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RESEARCH ARTICLE

Trichomonas Tenax and *Entamoeba Gingivalis* Prevalence Among Palestinian Adults, A PCR-Based Cross-Sectional Study

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ABSTRACT

***Introduction*: Periodontal diseases affect the tissues responsible for tooth support within the oral cavity. These conditions are directly connected to tooth loss and other general health issues like cardiovascular diseases. Microorganisms associated with periodontal diseases have been extensively studied; however, parasites like *T. tenax* and *E. gingivalis* have been the least investigated and least frequently linked to periodontitis. This research aims to study the prevalence of these parasites in the Palestinian adult community.**

***Materials and Methods:* A cross-sectional study was designed to reveal the prevalence of both parasites. Cities to be included in the study were selected via cluster sampling. 291 saliva and plaque scraping samples were extracted from subjects who were eligible and accepted to enroll. Deoxyribonucleic acid (DNA) extraction was done, followed by Polymerase chain reaction (PCR) and agarose gel electrophoresis to detect parasite infection.**

***Results*: After conducting DNA extraction and PCR, all samples were negative for T. *tenax* and *E. gingivalis* infection.**

***Conclusion:* The negative results of this study were insufficient to establish the prevalence of the two parasites among Palestinian adults. Improved general and oral health may have reduced parasite prevalence. Further research is needed to identify other potential microorganisms directly linked to these oral diseases.**

Keywords: DNA extraction, *E. gingivalis*, PCR, Prevalence, *T. tenax*.

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1. Introduction

Parasites have infected humans for thousands of years, causing significant health, societal, and economic impacts. According to the World Health Organization (WHO), over 3 billion people worldwide are infected with one or more parasites [1]. On the other hand, the burden of oral disorders and their sequelae has potentially increased over the last three decades [2]. Severe periodontitis is the sixth most prevalent disease of those, affecting millions of people all over the globe [3], which requires special attention due to its impact on both dental and general health. It is likely initiated by microorganisms interacting with commensal microbiota, host susceptibility, and the host environment [4]. Two main protozoa, *Trichomonas tenax* and *Entamoeba gingivalis*, have been identified in human periodontal tissues. Their role in oral health remains controversial, some research supports a link between these protozoan infections and periodontal diseases [5], [6]. Conversely, other studies refute this connection, denying a direct link to oral health issues [7].

Since the 1960s, *T. tenax* has been noted in periodontitis literature, but research has largely focused on bacterial causes, neglecting aspects like prevalence, diagnosis, and pathophysiology of *T. tenax* [8]. Formerly known as *Trichomonas buccalis* and *Trichomona elongata*, *T. tenax* are motile flagellates that live anaerobically in the oral cavities and respiratory tracts of humans, non-human primates, and domestic animals like cats and dogs [9], [10]. The presence of *T. tenax*, the smallest in the genus *Trichomonas* [9], [11], has been assisted by direct microscopy or culture of specimens from oral calculus or plaque, which gave a widely varying prevalence (0%–94.1%) [8], and poor

species identification, so more sensitive and specific techniques are needed to detect the presence of *T. tenax*, such as polymerase chain reaction (PCR), which was used only a few times for this purpose and became a reference method after proving reliable diagnostic outcomes [8], [12].

On the contrary, *E. gingivalis* was prescribed much earlier in the literature, for the first time by Gros in 1849 as the first Amoeba of the seven types that were found to infect humans, and later by Grasse in 1879 where it was isolated from gingivitis lesions and was named *Amoeba dentalis* or *Amoeba gingivalis* [13]. *E. gingivalis* is a non-cyst producer, motile trophozoite, that is found in the oropharynx, in the dental plaque, or gingival tissues [14]. Although periodontitis has been known since antiquity, parasites were identified as potential etiological pathogens only since 1914 with the detection of *E. gingivalis* within the dental plaque [13], [15], Smith and Barret were pioneers in establishing this relationship by successfully treating 17 patients with "oral entamebiasis" using the amoebicidal agent emetine [15].

A wider range of research found it a nonpathogenic, asymptomatic colonizer with a similar attitude to *Entamoeba histolytica* (*E. histolytica*) toward human epithelial cells in vitro [16]. Transmission is recorded to be through saliva droplets, food utensils, and kissing, and the average global prevalence is 37 %, the highest scores were in Jordan with 87%, and the lowest in Portugal and Iran with 3% [6], and 0.5 % [7], respectively. Diagnosis of *E. gingivalis* is done by microscopic examination of the dental plaque with the aid of stains. Unfortunately, *E. gingivalis* could be mistaken with *E. hystolitica*, or macrophages, because the cytoplasm contains ingested debris in both [17]. PCR made detection easier and resulted in more accurate prevalence rates [13]. Many countries all over the world [6], and Middle Eastern countries [6], [7], [18] had determined the prevalence of these parasites, but unfortunately, this is not the case in Palestine. This study aims to determine the prevalence of *T. tenax* and *E. gingivalis* among Palestinian adults.

2. Materials and Methods

2.1. Design and Population

A cross-sectional study was conducted to estimate the prevalence of *T. tenax* and E. *gingivalis* among Palestinian adults between March 2022 and December 2022, in the molecular laboratories of An-Najah National University (NNU), Nablus, Palestine. The samples were collected from three northern cities; Nablus, Tulkarm, and Jenin. The study included Palestinians aged more than 18 years who attended dental clinics for checkups or treatment, generally healthy subjects with no systemic diseases, and accepted to participate. Pregnant women, subjects who have been recently treated with antibiotics, and immunocompromised patients were all excluded.

The incidence in Palestine was expected to align with trends observed in culturally similar societies, such as Iraq, Jordan, Iran, and Turkey. The Palestinian Central Bureau of Statistics (PSBS) estimates that around 954,000 people are living in the three cities of Jenin, Tulkarm, and Nablus. Based on a 95% confidence level and a 5% margin of error, we utilized the OpenEpi info software to calculate the sample size which was 288. To prevent the use of poor-quality specimens, a total of 307 samples were gathered.

The West Bank was divided into clusters to select the cities for the study, and one group was chosen through simple random selection. For choosing the subjects of the study, a non-probable volunteer sample technique was used, each city was classified into districts, and one dental clinic was chosen randomly to represent that district, a number of 96–100 subjects was selected from the chosen clinics of each city.

2.2. Measurement Tools and Data Collection

The nested Polymerase Chain Reaction was one of the two measurement tools used in this study to reveal the prevalence of *T. tenax*, and the other was conventional PCR for *E. gingivalis* detection. Nested PCR is used in situations where it is important to increase the sensitivity and/or specificity of molecular testing, which uses two sets of primers and two successive PCR reactions [19].

Questionnaires were disseminated to participants who agreed to enroll in the study and included inquiries of demographic and general health status data. The instruments were sterile to meet infection control guidelines in dental clinics and were shown and explained to the participants before use. Samples were withdrawn using a sterile plastic swap to scrap teeth surfaces and collect plaque. The extracted plaque samples were preserved in Eppendorf tubes containing 500–1000 µl of the participant's saliva extracted with the aid of a clean disposable pipet. Then it was transported via specimen transport cold box with 4 ice backs that keep the temperature at 2°C–8°C, before being transferred to NNU molecular laboratories and saved at −80°C for later DNA extraction and amplification.

The Wizard® Genomic DNA Purification Kit by Promega was used for DNA extraction, and the instructions of the manufacturers were followed. 250 µl of the collected sample was added to 900 µl of Cell Lysis Solution incubated for 10 minutes and then centrifuged at 13000–16000 units of gravity (x g) for 20 seconds. The supernatant was discarded, and the remaining pellet was vortexed for 5 seconds. 300 µl of Nuclei Lysis Solution was added and mixed with the pellet by inversion, then Protein Precipitation Solution was added in a volume of 100 µl and vortexed for 20 seconds, again it was centrifuged at 13000–16000 x g, for 3 minutes. The supernatant was transferred to a new Eppendorf tube and mixed with 300 µl of isopropanol and centrifuged at 13000–16000 x g, for one minute. The resulting supernatant was discarded and 300 µl of 70% ethanol was added and centrifuged as in the previous steps for one minute, then ethanol was aspirated, and the pellets were left to air dry for 15 minutes. Finally, the DNA Rehydration Solution was added in a volume of 100 µl and incubated for one hour at 65°C, after this last step the samples were ready for PCR reaction and were preserved under −80°C to be used later. The DNA extraction product of sample number 19 from each 20 samples was run on agarose gel for electrophoresis to verify the validity of the extraction procedure.

The nested PCR reaction for *T. tenax* went through two phases; first outer primers that will bind outside the specific DNA and amplify many parts that may include non-target parts, and second inner primers that will bind to the precise target DNA previously amplified [20]. Amplification of DNA was done by TRC1-F (primer sequence: 5′-GGTAATTCCAGCTCTGCG-3′) and TRC1-R (primer sequence: 5′-TGGTAAGTTTCCCCGTGT-3′) first primers, and then by TRC2-F (primer sequence: 5′-GTTA AAACGCCCGTAGTC – 3′) and TRC2-R (primer sequence: 5′-CCAG AGCCCAAGAACTAT-3′) as the second primers. The target gene of *T. tenax* was the 18S rRNA gene [19], with a product size of approximately 400 base pairs [19]. *Trichomonas vaginalis* was used as a positive control for DNA extraction and PCR reaction of *T. tenax* [19].

The conventional PCR reaction for *E. gingivalis* included the forward primer EGO-1 (primer sequence: 5′-GAATAGGCGCATTTCGAACAGG) and the reverse primer EGO-2 (primer sequence: 5′-TCCC ACTAG-TAAGGTACTTACTC) [21]. The target gene for *E. gingivalis* was the SSU rDNA gene [13], [21], [22], with a product size of 1400 base pairs [21], [22]. *E. histolytica* and *E. disbar* were used as a positive control for this PCR reaction [21], [23].

2.2.1. PCR Reaction for T. tenax

A total volume of 20 µl composed of 1 µl of extracted DNA, with 0.3 µM of each primer (TRC1-F and TRC1-R), 0.2 mM of each dNTP, 2.0 µl of 10x PCR buffer, and 2.0 U of Taq DNA polymerase. The PCR thermocycler was set up to carry out the following steps: first, Initial denaturation at 98°C for two minutes, then a cycle of 98°C for 10 seconds, then 53°C for 30 seconds and 68°C for 30 seconds, this cycle was repeated for 20 times. And finally, the last extension step for 5 minutes at 68°C [19]. The subsequent step of the second primer's reaction was 20 µl composed of: 0.4 µl of the DNA produced by the first primer's reaction, 0.3 µM of each primer (TRC2-F and TRC2-R), 0.2 mM of each dNTP, 2.0 µl of 10x PCR buffer, and 2.0 of Taq DNA polymerase. Later, 10 µl of each sample produced after the second PCR was dissolved in 1.5% agarose gel for further nucleic acid electrophoresis, 100bp DNA Ladder RTU kit manufactured by BIO-HELIX® was used. If the resultant electrophoresis product were about 400 base pairs in length, then the sample would be considered positive for *T. tenax* infection.

2.2.2. PCR Reaction for E. gingivalis

20 µl total volume composed of 2 µl of extracted DNA, 1 µl of each primer (EGO-1 forward primer and EGO-2 reverse primer), 10 µ PCR master mix, and 6 µl of distilled water. The PCR thermocycler was set up to perform the following steps: first, initial denaturation at 95°C for 30 seconds. Second, a cycle composed of 95°C for 30 seconds, then 60°C for 60 seconds, and 68°C for 60 seconds, the cycle in step number two was repeated 30 times and finally extension at 72°C for 5 minutes was done [21]. Later, 10 µl of each PCR product was dissolved in 1.5 % agarose gel for further nucleic acid electrophoresis, 100bp DNA Ladder RTU kit manufactured by BIO-HELIX® was used. If the resultant electrophoresis product were about 1400 base pairs in length, then the sample would be considered positive for *E. gingivalis* infection.

2.3. Data Analysis Plan

Statistical analysis of the data was conducted using IBM SPSS v. 21.0 (Armonk, New York, the United States: IBM Corp).

2.4. Ethical Considerations

Participant's safety, health welfare, equality, and autonomy were guaranteed by gaining ethical approval from the Institutional Review Board (IRB) [Reference #: Mas. Feb. 2022/7]. Participant privacy was ensured during examinations in a licensed dental clinic by a qualified dentist. Confidentiality was upheld by coding samples and questionnaires, with access restricted to the researcher for analysis.

3. Results

307 samples were collected, 291 were eligible for DNA extraction and PCR reaction, and 16 samples were rejected and excluded from the experimental and statistical analysis.

3.1. Demographic Variables, Habits, and Medical Conditions

Of the 291 eligible samples, 67.7% were female. Almost half of the participants confirmed that they had no income, while almost one-third earned above the Palestinian income average established by the PCBS. Other variables are illustrated in Table I.

3.2. Dental Visits and Oral Health Practices

Table II shows that more than 54% of the 291 participants are irregular attendees of dental clinics. An important indication of oral health awareness was the

TABLE I: Demographic Variables, Habits, and Medical Conditions

Variables		N = 291 patients
Age	Median (Min-Max)	39.0 (18.0–80.0)
Gender: n (%)	Males	94 (32.3%)
	Females	197 (67.7%)
Marital status: n (%)	Single	70 (24.1%)
	Married	219 (75.3%)
Monthly income: n (%)	No income	152 (52.2%)
	Less than 2000 Shekel	48 (16.5%)
	More than 2000 Shekel	91 (31.3%)
Smoking: n (%)	Yes	84 (28.9%)
	No	207 (71.1%)
Number of cigarettes: n (%)	1–10	29 (34.5%)
	11–19	30 (35.7%)
	More than 20	25 (29.8%)
Diabetes mellitus: n (%)	Yes	41 (14.1%)
	No	250 (85.9%)
HA1C: n (%)	No diabetes	250 (85.9%)
	≤7	23 (7.9%)
	>7	18 (6.2%)

TABLE II: Dental Visits and Oral Health Practices

Variables		N = 291 patients
Dental visits: n (%)	Once/year	98 (33.7%)
	Twice/year	35 (12%)
	Irregular visits	158 (54.3%)
Reason for Attending dental clinics	Fillings/RCT*/Prosthesis	139 (47.8%)
	Extraction	55 (18.9%)
	Checkup	44 (15.1%)
	Scaling and polishing	39 (13.4%)
	Bleaching	34 (11.7%)
	Periodontal treatment	2 (0.7%)
Tooth brushing: n (%)	Once/day	144 (49.5%)
	Twice/day	71 (24.4%)
	Irregular	76 (26.1%)

Note: *RCT: Root Canal Treatment.

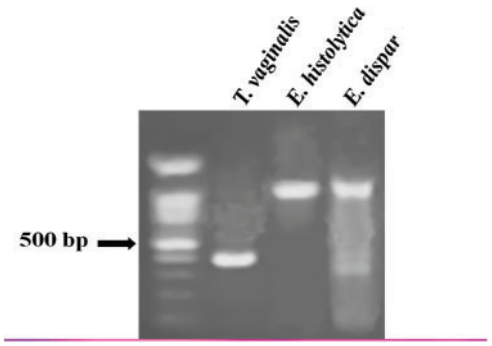

Fig. 1. Positive control for T. tenax and E. gingivalis.

number of teeth brushing per day, nearly half of the participants (49.5%) practiced teeth brushing once daily.

3.3. *Parasites Infection Rates*

After DNA extraction, PCR, and gel electrophoresis, all 291 samples tested negative for *T. tenax* and *E. gingivalis*, with no bands visible on the agarose gel. *E. histolytica* and *E. dispar* were used as positive controls for *E. gingivalis*, and *T. vaginalis* for *T. tenax*. The same extraction technique and kit produced visible positive results on gel electrophoresis, with bands of approximately 400 bp for *T. tenax* and 1400 bp for *E. gingivalis* (see Fig. 1).

4. Discussion

All samples tested negative for *T. tenax* and *E. gingivalis*. Although researchers often focus on publishing positive results, negative findings are equally valuable. As the saying goes, 'no result is a result' when research validity is upheld. In this study, multiple verification methods were employed to minimize bias, ensuring the reliability of the findings.

As a verification step of the DNA extraction procedure, electrophoresis for the DNA extraction product was done, 10 µl of the extracted DNA of sample number 19 of each 20 samples was mixed with 2 µl of the DNA staining reagent Novel Juice from BIO-HELIX® and loaded in 0.7% agarose gel, this means results of 15 samples, all came positive for DNA presence. As mentioned before, positive controls for the parasites under investigation were used; the DNA was extracted with the same kit, and PCR was performed using the same primers (Fig. 1).

Although our results were negative, they align with several published studies, as these are not the first null findings reported on the prevalence of these two parasites. Maraghi *et al*. [7] found a zero prevalence of *T. tenax* in 200 subjects, despite that the participant patients had been diagnosed with periodontitis. Maraghi and his team conducted a microscopic examination of both wet mount preparations and cultures of the saliva and plaque samples, and both showed no flagellates. With a prevalence of 0.5%, E. gingivalis infection was detected in only one out of 200 periodontitis patients. This finding led the author to hypothesize the involvement of other causative organisms in periodontitis and gingivitis [7].

Another systematic review highlighted the insufficient evidence and data on the role of *T. tenax* in periodontitis and recommended further research to identify other potential causative agents of periodontal diseases [5]. Similarly, many other researchers in separate studies had emphasized the lack of causality evidence between *E. gingivalis* infection and the development of periodontal diseases [14], [24]. In a case-control study by Rahdar *et al.*, direct microscopic examination and investigation by PCR were used to detect *E. gingivalis* among periodontitis cases and compare it to the control participants who had healthy periodontium, the results showed no significant difference between the two groups and *E. gingivalis* again was not the etiology of the periodontal inflammation the cases were diagnosed with [25].

These results came compatible with one of the earliest studies investigating the oral amoeba participation in gingival diseases by Dao and his colleagues, the study was conducted on gingival plaque scrapings of 113 periodontal patients and 96 healthy participants and no significant difference was found in its prevalence among healthy and diseased groups [17]. *T. tenax* was found to be zero or near zero in previous multiple studies among different participant categories [5], [26], [27], and the same negative results were noted in other research regarding *E. gingivalis* prevalence [13], [22]. In numerous studies, the parasite *T. tenax* was found to be associated with necrotic cervical lymph nodes [28] and lung abscesses [29], this may connect the presence of this parasite to more severe cases of periodontitis where necrosis of periodontal tissues is detected like necrotizing ulcerative periodontitis or necrotizing ulcerative gingivitis, and none of the cases in this study were diagnosed with such severe forms.

Improved general hygiene, better access to self-cleaning products, increased education and awareness, enhanced sanitation and housing, improved medical services, and the widespread use of antimicrobials like Metronidazole (one of the therapeutic options for these protozoa) [30], may all contribute to controlling these parasite infections as well as other infections. Nowadays, more burden in the health province is connected to non-infectious diseases, according to the WHO global burden of diseases study, non-communicable diseases counted for more than 86% of years lived with disability, and alone have participated in more than 38% of total deaths among adolescents [31].

Improved oral hygiene, supported by the widespread use of manual and electric toothbrushes, flosses, and waterjets, along with the availability of toothpastes and mouthwashes containing antiplaque agents like triclosan and chlorhexidine has contributed to better control of the oral pathogens [32], [33]. Further, the growing number of dentists and dental clinics has improved access to dental care for Palestinians. According to the Palestinian Ministry of Health and the Palestinian News and Info Agency, the West Bank has 4126 dentists serving a population of over 3,190,000, resulting in a ratio of one dentist per 773 residents, which is relatively high.

Another consideration is that the optimal pH for oral flagellates, like *T. tenax*, is between 7 and 7.5 [11]. However, the pH of dental plaque, a primary cause of periodontal disease, typically ranges from 6 to 7 and can drop to a critical level of 5.5 after consuming starchy carbohydrates and sugary drinks. *T. tenax* sensitivity to pH changes, coupled with the frequent decline in plaque pH due to modern dietary habits, may impact its ability to thrive in the oral environment.

The gender disparity observed in this study aligns with findings from other research, which indicates that men are generally less engaged in preventive and treatment practices compared to women [34]. This partly explains why approximately 70% of participants at the dental clinics in this study were female.

The research faced limitations, including the inability to collect samples from all cities in the West Bank. Data on health hygiene practices and dental visits were based on participant's responses. Additionally, the study's participant pool, primarily dental clinic attendees with a higher proportion of females, may not fully represent the broader Palestinian population. One of the strengths of this study was the sample size of 291. Additionally, the use of molecular identification through PCR, a highly sensitive laboratory technique, along with rigorous verification steps in DNA extraction and PCR reactions, enhanced the validity of the methodology.

5. Conclusion

In conclusion, the study's negative results were inadequate to determine the prevalence of the two parasites in Palestinian society, the role of these parasites in periodontal diseases may be overstated compared to other etiological factors; therefore, further studies focusing on those parasites and other potentially causative agents and their prevalence are recommended.

Acknowledgment

We want to thank the participants for dedicating their time and demonstrating patience during their involvement in the research.

Ethics Approval and Consent to Participate

All procedures were carried out in compliance with relevant national guidelines and regulations, laws, and the Declaration of Helsinki. The study received approval from the Institutional Review Board committee at An-Najah National University (Reference #: Mas. Feb 7, 2022). All subjects involved in the study were invited to participate voluntarily and signed an informed consent.

Availability of Data and Materials

The datasets generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

TB and WB contributed to the study's concept, design, and methods and supervised its implementation. critically reviewed and revised the manuscript. TB collected data. TB performed data analysis and wrote the first draft of the manuscript. The final version of the manuscript was revised and approved by WB and TB.

Declaration of Generative AI and AI-assisted technologies in the writing process

While preparing this article, the authors used ChatGPT to improve readability and language. Following the use of this tool, the authors reviewed and revised the content as necessary and assumed full responsibility for its publication.

Conflict of Interest

Authors declare that they do not have any conflict of interest.

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