International Journal of Biomedicine 15(3) (2025) 545-551
http://dx.doi.org/10.21103/Article15(3)_OA15

ORIGINAL ARTICLE

Dentistry

INTERNATIONAL JOURNAL OF BIOMEDICINE

Molecular Identification of *Entamoeba gingivalis* and Its Association with Periodontitis in Diabetic Patients: A PCR-Based Study Targeting the ITS2 Region

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Abstract

Entamoeba gingivalis is an anaerobic protozoan increasingly associated with periodontitis, particularly in individuals with systemic conditions such as diabetes mellitus. This cross-sectional study investigated the prevalence of *E. gingivalis* in 160 Thai patients with periodontitis and analyzed associated risk factors. Microscopy detected the parasite in 15.0% of patients, PCR in 25.6%, and sequencing confirmed the infection in 22.5%, all of which were Subtype 1. Infection was significantly more common among diabetic patients (41.3%) than non-diabetics (3.8%) (P<0.001). Multivariate analysis identified type 2 diabetes (adjusted OR = 20.77; 95% CI: 5.39–80.09), alcohol use (adjusted OR = 3.72; 95% CI: 1.01–13.68), and other underlying diseases (adjusted OR = 1.49; 95% CI: 1.07–2.06) as independent risk factors. PCR demonstrated superior diagnostic performance compared to microscopy. These findings support a potential pathogenic role of *E. gingivalis* in periodontitis, especially among patients with type 2 diabetes or behavioral risk factors. (**International Journal of Biomedicine. 2025;15(3):545-551.**)

Keywords: *Entamoeba gingivalis* • periodontitis • diabetes mellitus • internal transcribed spacer 2 • polymerase chain reaction

For citation: Sritongklang W, La N, Boonsuya A, Arunsan P, Trasaktaweesakul T, Asavaritikrai P, Rattanapitoon NK, Rattanapitoon SK. Molecular Identification of *Entamoeba gingivalis* and Its Association with Periodontitis in Diabetic Patients: A PCR-Based Study Targeting the ITS2 Region. International Journal of Biomedicine. 2025;15(3):545-551. doi:10.21103/Article15(3)_OA15

Abbreviations

ITS2, internal transcribed spacer 2; **PCR**, polymerase chain reaction; **T2D,** type 2 diabetes.

Introduction

Entamoeba gingivalis is an anaerobic protozoan commonly detected in the periodontal pockets of individuals exhibiting poor oral hygiene, periodontal disease, or compromised immune status.[1-5] Historically regarded as a commensal organism, recent studies suggest that *E. gingivalis* may play a pathogenic role in the progression of periodontitis, a chronic inflammatory disease affecting the supporting structures of teeth. Its close phylogenetic relationship to *E. histolytica*, a recognized human pathogen, further emphasizes the need to reassess the clinical relevance of *E. gingivalis*.

Periodontitis represents one of the most widespread oral health conditions globally and is strongly associated with systemic diseases, notably type 2 diabetes (T2D). The bidirectional relationship between diabetes and periodontitis is well documented: diabetic individuals are predisposed to increased periodontal destruction, while severe periodontitis can impair glycemic control and exacerbate diabetic complications. This interplay likely reflects shared pathophysiological pathways involving microbial dysbiosis, altered host immune responses, and systemic inflammation.

The anaerobic microenvironment created by periodontal inflammation provides an ideal niche for facultative and obligate anaerobic organisms, including *E. gingivalis*. However, conventional diagnostic approaches, such as light microscopy and staining, have limited sensitivity and may underestimate the true prevalence of this protozoan. In contrast, molecular diagnostic methods, especially PCR targeting the internal transcribed spacer 2 (ITS2) region, offer enhanced sensitivity and specificity for detecting *E. gingivalis* in clinical samples.

Despite these methodological advances, data on the prevalence of *E. gingivalis* among periodontitis patients, particularly those with diabetes, remain scarce, especially in Southeast Asian populations. Building on prior research, including the work of Boonsuya et al.,[6] the present study aims to elucidate the prevalence of *E. gingivalis* in Thai patients with periodontitis, both with and without T2D, utilizing both PCR and conventional microscopy. Furthermore, this study examined potential associations between *E. gingivalis* infection and host factors, including diabetes status and alcohol consumption. The findings are intended to enhance epidemiological understanding and inform diagnostic and therapeutic strategies for managing periodontal disease in high-risk population groups.

Materials and Methods

Study Design and Population

This cross-sectional analytical study was conducted between January 2021 and December 2024 in Banmai Subdistrict, Nakhon Ratchasima Province, Thailand. The primary objective was to assess the prevalence of *Entamoeba gingivalis* among patients with periodontitis and to explore its associations with systemic conditions, particularly T2D, and behavioral risk factors. A total of 160 individuals diagnosed with periodontitis were purposively recruited from a dental clinic affiliated with a regional public health center. The study population was divided into two equal groups: 80 patients with confirmed T2D and 80 patients without T2D, matched as closely as possible by age and sex.

The inclusion criteria included adults aged 25–100 years with a diagnosis of periodontitis, as defined by the 2017 World Workshop classification.[7] Exclusion criteria were recent antibiotic use (within the past 3 months), current antifungal or antiparasitic treatment, pregnancy, immunosuppressive conditions other than diabetes, and unwillingness to participate. All participants underwent a comprehensive periodontal examination. Demographic data and systemic health information were collected through structured interviews and reviews of medical records. Behavioral factors such as smoking and alcohol consumption were assessed using validated questionnaires.

Sample Collection

Gingival crevicular fluid (GCF) samples were collected from each participant. Following the isolation of the target area with cotton rolls and gentle air-drying, sterile absorbent paper points were inserted into the gingival sulcus for 30 seconds to absorb any remaining fluid. The paper points were then immediately transferred into individually coded, sterile microcentrifuge tubes and stored at −80°C until further analysis.

Parasitological Examination

Initial screening for *E. gingivalis* was performed using Gomori's trichrome staining technique.[8] Fixed smears of the GCF were stained and examined under a light microscope at 1000× magnification. Identification was based on classical morphological features, including amoeboid trophozoites ranging from 10–35 μm in diameter, with prominent pseudopodia, a central karyosome, peripheral chromatin, and cytoplasm containing ingested bacteria. Each slide was independently evaluated by three experienced parasitologists, who were blinded to the clinical status of the participants.

DNA Extraction and PCR Test

Genomic DNA was extracted using the QIAamp DNA Mini Kit® (Qiagen, Germany), following the manufacturer's protocol with minor modifications to enhance DNA yield from paper-point substrates.

Briefly, 400 μL of AL buffer and 20 μL of Proteinase K were added to each tube containing the sample, followed by incubation at 56°C for 30 minutes. DNA was subsequently purified using spin-column centrifugation and eluted in 75 μL of AE buffer. The extracted DNA was stored at −20°C until further analysis.

Detection of *Entamoeba gingivalis* was carried out using conventional polymerase chain reaction (PCR) targeting the internal transcribed spacer 2 (ITS2) region with species-specific primers: forward 5′-GAATAGGCGCGCATTTCGAACAGG-3′ and reverse 5′-TCCCACTAGTAAGGTACTACTC-3′. Each 25 μL PCR reaction mixture comprised 5 μL of DNA template, 3 μL of primers (1.5 μL each), 10 μL of PCR master mix (including $MgCl_2$, dNTPs, and Taq DNA polymerase), and 7 μL of nuclease-free water.

PCR amplification was performed under the following cycling conditions: an initial denaturation at 95°C for 7.5 minutes; 40 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds; followed by a final extension at 72°C for 5 minutes.

PCR products were separated by electrophoresis on 1.5% agarose gels stained with ethidium bromide and visualized under ultraviolet illumination. A 100bp molecular weight ladder (GeneRuler, Roche, Germany), along with positive and negative controls, was included in each run for quality assurance.

Subtyping and Sequencing

Samples that tested positive by ITS2-PCR were subsequently subjected to subtype-specific PCR to

distinguish between *E. gingivalis* Subtypes 1 and 2. The primers used were as follows: for Subtype 1, forward 5′-TACCATACAAGGAATAGCTTT-3′ and reverse 5′-GTGAAACAATAGAAGAAGGAAATGG-3′; for Subtype 2, forward 5′-GAGACAATCCCAGTTGTTTGTAC-3′ and reverse 5′-TACGTCCCTGCCCTTTGTAC-3′.

PCR amplification conditions were identical to those described previously. The resulting PCR products were purified using the QIAquick Gel Extraction Kit and submitted for Sanger sequencing.

The obtained sequences were analyzed using the NCBI BLAST tool to confirm species identity and compared with reference sequences available in the GenBank database. Multiple sequence alignment was conducted using ClustalW in the BioEdit software package to determine the *E. gingivalis* subtype and assess genetic similarity.

Statistical Analysis

All statistical analyses were conducted using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were used to summarize demographic, clinical, and parasitological data. Group differences in *E. gingivalis* prevalence were assessed using Chi-square or Fisher's exact test as appropriate. Binary logistic regression was used to identify independent predictors of E. gingivalis infection. Variables included in the model were diabetes status, underlying systemic disease, alcohol consumption, smoking status, age group, and gender. Results were reported as crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs). The diagnostic performance of microscopy and PCR was evaluated using sensitivity, specificity, and Cohen's kappa coefficient, using sequencing-confirmed infection as the reference standard. A *P*-value of <0.05 was considered statistically significant.

Results

A total of 160 patients diagnosed with periodontitis were enrolled in the study, comprising 80 patients with T2D and 80 patients without T2D. The presence of *E. gingivalis* was initially investigated through parasitological examination. Microscopic analysis revealed the parasite in 24 participants, including 22 diabetic patients and only 2 non-diabetic individuals.

Subsequent molecular detection via PCR targeting the ITS2 region identified *E. gingivalis* DNA in 41 participants (25.6%), of whom 37 were diabetic and 4 were non-diabetic. Sequence alignment of the amplified ITS2 region confirmed infection in 36 cases (22.5%), all of which were classified as Subtype 1. No cases of Subtype 2 were detected. Demographic and clinical characteristics of participants in relation to *E. gingivalis* infection are summarized in Table 1.

Among the 160 participants, the majority were female (70.0%), with an age range of 25 to 100 years. The prevalence of infection was higher in females (24.1%) than in males (18.8%), although this difference was not statistically significant (*P*=0.457). By age group, infection was most common among participants aged 45–64 years (11.9%), followed by those aged 65–84 years (9.4%). However, no statistically significant trend was observed across age groups (*P*=0.227). A significant association was identified between *E. gingivalis* infection and the presence of underlying diseases (*P*=0.006). Patients with hypertension (69.4%) and hypercholesterolemia (63.6%) demonstrated higher infection rates than those without comorbidities (14.7%). Diabetes mellitus emerged as the strongest associated risk factor: 41.3% of diabetic participants tested positive compared to only 3.8% of non-diabetics (*P*<0.001). Alcohol consumption was also significantly associated with infection. Among participants who reported alcohol use, 50.0% tested positive for *E. gingivalis*, compared to 20.7% of non-drinkers (*P*=0.031). No statistically significant association was found with smoking status (*P*=0.517).

Table 1.

Demographic and clinical characteristics and prevalence of Entamoeba gingivalis infection.

Variables	Patients n (%)	*E. gingivalis* infection		*P*-value
		Yes, n (%)	No, n (%)	
Gender				
Male	48 (30.00)	9 (18.75)	39 (81.25)	0.457
Female	112 (70.00)	27 (24.11)	85 (75.89)	
Age				
25-44 years	26 (16.25)	2 (1.25)	24 (15.00)	0.227
45-64 years	72 (45.00)	19 (11.88)	53 (33.13)	
65-84 years	61 (38.13)	15 (9.38)	46 (28.75)	
85-100 years	1 (0.63)	0 (0.00)	1 (0.63)	
Underlying diseases				
No	102 (63.75)	87 (85.29)	15 (14.71)	0.006
Hypertension	36 (22.50)	25 (69.44)	11 (30.56)	
Hypercholesterolemia	11 (6.88)	7 (63.64)	4 (36.36)	
Other	11 (6.88)	5 (45.45)	6 (54.55)	
Diabetes mellitus				
Yes	80 (50.00)	33 (41.25)	47 (58.75)	0.000
No	80 (50.00)	3 (3.75)	77 (96.25)	
Alcohol use				
Yes	10 (6.25)	5 (50.00)	5 (50.00)	0.031
No	150 (93.75)	31 (20.67)	119 (79.33)	
Smoking status				
Yes	6 (3.75)	2 (33.33)	4 (66.67)	0.517
No	154 (96.25)	34 (22.08)	120 (77.92)	

The diagnostic performance of microscopy and PCR was evaluated using sequencing-confirmed infection as the reference standard (Table 2). Microscopy demonstrated a sensitivity of 66.67% and specificity of 100%, with moderate agreement (Kappa = 0.593, *P*<0.001). PCR showed superior diagnostic accuracy, with 100% sensitivity, 95.96% specificity, and substantial agreement with sequencing (Kappa = 0.749, *P*<0.001. While PCR identified all confirmed infections, a small number of false positives were detected.

Table 2.

Detection of Entamoeba gingivalis infection by parasitological and molecular methods.

Methods	Periodontitis with T2D n (%)	Periodontitis without T2D n (%)	Total n (%)	Sensitivity (%)	Specificity (%)	Measure of Agreement Kappa	*P*-value
Microscopy	22 (86.67)	2 (13.33)	24 (15.00)	66.67	100	0.593	0.000
PCR based ITS2	33 (91.67)	3 (8.33)	36 (22.50)	100	95.96	0.749	0.000
Subtype1	33 (91.67)	3 (8.33)	36 (22.50)				
Subtype 2	0	0	0				

Table 3.

Association between Entamoeba gingivalis infection and clinical variables analyzed by binary logistic regression.

Variables	Patients n (%)	Presence of *E. gingivalis* infection			
		Crude OR	95% CI	Adjusted OR	95% CI
Gender					
Male	48 (30.00)	1.38	0.59–3.20	1.28	0.49–3.30
Female	112 (70.00)				
Age					
25-44 years	26 (16.25)	2.38	0.67–2.96	0.57	0.26-1.26
45-64 years	72 (45.00)				
65-84 years	61 (38.13)				
85-100 years	1 (0.63)				
Underlying diseases					
No	102 (63.75)	1.58	1.21-2.07	1.49	1.07-2.06
Hypertension	36 (22.50)				
Cholesterol	11 (6.88)				
Other	11 (6.88)				
T2D					
Yes	80 (50.00)	18.02	5.23–62.05	20.77	5.39-80.09
No	80 (50.00)				
Alcohol use					
Yes	10 (6.25)	3.84	1.05–14.10	3.72	1.01-13.68
No	150 (93.75)				
Smoking					
Yes	6 (3.75)	1.77	0.31–10.05	1.8	0.31-10.49
No	154 (96.25)				

Binary logistic regression was conducted to identify factors independently associated with *E. gingivalis* infection (Table 3). Diabetes mellitus was the most significant predictor, with an odds ratio (OR) of 20.77 (95% CI: 5.39–80.09, $P<0.001$), indicating a markedly elevated risk of infection among diabetic patients. Alcohol consumption also showed a statistically significant association, with an OR of 3.72 (95% CI: 1.01–13.68, $P=0.047$). The presence of any underlying disease was independently associated with infection (OR = 1.49; 95% CI: 1.07–2.06, $P=0.019$), suggesting a potential compounding effect of comorbid conditions on susceptibility.

In contrast, gender, age group, and smoking status were not significantly associated with *E. gingivalis* infection in the multivariate model. Although younger participants (25–44 years) initially showed an increased crude OR, this association reversed after adjustment (OR = 0.57; 95% CI: 0.26–1.26), reflecting the influence of confounding variables.

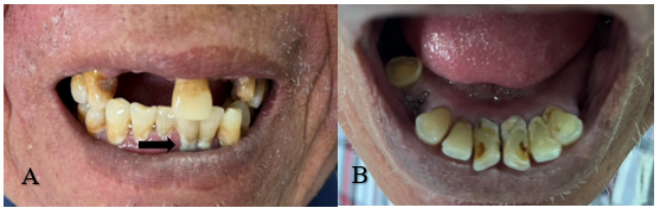

Figure 1. *Representative images of periodontal disease in patients exhibiting severe gingival inflammation, clinical attachment loss, and increased probing pocket depths. Black arrows indicate the sites from which dental plaque samples were collected. (A) Periodontitis in a patient with T2D, (B) Periodontitis in a patient without T2D.*

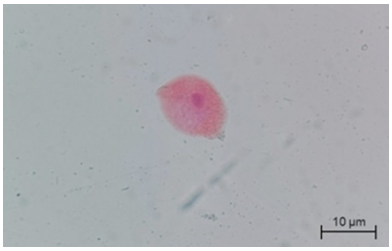

Figure 2. *Entamoeba gingivalis detected in a periodontitis patient with diabetes mellitus, visualized using Gomori's trichrome staining and observed under light microscopy.*

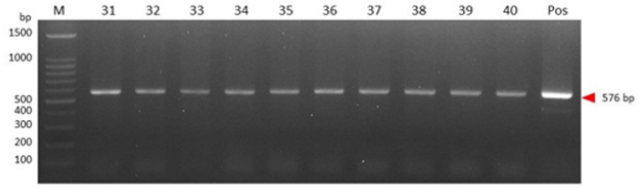

Figure 3. *Gel electrophoresis of PCR-amplified products targeting the gene for Entamoeba gingivalis detection. Red arrows indicate the specific 576 bp amplicons. M: 100 bp DNA ladder molecular size marker; positive control: E. gingivalis genomic DNA; lanes 31–40: positive samples.*

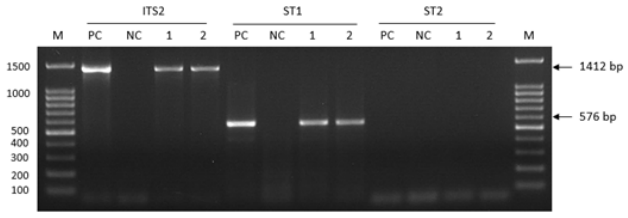

Figure 4. *Gel electrophoresis of PCR-amplified products targeting genes for Entamoeba gingivalis subtype 1 detection. Black arrows indicate the specific amplicons of 576 bp and ITS2 1412 bp fragments. M: 100 bp DNA ladder molecular size marker; positive control: E. gingivalis genomic DNA.*

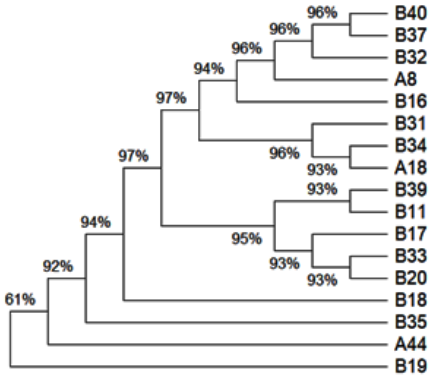

Figure 5. *Sequence alignment of the amplified Entamoeba gingivalis ITS2 region. Sequences obtained from PCR products were aligned using ClustalW in BioEdit software. The alignment confirms the presence of E. gingivalis by demonstrating high similarity to reference sequences retrieved from GenBank. The identified ITS2 sequences exhibited strong homology with known E. gingivalis strains, thereby validating the molecular detection method employed in this study. Conserved regions and sequence variations within the ITS2 region are highlighted, supporting accurate pathogen identification.*

Discussion

The present study provides important insights into the prevalence and diagnostic accuracy of *E. gingivalis* in Thai patients with periodontitis, with a particular focus on individuals with T2D. By integrating microscopy, PCR, and sequencing, this investigation provides robust data on the burden of *E. gingivalis* infection and its potential role as an opportunistic protozoan pathogen in periodontal disease. The detection rate by PCR was 25.6%, with 22.5% of cases confirmed by sequencing, highlighting the protozoan's under-recognized presence in the oral cavity and underscoring the microbial complexity in periodontitis pathogenesis.

One of the most striking findings was the significantly higher prevalence of *E. gingivalis* infection among diabetic patients (41.3%) than among non-diabetics (3.8%). This supports existing hypotheses that systemic conditions, such as T2D, predispose patients to protozoal colonization. Hyperglycemia has been shown to impair neutrophil function, reduce phagocytosis, and disrupt the cytokine environment in the periodontium, which may facilitate colonization by opportunistic organisms, including *E. gingivalis*. These findings are consistent with those of Boonsuya et al.[6] as well as studies from the Middle East and South America.[9,10] In addition to T2D, alcohol consumption was independently associated with infection. Half of the alcohol consuming participants tested positive for *E. gingivalis*, a finding that remained significant in multivariate analysis. Although underexplored in the literature, it is plausible that alcohol disrupts the oral microenvironment by decreasing salivary flow, altering mucosal immunity, and promoting biofilm dysbiosis, thereby enhancing susceptibility to protozoal colonization. These mechanisms remain speculative and warrant further investigation.

Beyond these factors, our multivariate model revealed that other underlying diseases—principally hypertension and hypercholesterolemia—were also independently associated with *E. gingivalis* infection (adjusted OR = 1.49; 95% CI: 1.07–2.06). Although direct evidence remains scarce, numerous studies link cardiometabolic disorders to heightened periodontal inflammation. Graziani et al.[11] reported an approximately 50% increase in hypertension risk among patients with moderate to severe periodontitis, while an umbrella review by Mauri-Obradors et al.[12] found consistent associations between dyslipidemia and periodontal disease that improve after periodontal therapy. Systemic vascular dysfunction and chronic low-grade inflammation, characteristic of these conditions, may compromise gingival microcirculation and innate immunity, thereby fostering protozoan persistence. Indirect support arises from immunocompromised cohorts; in Brazilian HIV-infected patients, Costa et al.[13] detected *E. gingivalis* in 63.4% of individuals, underscoring the role of systemic immune perturbation—regardless of etiology—in promoting protozoal carriage. Collectively, these observations suggest that multisystem comorbidity can have a subtle yet significant influence on the oral protozoan burden.[14,15]

In contrast, no significant associations were observed between *E. gingivalis* infection and demographic variables such as age, gender, or smoking status. Although slightly higher infection rates were observed among females and participants aged 45–64 years, the differences were not statistically significant. Similar trends have been reported in other Southeast Asian studies, but were also non-significant, suggesting that behavioral and systemic health factors may play a more decisive role in infection risk than demographic characteristics.

This study also contributes to the understanding of diagnostic accuracy for *E. gingivalis*. Microscopy using Gomori's trichrome staining yielded a positivity rate of 15.0%, compared with 25.6% by PCR and 22.5% confirmed by sequencing. Although microscopy demonstrated excellent specificity (100%), it had moderate sensitivity (66.7%). This aligns with prior work highlighting the limitations of microscopy in detecting oral protozoa.[16] ITS2-targeted PCR, followed by sequencing, enhanced diagnostic precision and enabled subtyping. All sequence-confirmed cases were Subtype 1, with no detection of Subtype 2, suggesting a predominant circulation of Subtype 1 in this Thai population. Similar subtype patterns have been reported in Iran and Egypt,[17,18] implying possible geographic variation.

To our knowledge, this is one of the first Thai studies to utilize ITS2 sequencing to confirm *E. gingivalis* infection in periodontitis patients. Previous local investigations relied mainly on microscopy or unconfirmed PCR.[6] The molecular approach presented herein improves prevalence estimates and provides subtype-level epidemiological insights. The balanced inclusion of diabetic and non-diabetic participants further strengthens the comparative analysis, addressing a common limitation in earlier research.

Clinically, these findings suggest that protozoa, such as *E. gingivalis*, may contribute to periodontal inflammation and tissue destruction alongside bacterial pathogens. *E. gingivalis* is known to produce proteolytic enzymes and phagocytose host cells, causing direct tissue damage.[16] Its persistence in periodontal pockets and ability to evade immune responses may sustain chronic inflammation and impair treatment outcomes. While current paradigms emphasize bacterial etiology, our results advocate for a broader consideration of eukaryotic pathogens.

Molecular diagnostics, though not yet standard in clinical dentistry, may enhance early detection, particularly among high-risk groups such as patients with T2D or multiple comorbidities. The adjunctive use of antiprotozoal agents in combination with mechanical debridement warrants exploration in clinical trials.

Despite its strengths, including molecular confirmation, subtype analysis, and stratification by systemic risk factors, this study has limitations. The cross-sectional design precludes causal inference; thus, *E. gingivalis* may be a consequence rather than a driver of disease. Additionally, the plaque index, oral hygiene practices, and diabetes duration were not recorded, which may confound the outcomes. Future longitudinal studies employing comprehensive microbiological profiling, including *Trichomonas tenax* and metagenomic approaches, are needed to elucidate the broader role of protozoa in periodontal disease.

In conclusion, this study demonstrates a considerable prevalence of Entamoeba gingivalis infection in Thai periodontitis patients, particularly those with T2D, alcohol use, or additional cardiometabolic conditions. The findings highlight the value of molecular diagnostics in detecting oral protozoa and underscore the influence of systemic and behavioral factors on infection risk. As the microbial landscape of periodontal disease continues to expand, the potential pathogenic role of protozoa such as *E. gingivalis* should not be overlooked. Further research is required to clarify their clinical relevance and therapeutic implications in periodontal care.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgment

This work was supported by the Institute of Research and Development (IRD), Parasitic Disease Research Center (PDRC) under the grant, Suranaree University of Technology (SUT), The One Research One Graduate(OROG) scholarship, Suranaree University of Technology (SUT), Thailand Science Research and Innovation (TSRI), and National Science, Research and Innovation Fund (NSRF) (NRIIS number 195617).

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