การจำแนกและประเมินเชื้อ *Trichomonas tenax* ในแผ่นคราบจุลินทรีย์ใต้เหงือกในผู้ป่วยโรคปริทันต์

นางพันธ์ทิพย์ จิตรพิทักษ์เลิศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
สาขาวิชาเวชศาสตร์ปริวรรต
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2566

IDENTIFICATION AND EVALUATION OF TRICHOMONAS TENAX IN SUBGINGIVAL BIOFILM OF PERIODONTAL DISEASE PATIENTS

MRS. PANTIP CHITPITAKLERT

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in Translational Medicine
Suranaree University of Technology
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IDENTIFICATION AND EVALUATION OF TRICHOMONAS TENAX IN SUBGINGIVAL BIOFILM OF PERIODONTAL DISEASE PATIENTS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Degree of Doctor of Philosophy

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พันธ์ทิพย์ จิตรพิทักษ์เลิศ : การจำแนกและประเมินเชื้อ *Trichomonas tenax* ในแผ่นคราบจุลินทรีย์ใต้เหงือกในผู้ป่วยโรคปริทันต์ (IDENTIFICATION AND EVALUATION OF TRICHOMONAS TENAX IN SUBGINGIVAL BIOFILM OF PERIODONTAL DISEASE PATIENTS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ แพทย์หญิงชวัลญ์ญา รัตนพิทูลย์, 85 หน้า.

คำสำคัญ: โปรโตซัวในช่องปาก/เชื้อทริโคโมแนส ทิแนค/โรคปริทันต์/โพลีเมอเรสเชนรีแอคชั่น/ความชุกของการติดเชื้อ

Trichomonas tenax (*T. tenax*), เป็นเชื้อโปรโตซัวชนิดหนึ่งที่มีหนวด (flagellated) พบในช่องปากของมนุษย์ มีความสามารถและเกี่ยวข้องกับการเกิดการอักเสบของเนื้อเยื่อรอบฟันและมีผลต่อการลดภูมิคุ้มกันในร่างกายมนุษย์ เป็นสาเหตุให้เกิดการทำลายของเนื้อเยื่อในช่องปากและการสูญเสียฟันจากการติดเชื้อเรื้อรัง ปัจจุบันยังขาดข้อมูลล่าสุดของการติดเชื้อ *T. tenax* ในประเทศไทย วัตถุประสงค์ของการวิจัย เพื่อตรวจหาความชุกของการติดเชื้อ *T. tenax* ในผู้ป่วยโรคปริทันต์ โดยใช้เทคนิค Polymerase Chain Reaction (PCR) ในบริเวณ 18S ribosomal RNA และศึกษาปัจจัยเสี่ยงที่เกี่ยวข้องกับการติดเชื้อโปรโตซัวนี้ รูปแบบการศึกษาวิจัยเป็นแบบเปรียบเทียบชนิดตัดขวาง ในกลุ่มผู้ป่วยโรคปริทันต์ จำนวน 250 ราย ระหว่างปี ค.ศ.2022-2023 ที่มารับบริการที่ศูนย์สุขภาพช่องปาก โรงพยาบาลมหาวิทยาลัยเทคโนโลยีสุรนารี จังหวัดนครราชสีมา ประเทศไทย โดยการตรวจช่องปากอาสาสมัครและเก็บตัวอย่างแผ่นคราบจุลินทรีย์ เพื่อตรวจหาเชื้อ *T. tenax* ด้วยวิธี PCR ในบริเวณ 18S ribosomal RNA สถิติที่ใช้ในการวิเคราะห์ความสัมพันธ์ของปัจจัยที่เกี่ยวข้องกับการติดเชื้อ *T. tenax* ด้วยค่าไคสแคว์ หรือฟิชเชอร์แอคเซสเทส และการวิเคราะห์การถดถอยโลจิสติกแบบไบนารี ผลการศึกษาพบว่าการติดเชื้อ *T. tenax* ในผู้ป่วยโรคปริทันต์ คิดเป็นร้อยละ 16.8 (42/250) โดยพบในผู้ป่วยโรคปริทันต์อักเสบและโรคเหงือกอักเสบ คิดเป็นร้อยละ 76.2 (32/42) และร้อยละ 23.8 (10/42) ตามลำดับ การติดเชื้อโปรโตซัว *T. tenax* เกี่ยวข้องกับผู้ป่วยโรคปริทันต์อย่างชัดเจน (χ^2 = 31.25, p<0.001) และเกี่ยวข้องกับดัชนีการตรวจ คัดกรองโรคปริทันต์ในช่องปากด้วย Periodontal Screening and Recording index (χ^2 = 6.28, p<0.05) เมื่อวิเคราะห์ด้วยสถิติความถดถอยโลจิสติกแบบไบนารี พบปัจจัยเสี่ยงในผู้ป่วยโรคปริทันต์อักเสบมีโอกาสติดเชื้อ *T. tenax* สูงกว่าผู้ป่วยโรคเหงือกอักเสบ มีความเสี่ยงสูงถึง 9.67 เท่า อย่างมีนัยสำคัญทางสถิติ (OR_{adj} = 9.67, 95%CI = 4.01 – 23.31) ดังนั้น การวิจัยครั้งนี้ ยืนยันว่าการติดเชื้อ *T. tenax* มีความสัมพันธ์กับกลุ่มผู้ป่วยโรคปริทันต์อักเสบ ทันตแพทย์ควรตระหนักและให้ความสำคัญ ในการศึกษาเชื้อ *T. tenax* ใน

ผู้ป่วยโรคปริทันต์อักเสบ นอกจากนี้ การศึกษาต่อไปจำเป็นต้องมีการศึกษาอุบัติการณ์ด้านระบาดวิทยาของการติดเชื้อ *T. tenax* ในช่องปากอย่างต่อเนื่องและศึกษากลไกของการ ก่อโรคเพื่อการดูแลรักษาที่ถูกต้อง

สาขาวิชา เวชศาสตร์ปริวรรต
ปีการศึกษา 2566

ลายมือชื่อนักศึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

PANTIP CHITPITAKLERT : IDENTIFICATION AND EVALUATION OF TRICHOMONAS TENAX IN SUBGINGIVAL BIOFILM OF PERIODONTAL DISEASE PATIENTS. THESIS ADVISOR : ASSOC. PROF. SCHAWANYA RATTANAPITOON, M.D., 85 PP.

Keyword: Oral protozoa/*Trichomonas tenax*/periodontal disease/Polymerase Chain Reaction (PCR)/prevalence

Trichomonas tenax, an oral flagellated protozoan of humans, has been recognized a potential to involve the inflammation of periodontal tissues and decrease immunity that caused the tissue damage and tooth loss from the chronic infection. Currently, we lack of *T. tenax* infection on data update in Thailand. Therefore, this study aimed to identify the prevalence of *T. tenax* infection in periodontal disease patients by Polymerase Chain Reaction (PCR) method to amplify the 18S ribosomal RNA (18S rRNA) gene and determine the risk factors to associate with the presence of this protozoan. A cross-sectional analytical study was conducted among 250 patients with periodontal disease, during 2022 to 2023 who underwent oral health center of Suranaree University of Technology Hospital, Nakhon Ratchasima province, Thailand. The dental plaque specimens were collected and examined for the presence of *T. tenax* using PCR-based 18S rRNA gene. The occurrence of risk factors to reveal *T. tenax* infection was analyzed by the Chi-square test, Fisher's exact test and binary logistic regression. Results revealed that *T. tenax* infection in periodontal disease patients was 16.8% (42/250), in periodontitis and gingivitis patients were 76.2% (32/42) and 23.8% (10/42), respectively. The presence of *T. tenax* associated with periodontal disease patients ($\chi^2 =$ 31.25, p<0.001) and Periodontal Screening and Record (PSR) index ($\chi^2 =$ 6.28, p<0.005). In binary logistic regression analysis, it was found that periodontitis patients had a significantly higher risk of *T. tenax* infection, 9.67 times greater than gingivitis patients (OR_{adj} = 9.67, 95% CI = 4.01 – 23.31). This study confirmed that *T. tenax* infection was associated with periodontitis patients. Dentists should concern about these oral protozoans in periodontitis patients. Furthermore,

epidemiologic studies of *T. tenax* are still needed and essential to be investigated the mechanism of pathogenesis from *T. tenax* infection.

School of Translational Medicine

Academic Year 2023

Student's Signature.....

Advisor's Signature.....

Co-Advisor's Signature.....

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Pantip Chitpitaklert

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LIST OF ABBREVIATIONS

ADA	=	the American Dental Association
AAP	=	the American Academy of Periodontology
Adj OR, OR_{adj}	=	Adjusted Odd Ratio
bp	=	base pairs of DNA
CDA	=	The Canadian Dental Association
Co., Ltd.	=	company limited
CPA	=	the Canadian Periodontist Association
Cru OR, OR_{cru}	=	Crude Odd Ratio
BLAST	=	Basic Local Alignment Search Tool
BMI	=	Body Mass Index
BOP	=	bleeding on probing
DNA	=	deoxyribonucleic acid
DM	=	Diabetes melitus
et al.	=	and others
HT	=	Hypertension
ITS	=	internal transcribed spacer
NCBI	=	National Center for Biotechnology Information
RNA	=	ribonucleic acid
PCR	=	Polymerase Chain Reaction
PDRC	=	the Parasite Department and Research Center
PPD	=	periodontal pocket depth
PSR Index	=	Periodontal Screening and Recording Index
Tm	=	Melting temperature
T. tenax	=	*Trichomonas tenax*
SUT	=	Suranaree University of Technology
SUTH	=	Suranaree University of Technology Hospital

CHAPTER 1
INTRODUCTION

1.1 Background and Problem

Trichomonas tenax (*T. tenax*) is a motile-flagellated protozoan that can be found in the oral cavity and nasopharynx of humans. Initially, it is described as a commensal organism by Muller in 1773 (Matthew, Yang, Ketzis, Mukaratirwa, and Yao, 2023). *T. tenax* is characterized by its oval or ellipsoidal structure and the presence of four front-facing flagella., and a 5-6 μm wide and 12-20 μm long axostyle (Marty, Lemaitre, Kemoun, Morrier, and Monsarrat, 2017). Although primarily associated with oral infections, *T. tenax* has been implicated in various extraoral infections such as polymicrobial meningitis (Masur, Hook, and Armstrong, 1976), submaxillary gland salivary duct infection (Duboucher, Mogenet, and Périé, 1995), trichomoniasis infections of the lungs, and upper respiratory tract infections (Bellanger, et al. 2008). Moreover, its presence has been linked to periodontal diseases (Marty et al. 2017), with higher detection rates in dental plaque, calculus, and periodontal pockets compared to healthy periodontal tissue. *T. tenax* possesses enzymes that contribute to epithelial cell adherence and breakdown of periodontal tissues, along with cytokine induction from infected macrophage cells (Bisson, Dridi, and Machouart, 2019). Agreement with study of Ribeiro, Santos, and Benchimol (2015) conducted research highlighting the damaging effects of *T. tenax* on mammalian epithelial cells, demonstrating similarities with *T. vaginalis*, a closely related and pathogenic *Trichomonas species* found in the genitourinary tract. These findings support the classification of *T. tenax* as a parasite. Several studies have identified *T. tenax* in adult periodontal disease patients, with detection rates ranging from 6% to 56% (Ghabanchi, Zibaei, Afkar, and Sarbazie, 2010; Feki, Molet, Haag, and Kremer, 1981; Bracamonte-wolf et al. 2019). However, there is limited evidence regarding the presence of *T. tenax* in Thailand.

Dental biofilm refers to the accumulation of microbiota, food particles, and tissue cells found in supragingival or subgingival areas. While the focus of understanding

oral microorganisms in dental biofilm has predominantly been on bacteria, it is important to recognize the presence of other organisms, including fungi, archaea, and protozoa species such as *Trichomonas tenax*, a flagellated protozoan (Darveau, 2010). Despite the extensive research on oral bacteria, studies on oral parasites have been relatively limited (Baker, Bor, Agnello, Shi, and He, 2017). In previous reports, detection of *T. tenax* in dental biofilm samples was primarily achieved through microscopy, culture, or polymerase chain reaction (PCR) techniques. The PCR method is particularly advantageous as it offers increased sensitivity and specificity for the detection of protozoa, thus improving the identification and estimation of *T. tenax* prevalence in patients with periodontal disease (Bracamonte-wolf et al., 2019).

Periodontal diseases are the main chronic inflammation and infectious diseases in the oral cavity and the causes of tooth loss. They were divided into two major diseases, gingivitis and periodontitis. Clinical features of gingivitis can be characterized by the clinical signs: gingival redness, bleeding on probing (BOP), changes in texture and contour, calculus or plaque deposit on a tooth, and no alveolar bone loss. Clinical signs of periodontitis have gingival redness, swelling, inflammation, bleeding, alveolar bone loss, increased periodontal pocket depth (PPD), gingival recession due to root exposure, tooth mobility, and subsequent loss. The prevalence of periodontal diseases in worldwide-population is approximately 20-50% (Marty et al. 2017). According to the National Pathfinder Survey in 1999 reported that over 70% of elderly people in Japan had experienced periodontal disease progression. This high prevalence of periodontal disease in various age groups, including adolescents, adults, and the elderly, poses a significant public health concern. Risk factors associated with periodontal diseases include smoking, inadequate oral hygiene, diabetes, medication use, age, genetics, and stress. In addition, previous studies presented that “the infectious and inflammatory processes of periodontal diseases are closely related to systemic diseases, such as cardiovascular diseases, diabetes mellitus, arthritis, obesity, and Alzheimer’s disease” (Otomo-Corgel, Pucher, Rethman, and Reynolds, 2012; Mawardi, Elbadawi, and Sonis, 2015; Rahajoe et al. 2020). Thus, it is essential to provide timely and effective treatments for periodontal diseases.

From previous studies, protozoa have been associated with the occurrence of periodontal disease. Although several studies about the prevalence of *T. tenax* support the idea that the oral parasite was much greater in patients with periodontal diseases, in Thailand, the knowledge of *T. tenax* presence in periodontal disease patients is poorly known. Therefore, the study on *T. tenax* epidemiology is important to our better considerate of this parasite. For this study, the PCR technique is performed to detect the existence of *T. tenax* in patients, to provide updated prevalence information for this protozoan., and evaluate potential risk factors on the presence of *T. tenax*.

1.2 Research Objectives:

1) To determine the presence of *T. tenax* in subgingival biofilm using polymerase chain reaction (PCR) method
2) To identify the *T. tenax* in periodontal disease patients using the molecular technique such as DNA sequencing, alignment, and phylogenic analysis
3) To evaluate the risk factors associated with *T. tenax* infection in periodontal disease patients

1.3 Scope and limitation of the Study

This study mainly focuses on identifying *T. tenax* in subgingival biofilm and /or dental calculus in the participants who attended the Oral Health Center of Suranaree University of Technology Hospital (SUTH), Nakhon Ratchasima, Thailand from May 2022 to January 2023. Data collection was conducted on 250 samples. The identification of *T. tenax* was conducted by the polymerase chain reaction technique base 18s rRNA gene. PCR products were detected under 1.5% agarose gel electrophoresis analysis. Consequently, the positive bands of DNA were purified for DNA sequencing and aligned to analyze the genus and species using a phylogenetic tree.

1.4 Conceptual Framework

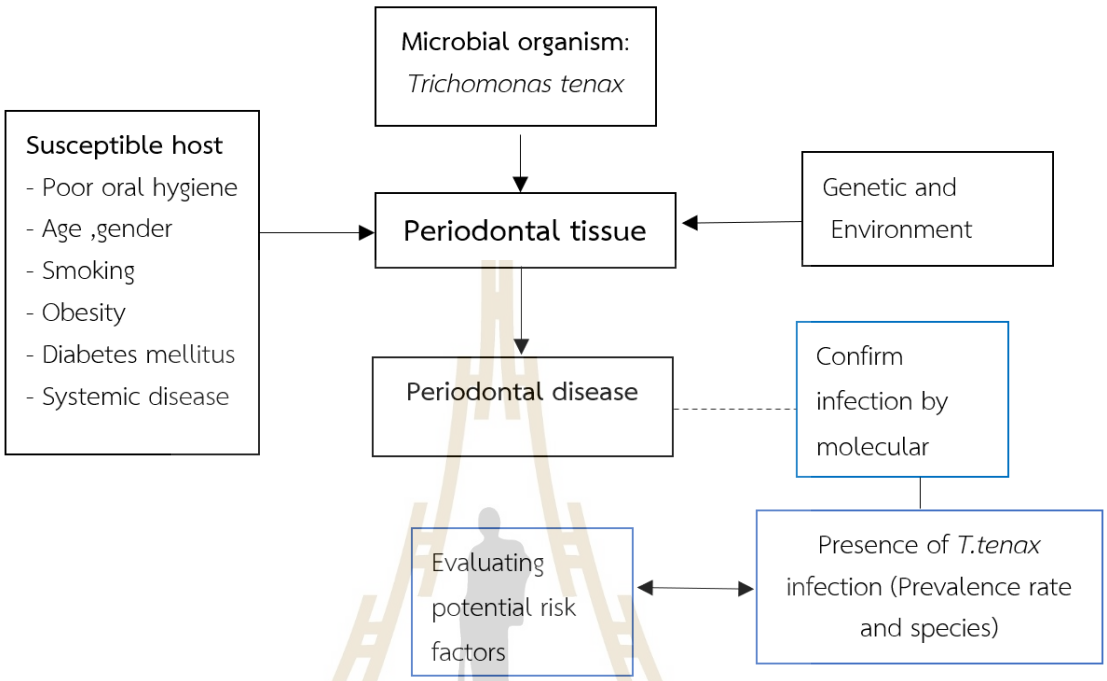


Figure 1.1 Diagram of Conceptual Framework

1.5 Contribution

This study led to epidemiological data on oral parasitic diseases for the health care worker. Little evidence was known about the prevalence rate and associated factors of *T. tenax* in oral health. This study was provided additional data and postulated new knowledge in the Thai context. Moreover, people must care for good oral hygiene.

CHAPTER 2

LITERATURE REVIEWS

2.1 Oral Protozoa: *Trichomonas tenax*

2.1.1 Morphology of *T. tenax*

One of the oral parasites in humans, *Trichomonas tenax* was first described as a commensal parasite organism in the human nasopharyngeal, and oral cavity by Muller (1773). It seems to have the capacity to be involved in the inflammation of poorly- cleaned periodontal tissues. *T. tenax*, is an anaerobic motile-flagellated protozoan that presents in the human oral cavity. It has measured about 5-6 µm wide, 12-20 µm long, and is ovoid shape (Marty et al. 2017). Morphologically, each trophozoite has a large nucleus, a posterior axostyle, four anteriorly-directed flagella, and an undulating membrane. This undulating membrane may look like small legs (Figure 2.1). It is a very similar morphology to *Trichomonas vaginalis* (*T. vaginalis*) observed in urogenital specimens (Marty et al.). The stage of actively feeding and breeding parasite protozoa is often referred to as the trophozoite form. There is no cyst stage. *T. tenax* trophozoite is a type of oral scavenger that feeds on bacteria, and local microorganisms located on the teeth, pyorrhea pockets, gingival margin, and tonsillar crypts.

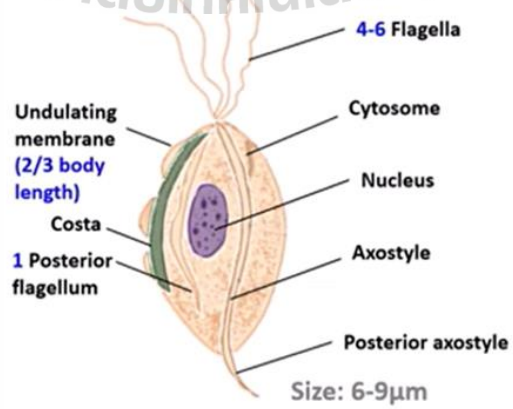


Figure 2.1 Morphology of *T. tenax* (Mehlhorn, 2015)

Reproduction takes place through repetitive longitudinal binary fission. In instances of inadequate oral hygiene, there is an escalation in oral microflora, leading to extensive reproduction. The presence of oral *T. tenax* should be established due to the ease with which the parasite can be transmitted by direct contact with mucosal membranes, saliva, droplet spray, kissing, contaminated utensil, and drinking water (Mallat et al. 2004) (Figure 2.2). However, the trophozoites of *T. tenax* are unable to survive the digestion process, and it is sensitive to pH and temperature environment.

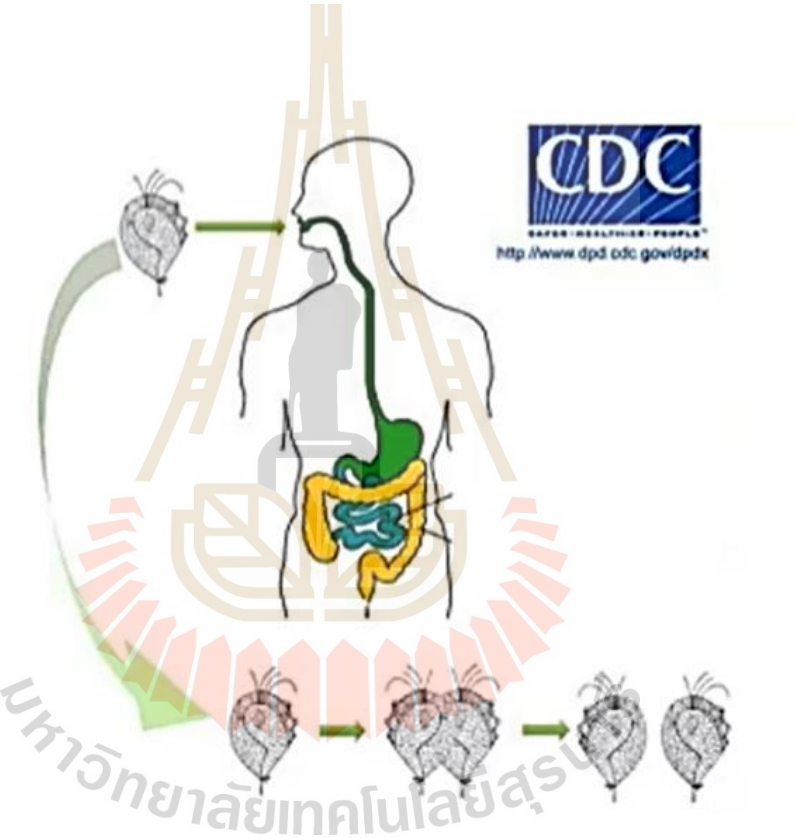


Figure 2.2 Transmission of *T. tenax* trophozoite can proceed through direct contact (Heinz Mehlhorn, 2015)

T. tenax is a unicellular eukaryotes protozoan and is classified in the same genus as *T. vaginalis (*Roberts and Schmidt, 2009*).* The taxonomy of *T. tenax* is displayed in table 2.1, as below

Table 2.1 Taxonomy of *T. tenax* (Guiry & Guiry, 2022)

Scientific classification of ***T. tenax***	
Domain	*Eukarya*
Kingdom	*Protozoa*
Phylum	*Metamonada*
Class	*Trichomonadea (Trichomonadophyceae)*
Order	*Trichomonadida*
Genus	*Trichomonas*
Species	*T. tenax*

Trichomonas is a genus of single-celled protozoan parasites belonging to the family *Trichomonadidae*. The classification of *Trichomonas species* is based on various characteristics, including morphology, behavior, host specificity, and genetic analysis. There are about the classification of *Trichomonas species*:

- *Trichomonas vaginalis:* This species is the causative agent of trichomoniasis, a common sexually transmitted infection in humans. Trichomonas vaginalis is primarily found in the urogenital tract, where it can cause inflammation and discomfort. It is the best-known species within the Trichomonas genus and has been extensively studied due to its medical importance.
- *Trichomonas tenax*: This species primarily inhabits the oral cavity and is commonly found in the gingival crevices and periodontal pockets. *Trichomonas tenax* is considered an opportunistic pathogen and is associated with poor oral hygiene and various oral diseases.
- *Trichomonas gallinae*: This species primarily infects birds, particularly pigeons and doves. It is responsible for a disease called "canker" in birds, which affects the respiratory and digestive tracts.
- *Trichomonas foetus*: This species infects the reproductive tracts of cattle, causing infertility and abortion in cows. It is a significant concern in cattle breeding and can lead to economic losses in the livestock industry.

- *Trichomonas hominis*: This species has been found in the human gastrointestinal tract, primarily in the large intestine. It is generally considered nonpathogenic and does not cause any specific disease in humans.
- *Trichomonas caviae*: This species is found in the gastrointestinal tract of rodents, particularly guinea pigs, where it is considered a nonpathogenic commensal organism.

It's important to note that the classification of *Trichomonas species* is continually evolving, and with advancements in genetic analysis and molecular techniques, the understanding of their diversity and relationships may change over time. Accurate identification and classification of these species are essential for understanding their biology, transmission, and potential pathogenicity in various hosts.

2.1.2 Epidemiology of *T. tenax*

T. tenax has been found in humans' oral cavity and other diseased organs, including sinusitis, tonsillitis, jaw abscess, cancer of the lingua, pleuropulmonary infection, and esophagus and the hypochlorhydria stomach (Albuquerque Júnior, Melo, Santana, Ribeiro, and Silva, 2011). It is believed to have entered the lower respiratory tract by aspiration of contaminated oropharyngeal secretions (Lewis, Doherty, Ribes, Seabolt, and Bensadoun, 2003; Gilroy et al. 2007). However, this parasite is more frequently detected in dental plaque (biofilm) and dental calculus from sites with periodontal disease than in periodontal healthy sites, which is capable of producing various enzymes that may contribute to epithelial cell adherence and periodontal tissue degradation as well interleukin-8 (IL-8) was produced from macrophage cell lines infected with parasite lysate (Bisson and Machouart, 2019).

In the previous studies, only two studies revealed the prevalence of *T. tenax* in Thailand. Boonthanom Moonmeungsan (2004) reported the presence of *T. tenax* was 14.63% in adult periodontitis patients and Saranya Siriba, Panee Chaksangchaichot, and Pongruj Pattaprasert (2016) found it only 1.1% in community screening.

Mehr, Zarandi, and Anush (2015) presented that the percent of infection to *T. tenax* in the case group was significantly higher than in the control group and the percent of infection to *T. tenax* was 26.9% in Down syndrome patients. Likewise,

Wantland and Lauer (1970) reported an approximately 4% infection range to this parasite, among children aged 2-12-year-old.

Brakamonte-Wolf et al. (2019), reported the presence of *T. tenax* was 56% in patients with periodontal disease, and 70% in periodontitis, 35% in gingivitis. The factors associated with the presence of *T. tenax* such as gender, age, smoking habit, and diabetes mellitus were non-statistically significant but the association between the presence of oral protozoan and periodontal disease and between *T. tenax* and Periodontal Screening and Recording (PSR) index were statistically significant. The authors recommended that screening for *T. tenax* needs to be considered in periodontitis patients. The higher PSR index (score 3 to 4) is mainly due to the potential risk of *T. tenax* infection.

Salah A Ali Mohammed and Ali B Mohsen ALwaaly (2019) reported the incidence of oral *T. tenax* in the holy province of Karbala at 8.09%. The incidence of *T. tenax* infection was compared with other factors such as sex, smoking habit, diabetes, and oral diseases. The results showed *T. tenax* infection in females was (11.01%) higher than in males (6.79%), while smokers had a higher rate of infection than nonsmokers (15.7% and 4.58%). The incidence of *T. tenax* infection among diabetes patients was higher than in non-diabetes (12.97 %, and 5.55%), and the incidence of *T. tenax* in oral disease patients was greater than in non-oral disease patients (12.58% and 5.17%).

Eslahi et al (2021) reported results of 17% a pooled prevalence in worldwide for *T. tenax* infection in the mouth. The highest prevalence was 56% in Chile, while the lowest prevalence was 3% in Kenya (Figure 2.3). The age group analysis found the infection with 15% the most common in the 46–55-year-old.

In the study conducted by Fadhil, Jwad, and Khalis Al-Masoudi (2022), the results showed that among 230 samples of dental plaque, *E. gingivalis* was detected in 60 cases (26.08%), and *T. tenax* was found in 37 cases (16.08%). Interestingly, the infection rate was higher in patients older than 30 years. Furthermore, *T. tenax* was detected in 81.08% of the urban population and 18.91% of the rural population, indicating a higher prevalence in urban areas. The study also found that

parasitic infections were more common in patients with chronic and periodontal diseases.

Concerning the above descriptions, *T. tenax* may be present in various oral and extra-oral locations with likely different pathologic outcomes. However, still there exist many gaps in the epidemiology and possible risk factors of *T. tenax* infection around the world.

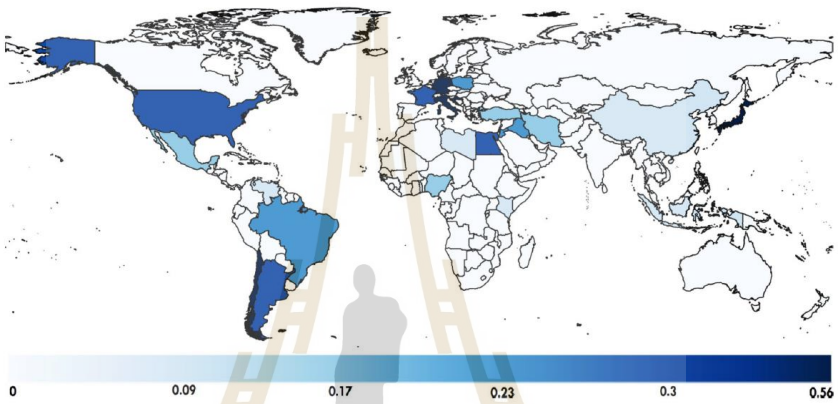


Figure 2.3 The blank map showed the global prevalence of *T. tenax* infection, dark blue was high prevalence and light blue was low prevalence, and a pooled prevalence of 17% worldwide for *T. tenax* infection in the mouth. (Eslahi et al. 2021)

2.1.3 Pathophysiology of *T. tenax* Infection

In 1773 Muller's initial discovery, Trichomonad parasites have a common commensal of the oral cavity and nasopharyngeal. Regarding recent literature, the presence of trichomonads in the human mouth could present highly prevalent in patients with periodontal disease, and its role is associated with periodontal disease.

For several decades of research, authors have been reported to cause bronchopulmonary infections, primarily in patients with other lung diseases or underlying cancers implicated with *T. tenax*, respectively (Mallet et al. 2004).

The microorganism is believed to enter the respiratory tract by aspiration from the oropharynx.

Dybicz, Perkowski, Sędzikowska, Baltaza, and Chomicz (2018) mentioned that *T. tenax* infection is often symptomatic, rather than a typical opportunistic disease. Infection of oral *T. tenax* in HIV patients is more relevant to the inflammatory process than to immunosuppressive patients.

Although the past studies by Muller (1773) mentioned that *T. tenax* is a commensal oral protozoan, the recent reviews controversial explain the highly proteolytic and collagen-degrading activity of this flagellate protozoan with destructive effects on the oral mucosa and periodontal tissue (Bózner and Demeš, 1991; Segovic, Buntak-Kobler, Galic, and Katunaric, 1998). The presence of many proteolytic enzymes in *T. Tenax* infection may affect pathogenicity in the human oral cavity.

Yamamoto, Asaga, Nagao, Igarashi, and Goto (2000) emphasized that the oral parasite, *T. tenax* could produce cathepsin B-like proteinases that proteinases affect to facilitate host penetration, digest host proteins, and interrupt the host immune system.

Moreover, Reiboro et al. (2015) described the capability of *T. tenax* to induce cytotoxic effects as well as damage and disruption of epithelial cells that is the first barrier protecting the periodontium and could promote and facilitate its invasion into the deepest connective tissue. The results of this study showed that the parasite is causing damage to different mammalian cells when in contact with target cells in vitro and has virulence similar to *T. vaginalis*.

According to a study by Hong et al. (2023), T. tenax was discovered to cause damage to gum epithelial cells by interrupting cell junctions, yet its influence on alveolar A549 cells and mucoepidermoid NCI-H292 cells was limited. Notably, *T. tenax* triggered the generation of IL-6 at a low multiplicity of infection (MOI) in gum, A549, and NCI-H292 cells. The researchers concluded that *T. tenax* can trigger cytotoxicity in gingival cells, disrupt cell junctions, and induce IL-6 production in both gingival and pulmonary cell lines.

Several in vitro studies demonstrated that *T. tenax* produces various proteinases and can cell adhesion and cellular damage, suggesting the pathogenicity

of *T. tenax* (Yamamoto et al. 2000, Marty et al. 2017). Nevertheless, there are fewer clinical and experimental studies regarding the pathogenicity of the oral protozoan.

2.1.4 Detection and Identification Methods of *T. tenax*

2.1.4.1 Conventional Techniques

In the 1960s the detection of the oral parasite, *T. tenax* first be investigated, and more interesting has been attached to the research from the 1980s to the present (Mary et al., 2017). In the past several studies were carried out to determine the prevalence of oral protozoan by conventional methods such as wet-mount preparations, direct smear methods, and Giemsa staining with microscopic observation to detect *T. tenax* (Marty et al. 2017). In addition, staining is useful for species identification, and culture techniques are routine use (Caliendo et al., 2005; Brooks, Carroll, Butel, and Morse, 2007).The microscopic methods are useful for detecting the typical motility and determining morphological characteristics of *T. tenax.* In the permanent preparation of the culture stained are all characteristic cell organelles stainable, enabling their detection without their typical motility. However, microscopic detection of *T. tenax* is not sensitive enough to distinguish between species based on morphological characteristics. Dybicz et al. (2018) suggested that conventional methods like microscopic observation and cultivation are 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*. In addition, *T. tenax* is rarely culturally (Cambon et al., 1979; Pardi, Perrone, and Mazzali, 2002). Recently, molecular tools have been developed for the faster detection of trichomonas.

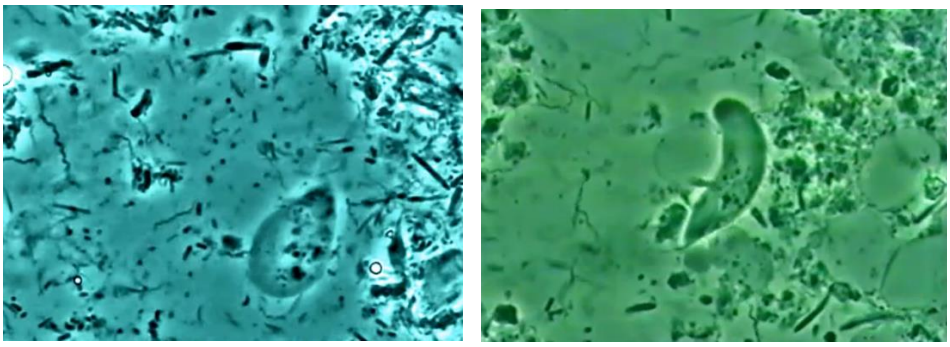


Figure 2.4 Morphology of *T. tenax*, the microscopic methods with 1,000X magnitude were useful for detecting the typical motility and determining morphological characteristics of *T. tenax*. (Mark Bonner, 2015)

2.1.4.2 Molecular Techniques

1) Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a lab method employed to amplify DNA sequences because of its excellent sensitivity and reproducibility (Mullis and Faloona, 1987). This method utilizes short DNA sequences known as primers to specifically target and amplify a particular portion of the genome. By repeatedly cycling the sample's temperature, it facilitates the DNA replication enzyme in duplicating the desired DNA sequence. Remarkably, this technique is capable of generating a billion copies of the target sequence within a matter of hours. PCR's amplification capacity enables the generation of substantial quantities of particular DNA products, detectable through various techniques.

The PCR method for detecting *T. tenax* has been published from 2004 to the present (Kurnatowska et al., 2004b; Athari et al., 2007; Mehr et al., 2015; Dybiicz et al., 2018; Brakamonte-Wolf et al.,2019). The authors described the development of a PCR assay the available tool for more rapid and specific detection and identification of *T. tenax* than conventional methods.

2) Primer Design

Primer-design techniques are crucial for enhancing PCR product yield and preventing the formation of non-specific products. PCR's effectiveness relies on its capability to amplify a precise DNA segment using primer pairs. (He, Marjamaki,

Soini, Mertsola, and Viljanen, 1994). Kamel and Abd-Elsalam (2003) presented, that primers should generally have the following properties: (1) Primers with a length of 18-30 nucleotides are usually best. Primers should be at least 18 nucleotides long to minimize the potential for problems at secondary hybridization sites on the vector or insert. Primers with long single-based runs should generally be avoided. (2) Melting temperature (Tm) should be between 52 and 68°C. The formula of Wallace et al. (1979) can be calculated temperature using; Tm = 2(A+T) + 4(G+C). (3) A GC content should be between 45 and 60 percent and provide information about the strength of annealing. (Dieffenbach, Lowe, and Dveksler, 1995).(4) Primers should not contain sequences of nucleotides that would allow one primer molecule to anneal to itself or the other primer used in PCR reactions (primer dimer formation). (5) Complementary primer sequences: no intra-primer homology beyond 3 base pairs for avoiding hairpin information. (6) Low specific binding at the 3' end in PCR primers is essential for the control of mispriming (Kwok et al., 1990)

The key to the PCR lies in the primer design, several parameters, such as primer length, GC content percentage, and 3'sequence, need to be optimized for successful PCR. Some of these parameters can be easily adjusted by hand, while others are best to run in a commercial computer program. (Kame and Abd-Elsalam, 2003).

In the previous study, Kikuta et al. (1997) reported the detection of *T. tenax* using primer design by the part of alignment in 18S rRNA gene sequence of *T. tenax* from GenBank accession number: D49495. Dybicz et al. (2018) performed the region of ITS-5.8S rRNA- ITS2 of different trichomonad species sequences and registered in GenBank served for the specific *T. tenax* primer design. According to the study of Brakamonte-Wolf et al. (2019), the primer used was designed from the DNA sequence of the *Trichomonas vaginalis* beta-tubulin gene (accession number: XM_001582993) for the detection of *T. tenax.*

3) Analysis of the PCR Products by Agarose Gel Electrophoresis

Gel electrophoresis separates charged molecules such as DNA, RNA, and proteins by size in the lab. Applying an electric current cause negatively charged DNA to migrate towards the positive electrode. (figure 2.6). Smaller molecules move faster and travel farther, while larger ones move slower and cover a shorter distance, resulting in size-based separation of molecules (figure 2.7).

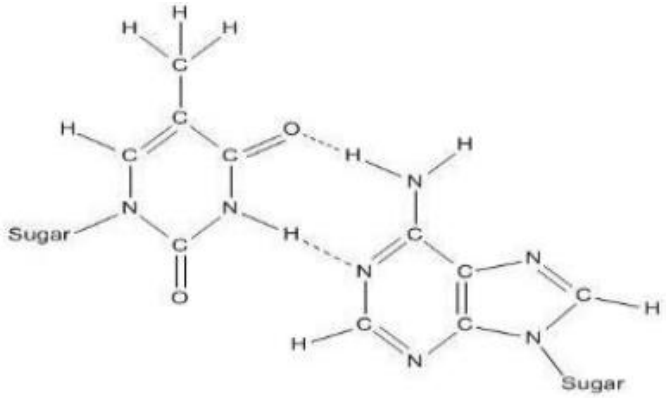


Figure 2.5 The diagram shows the structure of the DNA molecule. The hydroxyl group in phosphate is dissociated, and therefore the DNA molecule is always negatively charged (Yiran Cai, 2020).

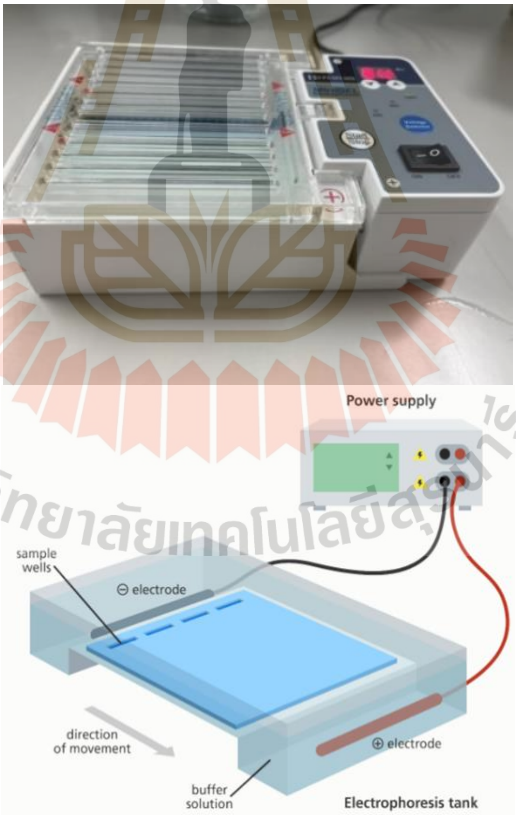


Figure 2.6 DNA Electrophoresis Equipment; Image credit: Genome Research Limited (https://www.yourgenome.org/facts/what-is-gel-electrophoresis)

A DNA marker, also known as a size standard or DNA ladder, is loaded into the first well of the gel. This marker contains fragments of known lengths, aiding in approximating the size of sample fragments. Dyes are used to visualize the separated DNA on the gel, appearing as bands. To visualize the DNA, the gel is stained with a dye that binds to it and is then illuminated with an ultraviolet transilluminator, making the stained DNA appear as bright bands. The sample DNA's size can be approximated by aligning it with the horizontal line traversing the DNA marker bands, allowing for estimation of the DNA's size in the sample by aligning it with the nearest marker band. (Yiran Cai, 2020; Sargar Aryal, 2022).

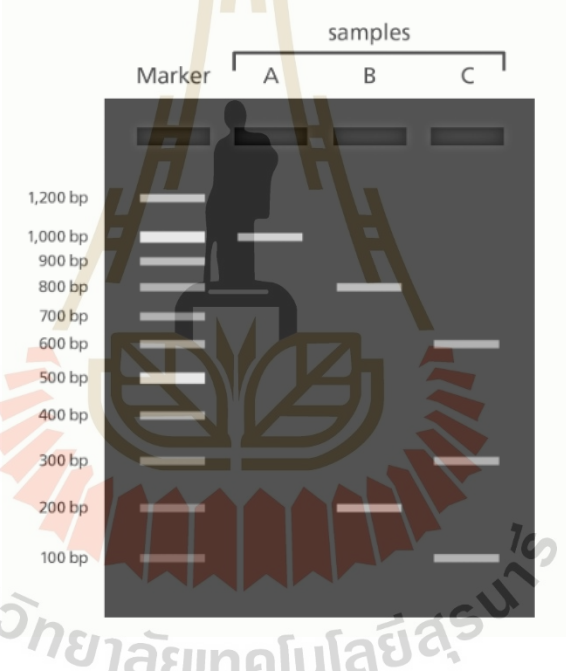


Figure 2.7 Illustration showing DNA band separated on a gel. The DNA fragment's length is measured against a marker containing a fragment of established length. Image credit: Genome Research Limited (https://www.yourgenome.org/facts/what-is-gel-electrophoresis)

2.2 Periodontal Diseases

Periodontal diseases are the main chronic inflammation and infectious diseases in the human oral cavity. The disease affecting the soft and hard tissue that supports

the teeth is broadly categorized as periodontal disease, with gingivitis and periodontitis as the most common presentations (Figure 2.8). The physiologic healthy state of gingiva can be characterized as the complete absence or very low levels of clinical inflammation in the tissues supporting the teeth. (Caton et al., 2018). In early periodontal disease, gums may turn red, swell, and bleed, known as gingivitis. Effective management can reverse this condition back to a healthy state (Caton et al.). In its more serious form of periodontitis, there's destruction of the periodontal tissues, including the ligament, cementum, and bone, along with the shifting of the epithelium. While inflammation and microbiota can be managed, tissue doesn't fully return to its original state (Caton et al.). Therefore, consistent oral hygiene maintenance is crucial.

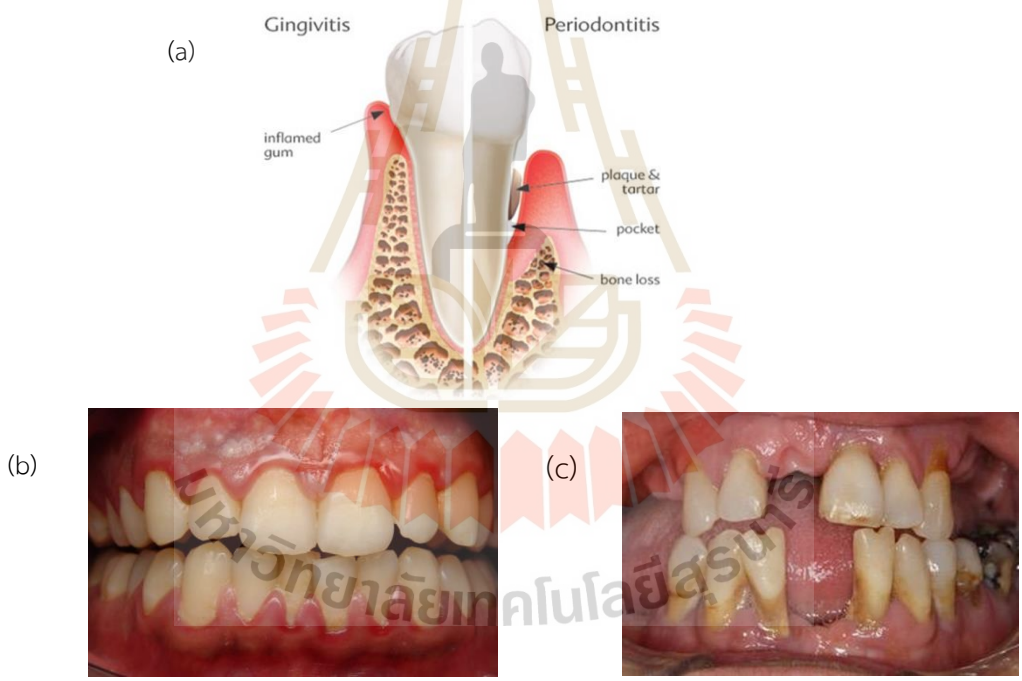


Figure 2.8 Periodontal diseases with gingivitis and periodontitis as the most common presentations; (a): Diagram of Periodontal Diseases; (b): Gingivitis; (c): Periodontitis; Source by https://nras.org.uk/resource/gum-disease

2.2.1 Classifications of the Disease

The classification of periodontal disease underwent significant changes following the 1999 International Workshop for classification of Periodontal Disease

and Conditions, as presented in Table 2.2. While periodontitis can vary in terms of age of onset and severity, current understanding recognizes that its underlying pathophysiology is fundamentally similar. Consequently, there is no substantial support for making distinctions based on these factors. A more recent development is the 2018 classification system, a collaborative effort between the American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP). During the revision process, the workshop established a comprehensive framework for categorizing periodontitis. This framework incorporates a multidimensional staging and grading system designed to adapt to new evidence over time. This system classifies disease severity into stages I through IV and progression rates into grades A through C, as detailed in Table 2.3 (Papapanou et al., 2018; Tonetti et al., 2018).

Table 2.2 Classification of Periodontal diseases and conditions (Armitage GC, 1999)

Gingival Diseases
- Plaque-induced gingival diseases
- Non-plaque-induced gingival diseases
Chronic Periodontitis
- Localized
- Generalized
Periodontitis as a manifestation of Systemic Disease
Necrotizing Periodontal Diseases
- Necrotizing Gingivitis (NUG)
- Necrotizing Periodontitis (NUP)
Abscess of the Periodontium
- Gingival abscess
- Periodontitis abscess
Periodontitis association With Endodontic Lesions
Developmental or Acquired Deformities and Conditions
- Localized Tooth-Related Factors That Modify or Predispose to Plaque-Induced Gingival Diseases or Periodontitis
- Mucogingival Deformities and Conditions Around Teeth
- Mucogingival Deformities and Conditions on Edentulous Edges

Gingivitis is associated with retained dental plaque and is the most common form of gingival disease. Dental plaque-induced gingivitis is the result of an interaction between the microorganisms found in dental plaque biofilm and the inflammatory host response. Microbial plaque is thus considered to be the primary etiology factor of gingivitis. Gingivitis can be modified by risk factors such as tobacco use, and drug and hormone changes occurring during pregnancy and teenage (Kinane 2001). It is fully reversible in otherwise healthy persons within weeks following the removal of local factors and reduction of the microbial load around the teeth. Non-plaque-induced gingivitis occurs in a small percentage of people. It mainly encompasses lesions of idiopathic etiology that can manifest in the gingiva (gum) such as lichen planus, pemphigoid, systemic condition, and trauma (Ubertalli and Hingham, 2020).

The clinical sign of gingivitis includes redness, swelling, and pain. The change in color of the gingival tissue is due to increase vascularity at the inflamed site. This increased vascularity leads to fluid accumulation and swelling of gingival tissue. Additionally, bleeding on probing is regarded as more sign of gingivitis than gingival color changing.

Table 2.3 Periodontitis Consensus Report (Papapanou, Sant et al. 2018; Tonetti et al., 2018)

Forms of Periodontitis

1. Necrotizing Periodontal Diseases
   a. Necrotizing Gingivitis
   b. Necrotizing Periodontitis
   c. Necrotizing stomatitis
2. Periodontitis as a manifestation of Systemic Disease
3. Periodontitis
   a. Stages: Base on severity and Complexity of Management
   b. Extent and distribution: localized; generalized; molar-incisor distribution
   c. Grades: Evidence or risk of rapid progression, anticipated treatment response
      i. Grade A: Slow rate of progression
      ii. Grade B: Moderate rate of progression
      iii. Grade C: Rapid rate of progression

The severity of periodontitis includes an amount of loss of gingival attachment, periodontal depth of pockets, and alveolar bone loss seen on x-ray (Ubertalli and Hingham 2020).

Clinical signs and symptoms of periodontitis, its typically lacks pain unless there's an acute infection in a periodontal pocket. Pain during meals can result from food getting stuck in these pockets. It's marked by excess plaque, redness, swelling, and exudate, with tender, bleeding gums and bad breath. As teeth loosen, especially when only a third of the root remains in the bone, chewing becomes painful.

2.2.2 Epidemiology of Periodontal Disease

Periodontal disease is a common illness that affects people of all ages, but it is more prevalent among the elderly. (Kinane et al., 2017; Tonetti et al., 2017). The disease can begin anywhere between early childhood and older adulthood. About 85% of the population is affected to a mild degree, but the most advanced cases are seen in < 5% of the population. In 2010, periodontitis affected 47% of adults aged 30 and above in the United States, and severe periodontitis had a global prevalence of 11%, with higher rates for gingivitis. (Nazir, 2017). According to the national Pathfinder Survey in 1999, over 70% of elderly people in Japan have experienced periodontal disease progression (Report on the Survey of Dental Diseases, Health Policy Bureau Ministry of Health and Welfare Japan 1999) and the 8th National Oral Health Survey, 2017 found 36.3% periodontal disease in aged 60-74 years in Thailand. In addition, periodontitis is a significant contributor to tooth loss in older adults, impacting their quality of life. A recent study found that among dentate US adults aged 30 years or older, 42% had periodontitis, with 7.8% experiencing severe forms of the disease. (Eke et al., 2020).

Clinical Risk factors, several factors have been linked to an increased incidence of periodontal diseases. These factors can be categorized into modifiable and non-modifiable components, all of which play a role in determining the clinical importance of periodontal diseases. (Nazir 2017). Examples of non-modifiable factors encompass elements such as aging and genetic predisposition, while modifiable factors consist of diabetes mellitus, psychological stress, smoking, alcohol consumption, and

inadequate oral hygiene. (Van Dyke and Sheilesh, 2005; Nazir, 2017). Most studies identified age, gender, socioeconomic status, oral hygiene, periodontopathic bacteria, smoking, and diabetes as being associated with periodontitis (Hyman and Reid, 2003).

The fundamental cause and development of periodontal disease are associated with microbial dysbiosis. (Sudhakara, Gupta, Bhardwaj, and Wilson, 2018). The oral cavity contains a diverse and intricate biofilm consisting of bacteria, viruses, fungi, and protozoans. Regarding *T. tenax*, some researchers have proposed its potential involvement in periodontal diseases. (Feki et al., 1981; Benabdelkader et al., 2019)

2.2.3 Measurements of Periodontal Disease by Periodontal Screening and Recording (PSR) Index

Early detection and prevention of periodontal disease are of utmost importance. To define and diagnose periodontal diseases, various indices have been developed, with common clinical measurements including bleeding on probing (BOP), periodontal pocket depth (PPD), and attachment loss. In 1994, the American Dental Association (ADA) and the American Academy of Periodontology (AAP) introduced the Periodontal Screening and Recording (PSR) index. Following suit in 1995, the Canadian Dental Association (CDA) and the Canadian Periodontist Association (CPA) updated and adopted the PSR index. Since then, this index has gained prominence among dentists and periodontists (Landry and Jean, 2002).

The PSR Index is based on three key parameters: gingival bleeding on probing, calculus accumulation, and depth of probing. It provides a detailed assessment of a patient's periodontal health (Bracamonte-Wolf et al. 2019). The primary purpose of this index is to aid dentists in identifying periodontal diseases during routine oral examinations, streamlining the screening process. Numerous studies have validated the PSR Index as a valuable tool due to its reproducibility, reliability, and efficiency (Landry and Jean, 2002). Some researchers have suggested modifications to the recording method to enhance its utility, both for screening purposes and for monitoring the outcomes of periodontal therapy.

Piazzini (1994) highlighted the effectiveness of PSR in estimating the severity of periodontal disease, demonstrating that it is, on average, nine times

faster than conventional evaluation methods. In fact, periodontal screening using PSR can be completed in under two minutes.

2.3 *T. tenax* and Periodontal Disease

The presence of *T. tenax* in periodontal diseases has been documented since the 1960s, with growing research attention from the 1980s onwards (Marty et al., 2017). Numerous studies on *T. tenax* prevalence reinforce the notion that this parasite is more commonly found in oral infections among patients with periodontal diseases than in those with good periodontal oral health. (Wantland and Lauer, 1970; Marty et al., 2017)

Bisson et al. (2019) pointed out that pulmonary trichomoniasis, an opportunistic infection attributed to *T. tenax*, is typically benign. Nevertheless, in individuals with inadequate oral hygiene, it can escalate into a more severe condition. Periodontal diseases, such as gingivitis and periodontitis, which arise due to imbalances in the oral microbiome, frequently serve as catalysts for *T. tenax* infections.

Furthermore, Marty et al. (2017) reported that the existence of *T. tenax* in the deep periodontal pocket could provide support for its involvement in periodontal dysbiosis and inflammation. The anaerobic conditions within the periodontal pocket might be a crucial element facilitating the colonization and proliferation of anaerobic *T. tenax*.

From previous studies, *T. tenax* have been associated with the occurrence of periodontal disease. Although several reports about *T. tenax* prevalence corroborate the notion that the occurrence of the parasite in oral infections was notably higher among individuals with periodontal diseases. In Thailand, the knowledge of this protozoan presence in periodontal disease patients is poorly known.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Study Design and Population

This cross-sectional analytical study was performed on periodontal disease patients who attended in Oral Health Center of Suranaree University of Technology Hospital (SUTH), Nakhon Ratchasima, Thailand between May 2022 to January 2023. A total of 250 participants aged 18 to 85 years old were invited to examine for *T. tenax* infection. All participants were kindly requested to complete the questionnaire and oral examination (Appendix C). Subgingival biofilm and/or dental calculus samples were collected and transported to the Parasite Department and Research Center (PDRC) of Suranaree University of Technology (SUT) laboratory to identify the presence of *T. tenax* by molecular methods. All participants were appropriately treated and advised to receive oral care. The study was performed according to the Helsinki Declaration and this trial protocol, which includes the study information and consent form, were approved by Suranaree University of Technology (EC-65-66) and the Ethical Committee of Nakhon Ratchasima Public Health Provincial office (KHE 2021-055) before starting the recruitment of all participants for the study (Appendix D). All participants were written informed consent. We were responsible for taking the appropriate consent form from the participants before recruiting them into the study. We were kept all subjects' data confidential. There was no commercial interest in any form related to this study.

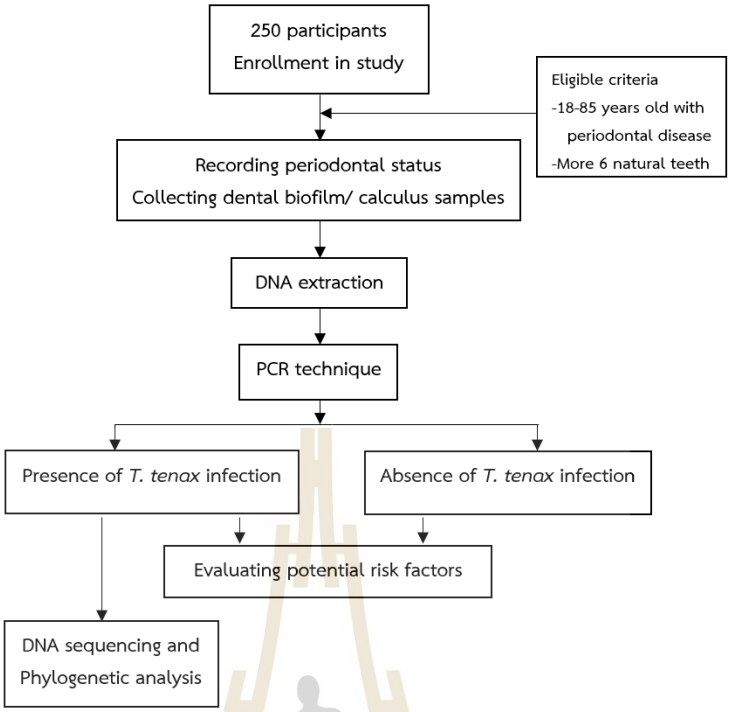


Figure 3.1 Flow diagram of the study design process

3.2 Sample Size Calculation

The population in this study was included periodontal disease patients who attended the Oral Health Center of SUTH for 6 months. The statistical formula for sample size calculation was calculated in a mainly cross-sectional analytical study by Vega et al., 2021, as below

$$n_0 = \frac{\left[Z_{1-\alpha 2} \times \sqrt{(r+1)P_M(1-P_M)} - Z_{1-\beta} \sqrt{r \times (1-P_1) + P_2(1-P_2)} \right]^2}{r \times (P_1 - P_2)^2}$$

Where:

n_0: Total sample size

P_1: anticipated proportion of positive exposed

P_2: anticipated proportion of positive unexposed

Pm= $(P_1+P_2)/2$

r= ratio of negatives to positives in relation to the outcome

100(1-a) %: confidence level

100(1-β) %: Statistical power

The sample size calculation is performed by our pilot study (30 samples) that the prevalence rate of *T. tenax* in periodontal disease patients is 33.3%, Percent outcome in exposed group = 50, Percent outcome in unexposed group = 30, Ratio (unexposed: exposed) = 1, Prevalence ratio = 50/30=1.67, Two-sided confidence interval 95% and Power of test 90%, Therefore, the sample size was 248 cases.

We were included more than the calculated sample size to increase the power of the study and also to have a more representative sample, the sample adjusted should be 250 cases, which was represent the population. A total of 250 samples were purposively selected from periodontal disease patients. Inclusion criteria were the clinical diagnosis of periodontal disease, more than six natural teeth, and the ability to perform routine oral care. Exclusion criteria for patients were the following: 1) having taken medication that could modify their oral microbiota 2) having received periodontal treatment during the last 3 months. Following this, 250 participants were selected based on their periodontal diagnosis of gingivitis or periodontitis.

3.3 Materials and Methods

3.3.1 General Characteristics of Participants

This study was provided questionnaire information. The participants were determined by demographic data such as age, gender, weight, high, Body Mass Index (BMI), education, status, and the risk factors such as smoking habits, alcohol consumption, diabetes mellitus, and other systemic diseases, according to the questionnaire form. Moreover, respondent information and verbal screening questionnaire as more convenient multiple choice were provided using a paper form (Appendix C).

3.3.2 Clinical Examination and Collection Samples

The clinical oral examination was performed to investigate the occurrence of periodontal disease by only one dentist and using the Periodontal Screening and Recording (PSR) index with standard procedure and supervision by periodontology dentist. The dentist examined the periodontal status of individual participants and conduct standard procedures for periodontal diagnosis including gingivitis and periodontitis. To fill out a periodontal chart, a dental assistant was a recorder of

examination findings, as the dentist calls out the measurement she was recorded in the chart.

All participants were received a full-mouth periodontal examination and clinical periodontal parameters were recorded: 1) Probing Pocket Depth (PPD) was measured as the distance from the free gingival margin to the bottom of the pocket 2) Bleeding on probing (BOP) was used to evaluate the clinical periodontal inflammation and stability 3) tooth mobility, and 4) Radiographic examination was performed in periodontitis patients. A full-mouth circumferential periodontal inspection with the World Health Organization probe was performed at six sites per tooth (mesiobuccal-MB, buccal-B, distobuccal-DB, mesiolingual-MLi, lingual-Li, and distolingual-DLi). At the end of the examination, participants were received information about their periodontal status.

Figure 3.2 Periodontal examination, A: on six sites per tooth, B: walking prob on a molar (Wilkins, 2004)

The PSR Index is a method for evaluating periodontal disease based on specific criteria: gingival bleeding on probing, the presence of tartar or calculus accumulation, and the measurement of periodontal probing depth. This index provides a comprehensive assessment of periodontal health, including additional factors such as tooth mobility, furcation involvement, mucogingival problems, and gingival recessions. When any of these abnormal conditions are detected, they are indicated with an asterisk (*) alongside the PSR score for the corresponding sextant of the mouth. To measure the periodontal status, a specialized instrument is used. This instrument

is equipped with a 0.5mm diameter ball tip and a colored band extending from 3.5mm to 5.5mm from the tip.

During the assessment, each tooth within a sextant is probed at six distinct sites, and a score is assigned on a scale from Code 0 to Code 4. However, only the highest score within that sextant is recorded. It's worth noting that sextants with fewer than two teeth present are marked with an 'X' and are not factored into the overall evaluation.

PSR code definitions (Landry and Jean, 1997), as below

Code 0 No clinical sign
Code 1 Gingival bleeding on probing
Code 2 Tattar or calculus accumulation on Supra and/or subgingival
Code 3 Periodontal pocket depth between 3.5 mm to 5.5 mm
Code 4 Periodontal pocket depth more than 5.5 mm
Code * Periodontal abnormalities present
Code X presents fewer than 2 teeth per sextant

Collection of the Subgingival Biofilms

The subgingival biofilms and/or dental calculus samples by individual were collected with sterile curette from the nearly cervical tooth then scrape, and dispersed in the buffer of Water for molecular biology (A7398, 0500: PanReac AppliChem). The samples were contained in a 1.5ml microtube and transported to the Parasite Department and Research Center (PDRC) of Suranaree University of Technology (SUT) laboratory within 2 hours of storage at a temperature of -20 ℃ before molecular identification.

3.3.3 Detection of *T. tenax* Infection using Molecular Methods

3.3.3.1 Polymerase Chain Reaction (PCR)

(1) Genomic DNA Extraction

According to the manufacturer's instructions, the genomic DNAs from dental biofilms and/or dental calculus samples were extracted by the

standard methods with the QIAamp DNA Micro Kit (Qiagen, Germany) [QIAcube Connect, 2016] (Appendix A). The procedure consists of 4 steps, as below

1) Lysing sample: Samples are lysed using proteinase K and buffer ALT.
2) Binding DNA: the conditions for blinding to the silica-gel membrane are Buffer AW1 and AW2.
3) Washing to eliminate residual contaminants: using first Buffer AW1 and then BufferAW2.
4) Eluting pure DNA: using a small volume of either Buffer AE or distilled water

For much detailed DNA extraction procedures are presented in Appendices.

Figure 3.3 DNA QIAamp DNA Micro kit: using QIAamp DNA Micro Handbook follow protocol for Isolation of genomic DNA from small volumes of blood

(2) Amplification of *T. tenax* using Polymerase Chain Reaction (PCR) based 18S rRNA gene

After DNA samples extraction by using the QIAamp DNA Micro Kit (Qiagen, Germany), PCR amplification were performed to detect the region of 18S rRNA gene of different trichomonad species sequences registered in GenBank database served for the species *T. tenax,* primer design to amplify a 775 bp by Kikuta et al., 1997. The forward primer was (PT3) 5'-AGTTCCATCGATGCCATTC-3', and the reverse

primer was (PT7) 5'-GCATCTAAGGACTTAGACG-3'. The PCR reaction was performed in a volume of 25 µL, containing: 1 µL of DNA template, 10X Taq DNA buffer 2.5 µL, dNTP 1 µL, $MgCl_2$ 1.5 µL, 1 µL of each primer, and 0.2 µL of Taq DNA polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (Table3.1).

The reaction was performed in a Thermal Cycler using the following reaction conditions: Heat lid at 112°C pre denaturation 94°C 3.5min, denaturation at 94 °C for 1 min., followed by 35 cycles of denaturation at 94 °C, primer annealing at 58 °C for 1 min., extension at 72 °C for 1 min., and a final stage of 72 °C for 4 min. (Table3.2)

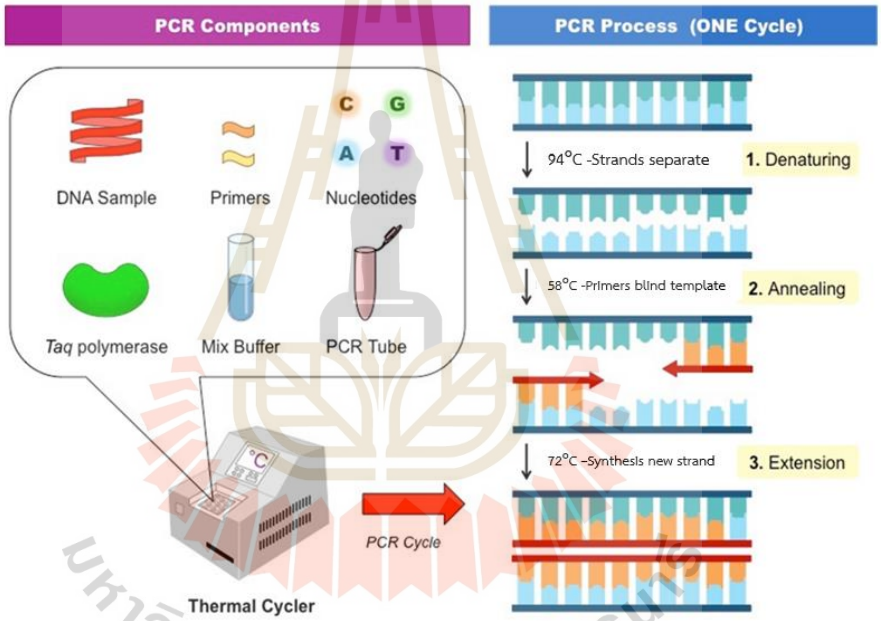


Figure 3.4 Molecular method by using Polymerase Chain Reaction (PCR) (https:// facellitate.com/advantages-and-disadvantages-of-pcr-technology/)

Table 3.1 PCR Amplification of *T. tenax* using PCR Master Mix

Component	Amount (µL)
Water	16.8
10X Taq buffer	2.5
dNTP	1
F primer	1
R primer	1
Taq DNA polymerase	0.2
$MgCl_2$	1.5
DNA template	1
Total	25 µL /tube

Table 3.2 PCR Amplification of *T. tenax* using Temperature and Timing

PCR amplification	
Heat lid	112°C
Pre-denaturation	94°C 3.5 min
Denaturation	94°C 1 min
Annealing	58°C 1 min
Extension	72°C 1 min
Final incubation	72°C 4 min
Store	4°C

(3) Evaluation of PCR products

The PCR products were separated by electrophoresis in 1.5 % agarose gel. Gel images were taken photograph on a UV transilluminator.

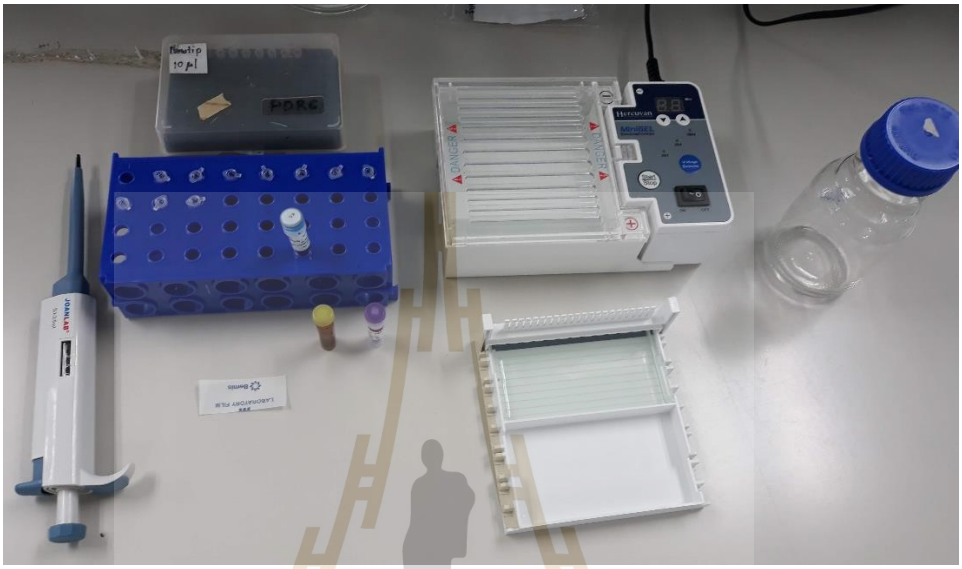


Figure 3.5 Agarose Gel Electrophoresis equipment

Figure 3.6 PCR product, gel image of *T. tenax*

3.3.3.2 DNA sequencing and Alignment

Purification of all positive PCR products were sent out for the direct sequencing services from U2Bio Co., Ltd., Thailand. (https://www.u2bio.co.th/). The dideoxy method (Sanger et al., 1977) were used to determine the nucleotide sequence of DNA, using an automated DNA sequencer. Each sequence was used to

search for homology with the DNA database using the BLAST algorithm (http://www.ncbi.nlmnih.gov/BLAST) in GenBank (www.ncbi.nlm.nih.gov/GenBank). The sequence analysis was performed using the BioEdit Sequence Alignment software program Editor 7.2.5. (Appendix A).

3.3.3.3 Phylogenetic analysis

The phylogenetic analysis was performed using MEGA–X version 11, comparing the obtained sequences with a reference set retrieved from the NCBI GenBank database (Appendix B).

3.4 Statistics analysis

All the data were coded and collected using the software "Microsoft Excel" and then analyzed using the statistical software package STATA version 17. Descriptive statistics were employed, encompassing frequency, percentage, mean, and standard deviations. Subsequently, the Chi-square test was applied to explore potential associations between two variables. In instances of small samples, Fisher's exact test was utilized. To model the likelihood of detecting *T. tenax* presence based on predictive variables, binary logistic regression analysis was conducted. The significance threshold was set at $p<0.05$, serving as a critical value to manage the risk of alpha error.

CHAPTER 4

RESULTS AND DISCUSSION

Results

4.1 The presence of *T. tenax* and associated risk factors

The present study was conducted in 250 patients; PCR was used to determine the presence of *T. tenax*. The product size was 775 bp by amplification of the specific18S rRNA gene for *T. tenax*. The PCR products were separated by electrophoresis in a 1.5 % agarose gel, as shown in Figure 4.3 – 4.4. The demographic data and the presence of *T. tenax* are summarized in Table 4.1-4.2. The 42 patients (16.8%) who were positive for *T. tenax* infection included 17 (40.48%) men and 25 (59.52%) women, with no association between patient gender and *T. tenax* infection ($\chi^2 = 0.15$, $p>0.05$). The highest frequency of *T. tenax* was observed in the 25-59-year-old group, with 21 positive patients (50%), followed by the 60-year-old and older group, with 16 positive patients (38.1%), and the less than 25-year-old group, with 5 positive patients (11.9%). *T. tenax* infection was not associated with the patient age group ($\chi^2 = 1.26$, $p>0.05$). Additionally, *T. tenax* infection was not found to be associated with BMI ($\chi^2 = 0.17$, $p>0.05$) or status ($\chi^2 = 2.16$, $p>0.05$), education ($\chi^2 = 3.54$, $p>0.05$), and brushing ($\chi^2 = 0.99$, $p>0.05$) in Table 4.4. The infection rate of *T. tenax* was associated with periodontal disease, which showed that the oral protozoan was present in 10 of 155 patients (23.8%) with gingivitis and in 32 of 95 patients (76.19%) with periodontitis. A statistically significant association between *T. tenax* infection and periodontal disease ($\chi^2 = 31.25$, $p=0.001$) was found. Oral motile 0protozoan frequency was presented in 4 of 39 (9.52%) smoking patients and 38 of 211 (90.48%) nonsmoking patients, indicating no association between smoking and the presence of *T. tenax* ($\chi^2 = 1.42$, $p>0.05$). The analysis between alcohol consumption and *T. tenax* infection was observed in 11 of 96 patients who drank alcohol and 31 of 154 patients who did not drink alcohol, which were not statistically significant ($\chi^2 = 3.18$, $p>0.05$). The presence

of *T. tenax* infection based on systemic disease was observed in 8 patients (19.05%) with underlying conditions and 34 patients (80.95%) with no underlying disease. However, there was no association between systemic disease and *T. tenax* presence (χ^2 =0.19, p>0.05).

In this study, the association between *T. tenax* infection and diabetes mellitus (DM), hypertension (HT), and comorbidities was analyzed. Out of 29 patients with DM, four patients were observed to have *T. tenax* infection, and out of 46 patients with HT, nine patients had *T. tenax* infection. Similarly, 4 out of 28 patients with comorbidities were found to have *T. tenax* infection. However, these associations were not statistically significant (DM: χ^2 = 0.21, p > 0.05; HT: χ^2 = 0.31, p>0.05; comorbidities: χ^2 = 0.14, p > 0.05).

Additionally, *T. tenax* infection was associated with the PSR index. Patients with a PSR index range of 0-2.0 were frequently detected in 5 of 64 patients (11.90%), range of 2.1-2.9 was detected in with 30 of 138 patients (71.43%), and a range of 3.0-4.0 was detected in with 7 of 48 patients (16.67%). The association between the presence of *T. tenax* and the PSR index was revealed (χ^2 =6.28, p<0.05).

Finally, this study employed binary logistic regression analysis to identify potential risk factors for *T. tenax* infection, assessing both crude odds ratio [OR_{cru}] and adjusted odds ratio [OR_{adj}]. The analysis found no significant associations between *T. tenax* infection and factors like age group, gender, BMI, status, education, brushing, systemic disease, smoking, or alcohol consumption (age group; OR_{cru}=1.34 (95% CI = 0.47-3.81), gender; OR_{cru}=1.14 (95% CI = 0.06-2.25), BMI; OR_{cru}=0.82 (95% CI = 0.32-2.10), status; OR_{cru}=0.60 (95% CI = 0.30-1.19), education; OR_{cru}=0.53 (95% CI = 0.27-1.03), brushing; OR_{cru}=2.73 (95% CI = 0.35-21.48), systemic disease; OR_{cru}=1.21 (95% CI = 0.52-2.79), smoking; OR_{cru}=1.92 (95% CI = 0.65-5.73), and alcohol consumption; OR_{cru}=1.95 (95% CI = 0.93-4.09). However, when comparing periodontitis patients to gingivitis patients, the OR_{cru} for *T. tenax* infection was 7.37 (95% CI = 3.41 – 15.89, p = 0.001), while the adjusted value was 9.67 (95% CI = 4.01 – 23.31, p = 0.001). These findings strongly suggest a significant link between *T. tenax* presence and periodontal disease (Table 4.5).

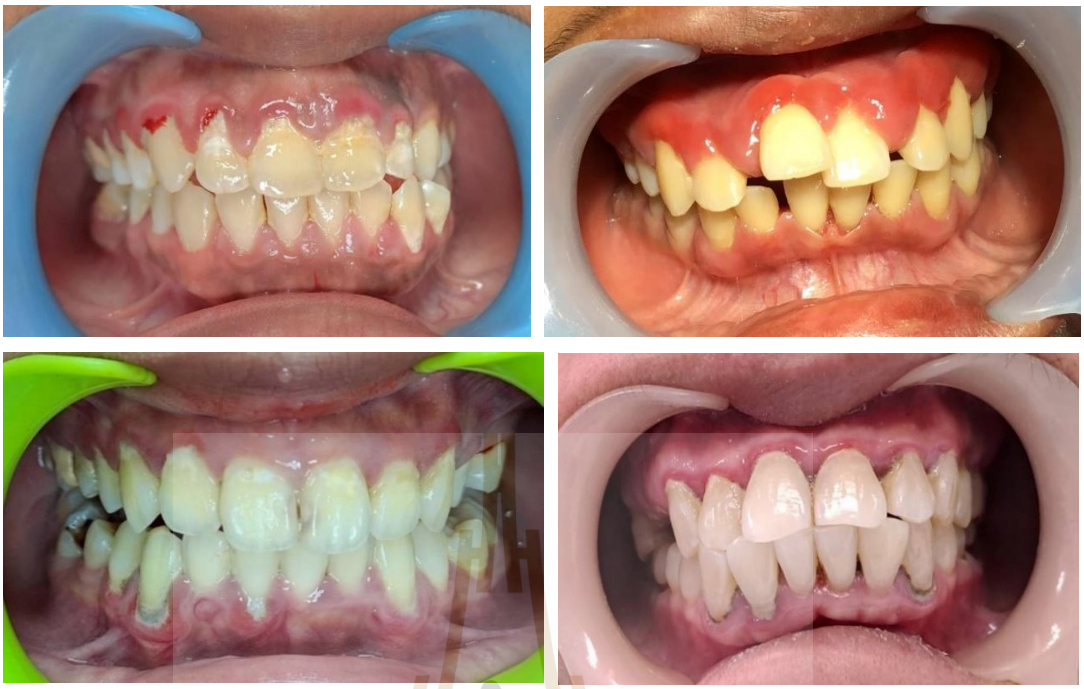


Figure 4.1 Representative image of periodontal disease. Characteristic image from a gingivitis patient, there is inflammation of the gums, causing bleeding with swelling, and redness.

Figure 4.2 Representative image of periodontal disease. Characteristic image from periodontitis patient, there is loss of alveolar bone, formation of deep gum pockets. The black arrows indicate periodontal lesions.

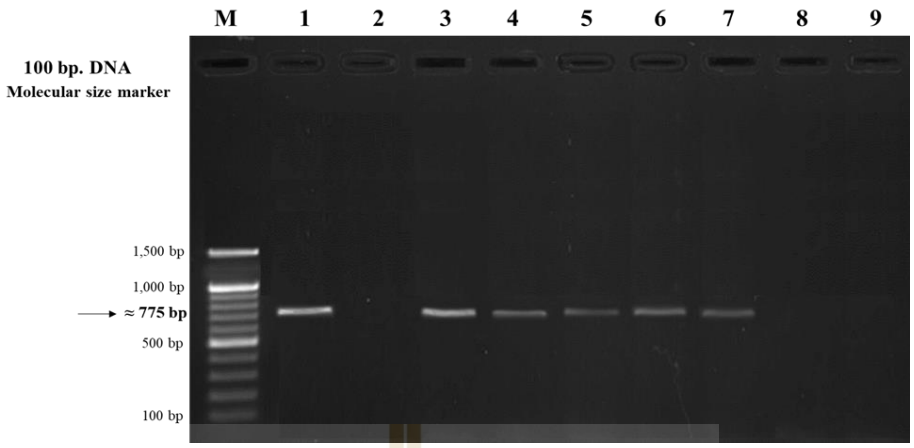


Figure 4.3 Agarose gel electrophoresis of *T. tenax* infection cases. Lanes1: positive control (*T. tenax* DNA); Lane 2: negative control; Lane 3-7: positive samples; Lane 8-9: negative samples; Lane M: DNA Ladder Molecular Size Marker tiangen®, 100 bp. The position of the PCR product is indicated by the arrow on the left of the gel.

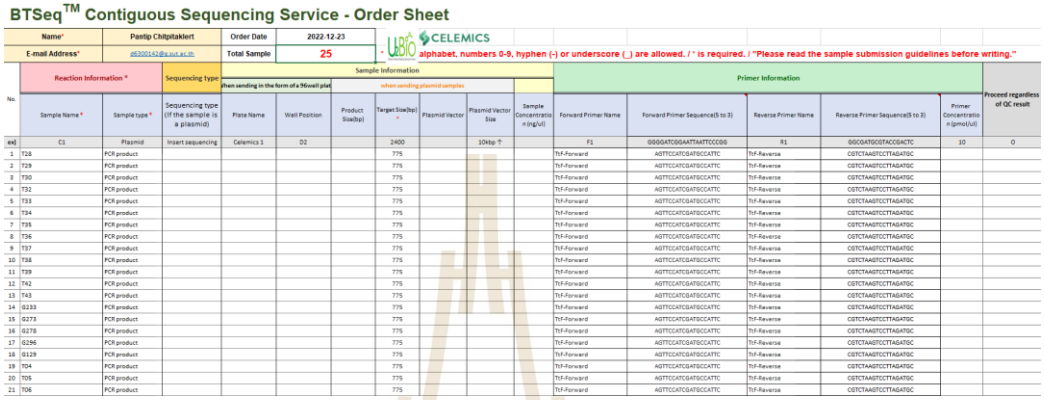
Figure 4.4 Representative image agarose gel electrophoresis of *T. tenax* infection cases. Lane M: DNA Ladder Molecular Size Marker tiangen®, 100 bp. The position of the PCR product is indicated by the arrow on the left of the gel. Lanes PC: positive control (T. tenax DNA); Lane NC: negative control; Lane 1-2, 5-12, 15-17: positive samples; Lane 3-4, 13-14, 18-19: negative samples.

4.2 DNA sequencing, alignment, and phylogenetic tree

PCR amplification was performed to detect the region of 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,1997). Then the PCR product was brought to run gel to check the PCR results by using 1.5 % agarose gel electrophoresis. From 42 samples of successful PCR products of amplification were sequenced by the Sanger method from the U_2Bio (Thailand) Co., Ltd (Figure 4.5). The sequence analysis was performed using the BioEdit Sequence Alignment Editor 7.2.5. Genotype identification was accomplished by conducting a nucleotide similarity search in Basic Local Alignment Search Tool (BLAST) to search for the most similar reference sequences to the *T. tenax* sequence (Appendix B). Strains of *T. tenax* were recognized at the species level, based on ≥99% identity with the reference sequences, deposited in the GenBank under the accession numbers D49495 (Kikuta, 1997). The results of the nucleotide sequences of the samples in this study were analyzed with *Trichomonas tenax* gene for SrRNA data (GenBank accession number D49495.1) from the GenBank database. It was found that Query coverage= 100%, Identity= 100% (1434/1434), E-value= 0.0 and results from *Trichomonas tenax isolate sputum11* (GenBank accession number JX443581.1) Query coverage= 100% Identity= 99.23% (1400/1400) E-value = 0.0 respectively. Phylogenetic analysis was performed using the MEGA-X software (version 11). The phylogenetic tree, of obtained *Trichomonas* genotypes from the present study together with sequences from GenBank, was constructed using the Maximum Likelihood method with bootstrap values (determined by 100 replicates) (Figure 4.6).

In this study, we successfully detected *T. tenax* in 42 dental plaque samples using PCR. All 42 samples showed a single 775 bp product of the 18S rRNA gene, confirming its agreement with the expected *T. tenax* product size (Figure 4.3-4.4), to validate the findings, sequence analysis and alignment were performed using the BioEdit Sequence Alignment and Basic Local Alignment Search Tool (BLAST) (Figure 4.6-4.7). For the phylogenetic analysis of *T. tenax* gene sequences, we utilized the Maximum Likelihood method model in Mega software (version 11). The isolates or strain names were obtained from GenBank, with corresponding accession numbers

indicated in parentheses (Figure 4.8). As a reference, we used *Entamoeba gingivalis* (accession number MW676260) as the out-group. Our *T. tenax* isolates in this study closely matched the reference sequence for *Trichomonas tenax* gene for SrRNA (Accession No 9495.1)



BTSeq™ Contiguous Sequencing Service - Order Sheet

CELEMICS

Name*	Pantip Chitpitaklert	Order Date	2022-12-23
E-mail Address*	a6300142@ku.ac.th	Total Sample	25

alphabet, numbers 0-9, hyphen (-) or underscore (_) are allowed. / * is required. / "Please read the sample submission guidelines before writing."

No.	Reaction Information *		Sequencing type	Sample Information							Primer Information					Proceed regardless of QC result
	Sample Name *	Sample type *	Sequencing type (If the sample is a plasmid)	Plate Name	Well Position	Product Size(bp)	Target Size(bp)	Plasmid Vector	Plasmid Vector Size	Sample Concentration (ng/ul)	Forward Primer Name	Forward Primer Sequence(5 to 3)	Reverse Primer Name	Reverse Primer Sequence(5 to 3)	Primer Concentration (pmol/ul)	
ex)	C1	Plasmid	Insert sequencing	Celemics 1	D2		2400		10kbp ↑		F1	GGGGATCGGAATTAATTCCCGG	R1	GGCGATGCGTACCGACTC	10	O
1	T28	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
2	T29	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
3	T30	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
4	T32	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
5	T33	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
6	T34	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
7	T35	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
8	T36	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
9	T37	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
10	T38	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
11	T39	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
12	T42	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
13	T43	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
14	G233	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
15	G273	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
16	G278	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
17	G296	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
18	G129	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
19	T04	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
20	T05	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		
21	T06	PCR product					775				Tnf-Forward	AGTTCCATCGATGCCATTC	Tnf-Reverse	CGTCTAAGTCCTTAGATGC		

Figure 4.5 Representative of PCR product were sent for DAN sequencing to the U_2Bio (Thailand) Co., Ltd.

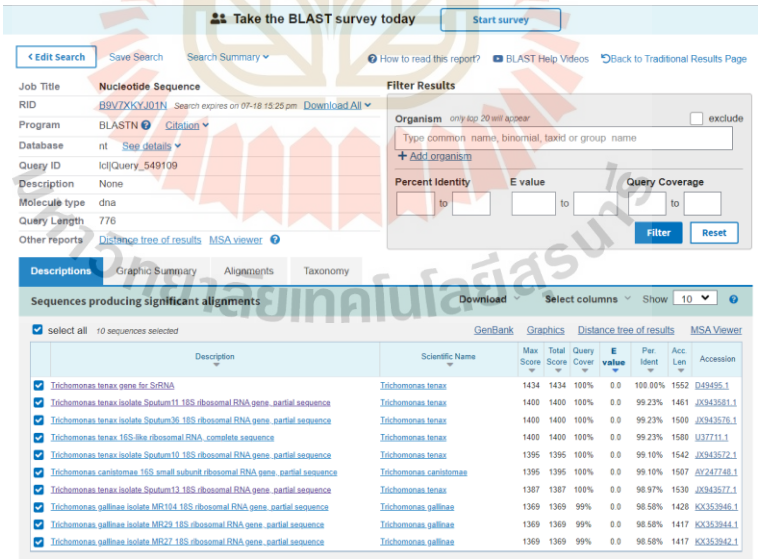


Figure 4.6 Genotype identification by Basic Local Alignment Search Tool (BLAST): comparative results of DNA sequencing of *T. tenax* in GenBank data base by BLAST method (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Table 4.1 The Characteristic Information of Variables

Variables		Number(n)	Percentage (%)
Age	Mean (SD): 47.62 (7.33)		
<25		40	16
25-59		131	52.4
≥60		79	31.6
Gender			
Male		108	43.2
Female		142	56.8
BMI	Mean (SD): 19.75 (3.65)		
<23		209	83.6
≥23		41	16.4
Status			
Single		141	56.4
Marriage		109	43.6
Education			
Under Bachelor degree		110	44
Bachelor degree and up		140	56
Systemic disease			
No underlying disease		196	78.4
Underlying conditions/disease		54	21.6
Smoking			
Yes		39	15.6
No		211	84.4
Alcohol consumption			
Yes		96	38.4
No		154	61.6
Brushing			
<2 time/day		14	5.6
>=2 time/day		236	94.4
Periodontal disease			
Gingivitis		155	62
Periodontitis		95	38
PSR index			
0-2		64	25.6
2.1-2.9		138	55.2
3-4		48	19.2
***T. tenax* infection**			
Yes		42	16.8
No		208	83.2

BMI: body mass index.; PSR index: the Periodontal Screening and Record index

Table 4.2 The Percentage of DM, HT, Comorbidities Samples

Variables	Number(n)	Percentage (%)
Diabetes mellitus		
Yes	29	11.66
No	221	88.40
Hypertension		
Yes	46	18.40
No	204	81.60
Comorbidities*		
Yes	28	11.20
No	222	88.80

*Comorbidities: patients who have a disease or condition also have one or more other diseases or conditions

Table 4.3 DM, HT, Comorbidities and *T. tenax* Infection

Variables	*T. tenax* infection		P-value
	Yes (n, %)	No (n, %)	
Diabetes mellitus			0.795
Yes	4(9.52)	25(12.02)	
No	38(90.48)	183(87.98)	X^2=0.212
Hypertension			0.662
Yes	9(21.43)	37(17.79)	
No	33(78.57)	171(82.21)	X^2=0.308
Comorbidities*			1.000
Yes	4(9.52)	24(11.54)	
No	38(90.48)	184(88.46)	X^2=0.143

*Comorbidities: patients who have a disease or condition also have one or more other diseases or conditions

Table 4.4 The Presence of *T. tenax* Infection and Variables

Variables	*T. tenax* infection		P-value
	Yes (n, %)	No (n, %)	
Age			0.591
< 25 years	5(11.9)	35(16.83)	
25-59 years	21(50)	110(52.88)	(X^2=1.259)
≥60 years	16(38.1)	63(30.29)	
Gender			0.735
Male	17(40.48)	91(43.75)	(X^2=0.153)
Female	25(59.52)	117(56.25)	
BMI			0.821
<23	36(85.71)	173(83.17)	(X^2=0.165)
≥23	6(14.29)	35(16.83)	
Status			0.173
Single	28(66.67)	113(54.33)	(X^2=2.164)
Marriage	14(33.33)	95(45.67)	
Education			0.064
Under Bachelor degree	24(57.14)	86(41.35)	(X^2=3.539)
Bachelor degree and up	18(42.86)	122(58.65)	
Systemic disease			0.837
No underlying disease	34(80.95)	162(77.88)	(X^2=0.194)
Underlying conditions/disease	8(19.05)	46()22.12	
Smoking			0.350
Yes	4(9.52)	35(16.83)	(X^2=1.416)
No	38(90.48)	173(83.17)	
Alcohol consumption			0.084
Yes	11(26.19)	85(40.87)	(X^2=3.181)
No	31(73.81)	123(59.13)	
Brushing			0.476
<2 time/day	1(2.38)	13(6.25)	(X^2=0.989)
>=2 time/day	41(97.62)	195(93.75)	
Periodontal disease			0.001*
Gingivitis	10(23.81)	145(69.71)	(X^2=31.251)
Periodontitis	32(76.19)	63(30.29)	
PSR index			0.039*
0-2	5(11.90)	59(28.37)	
2.1-2.9	30(71.43)	108(51.92)	(X^2=6.276)
3-4	7(16.67)	41(19.71)	

* p<0.05

Table 4.5 The presence of *T. tenax* infection and variables using binary logistic regression analysis

Variables	Presence of *T. tenax* infection			
	Crude OR (95% CI)	p-value	Adj OR (95% CI)	p-value
Age		0.531		
< 25 years	1		1	
25-59 years	1.34(0.469-3.806)		0.68(0.162-2.830)	0.593
≥60 years	1.78(0.600-5.266)		0.65(0.119-3.511))	0.615
Gender		0.695		
Male	1		1	0.534
Female	1.14(0.058-2.245)		0.73(0.272-1.964)	
BMI		0.681		
<23	1		1	1.000
≥23	0.82(0.323-2.104)		0.99(0.297-3.363)	
Status		0.137		
Single	1		1	0.299
Marriage	0.595(0.296-1.194)		0.58(0.210-1.616)	
Education		0.061		
Under Bachelor degree	1		1	0.088
Bachelor degree and up	0.53(0.270-1.034)		0.50(0.260-1.111)	
Systemic disease		0.656		
No underlying disease	1		1	0.245
Underlying disease	1.21(0.523-2.787)		0.52(0.170-1.569)	
Smoking		0.210		
Yes	1		1	0.467
No	1.92(0.645-5.730)		1.68(0.414-6.823)	
Alcohol consumption		0.068		
Yes	1		1	0.342
No	1.95(0.928-4.087)		1.59(0.612-4.124)	
Brushing		0.273		
<2 time/day	1		1	0.124
>=2 time/day	2.73(0.348-21.481)		5.66(0.623-51.421)	
Periodontal disease		0.001*		
Gingivitis	1		1	0.001*
Periodontitis	7.37(3.413-15.894)		9.67(4.008-23.314)	

* p-value <0.05

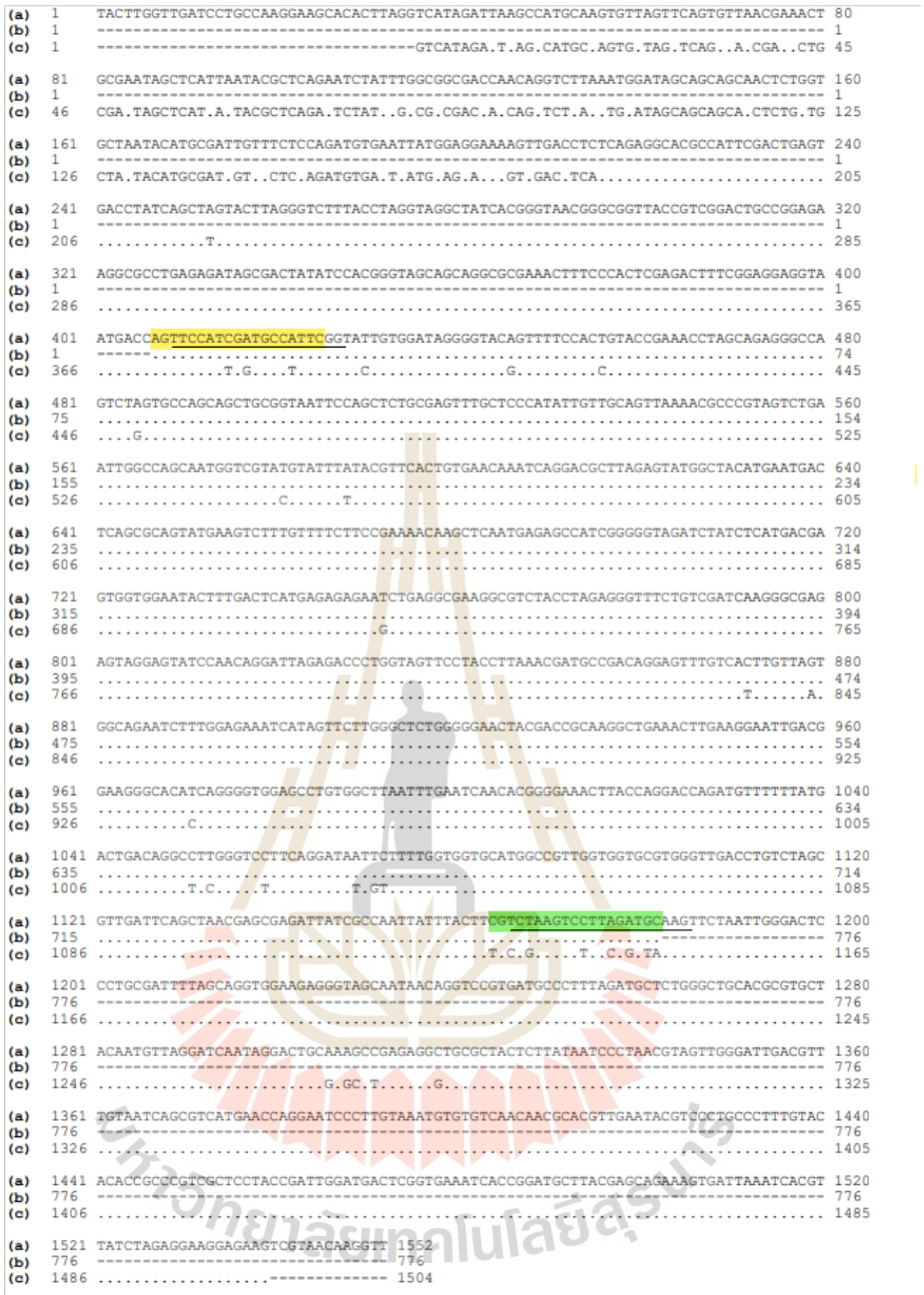


Figure 4.7 Representative of the sequence analysis, the alignment in 18S rRNA gene sequences of: (a) *Trichomonas tenax* (GenBank accession number: D49495) with close relative; (b) *Trichomonas tenax* (Sample in this study); and (c) *Trichomonas vaginalis* (ac:KM603336). The underline and highlight indicate the position of the primers. Dots indicate residues identical to those of *T. tenax*. Numbering was based on the data registered in GenBank.

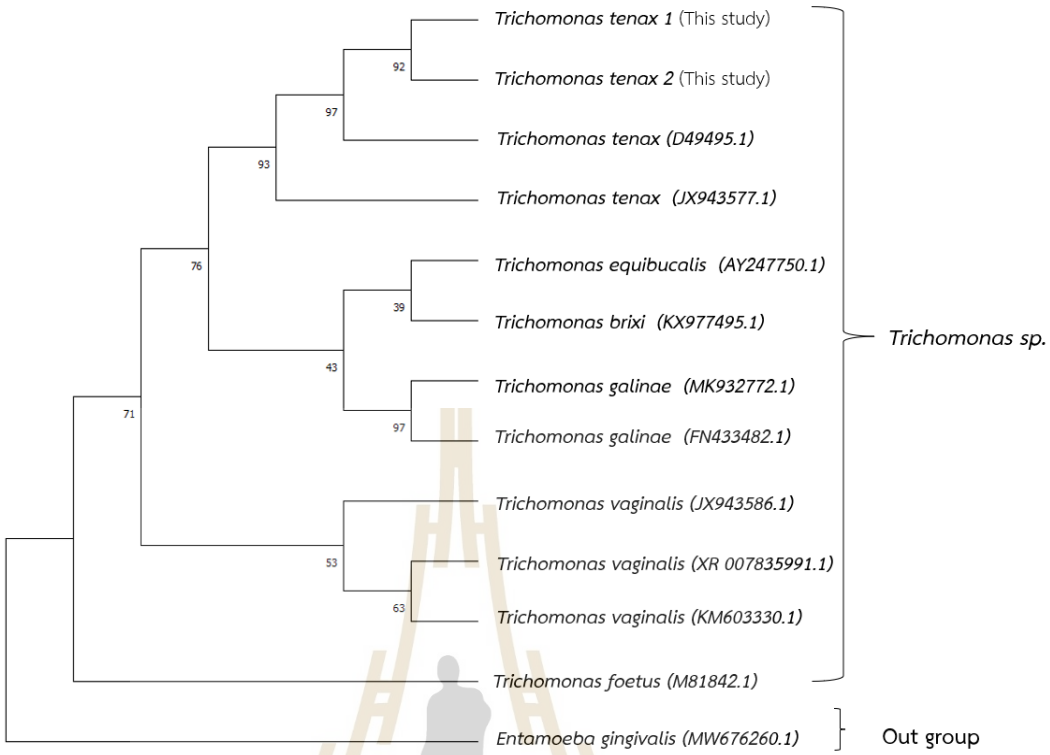


Figure 4.8 Molecular phylogeny of *Trichomonas tenax* in a group of parasites in *Trichomonade* species and *Entamoeba gingivalis* are individual outgroups. Phylogenetic analysis using: Maximum composite likelihood model; origin (node) represents the confidence (bootstrap values) from the swap sequence data. Nucleotide, 100 times analysis, using C model neighbor-joining (NJ) Program MEGA Version, Level 11

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* (Bracamonte-Wolf et al, 2019, Brooks et al., 2007). The sequence analysis in this study, the alignment in 18S rRNA gene sequences of *Trichomonas tenax* (samples in this study) and *Trichomonas tenax* (GenBank accession number: D49495) were close relatively.

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 16.8 % (42 of 250) in periodontal disease patients, including 76.2 % (32 of 42) in periodontitis patients and 23.8 % (10 of 42) in gingivitis patients. These results were most similar to those of previous studies that reported prevalence of 14.63%, 15.5%,13.54%, 13%, and 16.08 % (Boonthanom Moonmeungsan, 2004, Athari, Soghandi, Haghighi, and Kazemi., 2007, Dybicz et al., 2018, Eslahi et al.,2021, Fadhil et al., 2022). Additionally, another previous study reported a global pooled prevalence of *T. tenax* infection of 17% (95% CI = 14%-22%) (Eslahi et al.).

We identified the factors associated with *T. tenax* infection by performing bivariate analysis. The potential risk factors with *T. tenax* infection were periodontal disease ($p<0.001$) and PSR index (especially PSR index score ≥2, $p<0.05$). Multivariate analysis by binary logistic regression revealed that the statistically significant risk factors for *T. tenax* infection and periodontitis (OR_{adj} =9.67). 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, Bracamonte-Wolf et al.,2019, Benabdelkader et al., 2019, Yaseen et al.,2021, Matthew et al., 2023). The explanation of the study's results could be supported by Ribero 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. Our finding, indicated that *T. tenax* was more common in patients with periodontitis. These may be considered potential risk factors *T. tenax* infection. Additionally, the results were consistent with those of previous studies (Marty et al.,2017, Dybicz et al., 2018, Wolf et al.,2019, Feki et al.,1981, 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.

CHAPTER 5
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Trichomonas tenax is a parasitic protozoan that primarily resides in the oral cavity, particularly in the gingival crevices and periodontal pockets. It is considered an opportunistic pathogen and is associated with poor oral hygiene and various oral diseases. Epidemiological studies have provided valuable insights into the prevalence, transmission, and risk factors associated with *T. tenax* infections. The oral parasite, *T. tenax* have been reported worldwide, but their prevalence varies across different populations and geographic regions. Several studies have indicated a higher prevalence of *T. tenax* in developing countries, especially in areas with poor oral hygiene practices and limited access to dental care.

In this study, the presence of *T. tenax* was investigated using molecular techniques. Our *T. tenax* isolates showed a close match with the reference sequence for the *Trichomonas tenax* gene for SrRNA (Accession No 49495) available in the GenBank database, and it was found to be more prevalent in patients with periodontitis compared to those with gingivitis. The results strongly associate this oral protozoan with periodontal disease.

5.2 Recommendation for Research Study

The main limitation of this study is its cross-sectional design, which prevents us from establishing causality between variables. To overcome this limitation and gain a more comprehensive understanding, further longitudinal studies with a larger sample size are needed. Conducting a multicenter study would also increase generalizability, reduce selection bias and enhancing the validity of the findings. Such research efforts can provide valuable insights into the potential risk factors for *T. tenax* infection in the oral cavity.

Furthermore, ongoing research is focusing on the pathogenicity of oral parasitic diseases, and it is important to conduct comprehensive studies that involve the broader community in Thailand. This approach would help in understanding the prevalence, transmission, and impact of *T. tenax* infections in the population and guide preventive and therapeutic measures more effectively.

5.3 General Recommendation for Preventing the *T. tenax* Infection

Preventing *Trichomonas tenax* infection primarily involves maintaining good oral hygiene and adopting certain preventive measures to minimize the risk of transmission. Since *T. tenax* is primarily found in the oral cavity and is associated with poor oral hygiene practices, the following steps can help in preventing its infection:

Regular Oral Hygiene: Proper and regular oral hygiene practices are crucial in preventing *T. tenax* infection. This includes brushing teeth at least twice a day with fluoride toothpaste, flossing daily to remove plaque and food debris between teeth, and using mouthwash to reduce the microbial load in the mouth.

Routine Dental Check-ups: Regular dental check-ups and professional cleanings by a dentist or dental hygienist are essential. These visits allow for the early detection and management of any oral health issues, including *T. tenax* infection.

Avoiding Unprotected Oral Contact: T. tenax can be transmitted through close oral contact with an infected individual. Avoiding activities such as sharing utensils, toothbrushes, or kissing with an infected person can help prevent transmission.

Proper Care of Oral Devices: Oral devices like dentures, make sure to clean and disinfect them regularly as per your dentist's recommendations. Poorly maintained oral devices can harbor *T. tenax* and other harmful microorganisms.

Avoiding Risk Behaviors: Certain risk behaviors like smoking and substance abuse have been associated with an increased likelihood of *T. tenax* infections. Adopting a healthy lifestyle and avoiding such habits can reduce the risk of infection.

Maintaining a Healthy Immune System: A strong immune system can help fight off infections, including *T. tenax*. Maintaining a balanced diet, regular exercise, adequate sleep, and managing stress can all contribute to a healthy immune system.

Public Health Measures: Public health efforts to improve overall sanitation, access to clean water, and oral health education can also play a role in reducing the prevalence of *T. tenax* infections.

It's essential to note that while *T. tenax* is a pathogen and can be associated with oral diseases, it is generally considered nonpathogenic in healthy individuals. However, practicing good oral hygiene and taking preventive measures can contribute to better oral health and reduce the risk of various oral infections, including *T. tenax*.

5.4 Specific Recommendation for Dentists and Periodontists

T. tenax is a parasitic protozoan belonging to the family *Trichomonadidae*. It is commonly found in the oral cavity, particularly in the periodontal pockets and dental plaques of humans. The prevalence of *T. tenax* is distributed worldwide, but its prevalence varies in different populations. It is more commonly found in individuals with poor oral hygiene, and periodontal disease.

Risk factors: Several factors contribute to the susceptibility of *T. tenax* infections. Poor oral hygiene, characterized by inadequate practices such as infrequent brushing and flossing, creates an environment conducive to the growth and colonization of *T. tenax*. Additionally, the presence of oral devices like dentures, along with certain risk behaviors such as smoking or substance abuse, can increase the likelihood of transmission. Patients with periodontal disease, which is characterized by inflammation and infection of the gums and surrounding tissues, are also more susceptible to *T. tenax* infections. Furthermore, individuals with weakened immune systems, such as those affected by HIV/AIDS or undergoing immunosuppressive therapy, face an elevated risk of *T. tenax* infections.

Clinical Assessment: Evaluate patients for signs and symptoms such as inflamed or bleeding gums, bad breath, and oral discomfort. While these symptoms are non-specific, they can indicate the presence of an infection and prompt further investigation.

Diagnostic methods: The detection of the oral parasite, *T. tenax* using the microscopic methods are useful for detecting the typical motility and determining morphological characteristics of *T. tenax*. In the permanent preparation of the culture

stained are all characteristic cell organelles stainable, enabling their detection without their typical motility. Recently, molecular methods such as PCR and sequencing of their products have been applied for the accurate detection and identification of *T. tenax*.

Treatment and prevention: In cases where the infection is symptomatic or persistent, consider prescribing antiprotozoal medications such as metronidazole. Follow established dosing guidelines and inform patients about potential side effects. Treating underlying gum inflammation and infection can reduce susceptibility to *T. tenax* and other oral infections. Continuously educate patients about oral hygiene practices, the importance of regular dental visits, and strategies to prevent reinfection.

Collaboration: Work collaboratively with other healthcare professionals, especially if the patient's infection might be related to an underlying medical condition. This ensures comprehensive care.

It's important to tailor these recommendations to each patient's specific situation. A comprehensive approach that combines proper diagnosis, effective treatment, patient education, and preventive strategies will contribute to successful management of *T. tenax* infections in dental and periodontal practice.

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APPENDIX A

THE PROCEDURE OF POLYMERASE CHAIN REACTION

1. DNA Extraction

Equipment and chemical substants

1. QIAamp DNA Microkit DNA Extraction Kit
2. Autopipette and Tip size 1000 µl, 100 µl, 10 µl
3. Vortex machine
4. Heat block machine
5. Centrifuge
7. Absolute ethanol
8. 1.5 ml microcentrifuge tube

The procedure of DNA Extraction

1. Pipet 50 µl samples into a 1.5 ml microcentrifuge tube.
2. Add Buffer ATL 50 µl to a final volume of 100 µl.
3. Add 10 µl proteinase K.
4. Add 100 µl Buffer AL, close the lid, and mix by pulse-vortexing for 15 sec. To ensure efficient lysis, the sample, Buffer ATL, proteinase K, and Buffer AL must be thoroughly mixed to yield a homogeneous solution. If the volume of samples is lower than 10 µl, adding carrier RNA1 µl
5. Incubate at 56°C for 10 min.
6. Briefly centrifuge (2,500 rpm 1 minute) the 1.5 ml tube to remove drops from inside the lid.
7. Add 50 µl of ethanol (96–100%), close the lid, and mix thoroughly by pulse-vortexing for 15 sec. Incubate for 3 min at room temperature.
8. Briefly centrifuge the 1.5 ml tube to remove drops from inside the lid.
9. Carefully transfer the entire lysate from step 8 to the column (in a 2 ml collection tube) without wetting the rim. Close the lid, and centrifuge at 8000 rpm for 1 min. Place the column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the column is empty.

10. Carefully open the column and add 500 µl Buffer AW1 without wetting the rim. Close the lid, and centrifuge at 8000 rpm for 1 min. Place the column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

11. Carefully open the column and add 500 µl Buffer AW2 without wetting the rim. Close the lid, and centrifuge at 8000 rpm for 1 min. Place the column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. Contact between the column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the column. Take care when removing the column and collection tube from the rotor, so that flow-through does not come into contact with the column.

12. Centrifuge at full speed (14,000 rpm) for 3 min to dry the membrane completely. This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.

13. Place the column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the flow-through. Carefully open the lid of the column and apply 25 µl Buffer AE or distilled water to the center of the membrane. If high pH or EDTA affects sensitive downstream applications, use water for elution. Important: Ensure that Buffer AE or distilled water is equilibrated to room temperature (15–25°C). If using small elution volumes

14. Close the lid and incubate at room temperature (15–25°C) for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min. incubating the column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

DNA is eluted in buffer AE or water is immediately ready for use in amplification reaction or storage at -20°C. Purified DNA is free of protein, nucleases, and other impurities.

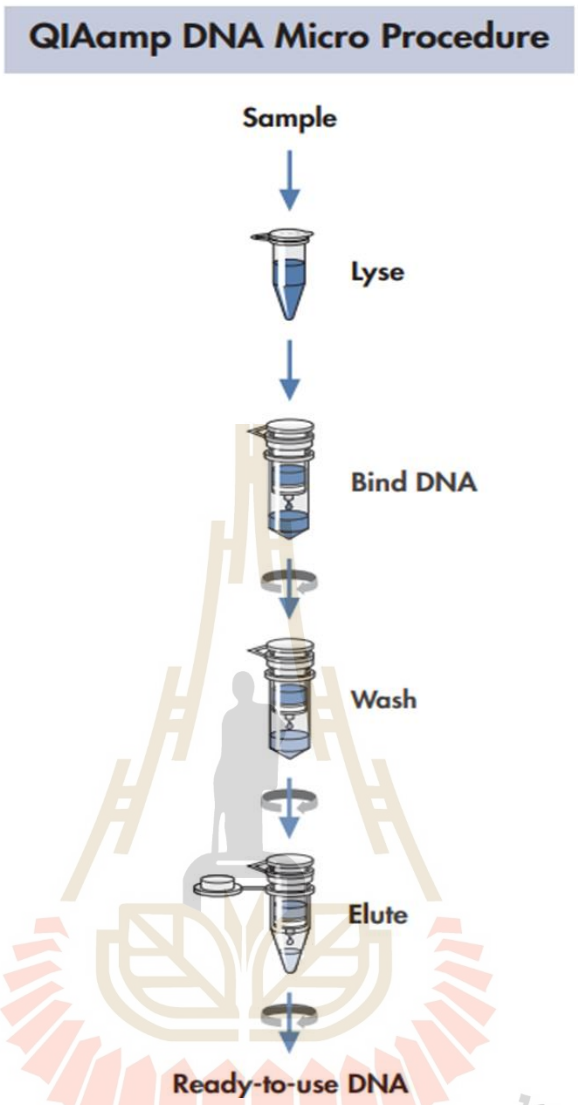


Figure A.1 QIAamp genomic DNA kits: www.qiagen.com, 2016

2. The procedure of Agarose Gel Electrophoresis

1. To prepare gel 0. 45mg in 30ml of 1XTAE, agarose powder is mixed with electrophoresis buffer, and heated in a microwave oven to melt it.

2. After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

3. After the gel has solidified, the comb is removed, taking care not to rip the bottom of the wells.

4. The gel, still in a plastic tray, is inserted horizontally into the electrophoresis chamber and is covered with 1X TAE buffer.

5. Samples containing DNA mixed with Maestrosafe and DNA loading dye are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied.

6. The current flow can be confirmed by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored blue, given its negative charge.

7. The distance DNA has migrated in the gel can be judged by visually monitoring the migration of the tracking dyes.

8. Gel images will be taken photograph on a UV transilluminator.

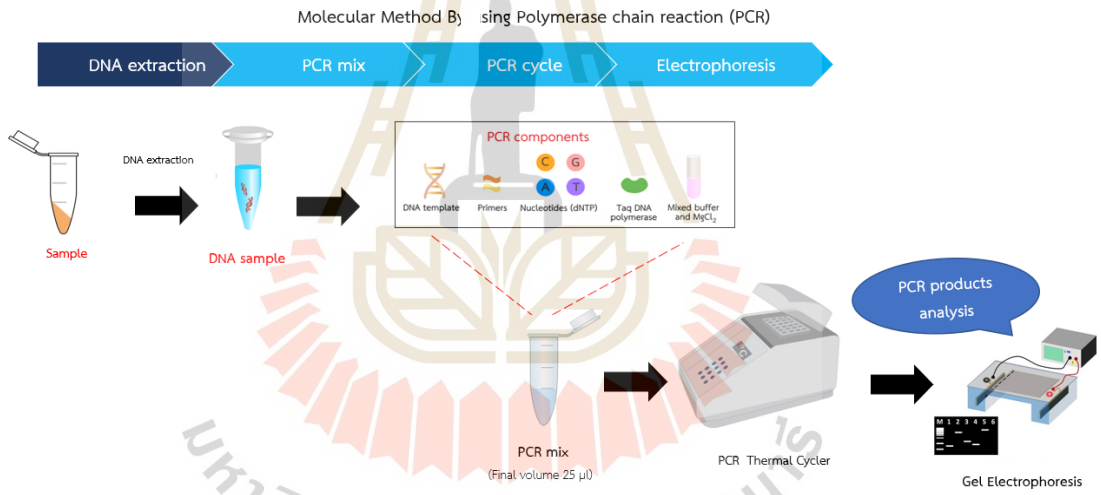


Figure A.2 Summary Step for PCR (https://study.com/skill/practice/analyzing-an-illustration-of-polymerase-chain-reaction-assessing-its-usefulness-in-dna-analysis-questions.html)

APPENDIX B

DNA SEQUENCING AND PHYLOGENETIC TREE

1. Sequence analysis

1.1 Prepare sequence sample for Nucleotide BLAST

1.2 Open NCBI website -->Nucleotide BLAST

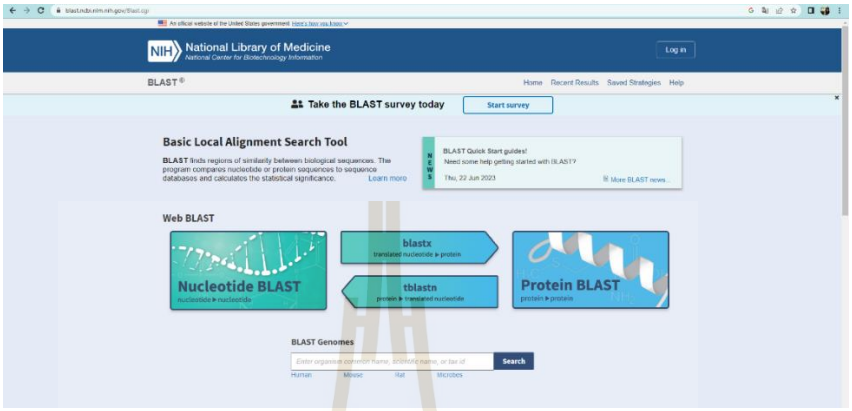


Figure B.1 BLAST findinding regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance.

1.3 Copy sequence sample, then click on BLAST button

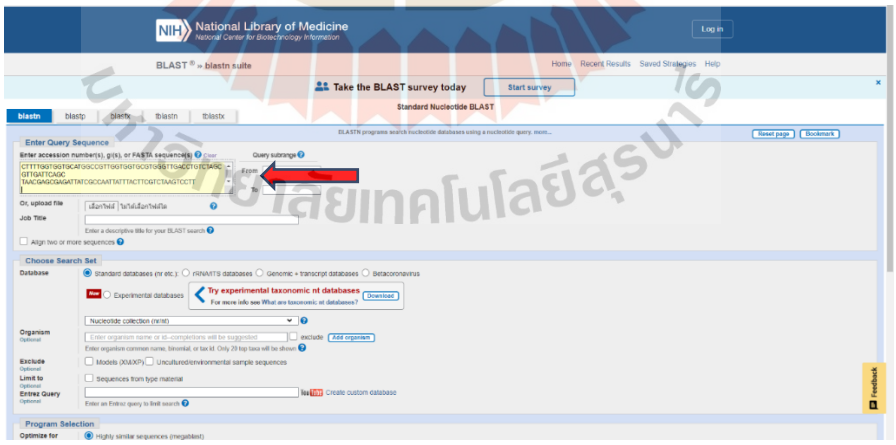


Figure B.2.1 Searching database Nucleotide collection (nr/nt) using Megablast (Optimize for highly similar sequences)

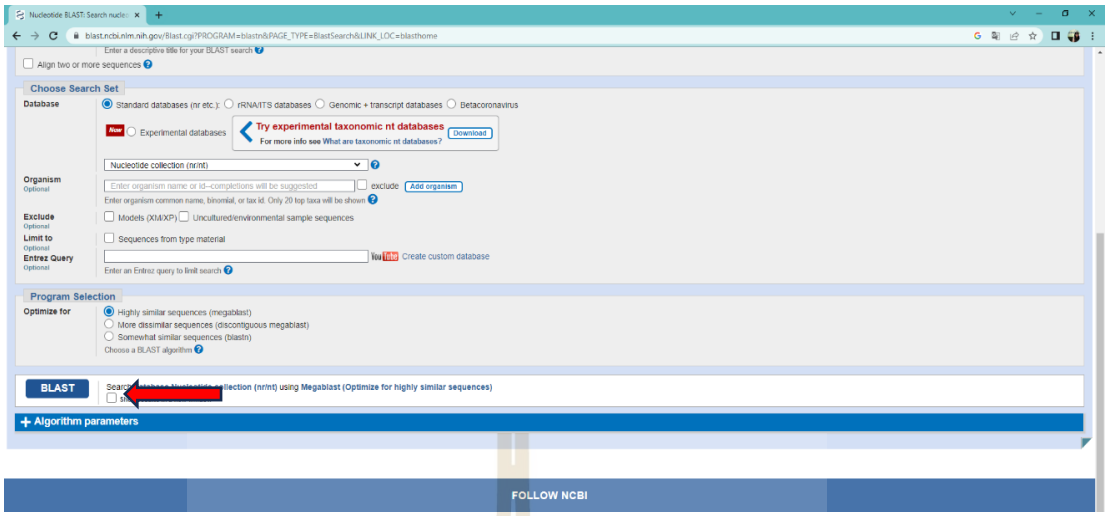


Figure B.2.2 Searching database Nucleotide collection (nr/nt) using Megablast (Optimize for highly similar sequences)

1.4 Results were shown many strains to closely with nucleotide sample

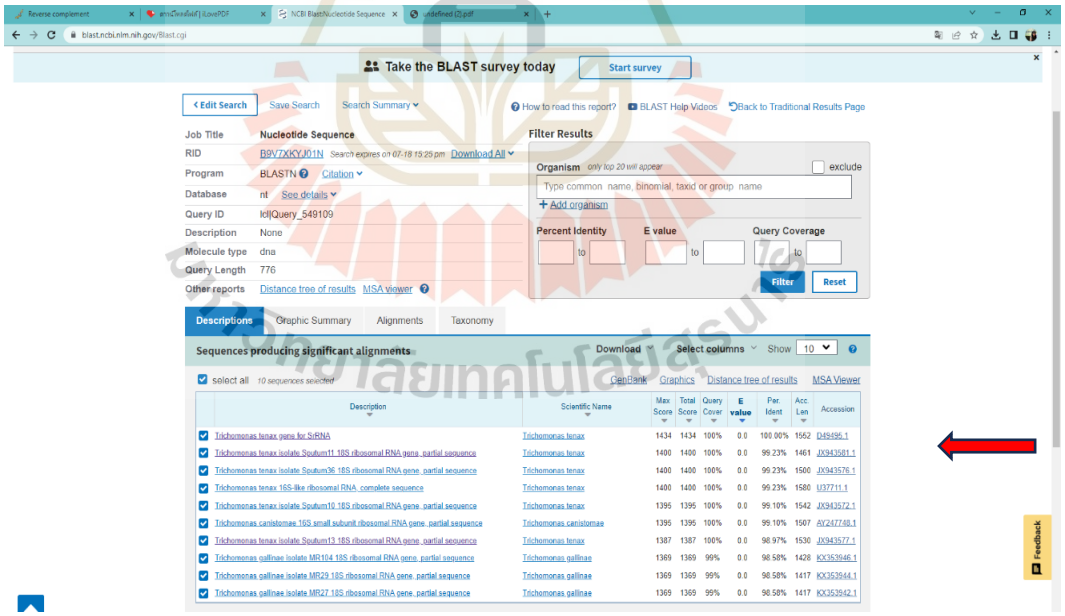


Figure B.3 Results of database Nucleotide collection (nr/nt) using Megablast (Optimize for highly similar sequences)

2. Alignment with BioEdit version 7.2

2.1 Open BioEdit program --> Open or import file (FASTA file of sample)

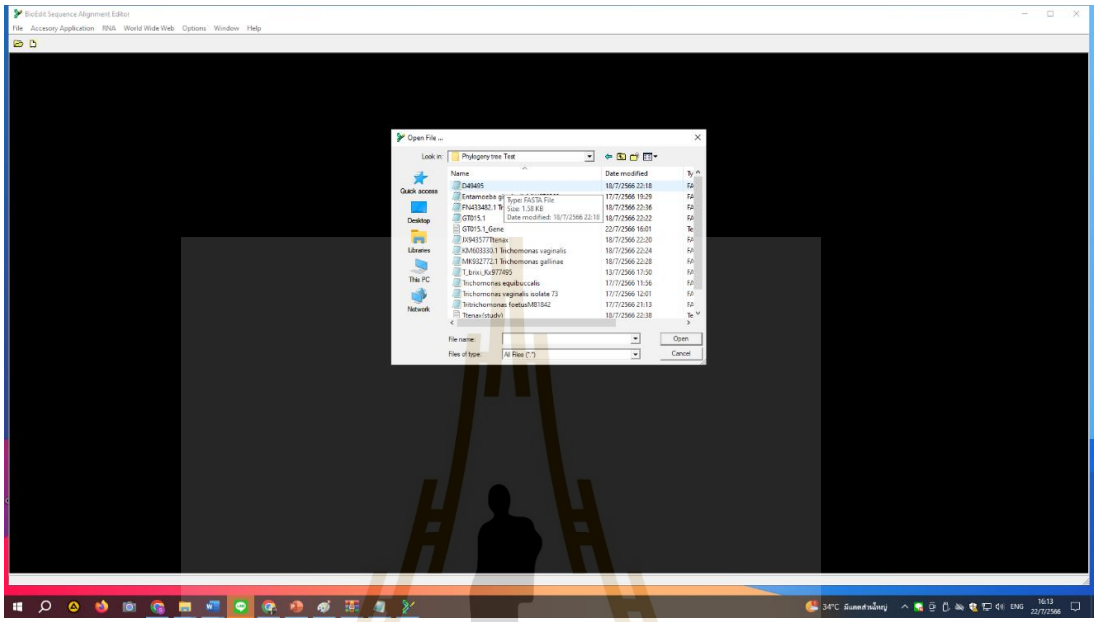


Figure B.4 Insert FASTA file in BioEdit program with opening file

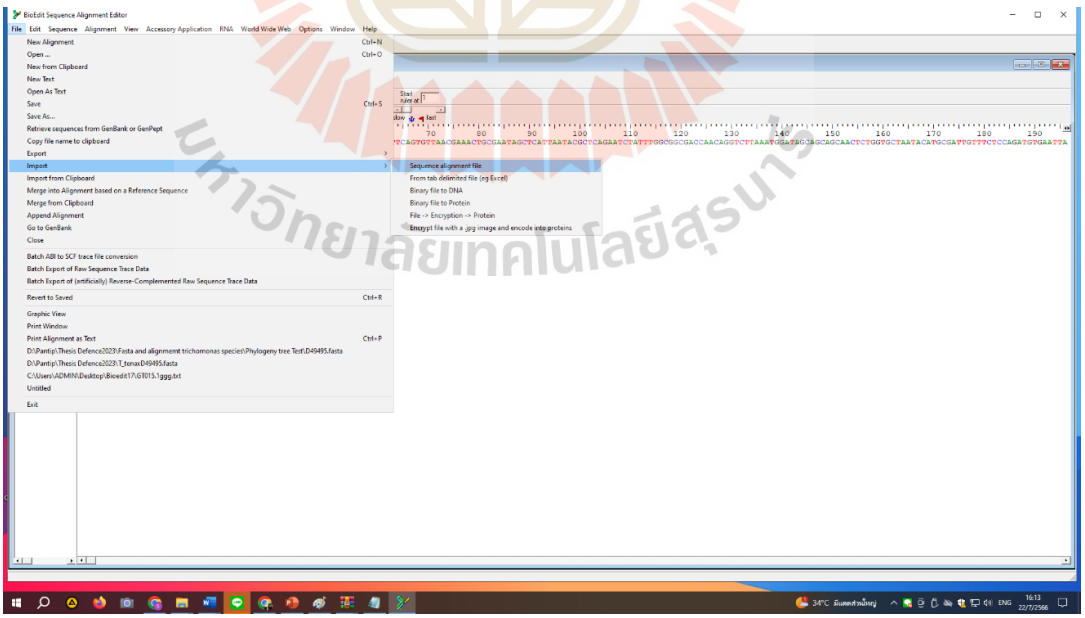


Figure B.5 Insert FASTA file in BioEdit program with import file

2.2 Nucleotide sequence of sample

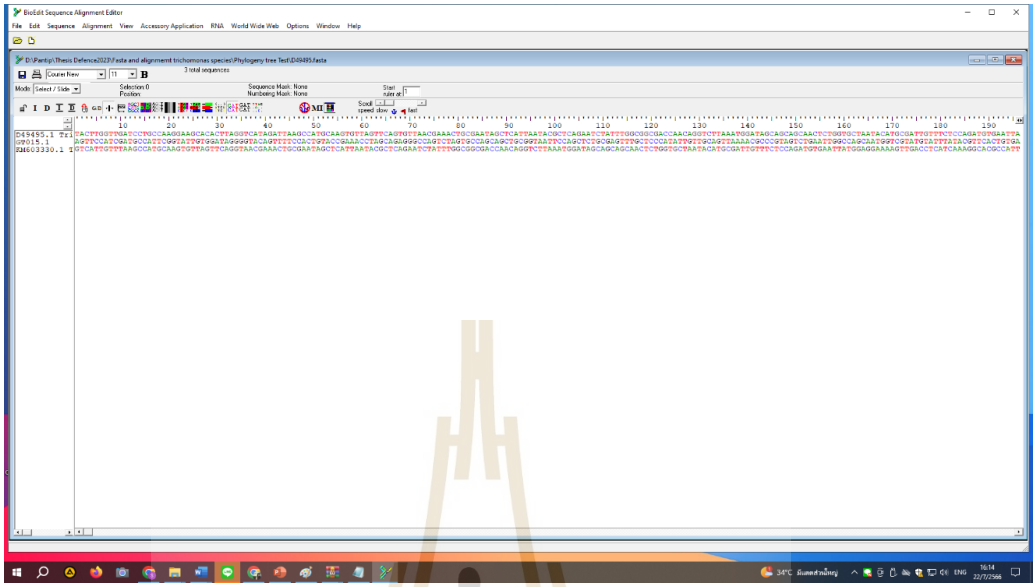


Figure B.6 Nucleotide sequence in BioEdit program

2.3 Select command “Assessory Application”--> CrustalW alignment-->Run ClustalW

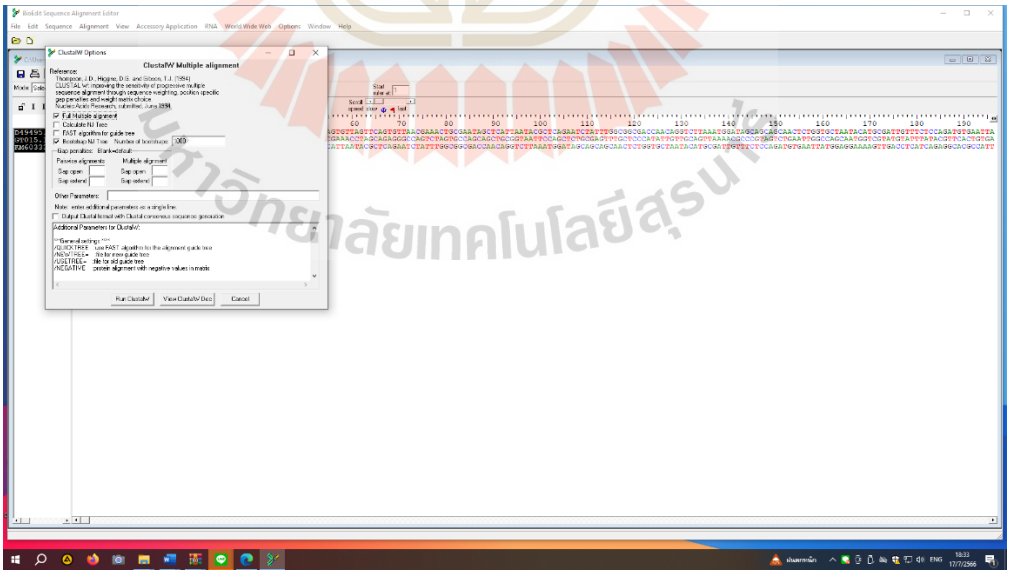


Figure B.7 Run ClustalW alignment

2.4 Select graphic view

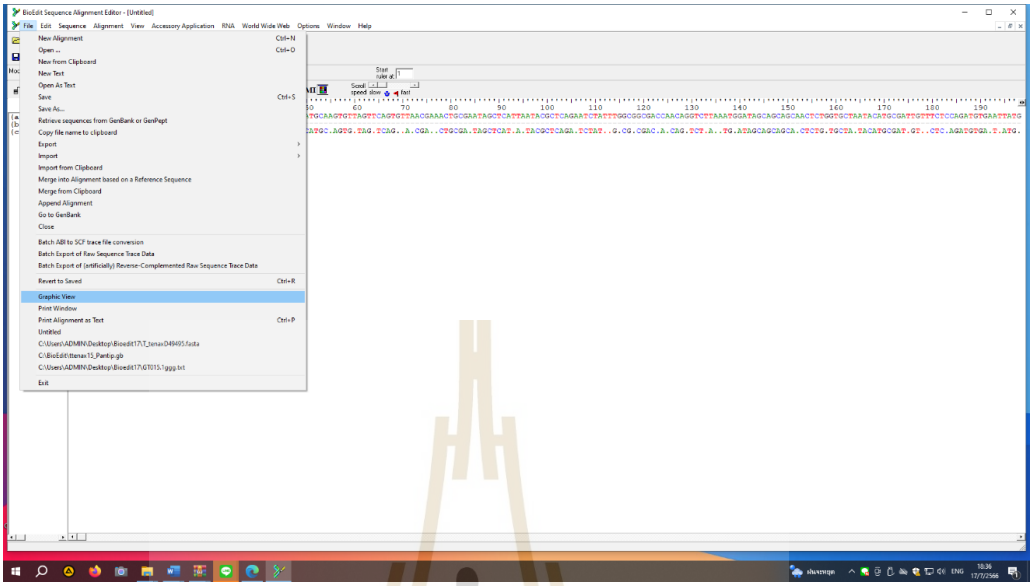


Figure B.8 Select graphic view for alignment view

2.5 Graphic view result

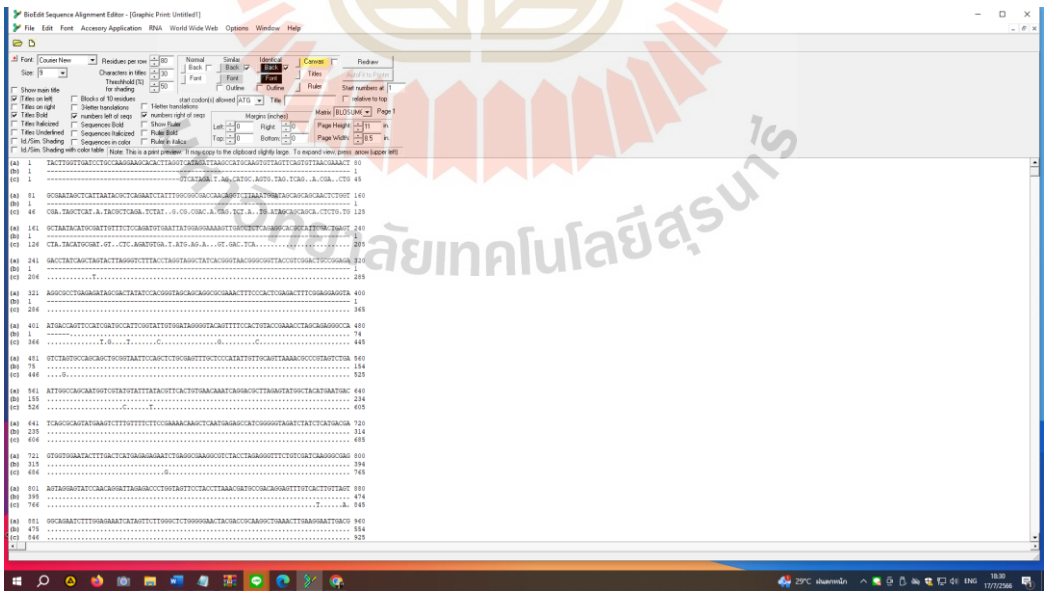


Figure B.9 Result of graphic view

4. Molecular evolutionary genetics analysis with MEGA X (version 11)

4.1 open MEGA X program --> Choose Align -->Edit/ Build Alignment --> Create a new alignment

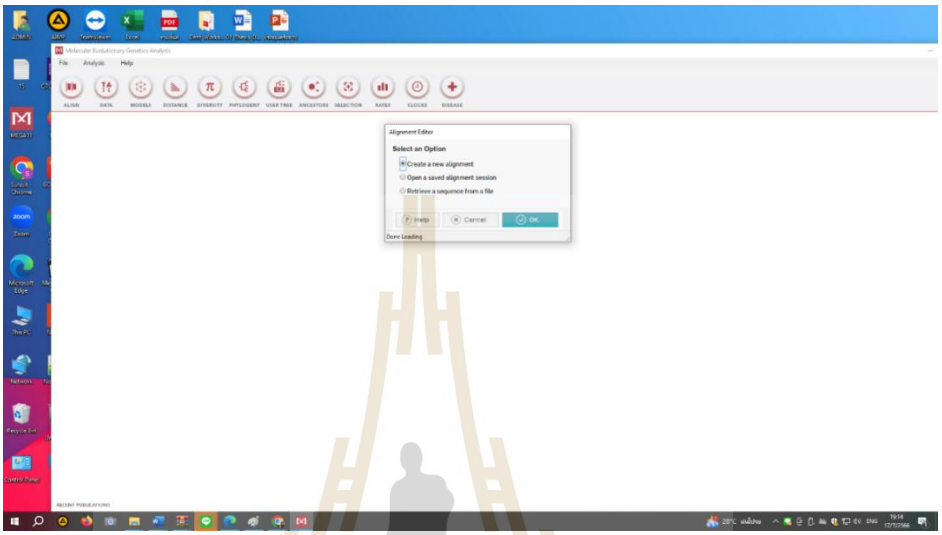


Figure B.10 Creating new alignment in MEGA X

4.2 Select data type for alignment -->DNA

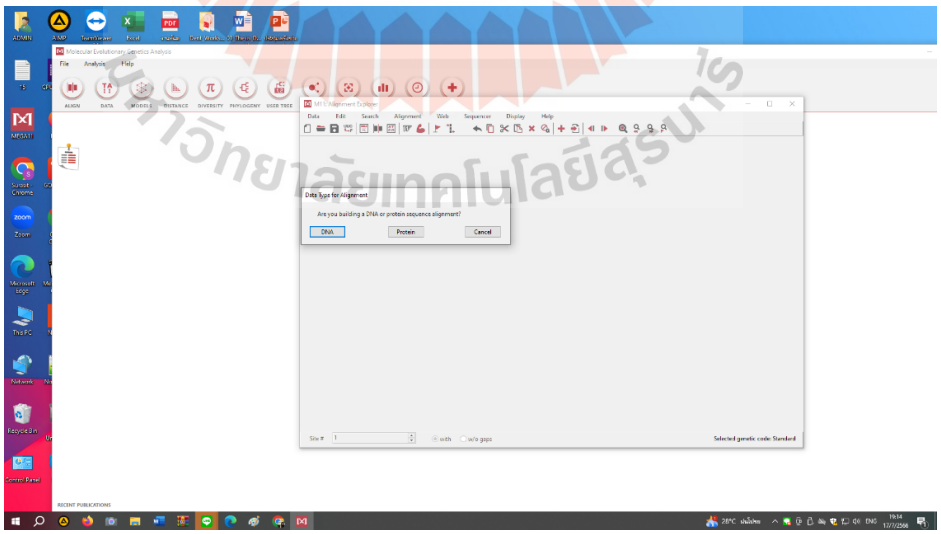


Figure B.11 Select data type for alignment with DNA

4.3 choose Edit --> insert sequence from file

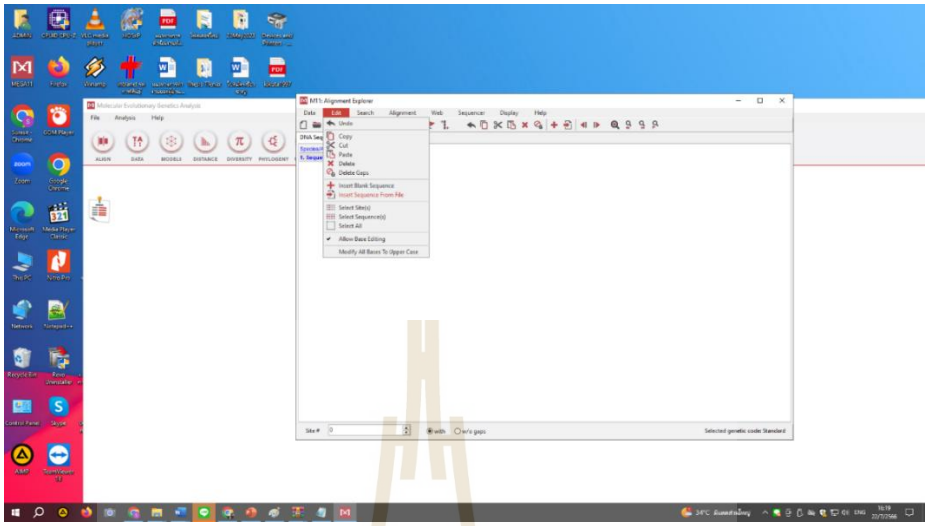


Figure B.12 insert sequence from file

4.4 Sample files as FASTA format

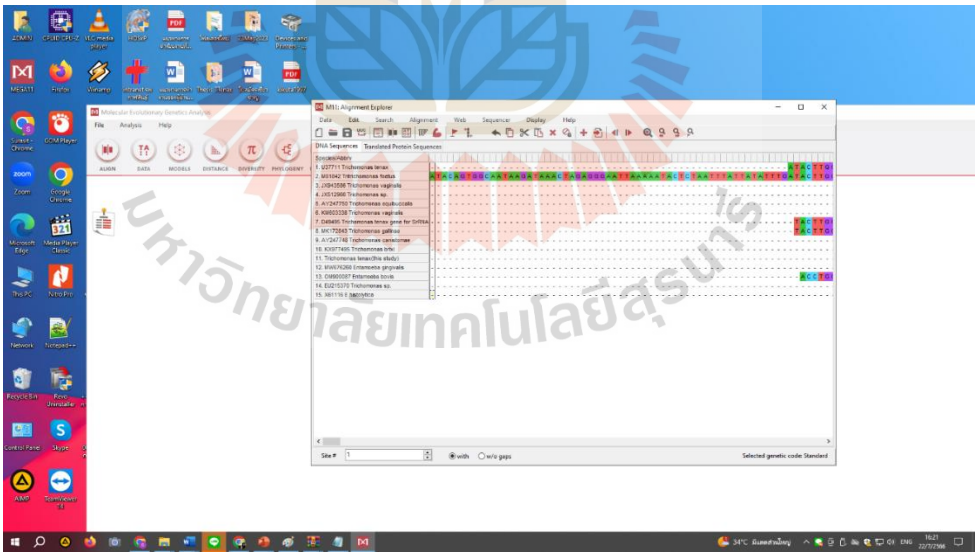


Figure B.13 DNA sequence from FASTA file

4.5 choose Alignment --> align by ClustalW or Align by muscle

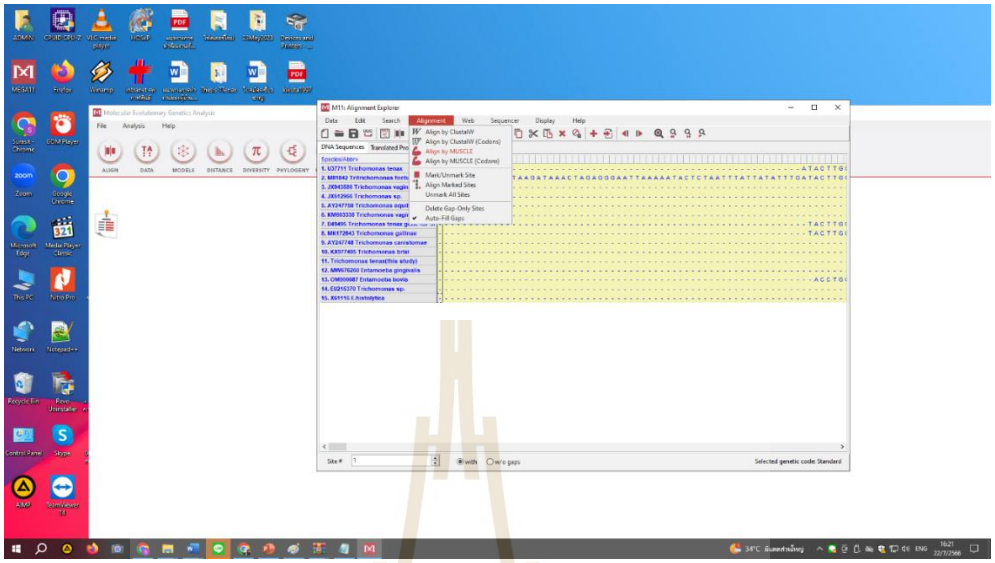


Figure B.14 select alignment by Align by muscle

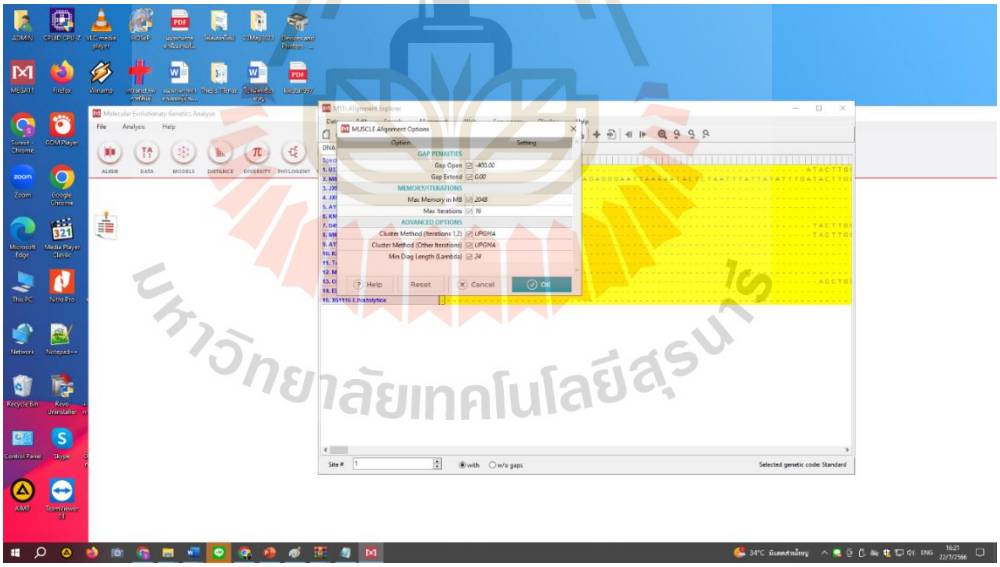


Figure B.15 MUSCLE alignment option

4.6 Result of alignment

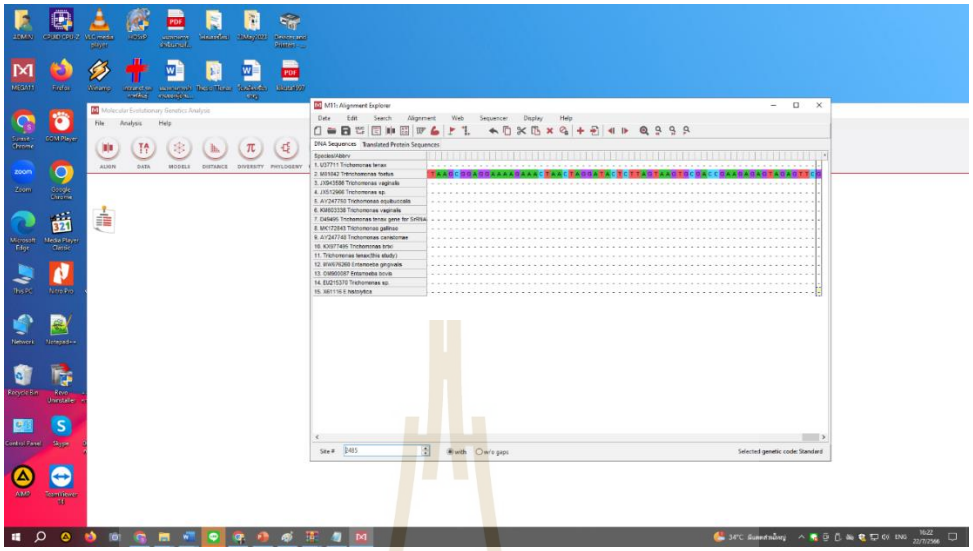


Figure B.16 Result of alignment

4.7 Select File --> Export Alignment --> MEGA Format

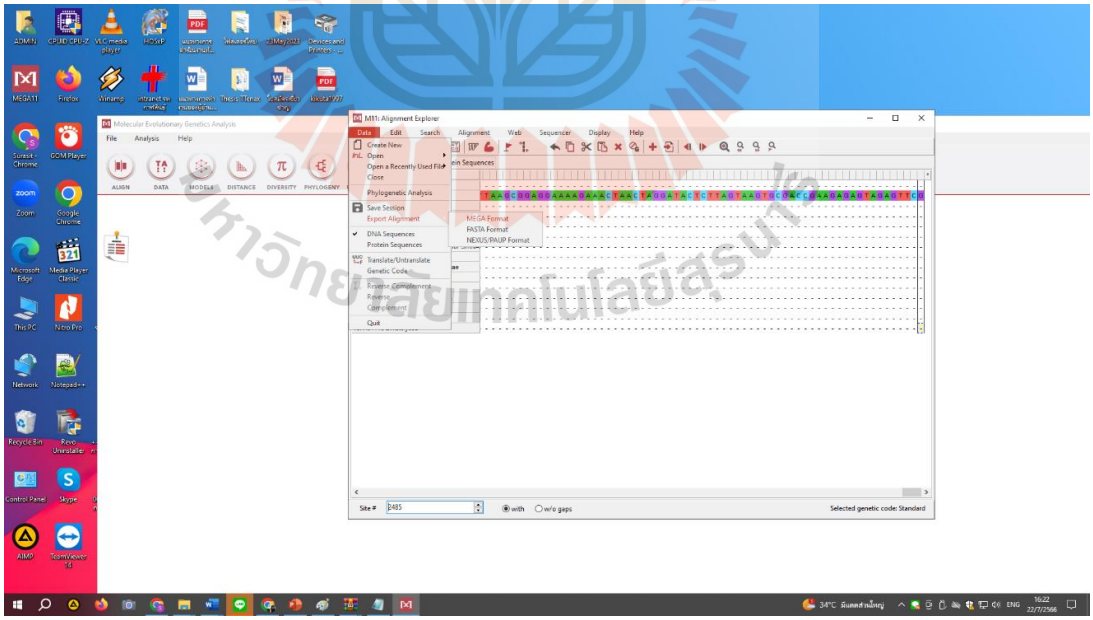


Figure B.17 Export alignment with MEGA format

4.8 select open file/ Session -->Phylogeny --> Construct/ Test Neighbor-Joining tree Phylogeny Reconstruction with Bootstrap method (No. of Bootstrap replication=100)

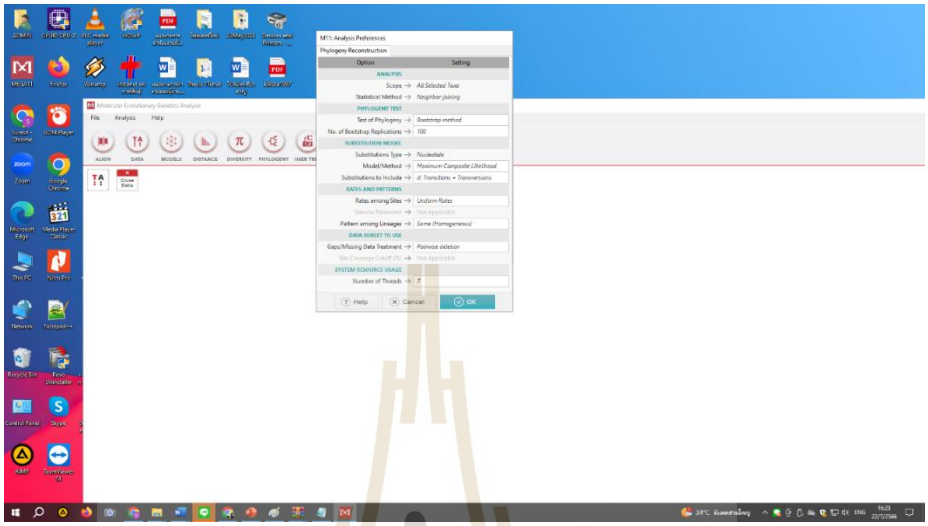


Figure B.18 Construction Phylogeny

4.9 Result of Neighbor- Joining Tree (NJtree) --->Evolution of a Phylogenetic Tree

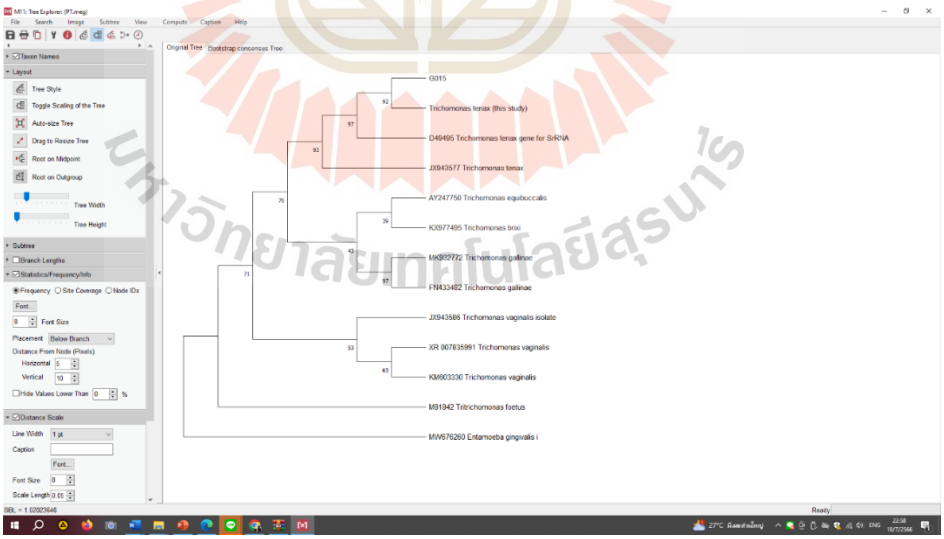


Figure B.19 Evolution of a Phylogenetic Tree

APPENDIX C

QUESTIONNAIRE AND RECORD FORM

แบบสอบถามเลขที่.....
วัน/เดือน/ปี ที่สัมภาษณ์ ...../...../.....

แบบสอบถามเพื่อการประเมินทันตสุขภาพ

ตอนที่ 1 ข้อมูลทั่วไป

คำชี้แจง : ให้กาเครื่องหมาย/ลงในช่องว่าง [ ] ที่ตรงกับความเป็นจริง

1. เพศ [ ] ชาย [ ] หญิง
2. อายุ.....ปี.....เดือน
3. น้ำหนัก.....กก. ส่วนสูง.....ซม.
4. การศึกษา [ ] ไม่ได้เรียน [ ] ประถมศึกษา [ ] มัธยมศึกษา [ ] ปวช./ปวส.
   [ ] ปริญญาตรี [ ] ปริญญาโท หรือสูงกว่า [ ] อื่นๆ (ระบุ).....
5. อาชีพหลัก
   [ ] ข้าราชการ/ พนักงานราชการ/ ลูกจ้างของรัฐ [ ] พนักงาน/ ลูกจ้างเอกชน
   [ ] เกษตรกร [ ] รับจ้างทั่วไป [ ] ค้าขาย [ ] แม่บ้าน/พ่อบ้าน
   [ ] ว่างงาน [ ] นักเรียน /นักศึกษา [ ] อื่นๆ.....
6. ท่านมีโรคประจำตัว หรือโรคทางระบบ ที่แพทย์ระบุ หรือไม่
   [ ] ไม่มี
   [ ] มี ได้แก่
      [ ] เบาหวาน [ ] ความดันโลหิตสูง [ ] ไขมันในเลือดสูง
      [ ] โรคหัวใจและหลอดเลือด [ ] โรคเครียด [ ] อื่นๆ (ระบุ).....

ตอนที่ 2 พฤติกรรมสุขภาพ

7. ท่านสูบบุหรี่ หรือยาเส้นหรือไม่
   [ ] ไม่เคยสูบ [ ] เคยสูบ แต่เลิกมาระยะเวลา.....ปี.....เดือน
   [ ] ยังสูบจนถึงปัจจุบัน เฉลี่ยวันละประมาณ.....มวน
8. ปัจจุบันท่านดื่มแอลกอฮอล์ หรือไม่
   [ ] ไม่ดื่ม [ ] ดื่มเฉพาะเทศกาล/ วันพิเศษ [ ] ดื่มประจำ
9. ท่านเคี้ยวหมากหรือไม่
   [ ] ไม่เคย [ ] เคย แต่เลิกแล้ว [ ] ยังเคี้ยวอยู่
10. ท่านแปรงฟันเป็นประจำ หรือไม่
    [ ] ไม่แปรงฟัน [ ] แปรงฟันวันละ 1 ครั้ง [ ] แปรงฟันอย่างน้อยวันละ 2 ครั้ง

12.การได้รับการตรวจและ/หรือรักษาทันตกรรม
   [ ] เป็นครั้งแรกในรอบ1-2 ปี [ ] ประจำทุกปี [ ] ประจำทุก6 เดือน

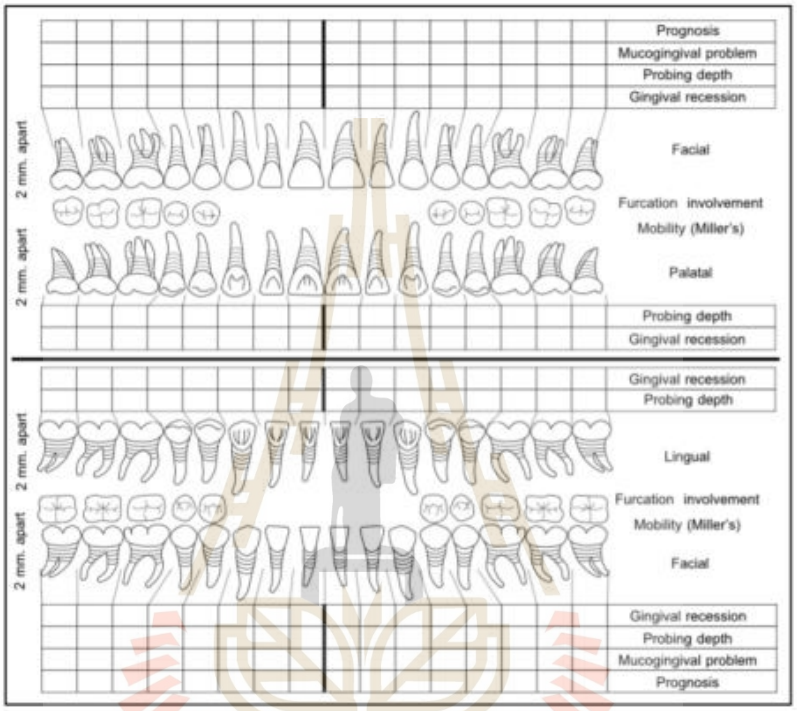
Version 2.0 (วันที่ 20 สิงหาคม 2565)

Figure C.1 Quesionaire Form

ID.....

D/M/Y ...../...../.....

Periodontal Examination Record



PSR Index

PSR code definitions (Landry and Jean, 1997), as below

Code 0 No clinical sign
Code 1 Gingival bleeding on probing
Code 2 Tattar or calculus accumulation on Supra and/or subgingival
Code 3 Periodontal pocket depth between 3.5 mm to 5.5 mm
Code 4 Periodontal pocket depth more than 5.5 mm
Code * Periodontal abnormalities present
Code X presents fewer than 2 teeth per sextant

Remark.....

.....

.....

.....

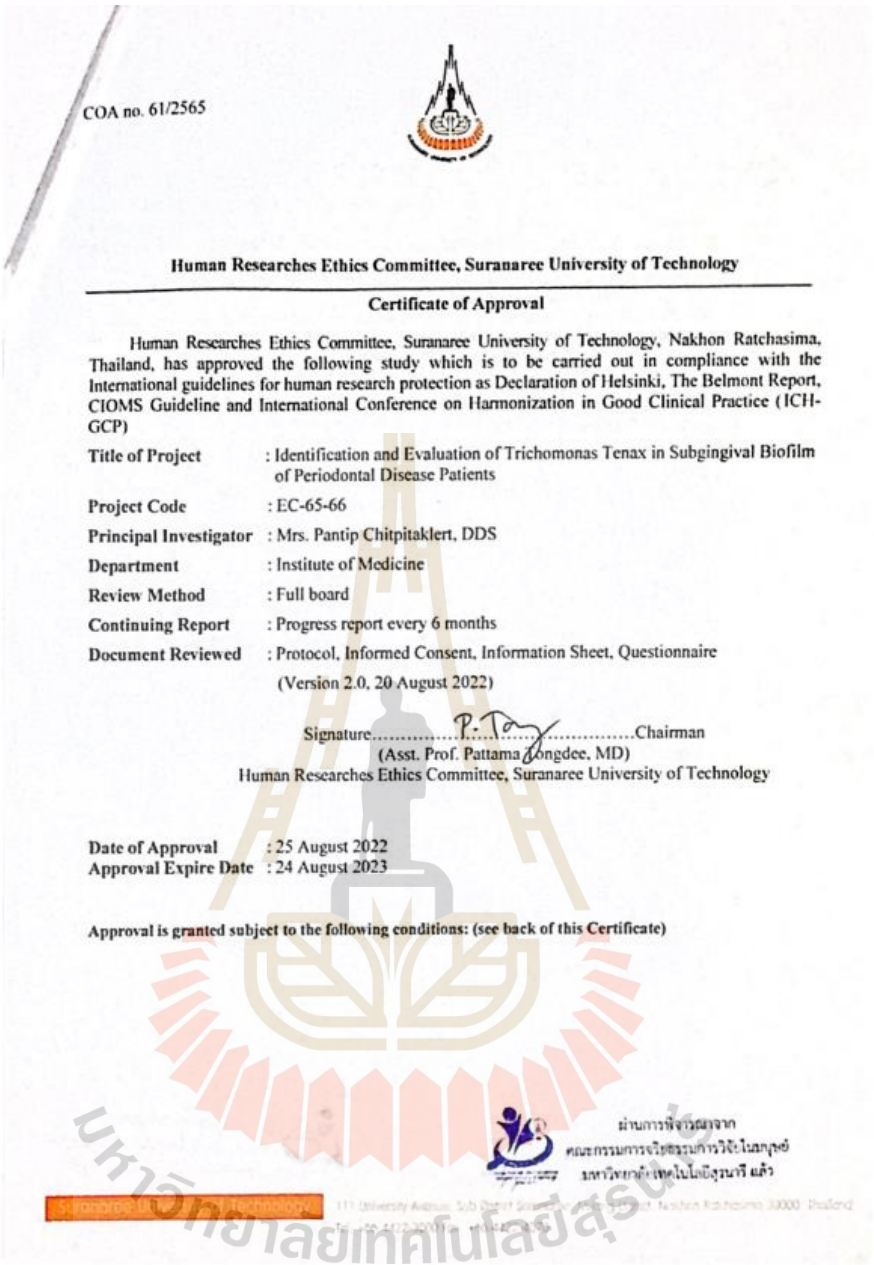
.....

Version 2.0 (วันที่ 20 สิงหาคม 2565)

Figure C.2 Record Form

APPENDIX D

ETHIC COMMITTEE APPROVED DOCUMENTS



COA no. 61/2565

Human Researches Ethics Committee, Suranaree University of Technology

Certificate of Approval

Human Researches Ethics Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand, has approved the following study which is to be carried out in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Title of Project : Identification and Evaluation of Trichomonas Tenax in Subgingival Biofilm of Periodontal Disease Patients

Project Code : EC-65-66

Principal Investigator : Mrs. Pantip Chitpitaklert, DDS

Department : Institute of Medicine

Review Method : Full board

Continuing Report : Progress report every 6 months

Document Reviewed : Protocol, Informed Consent, Information Sheet, Questionnaire (Version 2.0, 20 August 2022)

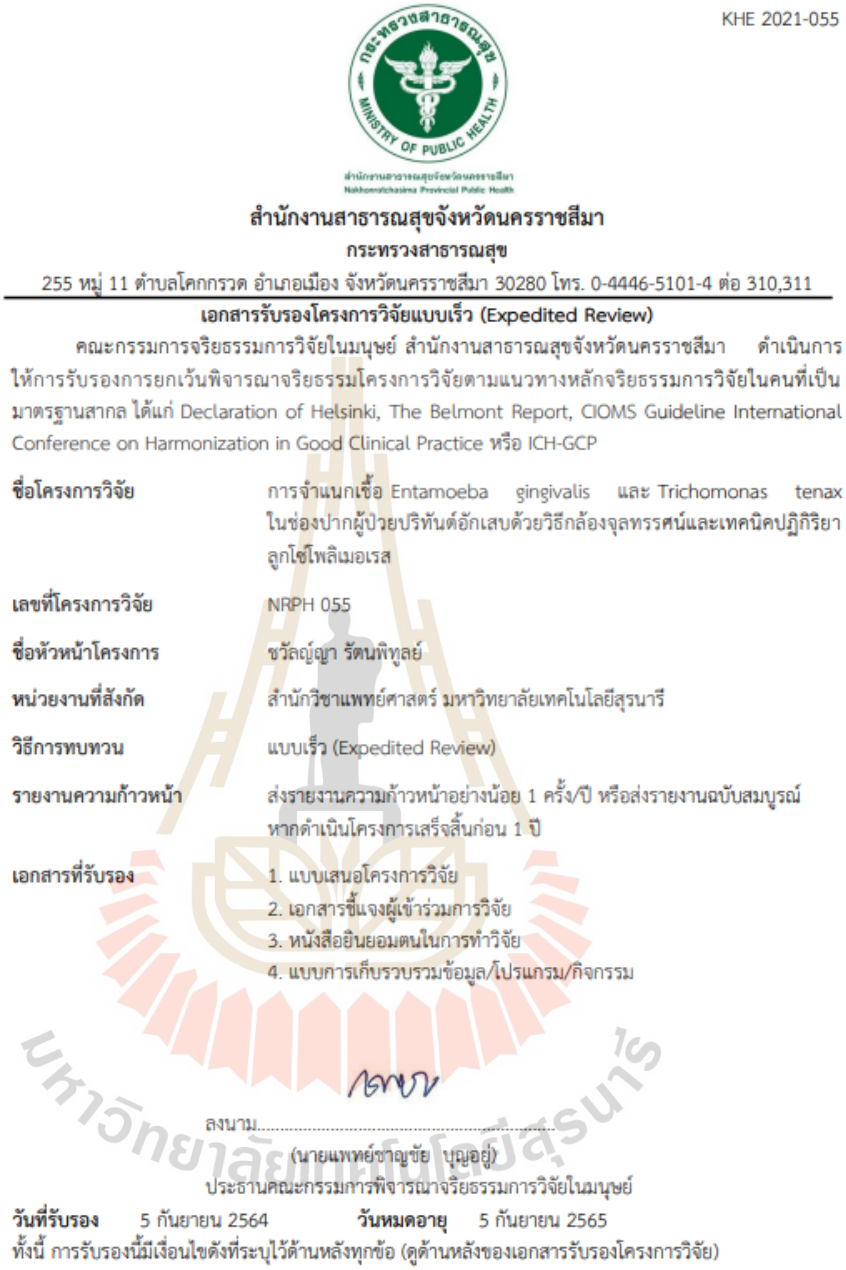
Signature.....Chairman
(Asst. Prof. Pattama Longdee, MD)
Human Researches Ethics Committee, Suranaree University of Technology

Date of Approval : 25 August 2022
Approval Expire Date : 24 August 2023

Approval is granted subject to the following conditions: (see back of this Certificate)

ผ่านการพิจารณาจาก
คณะกรรมการจริยธรรมการวิจัยในมนุษย์
มหาวิทยาลัยเทคโนโลยีสุรนารี แล้ว

Figure D.1 Ethic committee Approved from f Suranaree University of Technology (EC-65-66)



KHE 2021-055

สำนักงานสาธารณสุขจังหวัดนครราชสีมา
กระทรวงสาธารณสุข
255 หมู่ 11 ตำบลโคกกรวด อำเภอเมือง จังหวัดนครราชสีมา 30280 โทร. 0-4446-5101-4 ต่อ 310,311

เอกสารรับรองโครงการวิจัยแบบเร็ว (Expedited Review)

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ สำนักงานสาธารณสุขจังหวัดนครราชสีมา ดำเนินการให้การรับรองการยกเว้นพิจารณาจริยธรรมโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากล ได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการวิจัย	การจำแนกเชื้อ Entamoeba gingivalis และ Trichomonas tenax ในช่องปากผู้ป่วยปริทันต์อักเสบด้วยวิธีกล้องจุลทรรศน์และเทคนิคปฏิกิริยาลูกโซ่โพลิเมอเรส
เลขที่โครงการวิจัย	NRPH 055
ชื่อหัวหน้าโครงการ	ชวัลญ์ญา รัตนพิทูลย์
หน่วยงานที่สังกัด	สำนักวิชาแพทย์ศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี
วิธีการทบทวน	แบบเร็ว (Expedited Review)
รายงานความก้าวหน้า	ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี หรือส่งรายงานฉบับสมบูรณ์หากดำเนินโครงการเสร็จสิ้นก่อน 1 ปี
เอกสารที่รับรอง	1. แบบเสนอโครงการวิจัย 2. เอกสารชี้แจงผู้เข้าร่วมการวิจัย 3. หนังสือยินยอมตนในการทำวิจัย 4. แบบการเก็บรวบรวมข้อมูล/โปรแกรม/กิจกรรม

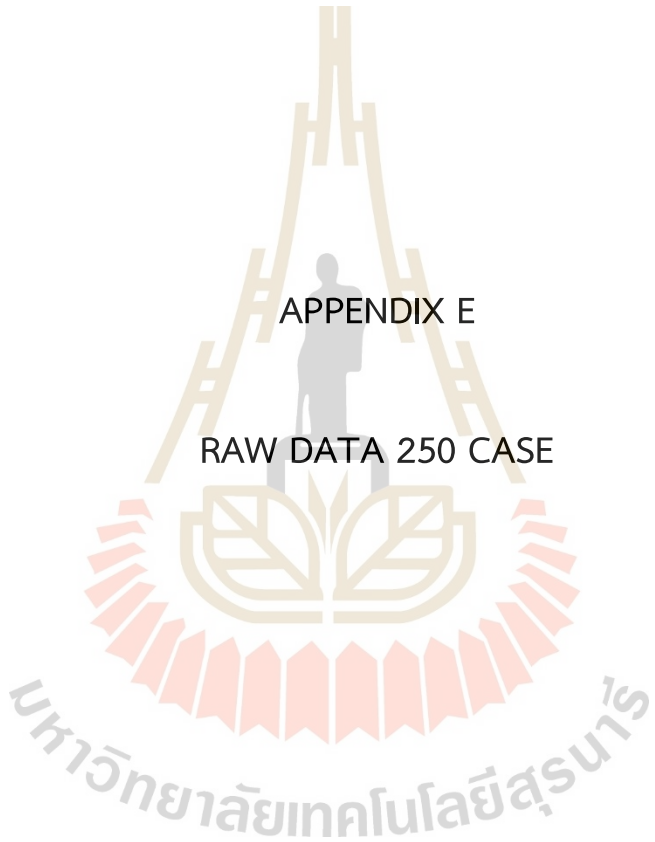
ลงนาม.....
(นายแพทย์ชาญชัย บุญอยู่)
ประธานคณะกรรมการพิจารณาจริยธรรมการวิจัยในมนุษย์

วันที่รับรอง 5 กันยายน 2564 **วันหมดอายุ** 5 กันยายน 2565
ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

Figure D.2 Ethic committee Approved from Nakhon Ratchasima Public Health Provincial office (KHE 2021-055)

APPENDIX E

RAW DATA 250 CASE



id	sex	age	weight	hight	bmi	status	dm	ht	comorbids	systemdz	smoking	alcohol	brushing	psrindex	t_tenaxinfection	pcr	education_gr	perio_gr	psrindex_3gr
1	1	61	91	170	26.8	1	0	1	0	1	0	0	2	2.5	1	1	2	2	2
2	2	50	61	158	19.3	1	0	0	0	0	0	0	2	2.5	1	1	1	2	2
3	1	69	70	165	21.2	1	0	1	0	1	1	1	2	2	0	0	2	1	2
4	1	64	64	163	19.6	1	1	1	1	1	0	1	2	2.7	0	0	2	2	2
5	2	57	62	154	20.1	2	1	0	0	1	0	0	2	1	0	0	2	1	1
6	1	71	57	162	17.6	1	0	1	1	1	0	0	1	2	0	0	2	1	2
7	2	35	52	167	15.6	2	1	1	1	1	0	0	2	2.5	1	1	1	2	2
8	2	70	75	159	23.6	1	1	1	1	1	0	1	2	1.5	0	0	1	2	1
9	2	61	67	154	21.8	1	0	1	1	1	0	0	1	2.3	0	0	1	2	2
10	1	53	85	165	25.8	1	1	0	0	1	0	1	2	1.7	0	0	1	2	1
11	2	53	60	154	19.5	2	0	0	0	0	0	0	2	2.5	0	0	2	2	2
12	2	46	68	134	25.4	2	1	0	0	1	0	0	2	4	0	0	1	2	3
13	1	24	65	168	25.4	2	0	0	0	0	1	0	2	1.2	0	0	2	1	1
14	2	57	57	167	22.4	1	0	0	0	0	0	0	2	1.5	0	0	1	1	1
15	2	57	53	155	17.1	1	0	0	0	0	0	0	2	1.2	0	0	1	1	1
16	2	48	65	155	21	2	0	0	0	0	0	0	2	1.5	0	0	1	2	1
17	2	58	82	168	24.4	2	1	1	1	1	0	0	2	4	0	0	2	2	3
18	1	60	63	173	18.2	1	0	0	0	0	0	0	2	3	0	0	2	2	3
19	1	68	75	165	22.7	1	0	0	0	0	0	1	2	3	0	0	2	2	3
20	2	71	65	152	21.4	1	1	1	1	1	0	0	2	1	0	0	2	1	1
21	1	66	65	167	19.5	2	0	1	0	1	0	0	2	1	0	0	2	1	1
22	2	51	57	167	17.1	1	0	0	0	0	0	0	2	2	0	0	2	1	2
23	2	46	47	155	15.2	2	0	0	0	0	0	0	2	2	0	0	2	1	2
24	2	45	72	165	21.8	1	0	0	0	0	0	0	2	2	0	0	2	1	2
25	2	25	78	165	23.6	2	0	0	0	0	0	1	2	2	0	0	1	1	2
26	2	36	55	152	18.1	2	0	0	0	0	0	0	2	2	0	0	2	1	2
27	2	46	70	155	22.6	1	0	0	0	0	0	0	2	2	0	0	1	1	2
28	1	65	80	168	23.8	1	0	1	0	1	0	1	2	2	0	0	2	1	2
29	1	57	84	177	23.7	1	1	0	0	1	1	0	1	3	0	0	2	2	3
30	2	70	80	162	24.7	1	0	0	0	0	0	0	2	1	0	0	2	1	1
31	1	18	65	170	19.1	2	0	0	0	0	0	1	2	1	0	0	1	1	1
32	1	82	54	160	16.9	1	0	0	0	0	1	0	1	4	0	0	1	2	3
33	2	68	54	149	18.1	1	0	0	0	0	0	0	2	2	0	0	2	1	2
34	2	42	50	153	16.3	2	0	0	0	0	0	1	2	3	0	0	2	2	3
35	2	38	49	159	15.4	2	0	0	0	0	0	0	2	1	0	0	2	1	1
36	1	36	61	170	17.9	2	0	0	0	0	1	1	2	2	0	0	2	1	2
37	2	38	55	152	18.1	1	0	0	0	0	0	0	2	3	0	0	2	2	3
38	1	39	60	170	17.6	2	0	0	0	0	0	1	2	4	0	1	2	2	3
39	2	77	48	155	15.5	1	0	0	0	0	0	0	2	2	0	0	1	1	2
40	1	47	70	179	19.6	2	0	0	0	0	0	1	2	1	0	0	1	1	1
41	1	38	77	175	23.6	1	0	0	1	1	0	0	2	2	0	0	2	1	2
42	2	52	54	150	18	1	0	0	0	0	0	0	2	2	0	0	2	1	2
43	1	52	52	154	16.9	2	0	0	0	0	0	0	2	1	0	0	2	1	1
44	1	70	77	172	22.4	1	0	0	0	0	0	1	2	1	0	0	2	1	1
45	1	50	60	170	17.6	2	0	0	0	0	1	1	2	2	0	0	2	1	2
46	1	31	75	175	21.4	2	0	0	0	0	1	1	2	3	0	0	1	2	3
47	1	28	55	174	15.8	2	0	0	0	0	1	1	2	2	0	0	1	1	2
48	2	45	63	155	20.3	2	0	0	0	0	0	0	2	2	0	0	1	1	2
49	2	52	54	150	18	1	0	0	0	0	0	0	2	4	0	0	2	2	3
50	2	60	63	162	19.4	1	0	0	0	0	0	0	2	1	0	0	2	1	1
51	2	48	72	163	22.1	1	0	0	0	0	0	0	2	1	0	0	2	1	1
52	2	60	52	159	16.4	2	0	0	0	0	0	0	2	2	0	0	1	1	2
53	1	62	78	160	24.4	1	0	0	0	0	0	1	2	1	0	0	2	1	1
54	2	68	55	160	17.2	1	0	0	0	0	0	1	2	2	0	0	2	1	2
55	2	44	69	165	20.9	2	0	0	0	0	0	0	2	2	0	0	2	1	2
56	2	49	49	162	15.1	1	0	0	0	0	0	0	2	2	0	0	1	1	2
57	1	56	85	174	24.4	1	0	1	1	1	1	1	2	4	0	0	2	2	3
58	1	72	53	161	16.5	1	1	0	1	1	0	1	2	3	0	0	2	2	3
59	1	35	82	175	23.4	2	0	0	0	0	0	1	2	2	0	0	1	1	2
60	2	63	70	158	22.2	2	1	1	1	1	0	1	2	2	0	0	2	1	2
61	2	63	67	169	19.8	2	0	0	0	0	0	1	2	2	0	0	2	1	2
62	2	65	49	153	16	1	0	0	0	0	0	0	2	3	0	0	2	2	3
63	2	49	52	152	17.1	1	0	0	0	0	0	0	2	2	0	0	2	1	2
64	2	24	36	153	11.8	2	0	0	0	0	0	0	2	2	0	0	1	1	2
65	2	48	57	168	17	1	0	0	0	0	0	1	2	2	0	0	2	1	2
66	1	24	63	171	18.4	2	0	0	0	0	0	0	2	2	0	0	1	1	2
67	1	59	88	182	24.2	1	1	1	1	1	0	1	2	2	0	0	2	1	2
68	1	52	70	169	20.7	1	0	0	0	0	1	1	2	2	0	0	2	1	2
69	2	55	51	160	15.9	1	0	0	0	0	0	0	2	2	0	0	1	1	2
70	1	45	70	160	21.9	2	0	0	0	0	0	1	2	2	0	0	2	1	2
71	1	61	78	170	22.9	1	0	0	0	0	1	1	2	2	0	0	2	1	2
72	1	61	72	160	22.5	1	0	0	0	0	1	1	2	2	0	0	1	1	2
73	1	76	77	159	24.2	1	0	0	1	1	1	0	2	4	0	0	1	2	3
74	2	51	68	164	20.7	2	0	0	0	0	0	0	2	1	0	0	2	1	1
75	1	66	72	165	21.8	1	0	1	0	1	0	1	2	2	0	0	2	1	2
76	2	44	60	155	19.4	2	0	0	0	0	0	1	2	4	0	0	1	2	3
77	1	41	70	172	20.3	2	0	0	0	0	0	1	2	1	0	0	2	1	1
78	2	62	64	160	20	1	0	0	0	0	0	0	2	3	0	0	1	2	3
79	1	29	85	175	24.3	2	0	0	0	0	1	1	2	2	0	0	1	1	2
80	1	72	72	168	21.4	1	0	0	0	0	0	1	2	2	0	0	1	1	2
81	1	24	90	180	25	2	0	0	0	0	0	1	2	2	0	0	2	1	2
82	2	59	50	152	16.4	1	0	0	0	0	0	0	2	2	0	0	1	1	2
83	2	18	89	160	27.8	2	0	0	0	0	0	0	2	2	0	0	1	1	2

Figure E.1 Raw Data and Demographic Characteristics of Samples

84	2	44	59	158	18.7	2	0	0	0	0	0	0	2	2	0	0	2	1	2
85	2	53	50	152	16.4	1	0	0	0	0	0	0	2	2	0	0	2	1	2
86	1	35	77	167	23.1	2	0	0	0	0	1	1	2	2	0	0	2	1	2
87	2	40	48	147	16.3	1	0	0	0	0	0	1	2	2	0	0	2	1	2
88	2	48	52	165	15.8	1	0	0	0	0	0	0	2	2	0	0	2	1	2
89	2	68	45	145	15.5	1	0	0	0	0	0	0	2	2	0	0	1	1	2
90	1	21	68	155	21.9	2	0	0	0	0	0	1	2	2	0	0	1	1	2
91	2	23	52	161	16.1	2	0	0	0	0	0	1	2	2	0	0	2	1	2
92	1	72	57	162	17.6	1	0	0	0	0	0	0	2	1	0	0	2	1	1
93	2	19	68	158	21.5	2	0	0	0	0	0	0	2	2	0	0	1	1	2
94	2	31	47	152	15.5	2	0	0	0	0	0	0	2	1	0	0	1	1	1
95	1	50	90	175	25.7	2	0	0	0	0	1	1	2	2.5	1	1	1	2	2
96	2	52	62	157	19.7	1	0	0	0	0	0	0	2	1	0	0	2	1	1
97	2	60	57	154	18.5	1	0	0	0	0	0	0	2	2	0	0	2	1	2
98	2	46	62	155	20	1	0	0	0	0	0	0	2	3	1	1	1	2	3
99	2	63	82	163	25.2	1	0	0	0	0	0	0	2	4	0	0	1	2	3
100	1	84	72	165	21.8	1	0	0	0	0	0	0	2	2.5	1	1	1	2	2
101	1	43	70	170	20.6	2	0	0	0	0	1	1	2	2	0	0	2	1	2
102	1	20	40	155	12.9	2	0	0	0	0	0	1	2	2.5	1	1	1	2	2
103	1	20	49	162	15.1	2	0	0	0	0	0	0	2	2.3	1	1	1	2	2
104	2	43	62	158	19.6	1	0	0	0	0	0	0	2	2	0	0	2	1	2
105	1	22	53	165	16.1	2	0	0	0	0	0	1	2	1	0	0	1	1	1
106	1	66	68	167	20.4	1	0	0	0	0	0	0	2	1	0	0	2	1	1
107	2	43	58	159	18.2	2	0	0	0	0	0	1	2	2	0	0	2	1	2
108	2	56	50	170	14.7	2	0	0	0	0	0	0	2	3	0	0	2	2	3
109	2	25	47	160	14.7	2	0	0	0	0	0	0	2	1	0	0	1	1	1
110	2	19	82	169	24.3	2	0	0	0	0	0	1	2	2	0	0	1	1	2
111	1	22	62	167	18.6	2	0	0	0	0	0	1	2	2	0	0	1	1	2
112	2	19	50	170	14.7	2	0	0	0	0	0	0	2	2	0	0	1	1	2
113	1	20	52	171	15.2	2	0	0	0	0	0	0	2	1	0	0	1	1	1
114	1	19	92	170	27.1	2	0	0	0	0	1	0	2	2	0	0	1	1	2
115	2	20	51	175	14.6	2	0	0	0	0	0	0	2	1	0	0	1	1	1
116	1	73	74	165	22.4	1	0	0	0	0	0	0	2	3.2	1	1	2	2	3
117	1	71	61	169	18	1	0	0	0	0	0	0	2	2.7	1	1	2	2	2
118	2	36	45	157	14.3	1	0	0	0	0	0	0	2	2.3	1	1	2	2	2
119	2	30	54	157	17.2	2	0	0	0	0	0	0	2	2.5	1	1	2	2	2
120	1	48	72	165	21.8	2	0	0	0	0	0	1	2	2.5	1	1	1	2	2
121	2	68	64	160	20	1	0	0	0	0	0	0	2	3	1	1	1	2	3
122	1	20	57	170	16.8	2	0	0	0	0	0	1	2	2.5	1	1	1	2	2
123	2	53	68	150	22.7	2	0	0	0	0	0	0	2	1	0	0	2	1	1
124	2	58	59	159	18.6	2	0	0	0	0	0	0	2	4	0	0	2	2	3
125	2	67	60	150	20	1	1	1	1	1	0	0	2	4	0	0	2	2	3
126	2	45	49	159	15.4	1	0	0	0	0	0	0	2	1	0	0	2	1	1
127	1	29	72	175	20.6	2	0	0	0	0	0	1	2	1	0	0	2	1	1
128	2	62	69	160	21.6	1	0	1	0	1	0	0	2	4	0	0	1	2	3
129	1	22	54	173	15.6	2	0	0	0	0	0	1	2	1	0	0	1	1	1
130	2	48	52	165	15.8	1	0	0	0	0	0	0	2	4	0	0	1	2	3
131	2	42	48	159	15.1	2	0	0	0	0	0	1	2	4	1	1	2	2	3
132	1	21	67	164	20.4	2	0	0	0	0	1	1	2	2	0	0	1	1	2
133	2	32	85	155	27.4	1	0	0	0	0	0	0	2	2	0	0	2	1	2
134	2	25	47	171	13.7	2	0	0	0	0	0	1	2	2	0	0	2	1	2
135	2	67	60	150	20	1	1	1	1	1	0	0	2	4	0	0	2	2	3
136	2	49	49	162	15.1	1	0	0	0	0	0	0	2	3	0	0	2	2	3
137	1	80	60	160	18.8	1	1	1	1	1	0	0	2	1	0	0	1	1	1
138	1	18	56	159	17.6	2	0	0	0	0	0	0	1	2	0	0	1	1	2
139	1	62	78	160	24.4	1	0	1	0	1	0	0	2	2	1	1	2	1	2
140	1	72	50	160	15.6	1	0	1	0	1	0	0	2	3	0	0	2	2	3
141	1	30	70	180	19.4	2	0	0	0	0	0	0	2	3	0	0	1	2	3
142	1	59	66	167	19.8	1	0	0	0	0	0	1	2	3	0	0	2	2	3
143	2	48	70	163	21.5	1	0	0	0	0	0	1	2	1	0	0	2	1	1
144	1	42	76	168	22.6	2	0	0	0	0	0	1	2	3	0	0	2	1	3
145	1	63	35	158	11.1	1	0	0	0	0	1	1	2	1	0	0	1	1	1
146	2	24	42	158	13.3	2	0	0	0	0	0	0	2	2	0	0	1	1	2
147	1	19	72	172	20.9	2	0	0	0	0	1	1	2	2	0	0	1	1	2
148	2	23	70	166	21.1	2	0	0	0	0	0	1	2	2	0	0	2	1	2
149	2	55	70	163	21.5	1	0	0	0	0	0	0	2	2	0	0	2	1	2
150	2	37	56	168	16.7	2	0	0	0	0	0	0	2	1	0	0	2	1	1
151	1	21	81	163	24.8	2	0	0	0	0	1	1	2	2	0	0	1	1	2
152	2	35	66	160	20.6	1	0	0	0	0	0	0	2	2	0	0	2	1	2
153	1	61	77	174	22.1	1	0	1	0	1	0	1	2	2	0	0	2	1	2
154	2	29	54	150	18	1	0	0	0	0	0	0	2	2	0	0	1	1	2
155	1	57	79	173	22.8	1	0	0	0	0	0	0	2	2	0	0	2	1	2
156	2	38	77	160	24.1	1	0	0	0	0	0	0	2	2	0	0	1	1	2
157	1	67	75	175	21.4	1	0	0	0	0	0	0	2	4	0	0	2	2	3
158	1	60	70	165	21.2	1	0	0	0	0	1	1	2	4	0	0	2	2	3
159	2	22	72	160	22.5	2	0	0	0	0	0	0	2	1	0	0	1	1	1
160	1	77	69	170	20.3	1	0	1	1	1	0	0	2	1	0	0	2	1	1
161	2	49	69	155	22.3	1	0	0	0	0	0	1	2	2	0	0	2	1	2
162	1	73	74	165	22.4	1	0	0	0	0	0	0	2	4	0	0	2	2	3
163	2	34	60	158	19	2	0	0	0	0	0	1	2	4	1	1	2	2	3
164	2	41	61	162	18.8	1	0	0	0	0	0	0	2	2	0	0	2	1	2
165	2	23	110	166	33.1	1	1	0	0	1	0	0	2	2	0	0	1	1	2
166	2	29	55	155	17.7	1	0	0	0	0	0	0	2	1	0	0	2	1	1
167	2	61	59	159	18.6	1	0	0	0	0	0	0	2	2	0	0	2	1	2

Figure E.1 Raw Data and Demographic Characteristics of Samples (Continued)

168	1	64	65	160	20.3	1	1	0	0	1	0	1	2	2	0	0	2	1	2
169	2	20	60	170	17.6	2	0	0	0	0	0	0	2	1	0	0	1	1	1
170	2	50	52	162	16	1	0	0	0	0	0	0	2	1.5	0	0	2	2	1
171	2	54	67	152	22	2	1	1	1	1	0	0	2	1.67	0	0	1	1	1
172	1	30	70	180	19.4	2	0	0	0	0	1	1	2	2.3	0	0	1	2	2
173	1	22	60	165	18.2	2	0	0	0	0	0	0	2	2	0	0	1	1	2
174	2	79	60	155	19.4	1	1	1	1	1	0	0	2	2	0	0	1	2	2
175	1	39	89	168	26.5	2	0	0	0	0	1	1	1	2.3	0	0	1	2	2
176	2	65	70	163	21.5	1	0	0	0	0	0	0	2	1.8	0	0	2	1	1
177	1	58	62	160	19.4	1	0	0	0	0	1	0	2	1.67	0	0	2	1	1
178	1	62	85	171	24.9	1	0	1	0	1	1	0	2	3	0	1	2	2	3
179	2	59	56	153	18.3	1	0	0	0	0	0	0	2	2	0	0	1	1	2
180	2	65	50	153	16.3	1	0	0	0	0	0	0	2	1	0	0	2	1	1
181	1	62	85	171	24.9	1	0	1	0	1	1	0	2	3	0	0	2	2	3
182	1	57	60	169	17.8	1	0	1	0	1	1	1	1	3.3	0	0	1	2	3
183	1	38	70	170	20.6	1	0	0	0	0	1	1	1	2.16	1	1	1	2	2
184	2	56	51	155	16.5	1	0	0	0	0	0	0	2	2.3	0	0	2	2	2
185	2	44	62	153	20.3	2	0	0	0	0	0	0	2	3	0	0	1	2	3
186	2	28	51	153	16.7	1	0	0	0	0	0	0	2	2	0	0	1	1	2
187	2	43	50	149	16.8	2	0	0	0	0	0	0	2	2.16	0	0	2	2	2
188	1	64	60	170	17.6	1	0	0	0	0	1	1	1	2.6	0	0	2	2	2
189	1	29	52	172	15.1	1	0	0	0	0	0	0	2	1.3	0	0	1	1	1
190	2	66	50	150	16.7	1	0	1	0	1	0	0	2	1	0	0	1	1	1
191	2	48	60	156	19.2	1	0	0	0	0	0	1	2	1	0	0	2	1	1
192	2	56	54	150	18	1	1	1	1	1	0	1	2	3.5	0	0	1	2	3
193	2	19	70	160	21.9	2	0	0	0	0	0	1	2	2	0	0	1	1	2
194	1	19	55	177	15.5	2	0	0	0	0	0	1	2	1.8	0	0	1	1	1
195	1	20	57	177	16.1	2	0	0	0	0	0	1	2	2	0	0	1	1	2
196	2	29	49	160	15.3	2	0	0	0	0	0	0	2	1.8	0	0	2	1	1
197	2	29	99	165	30	1	0	1	0	1	0	1	2	1.67	0	0	1	1	1
198	2	21	53	159	16.7	2	0	0	0	0	0	0	2	1.8	0	0	1	1	1
199	1	71	96	168	28.6	1	1	1	1	1	0	1	1	3.3	0	0	2	2	3
200	1	50	69	168	20.5	2	0	0	0	0	1	0	1	1.8	0	0	1	1	1
201	2	29	60	160	18.8	2	0	0	0	0	0	0	2	2	1	1	2	1	2
202	1	43	112	178	31.5	2	1	1	1	1	0	0	2	1.8	0	0	2	1	1
203	2	52	85	150	28.3	1	0	1	0	1	0	0	2	2.67	0	0	2	2	2
204	1	68	66	172	19.2	1	0	0	0	0	0	1	2	1.16	0	0	1	1	1
205	2	67	66	153	21.6	1	1	1	1	1	0	0	2	2	0	0	2	1	2
206	2	31	44	161	13.7	2	0	0	0	0	0	0	2	1.2	0	0	2	1	1
207	2	55	60	158	19	1	0	0	0	0	0	0	2	2.3	0	0	2	2	2
208	2	22	75	170	22.1	2	0	0	0	0	0	0	1	2.83	0	0	1	2	2
209	1	21	62	170	18.2	2	0	0	0	0	0	1	2	2	0	0	1	1	2
210	2	45	43	149	14.4	2	0	0	0	0	0	0	2	2	0	0	2	1	2
211	2	59	53	160	16.6	1	0	0	0	0	0	0	2	1.67	1	1	2	1	1
212	2	43	67	163	20.6	2	0	1	0	1	0	1	2	3	0	0	2	2	3
213	1	19	61	174	17.5	2	0	0	0	0	1	1	2	1.67	1	0	1	1	1
214	1	60	51	167	15.3	1	0	0	0	0	0	1	2	1.5	1	1	2	1	1
215	1	64	67	174	19.3	1	0	1	0	1	1	1	2	2	0	1	2	1	2
216	1	62	81	166	24.4	1	1	1	1	1	1	1	2	2.5	0	0	2	2	2
217	1	30	55	163	16.9	1	0	0	0	0	0	1	2	2	0	0	2	1	2
218	2	27	66	155	21.3	2	0	0	0	0	0	1	2	1.83	0	0	2	1	1
219	2	25	50	163	15.3	2	0	0	0	0	0	1	2	2	0	0	2	1	2
220	1	60	68	161	21.1	1	0	0	0	0	1	1	2	3.17	0	0	2	2	3
221	2	20	53	165	16.1	2	0	0	0	0	0	0	2	2	0	0	1	1	2
222	1	18	60	172	17.4	2	0	0	0	0	0	0	2	2	0	0	1	1	2
223	2	66	57	158	18	1	0	0	0	0	0	0	2	2.5	0	0	1	2	2
224	2	38	48	162	14.8	1	0	0	0	0	0	0	2	2.17	0	0	1	2	2
225	2	55	60	158	19	1	0	0	0	0	0	0	2	2.17	0	0	2	2	2
226	1	41	100	170	29.4	2	1	1	1	1	1	1	1	3	0	0	1	2	3
227	2	36	65	160	20.3	1	1	0	0	0	0	1	2	1	0	0	2	1	1
228	1	44	71	169	21	2	0	0	0	0	0	1	1	2.8	0	0	1	2	2
229	1	61	74	175	21.1	1	0	0	0	0	0	1	2	2.16	1	1	1	2	2
230	1	59	69	170	20.3	1	0	0	0	0	1	1	2	1.67	0	0	2	1	1
231	2	60	58	159	18.2	1	1	1	1	1	0	1	2	3	1	1	2	2	3
232	2	65	59	157	18.8	1	0	1	0	1	0	0	2	2.3	1	1	2	2	2
233	2	70	61	155	19.7	1	0	0	0	0	0	0	2	2.5	1	1	1	2	2
234	1	70	79	160	24.7	1	1	1	1	1	1	0	2	2.2	1	1	2	2	2
235	2	57	80	162	24.7	1	0	1	0	1	0	0	2	2.5	1	1	2	2	2
236	2	68	67	160	20.9	1	1	1	1	1	0	0	2	2.5	1	1	1	2	2
237	1	35	62	172	18	1	0	0	0	0	0	0	2	2.3	1	1	1	2	2
238	2	52	46	145	15.9	2	0	0	0	0	0	0	2	2.5	1	0	1	2	2
239	2	45	45	158	14.2	1	0	0	0	0	0	0	2	2.67	1	1	1	2	2
240	2	67	55	150	18.3	1	0	0	0	0	0	0	2	2.4	1	1	1	2	2
241	2	42	74	155	23.9	2	0	0	0	0	0	0	2	2.3	1	1	1	2	2
242	2	58	74	165	22.4	1	0	1	0	0	0	0	2	2.3	1	1	1	2	2
243	2	74	63	150	21	1	0	0	0	0	0	0	2	2	1	1	1	1	2
244	2	52	70	153	22.9	1	0	0	0	0	0	0	2	1.67	1	1	1	1	1
245	2	22	48	166	14.5	2	0	0	0	0	0	0	2	2	1	1	1	1	2
246	1	64	67	157	21.3	1	0	0	0	0	0	0	2	3.1	1	1	2	2	3
247	1	62	90	169	26.6	2	0	1	0	1	0	1	2	2.2	0	0	2	2	2
248	1	77	53	157	16.9	2	0	0	0	0	0	1	2	2.67	0	0	2	2	2
249	2	37	63	160	19.7	1	0	0	0	0	0	0	2	1.67	1	1	2	1	1
250	2	42	58	160	18.1	1	0	0	0	0	0	0	2	2	1	1	2	1	2

Figure E.1 Raw Data and Demographic Characteristics of Samples (Continued)

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During publication on Tropical Biomedicine Journal

2. Poster Presentation:

Oral Parasitic Protozoan Trichomonas tenax in The Periodontal Disease Patients: Hospital Based Cross-sectional Descriptive Study in Northeast Thailand

Authors: Pantip Chitpitaklert, Alisa Boonsuya, Nathkapach K. Rattanapitoon , Patpicha Arunsan and Schawanya K. Rattanapitoon at The 19th Asia Pacific Congress of Clinical Microbiology and infection (APCCMI) in July 6-8,2023 COEX, Seoul, Korea