virulence of oral pathogenic microbiota and an inappropriate immune response (Socransky & Haffajee, 1992; Darveau, 2010). A complex composite of oral microbiota; bacteria, viruses, fungi, and oral protozoa may cause oral disease such as gingivitis and periodontitis. Dental biofilm is found in supragingival or subgingival areas and comprises a much more diverse microbiota, such as bacterial, fungal, archaeal, and protozoan species, including the flagellated protozoan *T. tenax* (Darveau,2010). In most previous reports, dental biofilm samples of *T. tenax* were detected by microscopy, culture, and PCR techniques. The PCR method is a more sensitive and specific technique for protozoa detection and can improve the identification and estimation of the *T. tenax* prevalence in periodontal patients (Bracamonte-Wolf *et al*., 2019).

Several studies determined the presence of *T. tenax* in periodontal patients, and the detection of *T. tenax* varied from 3 to 56% (Feki *et al*., 1981; Ghabanchi *et al*., 2010; Bracamonte-Wolf *et al*., 2019). Eslahi *et al*. (2021) reported that the prevalence of T. *tenax* was 17% worldwide. A study of *T. tenax* infection from South America and Asia reported a prevalence rate ranging from 13 to 23% (Eslahi *et al*., 2021). In Thailand, Moonmeungsan (2004) revealed that the prevalence of *T. tenax* was 14.63% in periodontitis patients, and Siribal *et al*. (2016) found a prevalence rate of only 1.1% in elderly patients. Despite global variations, *T. tenax* is rarely reported in Thailand. Our study aimed to investigate *T. tenax* infection epidemiology using molecular methods in a population aged 18 to 85 years, with a larger sample size than previous Thai studies. Thus, this study examined *T. tenax* prevalence and risk factors in periodontal patients using PCR.

MATERIALS AND METHODS

Study settings and data collection

A cross-sectional study was conducted among periodontal patients aged 18-85 years who attended the dental clinic of the Oral Health Center, Suranaree University of Technology Hospital, Nakhon Ratchasima Province, northeastern Thailand, from 2021 to 2022. The sample size was calculated with the formula of Daniel (1999):

$$n = \frac{Z^2 P\,(1 - P)}{d^2}$$

In brief, n is the required sample size, and Z is the statistic for a level of confidence. For the level of confidence of 95%, which is conventional, the Z value is 1.96, P is the expected prevalence or proportion (P is considered 0.17) (Eslahi *et al*., 2021). d is precision (d = 0.05 in considered to indicate good precision and smaller error of estimate). To minimize errors and increase the reliability of the study, the target sample size was increased by 5%. The sample size estimation was 230. Purposive sampling was used to obtain an essential sample size of 230 participants. The inclusion criteria were the clinical diagnosis of periodontal disease, aged 18-85 years and a minimum of 6 natural teeth, and the exclusion criteria were having received periodontal treatment such as scaling and root planning during the last six months. All patients received periodontal examinations, and questionnaires were administered to assess gender, age, past medical history, comorbidities risk factors such as smoking and alcohol consumption. The Periodontal Screening and Record (PSR) index was used to record periodontal status with WHO probes by a dentist. The procedure was supervised by a periodontology dentist. All the participants signed informed consent, which was approved by the Ethics Committee of Nakhon Ratchasima Public Health Provincial Office and Human Research Ethics Office of Suranaree University of Technology, reference number NRPH 055 (KHE2021-055), and EC-65-66. Dental plaque and/or calculus were obtained from the subgingival sulcus or periodontal pocket in each patient using a sterile curette, and then samples were deposited into an independent microtube containing 1.5 mL of biomolecular water. Total samples were sent to the laboratory of the Parasite Department and Research Center (PDRC) at the Suranaree University of Technology (SUT) as soon as possible.

DNA extraction

Genomics DNAs from the dental plaque specimens were extracted using QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was re-suspended in 25 µL of the elution buffer and quantified using NanoDrop spectrophotometry (Thermo Fisher Scientific, Wilmington, USA) and immediately used or stored at -20°C until PCR proceeded.

PCR conditions

PCR amplification was performed to detect the 18S rRNA gene. The targeted sequence was registered in GenBank database and served as the species of *T. tenax* to design the specific primer [Ttf (5'-AGTTCCATCGATGCCATTC-3') and Ttr (5'-GCATCTAAGGACTTAGACG-3')] and generate the amplicons of 775 bp (Kikuta *et al*., 1997). The PCR reaction was performed in a total volume of 25 µL that contained 1 µL of DNA template, 10X Taq DNA buffer 2.5 µL, dNTP 1 µL, MgCl2 1.5 µL, 1 µL of each primer, and 0.2 µL of Taq DNA polymerase (Thermo Fisher Scientific, Inc, Waltham, MA, USA). The reaction was performed in a Thermal Cycler (G-STORM™) using the following reaction conditions: (i) initial denaturation at 94°C for 5 min (ii) 35 cycles at 94°C, primer annealing at 52°C for 30 sec, extension at 72°C for 45 sec, and (iii) final extension at 72°C for 7 min. The sample tubes were maintained at 4°C until its removal from the thermocycler. The amplification products size were determined

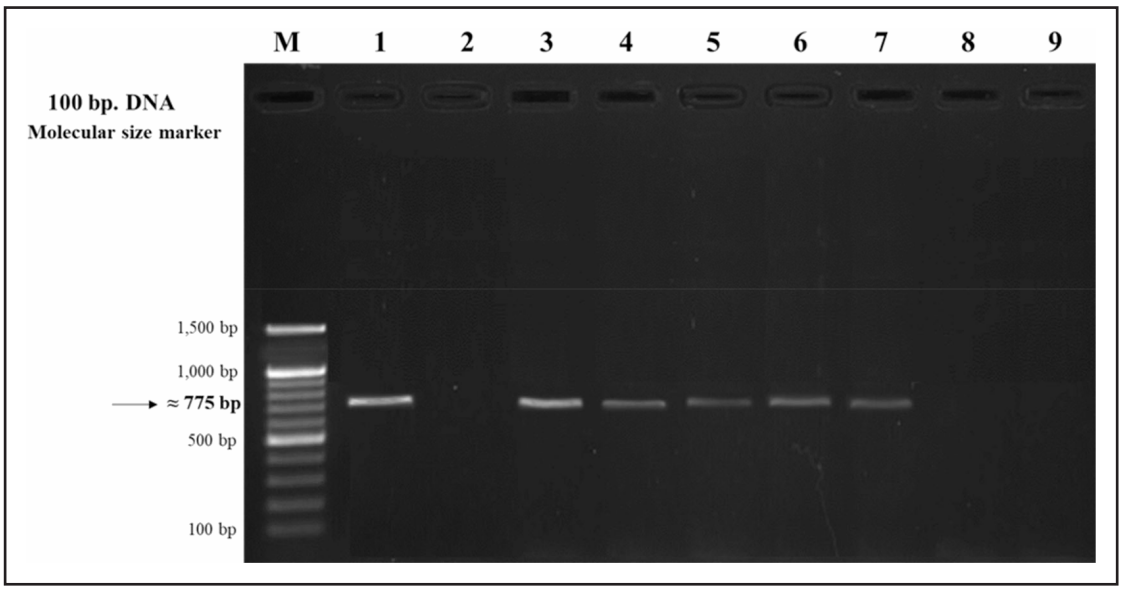

Figure 2. Agarose gel electrophoresis of *T. tenax* infection cases. Lanes 1: positive control (*T. tenax* DNA); Lane 2: negative control; Lane 3-7: positive samples; Lane 8-9: negative samples; Lane M: DNA Ladder Molecular Size Marker tiangen®, 100 bp. The position of the PCR product is indicated by the arrow on the left of the gel.

by 1% agarose gel electrophoresis using MaestroSafe nucleic acid (Maestrogen, Hsinchu, Taiwan) as the pre-stained loading dye. After that, the amplification products were visualized and photographed under UV light using gel documentation, Vilber Smart Imaging (Vilber, Marne-la-Vallée, France).

Statistical analysis

Data were analyzed by STATA/SE version 17.0 for Windows (StataCorpLLC, USA). Data are expressed as frequencies, and percentages, and chi-square test was used to assess the association between the variables. Binary logistic regression analysis was used to obtain the adjusted odds ratio (OR_{adj}) of *T. tenax* detection based on predictive variables. Values of $P<0.05$ were considered statistically significant.

RESULTS

The present study was conducted in 230 patients; PCR was used to determine the presence of *T. tenax*. The product size was 775 bp by amplification of the specific 18S rRNA gene for *T. tenax*. The PCR products were separated by electrophoresis in a 1% agarose gel, as shown in Figure 2. The demographic data and the presence of *T. tenax* are summarized in Table 1. The 31 patients (13.48%) who were positive for *T. tenax* infection included 16 (51.61%) men and 15 (48.39%) women, with no association between patient sex and *T. tenax* infection (χ^2=2.704, $p>0.05$). The highest frequency of *T. tenax* was observed in the 25–59-year-old group, with 18 positive patients (58%), followed by the 60-year-old and older group, with 8 positive patients (25.8%), and the 15-24-year-old group, with 5 positive patients (16.13%). *T. tenax* infection was not associated with the patient age group (χ^2=0.177, $p>0.05$). Additionally, *T. tenax* infection was not found to be associated with BMI (χ^2=0.274, $p>0.05$) or status (χ^2=0.012, $p>0.05$) (Table 1). The infection rate of *T. tenax* was associated with periodontal disease, which showed that the oral protozoan was present in 1 of 145 patients (3.23%) with gingivitis and in 30 of 85 patients (96.77%) with periodontitis. A statistically significant association between *T. tenax* infection and periodontal disease (χ^2=55.027, $p<0.001$) was found. Oral motile protozoan frequency was presented in 4 of 30 (12.90%) smoking patients and 27 of 200 (87.10%) nonsmoking patients, indicating no association

Table 1. The characteristic information and the presence of *Trichomonas tenax* infection

Variables	Samples n (%)	Presence of *T. tenax* infection		*P-value*
		Yes n (%)	No n (%)	
Age				
15–24 years	42 (18.26)	5 (16.13)	37 (18.59)	0.915
25–59 years	126 (54.78)	18 (58.06)	108 (54.27)	
≥60 years	62 (26.96)	8 (25.81)	54 (27.14)	
Gender				
Male	88 (38.26)	16 (51.61)	72 (36.18)	0.100
Female	142 (61.74)	15 (48.39)	127 (63.82)	
BMI				
≤25	213 (92.61)	28 (90.32)	185 (92.96)	0.601
>25	17 (7.39)	3 (9.68)	14 (7.04)	
Status				
Married	124 (53.91)	17 (54.84)	107 (53.77)	0.911
Single	106 (46.09)	14 (45.16)	92 (46.23)	
Systemic disease				
Comorbidities	66 (28.70)	7 (22.59)	59 (29.66)	0.418
No-underlying disease	164 (71.30)	24 (77.42)	140 (70.35)	
Smoking				
Yes	30 (13.04)	4 (12.90)	26 (13.07)	0.980
No	200 (86.96)	27 (87.10)	173 (86.93)	
Alcohol use				
Yes	95 (41.30)	15 (48.39)	80 (40.20)	0.389
No	135 (58.70)	16 (51.61)	119 (59.80)	
Periodontal disease				
Gingivitis	145 (63.04)	1 (3.23)	144 (72.36)	<0.001*
Periodontitis	85 (36.96)	30 (96.77)	55 (27.64)	
PSR index				
1.0–1.9	61 (26.52)	0 (0.00)	61 (30.65)	0.001*
2.0–2.9	133 (57.83)	23 (74.19)	110 (55.28)	
3.0–4.0	36 (15.65)	8 (25.81)	28 (14.07)	

*Significantly different P-value <0.05; BMI: body mass index; Comorbidities: patients who have a disease or condition also have one or more other diseases or conditions; PSR index: the Periodontal Screening and Record index.

Table 2. The presence of *T. tenax* infection and variables using binary logistic regression analysis

Variables	Samples n (%)	Presence *T. tenax* infection			
		Crude OR	95% CI	Adjusted OR	95% CI
Age (years)					
15 – 24	42 (18.26)	1			
25 – 29	126 (54.78)	1.23	0.43–3.56	0.32	0.029–3.573
≥60	62 (26.96)	1.10	0.33–3.61	0.25	0.019–3.351
Gender					
Male	88 (38.26)	0.53	0.25–1.14	0.246	0.062–0.981
Female	142 (61.74)	1			
BMI					
≤25	213 (92.61)	1.42	0.38–5.24	1.386	0.288–6.681
>25	17 (7.39)	1			
Status					
Married	124 (53.91)	0.96	0.45–2.05	0.49	0.143–1.702
Single	106 (46.09)	1			
Systemic Disease					
Comorbidities	66 (28.70)	1	0.59–3.54	0.306	0.099–0.942
No- underlying disease	164 (71.30)	1.45			
Smoking					
Yes	30 (13.04)	1	0.32–3.05	0.201	0.039–1.037
No	200 (86.96)	0.99			
Alcohol use					
Yes	95 (41.30)	1.40	0.65–2.98	0.92	0.253–3.342
No	135 (58.70)	1			
Periodontal disease					
Gingivitis	145 (63.04)	1	23.88–4738.49	239.885	23.801–2417.746
Periodontitis	85 (36.96)	336.39			
PSR index					
1.0 – 1.9	61 (26.52)	1			
2.0 – 2.9	133 (57.83)	26.16	1.56–438.19	1.060	0.486–2.315
3.0 – 4.0	36 (15.65)	36.68	2.05–657.82		

n: number of sample size; OR: odds ratio (an odds ratio of 1 indicates the reference group); CI: confidence interval; BMI: body mass index; Comorbidities: patients who have a disease or condition also have one or more other diseases or conditions; PSR index: the Periodontal Screening and Record index.

between smoking and the presence of *T. tenax* (χ^2=0.001, p>0.05). The analysis between alcohol consumption and *T. tenax* infection was observed in 15 of 95 patients who drank alcohol and 16 of 135 patients who did not drink alcohol, which were not statistically significant (χ^2=0.741, p>0.05). The presence of *T. tenax* infection based on systemic disease was observed in 7 patients (22.59%) with underlying conditions and 24 patients (77.42%) with no underlying disease. However, there was no association between systemic disease and *T. tenax* presence (χ^2=1.569, p>0.05).

Finally, *T. tenax* infection was associated with the PSR index. Patients with a PSR index range of 2.0–2.9 were frequently detected in 23 of 31 patients (74.19%), and a range of 3.0–4.0 was detected in with 8 of 31 patients (25.81%). The association between the presence of *T. tenax* and the PSR index was revealed (χ^2=13.523, p=0.001). Additionally, this study presented the crude odds ratio [OR_{cru}] and adjusted odds ratio [OR_{adj}] of the potential risk factors for *T. tenax* infection by binary logistic regression analysis. The ORs_{adj} for periodontitis patients compared with gingivitis patients, no underlying disease compared with comorbidities, and being male were 239.89 (95% CI=23.801–2417.746, p<0.001), 0.31 (95% CI=0.099-0.942, p=0.038), and 0.25 (95% CI=0.062–0.981, p=0.047), respectively. There was a significant association with an increased likelihood of the presence of *T. tenax* (Table 2).

DISCUSSION

Over the past decade, it has been reported that the oral cavity of humans is colonized by bacteria, fungi, and protozoa (Marty *et al*., 2017). The oral protozoan, *T. tenax* could be observed in humans with poor oral hygiene, and it may play a role in periodontal disease. Although previous studies by Muller (1773) mentioned that *T. tenax* is a commensal oral protozoan, recent reviews have controversially explained the highly proteolytic and collagen-degrading activity of this flagellate protozoan with destructive effects on the oral mucosa and periodontal tissue (Bózner *et al*., 1991; Segovic *et al*., 1998). The presence of many proteolytic enzymes in *T. tenax* infection may affect pathogenicity in the human oral cavity. In the past, several studies were carried out to determine the presence of *T. tenax* by conventional methods such as microscopic observation and cultivation, but these methods were time-consuming and insufficient for the differentiation of oral trichomonas species. Therefore, molecular methods such as PCR and sequencing of their products have been applied for the accurate detection and identification of *T. tenax* (Brooks *et al*., 2007; Bracamonte-Wolf *et al*., 2019). The present study used PCR to amplify a segment of 775 bp of the 18S rRNA gene for *T. tenax.* The results of this study also showed that the prevalence of *T. tenax* was 13.48% (31 of 230) in periodontal disease

patients, including 96.77% (30 of 31) in periodontitis patients and 3.23% (1 of 31) in gingivitis patients. These results were most similar to those of previous studies that reported prevalence of 14.63%, 15.5%,13.54%, and 13% (Moonmeungsan, 2004; Athari *et al*., 2007; Dybicz *et al*., 2018; Eslahi *et al*., 2021). Additionally, another previous study reported a global pooled prevalence of *T. tenax* infection of 17% (95% CI=14%–22%) (*Eslahi et al*., 2021).

We identified the factors associated with *T. tenax* infection by performing bivariate analysis. The factors that are significantly associated with *T. tenax* infection were periodontal disease (p<0.001) and PSR index (especially PSR index >=2) (p<0.001). Multivariate analysis by binary logistic regression revealed that the statistically significant risk factors for *T. tenax* infection included male sex (OR_{adj}=0.25), no underlying disease (OR_{adj}=0.31), and periodontitis (OR_{adj}=239.89). The association between *T. tenax* infection and the sex of patients was statistically significant (OR_{adj}=0.25, 95% CI=0.062 – 0.981, p=0.047) indicating that being male was a low risk factor against for *T. tenax* infection. This study agreed with other studies that *T. tenax* was more commonly observed in females (Albuquerque Jɲnior *et al*., 2011; Onyido *et al*., 2011). Conversely, Eslahi et *al*. (2021) revealed that being male was a possible risk factor for *T. tenax* infection, statistically non-significant (OR=1.02. 95% CI=0.68-1.52). *T. tenax* infection can affect both males and females, although certain factors may contribute to a higher prevalence in females. Hormonal factors, oral hygiene practices, and anatomical differences, such as saliva composition or pH levels, may increase inflammation and susceptibility to *T. tenax* infection, potentially leading to a higher prevalence in females. The presence of *T. tenax* infection was also correlated with no systemic disease (OR_{adj}=0.31, 95% CI=0.099-0.942, p=0.038), indicating that no underlying disease was a possible protective factor against *T. tenax* infection. The comorbidities or chronic disease required medical attention or medical care, such as diabetes, hypertension, dyslipidemia, cardiovascular disease, obesity, and kidney disease, which influenced the status of oral health. A high occurrence of *T. tenax* in patients with systemic disease, immunosuppressive therapy, and renal transplant was described that impaired the function of the immune system and affected the condition of the periodontium (Mehr *et al*., 2015; Dybicz *et al*., 2018).

The presence of *T. tenax* in periodontitis patients was higher than that in gingivitis patients, in accordance with results in other studies (Athari *et al*., 2007; Marty *et al*., 2017; Benabdelkader *et al*., 2019; Bracamonte-Wolf *et al*., 2019; Yaseen *et al*., 2021; Matthew *et al*., 2023). The explanation of the study's results could be supported by Ribeiro *et al*. (2015) and Marty *et al*. (2017), who revealed that the occurrence of *T. tenax* in the deep periodontal pocket may substantiate this role in periodontal dysbiosis. This anaerobic environment in the periodontal pocket may be a critical factor for *T. tenax* colonization, and oral parasites appears to induce cytotoxic effects, inducing membrane damage and cell apoptosis. Over the past two decades, this was the first study to identify the oral flagellate *T. tenax* in an adult population in Thailand. Our findings, indicated that *T. tenax* was more common in patients with periodontitis, patients with underlying disease, and females. These may be considered potential risk factors *T. tenax* infection. Moreover, our findings were consistent with those of previous studies (Feki *et al*.,1981; Dybicz *et al*., 2018; Marty *et al*., 2017; Bracamonte-Wolf *et al*., 2019; Yaseen *et al*., 2021). However, we studied a population who lived near university, likely because the academic community and dental services can be easily accessed. Therefore, the prevalence of *T. tenax* in the human oral cavity may vary based on the of the population, community setting, oral hygiene habits, economic limitations, and perception of health.

In conclusion, this study observed the presence of *T. tenax* by molecular techniques, and the oral protozoan was more prevalent in patients with periodontitis than in gingivitis patients. This oral parasite was closely associated with periodontal disease. The present study reported exploratory results and could be improved by increasing the sample size or conducting a multicenter study. Which would decrease the risk of selection bias and recall bias. Thus, we could more accurately examine potential risk factors for *T. tenax* infection in the oral cavity. Furthermore, the pathogenicity and potential risk factors for oral protozoa are continuing to be studied and require appropriate therapeutic methods for oral parasitic infection.

ACKNOWLEDGEMENTS

The author would like to thank the Suranaree University of Technology's grant "SUT Scholarship for Graduate Students. This study was supported by Suranaree University of Technology (SUT), the SUT Research and Development Fund, Thailand Science Research and Innovation (TSRI), and National Science, Research, and Innovation Fund (NSRF) (NRIIS number 160366). The authors would like to thank and greatly appreciate all subjects who participated in this research.

Conflict of interest

The authors declare no competing interests.

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