Faculty of Resource Science and Technology

A Preliminary Study on the Prevalence of Red Complex Periodontal Bacteria Among Sarawakian Young Adults

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A Preliminary Study on the Prevalence of Red Complex Periodontal Bacteria Among Sarawakian Young Adults

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Oral cavity is a habitat for a diverse bacterial species that is commensal to the host. However, a slight change in the oral equilibrium may lead to periodontal disease which is the inflammation of the gingival tissue that can lead to tooth loss and supporting gingivae tissue destruction as the severity precedes. In Malaysia, the documentation on periodontal pathogens is still lacking. Therefore, this research would determine the prevalence of red complex bacteria among Malaysian young adults and the risk predictions of getting periodontal disease. A total of thirty-three saliva samples (23 gingivitis, 10 healthy) were collected from young adults of age 18 until 30 years old from Sarawak General Hospital, Kuching, Sarawak. Three different DNA extractions were used to compare the DNA concentration and purity. Next, 16S rRNA gene was amplified via PCR followed by species-specific PCR for red complex bacteria detection. Statistical data was analysed using GraphPad Prism 8.4.1. Despite the low DNA concentration obtained using phenol-chloroform-isoamyl method (3.42 ng/µL) and Norgen kit (5.75 ng/µL), 16S rRNA gene was amplified successfully with little inhibitions as the value of protein assessment for both PCIA (A260/280: 1.51) and Norgen kit (A260/280: 1.77) methods are closest to ideal. Out of the 33 samples tested, *T. forsythia* were frequently detected in gingivitis sample (56.5%). Up to 30.0% of the healthy samples were found positive for both *P. gingivalis*, followed by *T. forsythia* (20.0%). In associating gender to positive detection of red complex bacteria, *T. forsythia* recorded the highest detection rate of 52.2% among a total of 23 female subjects. In male subjects, *T. forsythia* (30.0%) and *P. gingivalis* (10.0%) were successfully identified. This study shows that at least one member of the red complex is found in the oral sample regardless of periodontal health status and gender that maybe useful as an additional evidence for prognosis of periodontal disease and its severity.

Keywords: Periodontal disease, red complex bacteria, saliva, 16S rRNA, PCR

Kajian Rintis Mengenai Kelaziman Bakteria Kompleks Merah Dalam Kalangan Anak Muda Sarawak

ABSTRAK

Rongga mulut adalah habitat bagi pelbagai bakteria yang komensal dengan perumah. Walau bagaimanapun, sedikit perubahan pada keseimbangan ekosistem boleh menyebabkan penyakit periodontal. Di Malaysia, dokumentasi mengenai patogen periodontal masih kurang. Oleh itu, penyelidikan ini akan menjelaskan kelaziman bakteria kompleks merah dalam kalangan belia Malaysia dan risiko mendapat penyakit periodontal. Di Malaysia, dokumentasi mengenai patogen periodontal masih kurang. Oleh itu, penyelidikan ini akan menentukan kelaziman bakteria kompleks merah di kalangan orang dewasa muda Malaysia dan ramalan risiko mendapat penyakit periodontal. Sebanyak tiga puluh tiga sampel air liur (23 radang gusi, 10 sihat) dikumpulkan dari orang dewasa muda berusia 18 hingga 30 tahun dari Hospital Umum Sarawak, Kuching, Sarawak. Tiga ekstraksi DNA yang berbeza digunakan untuk membandingkan konsentrasi dan ketulenan DNA. Seterusnya, gen 16S rRNA diamplifikasi melalui PCR diikuti PCR spesis-spesifik untuk pengesanan bakteria kompleks merah. Data statistik dianalisa menggunakan GraphPad Prism 8.4.1. Walaupun konsentrasi DNA adalah rendah untuk kaedah fenol-kloroform-isoamil (3.42 ng/ µL) dan kit Norgen (5.75 ng/ µL), gen 16S rRNA berjaya diamplifikasi kerana nilai evaluasi protein untuk kedua-dua kaedah, PCIA (A260/ 280: 1.51) dan kit Norgen (A260/ 280: 1.77) adalah hampir dengan ideal. Dari 33 sampel yang diuji, T. forsythia sering dikesan pada sampel gingivitis (56.5%). Sebanyak 30.0% sampel sihat didapati positif untuk kedua-dua P. gingivalis, dan diikuti oleh T. forsythia (20.0%). Dalam menghubungkaitkan jantina dengan pengesanan positif bakteria kompleks merah, T. forsythia mencatatkan kadar pengesanan tertinggi 52.2% dari kalangan sejumlah 23 subjek wanita. Manakala untuk lelaki, T.

forsythia (30.0%) dan P. gingivalis (10.0%) berjaya dikesan. Kajian ini menunjukkan bahawa sekurang-kurangnya satu anggota kompleks merah terdapat dalam sampel oral tanpa mengira status kesihatan periodontal dan jantina yang mungkin berguna sebagai bukti tambahan untuk prognosis penyakit periodontal dan keparahannya.

Kata kunci: *Penyakit periodontal, bakteria kompleks merah, air liur, 16S rRNA, PCR*

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LIST OF ABBREVIATIONS

%	Percentage
-ve	Negative
+ve	Positive
°C	Degree of Celsius
µL	Microlitres
16S rDNA	16S ribosomal deoxyribonucleic acid
16S rRNA	16S ribosomal ribonucleic acid
bp	Basepair
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
H_2S	Hydrogen sulphide
HOMINGS	Human Oral Microbe Identification using Next Generation Sequencing
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LAP	Localized aggressive periodontitis
mL	Millilitre
mm	Millimetre
MY	Malaysia
ng/ µL	Nanogram per microliter
NH_4	Ammonium

PCIA	Phenol: Chloroform: Isoamyl Alcohol
P. gingivalis	*Porphyromonas gingivalis*
RFLP	Restriction Fragment Length Polymorphism
rpm	Rotation per minute
RT-PCR	Reverse-transcription polymerase chain reaction
SDS	Sodium dodecyl suphate
spp.	Several species
T. denticola	*Treponema denticola*
T. forsythia	*Tannerella forsythia*
TE	Tris Ethylenediamaine Tetraacetic Acid
Tris-HCl	Tris-hydrochloride
UNIMAS	Universiti Malaysia Sarawak
USA	United States of America
UV	Ultraviolet
UV/ VIS	Ultraviolet/visible

CHAPTER 1

INTRODUCTION

1.1 Study Background

Human general health and well-being can be reflected by a healthy condition of the mouth which is well-maintained by a good oral regime. The human oral cavity is a habitat to hundreds of microbial species, which are mostly commensal and they work in synergy to create a balanced ecosystem in the mouth (Belda-Ferre et al., 2012). The association between humans and their oral microflora changes concurrently throughout different stages in life starting right after birth until the old age. Recently, it has been apparent that diverse inter-species interactions with the host can contribute to the shift the oral ecosystem from health to diseased (Jenkinson & Lamont, 2005). However, there are a few that contribute to the progression of oral diseases, namely periodontal disease and dental caries (Marsh, 2010).

Periodontal disease is one of the diseases that can be found to affect up to one-third of the human race population (Arora et al., 2014). It is the most prevalent diseases among children and adolescents and involves mainly gingivitis (Meyle et al., 2001; Oh et al., 2002). Since gingivitis is a reversible condition, not all cases of gingivitis will advance to the severe state called periodontitis (Gafan et al., 2004). Periodontal disease is infectious and the development is known to have resulted from the presence of a complex bacterial biofilms that forms on and around teeth, causing an inflammatory host reaction (Al-Ghutaimel et al., 2014; Galimanas et al., 2014). The hallmarks of periodontitis include gingival tissue bleeding, suppuration, spacing of teeth, destruction of supportive alveolar bone and severe

tooth loss (Suzuki et al., 2013; Arora et al., 2014). It has been reported that in the human oral cavity, there are over 700 bacterial species living harmoniously together of which about 400 species were found in the periodontal pockets (Paster et al., 2006; Siqueira et al., 2009). While most of these bacteria are commensals, there are a few potential pathogens that could cause systemic disease (Paster et al., 2001).

Over the past couple of decades, the study and understanding of these microbiotas by relating them with the different forms of periodontal disease have been made possible with the advancement in technology and molecular identification approaches. These approaches such as RFLP, DNA hybridization, RT-PCR and sequencing techniques has reduced the need for labour-intensive and time-consuming works (Siqueira et al., 2009). Besides that, the introduction of high-throughput DNA sequencing technology has resolved the problems with difficult-to-culture bacteria and allowing analysis of microbial colonization patterns and community composition in the oral cavity (Chen et al., 2018).

Despite the extensive research done, there is still limited knowledge of the microbes that are linked to periodontal disease (Liu et al., 2012). The complexity of oral microbial community due to multilevel species interactions and inability to classify a single etiological agent as in Koch's postulates diseases makes it difficult to identify the potential oral pathogens (Belda-Ferre et al., 2012). To be considered as a potential pathogen, a microorganism has to meet these criteria that include amplified population at affected sites of diseased individuals, apparent reduction or elimination once treated, capable of triggering host's immune response, can cause disease when introduced to animal models and produces virulence factors to cause severe inflammation (Popova et al., 2014).

Apart from that, the limitations of sampling and detection methods could cause certain species which is present in low frequency in the oral cavity of healthy subjects to remain undetectable (Wade et al., 2011). It is also challenging to do cultivation in the laboratory due to the possibility that the microorganisms are unable to survive beyond its natural community (Jenkinson et al., 2005). Whole saliva collection has limitation in sample quality such as the possibility of external contaminants, that need to be reduced during the sample collection step, and the presence of too much protein in the sample that could indicate an underlying infection. The presence of large quantities of protein or foreign contaminants that is carried over to the final extracted DNA can cause inaccurate nucleic acid quantification (Goode et al., 2015).

1.2 Problem Statement

Many studies have been carried out worldwide to compare the bacterial composition of healthy and diseased oral cavity (Aas et al., 2005; Jenkinson & Lamont, 2005; Dewhirst et al., 2010; Belda-Ferre et al., 2012; Xu & Gunsolley, 2014). A few oral bacteria species including red complex are risk indicators for the development of periodontal disease (Mehta, 2015). The red complex bacteria which are anaerobic, gram-negative bacteria are found responsible for causing periodontal disease. Its presence serves as an indication of the disease severity as they are commonly linked to the advanced state (Tamura et al., 2006). The presence of periodontal disease has also been linked to different systemic disease because this condition often leads to bacteremia, which is the invasion of bacteria into the bloodstream (Kurita-Ochiai et al., 2015; Segura et al., 2015). Therefore, it is crucial to understand the microbiological aspects to control periodontal inflammation in individuals.

However, this study focuses primarily on the natives of Sarawak, mainly the locals in Kuching area. This research would provide a good insight into the prevalence of red complex bacteria in both healthy and diseased patients to be compared with the data from previous studies of other countries. Besides that, the data would be useful as a reference for predicting the risk of acquiring periodontal disease of individuals.

1.3 Objectives

For this research, the objectives are:

i. To compare the DNA yield and purity obtained from different extraction methods for periodontal saliva samples

ii. To detect the presence of red complex bacteria (*Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticola)* among young adults with periodontal disease and healthy periodontal status

1.4 Chapter Summary

This thesis is organised into five chapters, in which Chapter 1 comprised of the general introduction of the study, research problem statements and also the main objectives. Chapter 2 discusses the backgrounds of the research on periodontal disease and study organisms, which are the red complex bacteria, namely *Porphyromonas gingivalis, Tannerella forsythia*, and *Treponema denticola.* This chapter explains the different stages of periodontal disease, the microbial complexes in the oral cavity and risk factors of periodontal disease.

The next chapter is entitled “Optimization of three different methods used in saliva DNA extraction”. The three different methods used to isolate bacterial DNA from the clinical samples such as phenol-chloroform, Norgen kit, and chelex-100 resin, were elaborated in this chapter. This study included the pre-sampling and sample collection guidelines. This chapter also included the utilization of 16S rRNA sequence amplification to detect the occurrence of oral bacteria in the genomic DNA extracted. The primer set 1492R and 27F were used which produced an amplicon size of approximately 1500 bp.

The detection of red complex bacteria (*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola)* via specific primer set is reported in Chapter 4 which entitled “Detection of *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* via PCR in saliva of healthy and diseased young adults”. This chapter compares the distribution pattern of *P. gingivalis, T. forsythia* and *T. denticola* as well as their presence in the saliva of diseased and control patients. The occurrence of this species in different sexes was also investigated. Three different sets of sequence-specific primer were utilized and PCR product size of approximately 197 b for *P. gingivalis,* 641 bp for *T. forsythia* and 311 bp for *T. denticola* were amplified, respectively. After the successful detection of each member species of red complex in the samples, the relationship between gender difference and periodontal status to the presence of each species was observed and explained using binary logistic regression analysis.

Finally, in Chapter 5, general conclusions and recommendations for future studies are discussed. This chapter summarizes the significance of findings on red complex bacteria and its association to different gender groups and periodontal health status as well as

suggestions for improvements. All the figures and tables are placed within the main text of every chapter and the reference list which contained all the cited references are placed at the last section of this thesis.

CHAPTER 2

LITERATURE REVIEW

2.1 Periodontal Disease

The human oral cavity is populated by staggering bacterial communities of more than 700 phylotypes that are able to adhere to the respiratory tissues and digestive organ (Paster et al., 2006). The modifications of the oral ecosystem due to a gradual increase of periodontal pathogens leads to dysbiosis and initiation of periodontal disease (Bourgeois et al., 2019). Dysbiosis involves a shift in the multiple-species complex or a single species within a microbial population that tips the microbiota balance and eventually causing destructive inflammations (Olsen et al., 2017).

Periodontal disease is an infectious disease caused by the accumulation of bacterial biofilms that forms on and around teeth that can lead to the destruction of adjacent tissues and supportive bone (Suzuki et al., 2013; Al-Ghutaimel et al., 2014; Galimanas et al., 2014). Periodontal disease is divided into two types, which are gingivitis and periodontitis (Al-Ghutaimel et al., 2014). It can progress from mild, reversible gum inflammation (gingivitis) to destructive, irreversible periodontitis (Bourgeois et al., 2019).

Generally, the shift from healthy gum condition to periodontal disease occurs due to dental biofilm development (Figure 2.1). After dental biofilm is formed, the neutrophils are secreted by the host immune cells which, in turn, initiate the first gingivae inflammation. This condition then is accompanied by the secretion of T cells and macrophages. As the

immune cells secretion increases, periodontitis develops and progresses into deeper periodontal pocket region. During periodontitis stage, B cells and plasma cells makes up most of the lymphocytes production (Kriebel et al., 2018). The progression of periodontal disease is linked to modifiable (lifestyle and habits) and unmodifiable (genetic predisposition) risk factors (AlJehani, 2014). Since periodontal disease is a multi-species infection, this leads to complications in prescribing suitable periodontal treatment (Sbordone & Bortolaia, 2003).

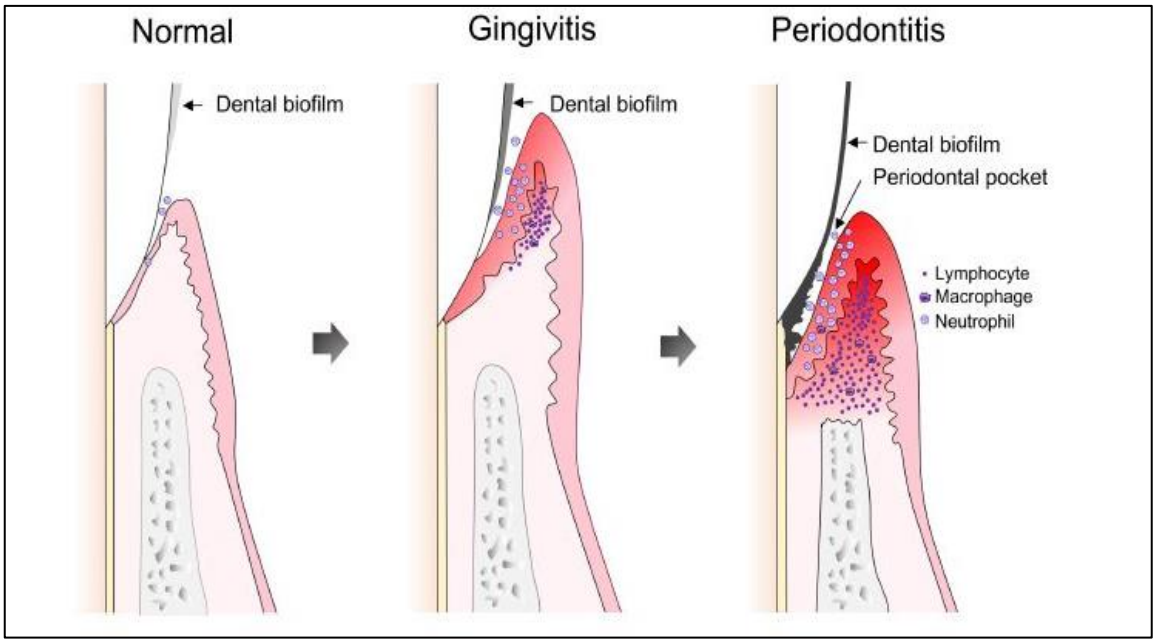

Figure 2.1: The progression of gingivitis and periodontitis following the formation of biofilm on tooth surface (Kriebel et al., 2018).

2.1.1 Gingivitis

A healthy gingivae or gum tissues is characterised by its pale pink, and firm tissues attached to the teeth structure. Upon probing, a healthy gum is not prone to bleeding (Cope & Cope, 2011). The inflammation of the gingivae or known as gingivitis condition (Figure 2.1), occurs when the action of the inflammatory cells is compromised and adjacent gum

margin is damaged (Cope & Cope, 2011). The development of biofilm on tooth surface can induce irritation to the gingival tissues with the formation of dental pockets due to gum recession, swollen gum, and more gingival crevicular fluid being produced (Larsen & Fiehn, 2017). Gingivitis is mild and reversible whereas periodontitis is the irreversible and destructive form of the disease (Figure 2.2) because it affects the underlying supporting structures of the teeth (Al-Ghutaimel et al., 2014). The mild stage of gingivitis includes tenderness of the gum and bleeding on brushing (Allan, 2012). When bacterial proliferation takes place due to poor oral regime (Cope & Cope, 2011), the change in bacterial population causes a slight alteration to the ecology thus to contribute to the gum inflammation (Larsen & Fiehn, 2017). *Actinomyces, Bacteroides, Fusobacterium,* and *Prevotella* species are among the common genus associated to the development of gingivitis (Lamont & Jenkinson, 2010).

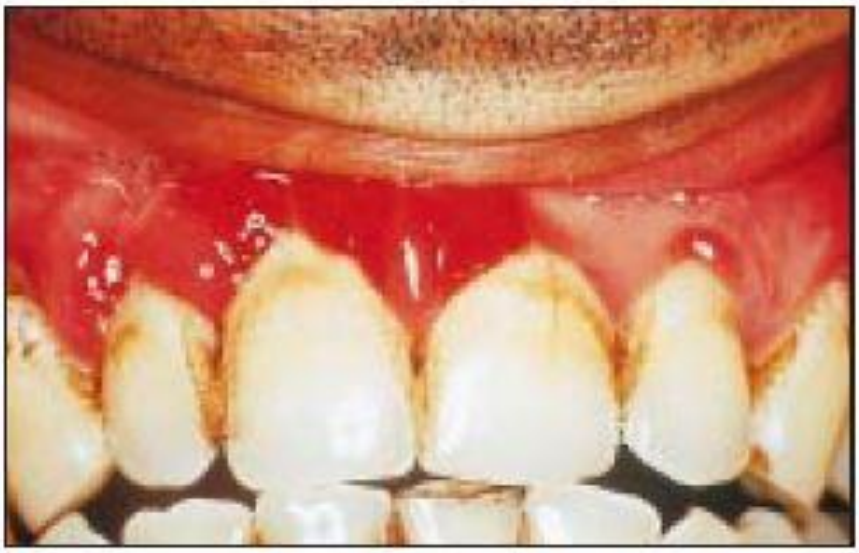

Figure 2.2: Gum swelling as observed in gingivitis patient (Coventry et al., 2000).

2.1.2 Periodontitis

Periodontitis is associated to the tenderness and destruction of the tooth-supporting tissues (Figure 2.3). The implicated microorganisms for periodontitis include *Aggregatibacter, Fusobacterium, Porphyromonas, Prevotella, Treponema, and Tannerella* (Lamont & Jenkinson, 2010). The progression and development of these diseases are caused by polymicrobial infections in the mouth. Rarely such infections are caused by single species, yet exception is made for localized aggressive periodontitis (LAP) as this disease is predominated by *Aggregatibacter actinomycetemcomitans* (Lamont & Jenkinson, 2010). As periodontitis progresses to the advanced stage, the gum starts to pull away and destruction of the supportive alveolar bone occurs which result in tooth mobility and finally tooth loss (Allan, 2012). The exudate from gingival pockets carries periodontal pathogens and other inflammatory cellular components is involved in the destructive mechanism linked with periodontitis through bacterial biofilm formation and inflammatory host immune response (Arigbede et al., 2012).

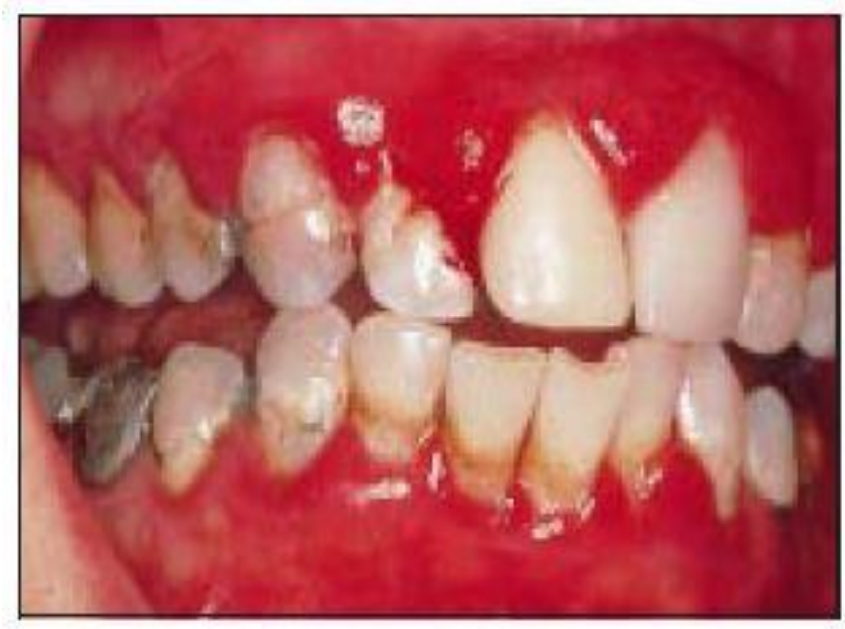

Figure 2.3: Periodontitis with noticeable gum recession (Coventry et al., 2000).

There are various hypotheses made by researchers since the early discovery of oral microbes (Figure 2.4). The first hypothesis was by Macdonald and his team in 1950s which discussed the non-specific plaque hypothesis that proposed the idea of abundance of plaque as the cause of dental infections. The next hypothesis was the "Specific Plaque Hypothesis" (1980s) suggesting that a few species of streptococci and lactobacilli were responsible for the disease progression. The "Concept of Red Complex" (1980-1990s) was then introduced with the idea that a cluster of subgingival microorganisms causes gingival tissue inflammation. In 2003, a hypothesis known as "Ecological Catastrophe Hypothesis" (2003), shed a light on the importance of balance microflora ecosystem as a slight disturbance may lead to disease. A new hypothesis was then derived in 2011 by Hajishengallis and his collegues, called as "Keystone-Pathogen Hypothesis" suggests that when there is an increase in the normal microflora population, it could trigger tissue inflammation. Until a decade ago, a new etiology of periodontitis was made by Hajishengallis that focuses on the polymicrobial synergy and dybiosis model (Rosier et al., 2014). Currently, there is a hypothetical theory that link low abundance of keystone pathogens as the cause to highly populated oral commensals. The slight disturbance in the ecosystem thus lead to dysbiotic condition (Jain et al., 2018).

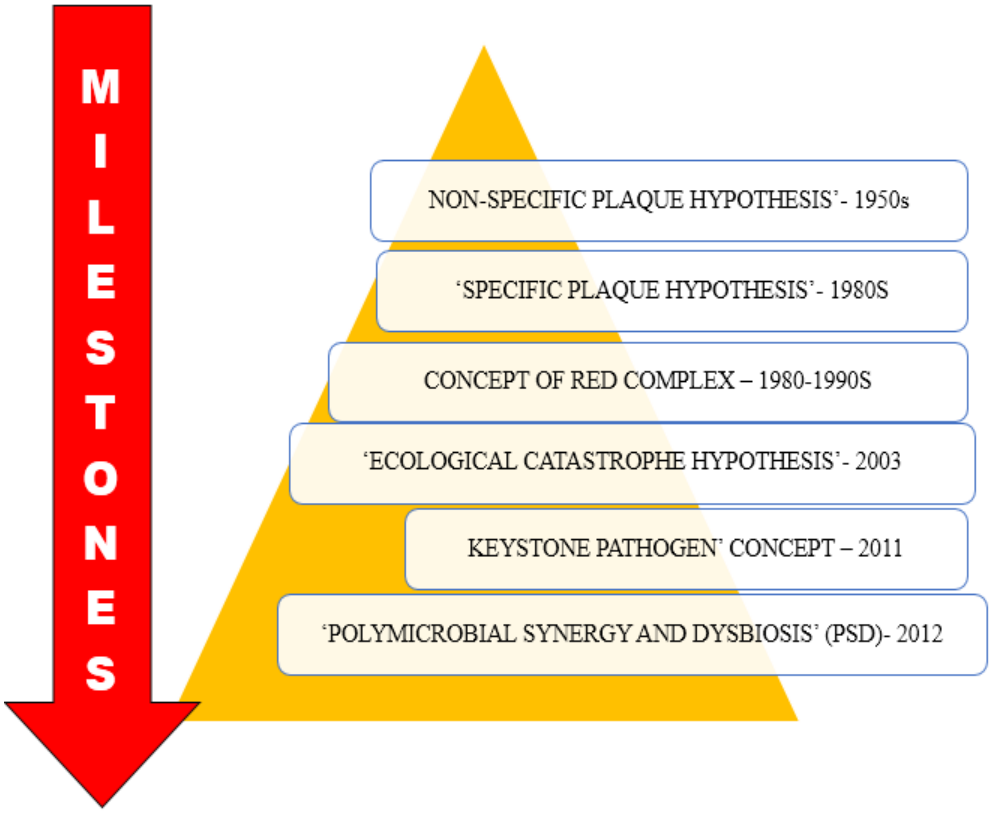

Figure 2.4: Milestones in the hypothesis for etiology of periodontitis (Jain et al., 2018).

2.2 Saliva

Saliva is a clear fluid secretion in the oral cavity that contains at least 108 cell/mL of microorganisms such as bacteria, protozoa, archaea and fungi (Marsh et al., 2016). Water constitutes of about 99% of the saliva components, and another 1% is for proteins and salts (Mittal et al., 2011). The major and minor salivary gland of the oral cavity are responsible for the mixed fluid secretion which also contains other intraoral shedding cells, microorganisms, expectorated bronchial, nasal secretions and food debris (Pedersen & Belstrom, 2019). Approximately, 93% of the salivary secretion comes from the major salivary glands and another 7% from the minor glands (Puy, 2006). It is sterile only at the moment it exits the salivary gland (Puy, 2006). On average, the normal saliva secretion is

between 500 mL to 1500 mL per individual (Mittal et al., 2011). The volume of salivary fluid secreted at one time is important because in the case of hypersalivation or hyposalivation condition, an individual general well-being and oral health can be affected (Puy, 2006).

Saliva offers more benefits in laboratory diagnosis that usually involved blood as the primary diagnostic fluid. Whole saliva is easy to collect as it does not require experienced personnel to handle the specimen (Mittal et al., 2011). Besides that, it is children-friendly due to the non-invasive protocol during the fluid collection process. Saliva analysis also is less expensive when doing a study for large populations (Mittal et al., 2011). As a diagnostic tool to monitor drug abuse and detect diseases (caries, periodontal disease, celiac disease, cystic fibrosis, Sjogren's syndrome and malignant tumour), saliva has demonstrated its usage as a promising alternative to blood specimen (Puy, 2006).

The main role of saliva is to provide moisture and lubricate the oral cavity and its surfaces. Bacterial growth and density is correlated to the moisture level on the surface of the tongue. A reduction in the bacterial numbers can be observed when the tongue surface is kept moisturized (Kobayashi et al., 2017; Su et al., 2019). Aside from that, it assists in the food ingestion, speech, act as a buffer fluid, and has antimicrobial function (Mittal el al., 2011). The antimicrobial action of saliva is present through a number of protein and peptides such as lysozyme, statherin, lactoferrin, lactoperoxidase, histatins and mucins, and antibodies (Pedersen & Belstrom, 2019).

2.3 Biofilm and Dental Plaque Formation

In the oral cavity, the intraoral structures are covered by a layer of microbial biofilm called plaque. Within the layers are cellular debris, bacterial communities and acellular components that are formed from dietary, salivary and mixed microbes sources. Dental plaque is a hard, creamy yellow deposit on tooth surface (Puy, 2006). The term "biofilm" has been used interchangeably with "dental plaque", a term used previously (Larsen & Fiehn, 2017). The development of periodontal disease begins with the biofilm formation (Jain et al., 2018). Biofilm formed on tooth surfaces can lead to dental caries, whereas biofilms accumulated on subgingival and supragingival sites can lead to periodontal disease (Larsen & Fiehn, 2017). The disease progression corresponds with the elevated microbiota population and also a gradual increase in appearance of species that are present in low numbers when in health. The subgingival biofilm composition differs significantly in one individual's oral cavity to another (Payne et al., 2019).

Bacterial plaque formation begins a couple of minutes after tooth brushing in which acellular layer (salivary proteins and macromolecules) is formed with a thickness of 2 until 10 µm (Puy, 2006). Salivary glycoproteins, known as the acquired pellicle, allows the weak adherence of oral bacteria on clean dental surface to form the initial biofilm layer (Larsen & Fiehn, 2017). This first stage of biofilm development that takes 4 until 24 hours allows the initial colonizers (mostly aerobes) to multiply in the layer. The early colonization of the thin biofilm layer is predominated by gram positive bacteria; *Streptococcus mitis* and *Actinomyces* species (Larsen & Fiehn, 2017; Jain et al., 2018). The concerted action of these two species in providing nutrition within the initial biofilm layer helps to provide an ideal anaerobic environment for late colonizers (Jain et al., 2018). After 24 hours, secondary

colonizers which are mostly anaerobic cocci and rods, start to accumulate in the deep gingival tissue until a period of 14 days leading to an increase in the thickness of biofilm layer (Puy, 2006; Larsen & Fiehn, 2017). The bridging species, *Fusobacteria*, are crucial to facilitate the formation of secondary dental biofilm because of its ability to communicate with both gram-negative and gram-positive bacteria (Jain et al., 2018). Gradually, stronger aggregation is made in the acquired pellicle via bacterial receptor-pairs which are the surface adhesins and glycoprotein receptor (Larsen & Fiehn, 2017). After two weeks, a mature dental plaque is formed that consists of a mixed population of viable and non-viable anaerobic bacteria. At this state, calculus may form due to mineralisation of the mature plaque (Puy, 2006).

In a summary, there are three different stages of dental biofilm life cycle (Figure 2.5) which are the planktonic phase, biofilm phase and dispersion phase (Berger et al., 2018). The first stage; adherence of free-floating bacteria to a surface is crucial for the development of biofilm layer. The attachment occurs randomly or via chemical stimulation. Depending on its environment, they can progress to biofilm or return to planktonic phase. In the second stage, the bacterial colonies accumulate to form biofilm layer. The last stage, that is the dispersion phase, biofilm development stops and pathogenic cells exit the layer to attach to other surfaces (Berger et al., 2018).

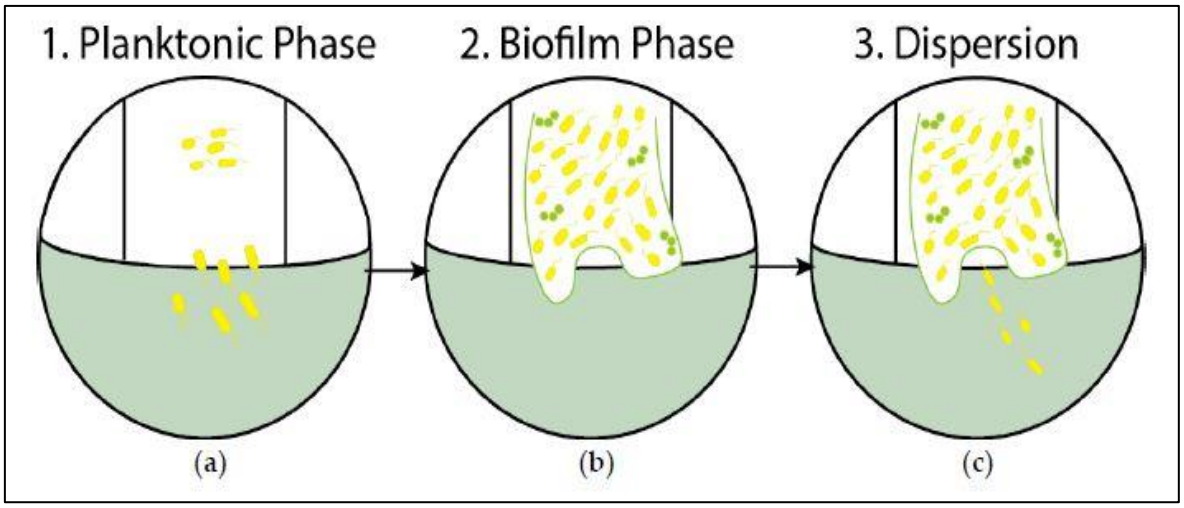

Figure 2.5: Dental biofilm development (Berger et al., 2018).

When there is a lack of oral hygiene practice to remove the biofilm layers, the initial layer called supragingival plaque will progress into the root and periodontal pocket to form subgingival biofilm (Larsen & Fiehn, 2017). Dental plaque biofilm of the supragingival and subgingival surfaces are composed of a dynamic multispecies bacterial interaction that are higher resistant to antibiotic and chemical therapy (Figure 2.6). Besides that, bacterial community in biofilm form are more capable of evading the host inflammatory cells (Gurenlian, 2007).

The daily oral practice of tooth brushing as well as chewing on gums helps to remove the biofilm layer. A study by Inui et al. (2019) demonstrated that chewing disrupts the adherence of early colonizer on tooth surface thus interfere with the progression of biofilm formation. When the biofilm causes gingival inflammation and severe periodontitis, the dental professionals will perform mechanical teeth cleaning and instruct the patients to practice oral hygiene regime that includes tooth brushing and flossing at least twice a day (Larsen & Fiehn, 2017).

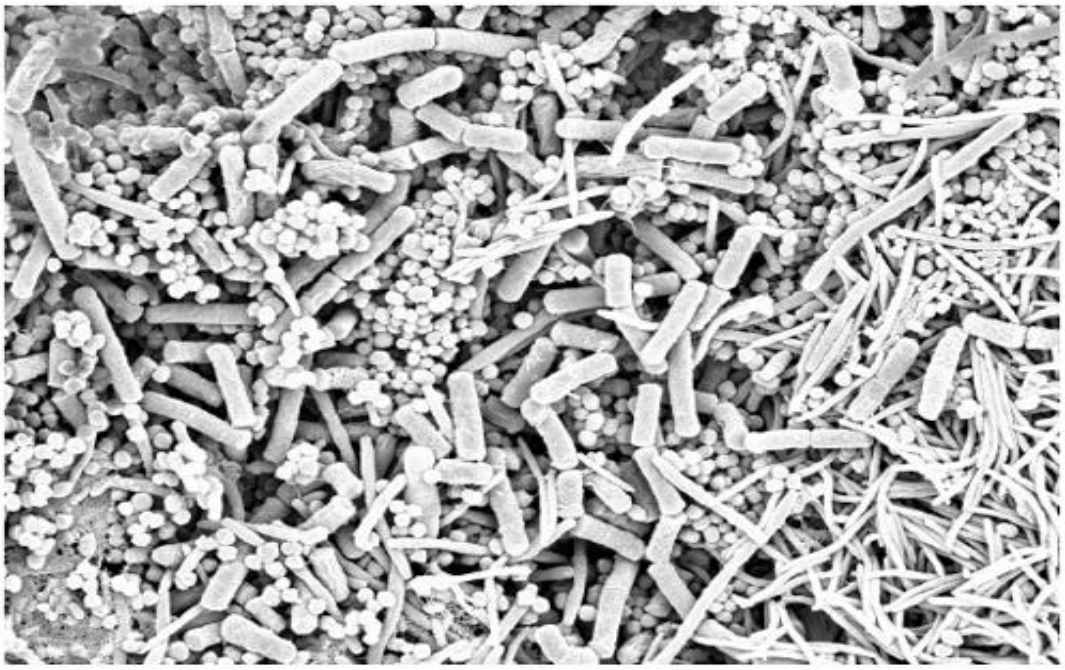

Figure 2.6: Scanning electronic microscope (SEM) image of biofilm grown for 10 days from the healthy subgingival plaque with polymicrobial matrix of *Streptococci* and *Actinomyces* predominates (Gurenlian, 2007).

2.4 Microbial Complexes in Periodontal Tissues

Oral bacteria have been linked to its survival and adherence to other organs (brain, heart valves, spleen, carotid atheromatous plaque, liver, pancreas, and bone) via bloodstream transportation (Van Dyke & van Winkelhoff, 2013; Scannapieco, 2013). It is common to detect similar oral bacteria with the gut microflora (Nagao & Tanigawa, 2019). The composition of oral microbiome is believed to be influenced by different factors that incudes genetics, geography, dietary habit, age and shared inhabitants (Burcham et al., 2020). It is of utmost importance for the dental practitioners to have ample knowledge on the relationships of the oral bacteria populations (commensalism, synergy or antagonism) as a guideline for choosing the right and targeted therapy because understanding the oral bacteria in isolation as opposed to in biofilm interaction is very difficult due to its dissimilar properties (Sbordone & Bortolaia, 2003).

The microbiota in the oral cavity are divided into different complexes (Table 2.1). Among the classified complexes, the bacterial species from purple, yellow and green complexes are linked to periodontal health, meanwhile the orange, red complex and unclassified species are counted as periodontal pathogens (Popova et al., 2014). These complexes are colour-classified by Socransky according to their virulence and their roles in causing periodontal inflammation as found in the subgingival site (Popova et al., 2014; Hashim, 2018). Within each complex, the species interact closely to each other as a community. It is rare to find the bacteria singly or in pairs in periodontal pockets as they are mostly found grouped together from the same complex (Sbordone & Bortolaia, 2003). Aside from that, complexes interaction does occur but only in a precise manner (Sbordone & Bortolaia, 2003).

Red complexes occasionally are found in the same sites as the orange complexes. As the number of colonies of red complex bacteria increase per site, the greater the detection of the orange complex microbial colonies (Mohanty et al., 2019). The clusters that are also commonly detected together are the yellow and green complexes but show weak inter-complexes relation with orange and red clusters. Conversely, the purple complex is more stand-alone as compared to the other complexes (Sbordone & Bortolaia, 2003).

Table 2.1: Microbial complexes in subgingival plaque (Sbordone & Bortolaia, 2003). Purple, yellow and green are related to healthy, normal oral microbiota whereas orange and red are closely-linked to disease state.

Cluster	Bacterial species
Purple cluster	*V. parvula, A. odontolyticus*
Yellow cluster	*Streptococci spp.: S. mitis, S. oralis, S. sanguis, S. gordonii, S. intermedius*
Green cluster	*E. corrodens, C. gingivalis, C. sputigena, C. ochracea, C. concisus, A. actinomycetemcomitans* serotype a
Orange cluster	*P. intermedia, P. nigrescens, P. micros, C. gragilis, C. rectus, F. periodonticum, F. nuc.nucleatum, F. nuc.vincentii, S. constellatus, E. nodatum, C. showae, F. nuc.polymorphum*
Red cluster	*P. gingivalis, T. forsythia (*formerly known as *B. forsythus), T. denticola*
Unclassified	*A. viscosus, Selenomonas noxia, A. actinomycetemcomitans* serotype b

From clinical point of view, classification of the yellow and the green complexes is based on the periodontal pocket depth and bleeding on probing (BOP) sites. The green and yellow complexes are closely linked to periodontal depth 4 mm and BOP-positive areas (Sbordone & Bortolaia, 2003). Once yellow and green colonization are found in a particular site, the subsequent colonization by orange and red complexes will usually follow after.

Therefore, in theory, the ability to alter the progression of colonization by yellow and green complexes could intervene the development of the orange and red clusters. However, this is difficult to achieve as the mechanism of interference of the colonies is yet to be fully understood (Sbordone & Bortolaia, 2003). Therefore, this highlights the distinct criteria of oral microbiology which are the complex ecology of oral environment and the multilevel species interaction between the microorganisms as the microorganisms exist in communities and not as monospecies (Lamont & Jenkinson, 2010).

2.5 Common Oral Bacteria Community in Health

The first discovery of oral microorganisms by Antony Van Leeuwenhoek (1632-1723), has led to the many discoveries and insights of today's oral microbiology. Among the earliest oral microorganisms discovered in the oral cavity includes cocci, spirochetes, and fusiform bacteria (Hashim et al., 2018). In general, it is common to discover a heathy oral site to be harboured by 20 until 50 bacterial species. At affected unhealthy sites, the likelihood to find the presence of multispecies is higher (Lamont & Jenkinson, 2010). According to Socransky et al. (1998) oral microbes' classification, generally, the purple, yellow and green complexes are among the oral bacteria that correlate to healthy periodontal condition (Popova et al., 2014).

Many studies revealed that the communities of commensal oral bacteria and the colonizers of healthy sites are significantly different from the ones identified in diseased subgingival and supragingival surfaces. Based on the localization of the microbial populations, lower densities of Gram-negative, pathogenic species are found in the healthy gingival sulcus than in periodontal pockets (Hashim et al., 2018). The gingivitis-free gum is

composed of predominant Gram-positive cocci species, some rod-shaped and filamentous Gam-positive bacteria and a few Gram-negative cocci. These bacteria work as a community that build-up 1 until 20 layers of supraginigival plaque (Sbordone & Bortolaia, 2003). Findings on healthy specimens demonstrated that an abundance of non-cultured saliva microorganisms is found to be from the genera *Prevotella, Porphyromonas, Streptococcus, Haemophilus, Aggregatibacter* and *Rothia* (Diaz et al., 2012; Pereira et al., 2012).

The genus Streptococcus can be found to be the most prevalent as compared to the other bacterial groups in healthy oral sites (Bik et al., 2010). Among the species reported are the *Streptococcus spp*. (*S. mutans, S. mitis, S. sanguis, S. oralis; Rothia dentocariosa; Staphylococcus epidermidis*), a number of facultative anaerobic, Gram-positive rods and filaments (Actinomyces spp.: *Actinomyces viscosus, Actinomyces gerencseriae, Corynebacterium spp*.) and very few Gram-negative cocci (*Veillonella parvula*; *Neisseria spp.*) phylotypes (Hashim, 2018).

Another molecular research also stated that aside from Streptococcus being the predominant genus, other genera found in healthy sulcus sites are *Abiotrophia, Gemella, Granulicatella, Neisseria, Rothia,* and *Prevotella* (Aas et al., 2005). A shared conclusion was made by Diaz et al. (2012) and Pereira et al. (2012) on bacterial distribution from healthy oral specimens. They demonstrated that an abundance of non-cultured saliva microorganisms is found to be from the genera *Prevotella, Porphyromonas, Streptococcus, Haemophilus, Aggregatibacter* and *Rothia* (Diaz et al., 2012; Pereira et al., 2012).

2.6 Bacterial Diversity Related to Periodontal Disease

It has been acknowledged that the communities in the oral cavity are polymicrobial (Jenkinson & Lamont, 2005; Galimanas et al., 2014). For many years, bacteria have been known to colonize the hard and the soft tissues of the oral cavity that influenced oral health and diseases. It has been estimated that over 700 bacterial species are harboured by the human oral cavity and of which about 400 species were found in the periodontal pockets (Paster et al., 2006; Siqueira et al., 2009). Different types of tissues, growth requirements and nutrients are needed in the growth of different communities. For certain bacteria, their prevalence is influence by the oral environments they reside in due to the ideal conditions for growth at that particular site. For example, the saliva and tongue shares a similar bacterial distribution as compared to the microbiota on teeth and periodontal pockets area (Paster et al., 2006). Every person harbours a unique oral bacterial community but in healthy individuals, the similarity is seen at genus level (Aas et al., 2005; Bik et al., 2010). While most of these bacteria are commensals, there are a few potential pathogens that could cause systemic disease (Paster et al., 2001).

In a study conducted by Serra e Silva Filho and colleagues (2014), they found that there were eight groups of bacteria distributed in the periodontal pockets of periodontitis patients which are *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes* and *Synergistetes.* Based on the results they obtained, it was found that *Bacteroidetes* suggested the most common phylotype present.

Similarly, previous research indicated that *Porphyromonas gingivalis (P. gingivalis)* and *Tannerella forsythensis (T. forsythensis)* from Bacteroidetes phylum whereas

Treponema denticola (*T. denticola)* from the Spirochaete phylum have been associated with periodontal disease (Tamura et al., 2006; Kang et al., 2009). These three bacteria have been classified as gram-negative anaerobic bacteria which have a red-complex that is responsible for causing the disease (Tamura et al., 2006).

2.7 Red Complex Bacteria

The classification of oral communities was first done by Socransky et al. in 1998 using cluster analysis and community ordination approaches on the subgingival microbial populations. The rationale of using different optical spectrum colours to classify the bacterial complexes have been questioned for numerous times. The most virulent is in the red complex and yellow is classified as commensals (Arora et al., 2014). Due to the polymicrobial synergy and dysbiosis model of periodontal disease, the complex characteristics and uncertain role of causative pathogens could be explained through their ability to invade the host immune response and disrupt the homeostatic equilibrium to dysbiosis state (Hajishengallis & Lamont, 2012).

The red complex consists of three-member species that are known as keystone pathogens in the development of periodontal disease, namely, *Porphyromonas gingivalis, Tannerella forsythia* (previously named *Bacteroides forsythus,* or *Tannerella forsythensis*) and *Treponema denticola* (Nayak et al., 2018*)*. Being late colonizers in biofilm community, these bacteria are densely found at periodontitis affected areas. The red complex bacteria are also commonly detected in sites where orange complex species are present. As for dental diagnosis, symptoms like bleeding on probing and increasing gingival pocket depth has been highly associated with the red complex species detection (Mohanty et al., 2019).

Aside from being detected in the oral biofilm of diseased patients, the red complex bacteria have also been identified in healthy oral sites (Ximenez-Fyvie et al., 2000). In a study done by Bik et al. (2010), all three members of red complex was detected in low abundance in the oral samples of recruited healthy individuals.

2.8 *Porphyromonas gingivalis*

Porphyromonas gingivalis is one of the members in the red complex bacteria and is strongly associated to periodontal disease (Papova et al., 2014). The average diameter of this coccobacillus anaerobe is 1 mm (Tan et al., 2014). This bacterium is categorized as a catalase-negative microorganism due to its incapability to metabolize carbohydrates (How et al., 2016). In periodontal pockets where the availability of sugar is limited, this species thrives on amino acid fermentation for its survival (Bostanci & Belibasakis, 2012).

This rod-shaped, non-motile, obligate anaerobic bacterium can be observed as colonies with black pigments when grown on blood agar media (Bostanci & Belibasakis, 2012; How et al., 2016). The black pigments as observed in *P. gingivalis* colonies (Figure 2.7) is related to the hemin (oxidized heme) build-up on its cell surface (Liu et al., 2004; Bostanci & Belibasakis, 2012; How et al., 2016). When grown in medium with reduced heme, *P. gingivalis* loses its ability as opportunistis periodontal pathogen hence relates the function of heme to its pathogenicity (Bostanci & Belibasakis, 2012). Also known as late colonizer in a polymicrobial biofilm community, *P. gingivalis* is often found in the areas within adjacent gingival tissue (Zijnge et al., 2011).

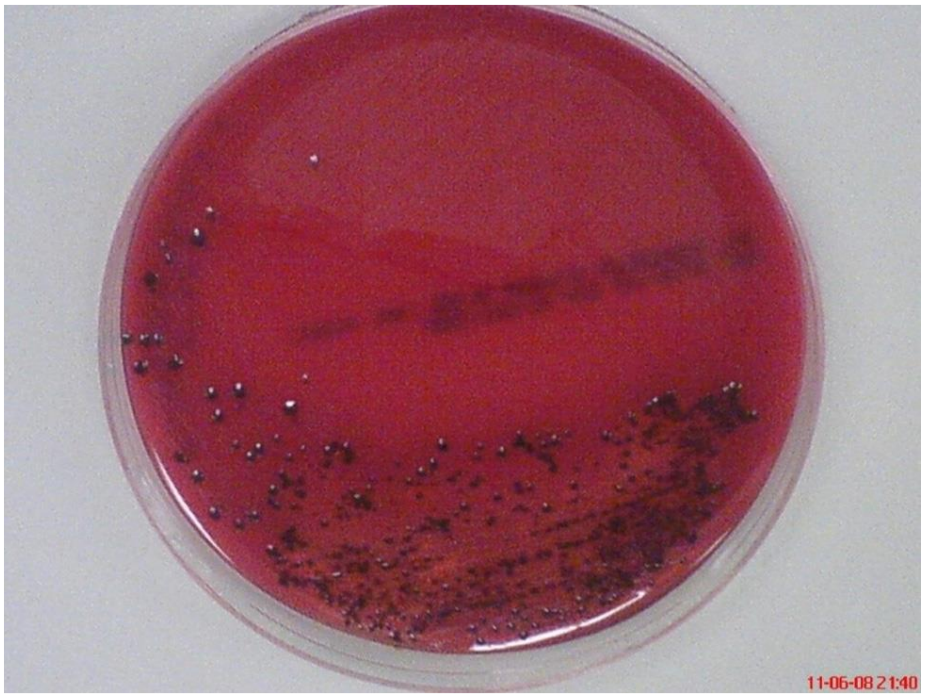

Figure 2.7: Colonies of *P. gingivalis* as cultured on horse blood agar (How et al., 2016).

In a study done by Verma et. al. (2010) using mice model, the monobacterial infection of *P. gingivalis* resulted in an enhanced alveolar bone destruction and an increased in the IgG antibody immune response. The capsule layer that protects *P. gingivalis* from phagocytosis, fimbriae and vesicles, has other functions which is the production of virulence factors. Among them are the proteases, collagenase, endotoxin, hemolysin, fatty acids, H_2S and NH_4 (Popova et al., 2014).

One of the strategies used by of this bacterium to elude the host defense mechanism is by upregulating the production of proteases called gingipains and exopeptidase, through the attachment its fimbriae to the host cells (Jain et al., 2018). The polypeptides produced by *P. gingivalis* are able to breakdown the host proteins and allow successful colonization

to happen (Jain et al., 2018). In dental biofilm especially in subgingival sites, it is common to find *P. gingivalis* together with *Treponema denticola* (Jain et al., 2018).

2.9 *Treponema denticola*

The motile, long spirochete *Treponema denticola* (Figure 2.8) is the most commonly isolated and characterized anaerobic oral species (Dashper et al., 2011). The average length of *T. denticola* is 5 to 20 mm (Tan et al., 2014). This gram-negative bacterium is linked to periodontal lesions that is frequently detected in advanced state of periodontitis and its presence is infrequently related to healthy or gingivitis gum.

During the development and progression of periodontal disease. *T. denticola* has been reported to be associated to the inflammation and swelling of the gingivae tissue and infection of the root canal. Intriguingly, it was documented that when grown in a single species biofilm, *T. denticola* loses its spiral structure (Zhu et al., 2013). However, in polymicrobial biofilm development with the presence of *P. gingivalis*, the synergy between these two anaerobes resulted in a retained spiral structure of *T. denticola* (Zhu et al., 2013; Ng et al., 2019).

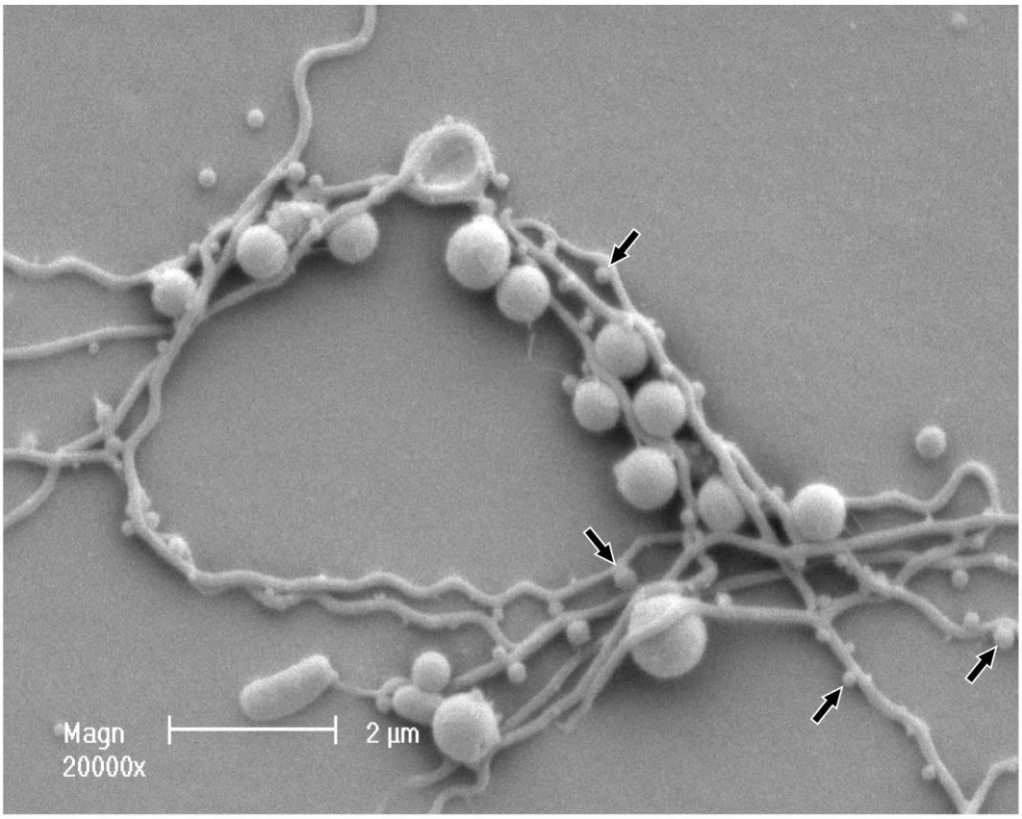

Figure 2.8: Scanning electron micrograph of continuous co-aggregated culture *P. gingivalis* and *T. denticola*. The arrows shown both putative *T. denticola* and *P. gingivalis* outer sheath vesicles along the length of *T. denticola* (Tan et al., 2014).

Since *T. denticola* is a late colonizer in the development of dental biofilm, it is vital for this species to adhere to other species such as *P. gingivalis, T. forsythia* (previously known as *B. forsythus*) and *Fusobacterium* species in order to embed itself to basement membranes (fibronectin, laminin, collagen, fibrinogen) and different cells (Kolenbrander et al., 2002).

Dental biofilm environment keeps fluctuating in its nutrient level, oxygen demand and pH level. Such transition in the surrounding exerts stress to *T. denticola.* In order to survive, this bacterium has its own counteractive mechanism. One of them is its ability to produce enzyme for glycogenesis under limited nutrient accessibility; an ability lacking in

other spirochetes (Seshadri et al., 2004). This bacterium depends mostly on selenium for growth, and a deficit in selenium can reduce its ability to handle oxidative stress. *T. denticola* has multiple *grdB* and *grdE* genes that allow this bacterium to obtain energy through the conversion of amino acids and related compounds (Seshadri et al., 2004). The proteolytic enzymes or proteases produced by *T. denticola* can damage the host immunoglobulins such as IgG, IgM and IgA and its complement proteins (Popova et al., 2014). Aside from compromising the host immune response, the protease called dentilisin provides necessary nutrients for its growth in anaerobic environment (Jain et., 2018). The pathogenicity of this bacteria is dependent on dentilisin, that acts as its cell-surface protein to breakdown host proteins and modulate the host resistance to disease (Nieminen et al., 2018).

2.10 *Tannerella forsythia*

Another bacterium that is often linked to the progression of periodontal disease is *Tannerella forsythia.* It has a rod-shaped structure and is a non-motile, Gram-negative anaerobic microorganism (Figure 2.9). The proteolytic enzyme produced by this bacterium is capable of altering the host immunoglobulins and complement factors, as well as triggering cell apoptosis (Popova et al., 2014).

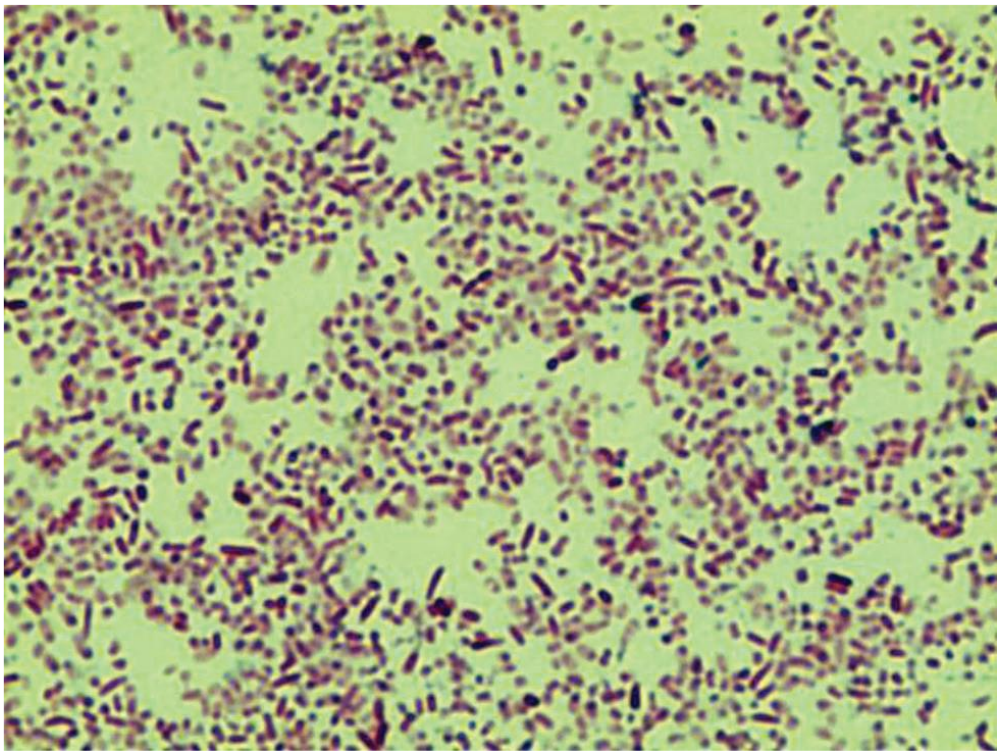

Figure 2.9: Gram's negative stain of rod-shaped *Tannerella forsythia* (Bankur et al., 2014).

It is frequently detected in high occurrence in chronic and recurring oral conditions such as gingivitis, chronic and advanced periodontitis (Sharma, 2000; Popova et al., 2014). It terms of nomenclature; this species has gone through a few classification changes. First known as *Bacteroides forsythus* (Tanner et al., 1986), then to *Tannerella forsythensis* (Sakamoto et al., 2002) and finally after considering its 16S rRNA phylogenetic analysis, a confirmed reclassification to *Tannerella forsythia* was made by Sakamoto et al. (2002) to avoid species nomenclature confusion (Maiden et al., 2003).

In a single-species biofilm culture, *T. forsythia* has shown to lose its virulence capacity (Honma et al., 2007; Sharma, 2010). A finding observed Sharma (2020), introduction of *T. forsythia* to animal model using single-species infection failed to initiate a significant lesion. It shows that the virulence of *T. forsythia* is dependent to the interaction

with other species in the dental biofilm. There is a possibility that this bacterium offers by-products that are useful for the growth of red complex bacteria (Sharma, 2010).

T. forsythia is classified as a periodontal pathogen as its characteristics is in agreement with the postulate criteria by Socransky et al. (1998). Firstly, this species is detected in increased abundance in periodontitis. Secondly, the antigens produced by this bacterium triggers host immune actions. Another characteristic is, this bacterium is capable of causing infection in animal models and lastly, it produces virulence factors that is capable of hastening the disease progression (Posch et al., 2012). A few virulence factors have been discovered that relates to its glycogen synthesis function including α-D-glucosidase and *N*-acetyl-β-glucosaminidase, glycosylated surface (S-) layer and a sialidase (Posch et al., 2012).

The complex growth nutrition needed by *T. forsythia* has made culture studies on this species not as well-studied as other periopathogens (Posch et al., 2012; Bankur et al., 2014). However, Bankur et al. (2014) managed to overcome this limitation by introducing N-acetyl muramic acid in the media culture. Colonies were found to grow fairly well on the media, indicating that the supplemented nutrient is vital for *T. forsythia* growth (Bankur et al., 2014).

2.11 Malaysian Oral Healthcare Scenario

In Malaysia, about 10% of the adults have been reported to suffer from poor oral health status, 15.7% among preschool children aged 5 until 6 years and 13.6% among 16-year-olds (Allan, 2012). In young children, the ignorance of good oral hygiene can cause pain and reduced the quality of life, nutritional status and their physical development (Allan,

2012). Based on national oral health survey done in the year 2000, it was reported that only 26% of 15 until 19-years-old have healthy periodontal tissues. Among those of 35 until 44 years of age, only 5% was found to have healthy periodontal tissues (Allan, 2012).

In a study conducted by Philip et al. (2009), there are many bacteria that are considered as important periodontal pathogens in the Malaysian oral cavity which is a mixed of gram-positive coccus and non-streptococci species. The gram positive cocci species include *Streptococcus (Strep.) pneumonia, Strep. pseudopneumonia, Strep. mitis, Strep. infantis, Strep. oralis* whereas non streptococci include *Staphylococcus aureus, Neisseria subflava, Rothia mucilaginosa, Kingella oralis* and *Actinomyces viscosus.* The samples that were collected from teeth surface, gingival crevice and tongue surface also contains non-pathogenic bacteria such *as Capnocytophaga granulosa, Strep. Sanguis and Lautrapia.* They are vital for the ecological balance of the oral cavity as well as for good oral health maintenance (Philip et al., 2009).

2.12 Molecular Approaches for Detection of Periodontal Bacteria

Over the last few decades, studies have been carried out to identify and detect the presence of periodontal bacteria primarily from the subgingival plaques of the oral cavity and saliva (Pereira et al., 2012; Galimanas et al., 2014; Serra e Silva Filho et al., 2014). Nearly 60% of the bacterial population that reside in the human mouth are not cultivable (Laserre et al., 2018). The detection and knowledge of bacterial diversity associated with periodontal disease is essential for the diagnosis and rational treatment of the disease. Molecular technologies have made tremendous discoveries for the identifications of strict anaerobic species in order to tackle the issues of periodontal disease (Sbordone & Bortolaia,

2003). To discover and understand the diversity of oral microbiome, the use of non-culture approaches is more reliable due to limitations of unculturable bacteria species (Hashim, 2018).

Several molecular approaches, such as checkerboard DNA-DNA hybridization, *in situ* hybridization or polymerase chain reaction (PCR), have been developed to overcome the limitation of uncultivable oral microorganisms and being used by researchers to identify periodontopathogenic bacteria (Liu et al., 2012; Kotsilkov et al., 2015; Laserre et al., 2018). The DNA extraction procedures and different PCR primers pairs used contribute to the differences in the ability to identify the diverse bacteria found in the oral samples (Bik et. al., 2010).

Terminal restriction fragment-length polymorphism (T-RFLP) is also another molecular approach which allows the assessment of complex bacterial communities (Sakamoto et al., 2003). Jinfeng et al. (2013) successfully reported the sequencing of 16 metagenomic samples obtained from saliva and plaques represented by four periodontal stages had strong correlation between community structure and disease status. In addition, they also reported that there was a significant amount of novel species and genes were identified in the metagenomic assemblies (Jinfeng et al., 2013).

The heterogeneity of the periodontal microbiome has been classified to over 400 species using 16S rRNA amplification, cloning and Sanger sequencing methods (Hashim, 2018). The use of a fast and accurate detection of bacteria is crucial in clinical laboratory in order to understand the causes of disease and types of treatment to offer. Conventional

method of species identification is less expensive but not all bacteria are able to be identified. Thus, specialised personnel and advance equipment are required especially for anaerobic microorganism or in the case of rare or novel species (Woo et al., 2008). The conventional phenotypic protocol depends on bacterial culture and its growth criteria as well as biochemical profile. Culture method is also time-consuming and laborious when the bacteria has slow growth rate. With the emergence of 16S rDNA sequencing, more data can be produced within 48 hours even for bacteria with slow growth (Woo et al., 2008).

In the past decade, studies on the complex diversity of oral microbiome has been made possible with the advent of phylogenetic analysis using 16S rRNA gene. The increasing interest in the development of culture-independent methods has made characterization of microbial community less of a hassle especially when dealing with the fastidious species and yet-to-be-discovered bacteria (Bik et al., 2010). The slow-growing and uncultivable species can be discovered using this powerful tool which is reliable due to its sensitivity and specificity (Ribeirio et al., 2011).

The usage of 16S small subunit ribosomal ribonucleic acid (rRNA) in molecular typing is because it is universally present as a conserved region in prokaryotes (Bik et al., 2010). Aside from its universal presence in almost all bacteria, the use of 16S rRNA for phylogeny analysis is also due to the stable function of the gene that remain unchanged over evolution. The fragment length of 16S rRNA which is approximately 1500 bp is also large enough to provide informatics data of a species (Patel, 2001). Universal 16S rRNA gene (Figure 2.10) amplification via polymerase chain reaction (PCR) is often utilized in molecular laboratory protocols for identifying ambiguous bacteria. Within the highly

conserved 16S ribosomal subunit, there are nine variable regions (V1-V9) that is unique to each genera and species. In determining the microorganism's phylogeny at genus or species level, quickly evolving regions give the highest resolution as compared to a more conserved region (Bukin et al., 2019). Recently, V5-V7 region has been used due to its ability to discriminate closely related and distinctly related microorganisms (Huber et al., 2019).

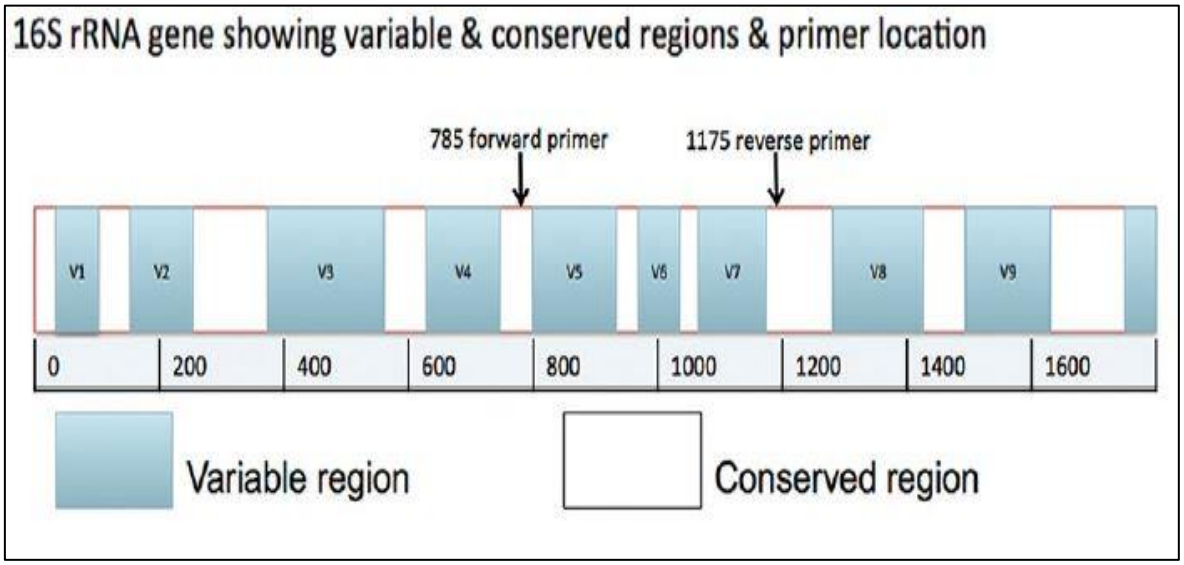

Figure 2.10: Schematic presentation of 16S rRNA gene showing variable region and conserved regions with the PCR primers placed at conserved regions (Patel et al., 2017).

In metagenome study of bacterial microbiota, after 16S rRNA PCR, it is preceded with DNA sequencing (e.g. targeting the V5-V7 regions) that allows the amplified sequences to be compared with a known genetic sequence database for bacterial species identification (Patel et al., 2017). In an oral bacteria research by Nasidze et al. (2009), partial 16S rRNA gene sequencing has successfully shown the highly diverse bacterial population in the healthy oral cavity of each individual from 12 geographic areas (Bik et al., 2010).

Despite the reliability of 16S rRNA subunit in bacterial strain identification, there is still uncertainties on the data obtained. A similarity score to a neighbour strain of less than 97% against the gene sequence database would indicate a new species, but a score of more than 97% similarity is not fully understood (Petti, 2007). This is because the value of greater than 97% may represent a novel species or a similar cluster to a previous taxon (Janda & Abbot, 2007). The detection of multiple similar or different duplicates of ribosomal operons can affect the accuracy of taxa numeration in environmental samples using 16S rRNA gene amplification and sequencing (Rosselli et al., 2016). Another option to analyse and identify bacterial species is by using metagenomics approach. However, metagenomics method is expensive, involves a large amount of reads and requires advanced computational capacity (Rosselli et al., 2016).

The accuracy and reliability of 16S rRNA gene detection can be compromised by a number of factors that can limit the outcome. In a positive bacterial culture of specimen, the inability to detect the 16S rRNA gene may arise due to the failure in gene amplification as the bacteria count in the sample is below the detection limit (Schuurman et al., 2004). PCR inhibitors can also disrupt the amplification of the target gene sequence. Besides, it is found that gram-postive bacteria is less likely to be successfully detected when using 16S rRNA gene target due to the bacterial thicker peptidoglycan cell wall that is harder to disrupt than gram-negative bacteria (Jenkins et al., 2012).

The recent technology which is the next generation DNA sequencing has brought about a major progress in the discovery of oral bacterial community in greater depth as compared to previous days that depend merely on culturing methods (Hashim, 2018). The Human Oral Microbe Identification using Next Generation Sequencing (HOMINGS), an Illumina-based metagenomic sequencing, has made it possible to identify up to 600 oral bacteria taxa in salivary microbiota analysis (Belstrom et al., 2016). Sequencing of the 16S ribosomal RNA is widely used due to its universality that has aided in the discovery of 11 phyla in the Bacteria domain (Hashim, 2018).

2.13 Factors Contributing to Oral Health and Periodontal Disease

The condition of health and disease in the assessment of the oral health of a person is determined by various factors. Among the factors include contributing influence by the hosts, biofilm growth, shift in the species community, imbalance in the nutrient and intraoral environment health that lead to the progression of the disease (Sbordone & Bortolaia, 2003).

In general, the contributing factors to periodontal disease can be classified into two namely modifiable and non-modifiable risk factors (AlJehani, 2014). Risk factor is a behavioural, biological or environmental aspect following a time-based progression, of which with its presence, can increase the probability of disease occurrence, while with its absence, can lessen the likelihood. In the case of disease progression, risk factor elimination may not reverse the condition (Van Dyke et al., 2005). Among the modifiable risk factors of periodontal disease are smoking, diabetes mellitus, microorganism colonization, socioeconomic status, stress and nutrition (AlJehani, 2014; Mehta, 2015).

One of the environmental factors and modifiable risk factors, particularly smoking can lead to the onset and development of periodontal disease (Pihlstrom et al., 2005). Smoking encourages the pathogenesis of subgingival biofilm development and offers a favourable habitat for periopathogens colonization (Jiang et al., 2020). It is found that cigarette smokers are five times at a higher risk to acquire chronic periodontitis than non-smokers (Kelbauskas et al., 2005). Individuals suffering from diabetes mellitus and having hyperglycemia are of greater chances to develop periodontal disease due to the oral environment that is conducive for bacterial growth (Singh & Mathur, 2012). In periodontal pathogenesis, not only one, but several microorganism work in synergy to cause disease. Until recently, among the identified pathogenic bacteria are *Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actimomycetemcomitans, Campylobacter rectus, Prevotella intermedia,* and *Spirochetes* species (Lang, 2009). In relation to socioeconomic status, there is a clear link between lower socioeconomic status to periodontitis and oral health. It can be observed that educated individuals with a stable household income has better periodontal health (AlJehani, 2014).

Another modifiable risk factor for periodontal disease is stress. It is well-documented that stress disrupts the optimal functioning of immune system by the release of stress-related hormone that can cause increase susceptibility to oral disease (Gunepin et al., 2018). In individuals with chronic stress, periodontal disease is found to be more pervasive (Mehta, 2015). When a person is under stress, it will also lead to changes in their nutritional choices. Impulsive eating due to stress usually involve the consumption of food in high fat and high carbohydrate content. Plaque formation is favourable by this type of eating habit (Gunepin et al., 2018). Individual behaviour such as eating junk food, alcohol abuse, poor oral practice,

lack of fluoride exposure and irregular visit to the dentist, too, contribute to the severity of this disorder (Allan, 2012).

Periodontal disease can also be caused by non-modifiable risk factors such as osteoporosis, host susceptibility, changes in aging, female hormones, and pregnancy (AlJehani, 2014; Mehta, 2015). The common criteria shared by both periodontitis and osteoporosis is both the diseases cause bone structure loss (Jagelaviciene and Kubilius, 2006). The effect of osteoporosis is seen in the progression of periodontal tissues breakdown and tooth mobility. It affects the dental procedures as alveolar bones becomes more fragile to loads of dental implants (Singh & Mathur, 2012). The effect of aging does relate to periodontal disease. Aging alters the periodontal tissues of elderly people as they have heightened sensitivity to changes in oral environment (Huttner et al., 2009). However, if good oral routine is practiced, the risk of having periodontal disease upon aging can be reduced, since it is not an exact risk factor (Reddy, 2006).

Aside from that, another non-modifiable risk factor for periodontal disease is host susceptibility to the disease. Each individual is genetically different in their response to disease conditions including response to oral microbiota changes. About 10 until 15% of a population has high susceptibility to periodontitis and its progression is rather quick (from mild to chronic) for this particular group (AlJehani, 2014).

Female hormones, too, is one of the non-modifiable risk factors. The hormonal changes that occurs throughout a female's lifetime can modify the periodontal health status of females (Lopez-Marcos et al., 2005). The female hormones may fluctuate during teenage

years, the menstrual phase, pregnancy, or menopause. The administration of oral contraceptives can also alter the hormone level (AlJehani, 2014). During pregnancy, a more prominent periodontal changes can be seen to females (AlJehani, 2014; Najeeb et al., 2016). Pregnant women are more incline to suffer from gingivitis, periodontitis and gingival hyperplasia. The increase oestrogen level may contribute to this condition during pregnancy. A proper eating habit and nutritional choices can be useful for the management of periodontal disease during pregnancy (Najeeb et al., 2016).

Besides that, there has been link found between periodontal disease with adverse pregnancy outcome and systemic diseases such as cardiovascular disease, stroke, pulmonary disease, and diabetes (Pihlstrom et al., 2005). However, little is known about the mechanisms of disease initiation and progression. There is also insufficient knowledge on the identification of high-risk forms of gingivitis that could lead to periodontitis and inadequate evidence on the effective disease prevention (Allan, 2012).

CHAPTER 3

OPTIMIZATION OF THREE DIFFERENT METHODS USED IN SALIVA DNA EXTRACTION

3.1 Introduction

Every part of the human body consists of microorganisms that are distinct for every anatomical sites of the host subject that interacts in a symbiotic manner (Dethlefsen et al., 2007). The oral cavity comes after gut as the part of the body with the highest and complex bacterial communities (Killian et al., 2016). It harbours to an estimate of over 700 bacterial species and of which about 400 species were found in the periodontal pockets (Paster et al., 2006). The interactions between the complex microbial populations serve as vital aspect for providing information on the state of human health and disease (Bik et al., 2010).

Up to the present time, blood is more commonly used as the biological starting material for molecular diagnostics. However, it has higher infectious risk and requires trained personnel for the fluid collection as opposed to using another alternative which is saliva that can also be used for direct-PCR method (Cascella et al., 2015). Besides that, there have been increasing studies on the potential of saliva DNA as a diagnostic fluid (Amado et al., 2008).

Human saliva encompasses of approximately 98% water with a vast mixture of nutrients such as electrolytes, mucus, antibacterial compound and enzymes (Looi et al., 2012). The bacteria from the saliva are from inner lining of the mouth and also from the food

being consumed or anything that is introduced into the mouth from the surroundings (Takeshita et al., 2016). Bacterial DNA is not the only component in the saliva as the DNA in saliva also originates from cells that shed from the intraoral surface and leukocytes (Looi et al., 2012).

Despite having benefit of ease in sample collection and easy storage, saliva sampling does have its disadvantages as well. The sample integrity of saliva is easily compromised when storage is made at freezing temperature. It may lead to structural changes and encourages the rapid DNA degradation causing problems to results of pharmacogenetic test and under certain circumstances may cause the recollection of samples from patients (Cascella et al., 2015).

In genomic research, the vital component is the genomic DNA. Once biological samples are collected, DNA extraction is the intial step before molecular assessments can be done. Therefore, it is of utmost importance to select the suitable protocol to achieve a pure yield of DNA which in turn benefits the subsequent downstream process especially PCR sensitivity. While there are a variety of DNA isolations procedures available, a lot of aspects need to be considered when choosing particularly when dealing with a massive sample size. In general, most DNA isolation techniques incorporate the usage of organic and non-organic reagents and involve centrifugations (Javadi et al., 2014).

Conventionally, culturing methods using solid and liquid media and biochemical tests have been incorporated for bacterial identifications. However, the approaches are labour-intensive, costly and arduous. As the technology advances in the recent decades,

molecular analyses are preferable because of its time efficient, sensitivity and accuracy in the results produced for detecting microorganism that are difficult to culture. One of the methods is polymerase chain reaction (PCR) amplification procedure (Tomazinho & Avila-Campos, 2007). The polymerase chain reaction (PCR) is a simple but powerful method that enables a specific DNA fragment to be amplified from a trace amount of DNA (Lorenz, 2012; Garibyan & Avashia, 2013).

In this study, saliva DNA was extracted from healthy and diseased patients using three procedures; phenol-chloroform isoamyl alcohol, chelex-100 resin and Norgen saliva DNA isolation kit. Optimization of final DNA elution volume was also done prior to assessment of DNA quality and purity via NanoDrop spectrophotometer followed by 16S rRNA PCR amplification. The universal 16S primer pairs (27F and 1492R) were used to amplify the bacterial target sequence. A successful amplification produced a product size of 1500 bp upon visualition under the UV transilluminator which indicated the success of optimization and feasible of the method for saliva DNA extraction. The findings of this study can provide a better understanding of the rationale of different DNA extraction in oral microbial research through the differences in DNA yield and quality obtained.

3.2 Materials and Methods

3.2.1 Sample Collection

Saliva samples were collected from nine (n=9) subjects aged from 18 to 30 years old. Two of them acted as control (healthy patients). Each subject was required to provide 2 mL of saliva in a given 15 mL Falcon tube. Within each prepared collection tube, an equal volume of 2 M Tris-EDTA buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0; 2% SDS) was

added. Prior to sampling, patient's information sheet and consent form were given to the patients. The information sheet contained brief information on the research and the risks during sampling which was prepared based on the guidelines provided by Clinical Research Center Malaysia. All the required items for sampling were prepared before sampling and delivered to the dentist on an agreed date and time. Ethical clearance for research was approved by medical ethics committee of Faculty of Medicine and Health Sciences UNIMAS under UNIMAS/NC.21.02/03.02 (72). The criteria for patients with a healthy gingival tissue status are having the absence of probing attachment loss, less than 10 percent bleeding in probing, no radiographic bone loss and periodontal probing depth of 3 mm and lesser. The feature commonly observed in gingivitis patients, that differentiate affected gingivae tissue from healthy ones is 10 percent or more bleeding on probing (Chapple et al., 2018).

3.2.2 DNA Extraction from Saliva Samples

Extraction were done by using different methods to compare the concentration and purity of DNA obtained. The methods used were modified phenol-chloroform method (Barkers et al., 1998), Chelex-100 resin (Tamura et al., 2006) and Norgen Saliva DNA Extraction kit that were done according to manufacturer's protocol. Different elution and pellet dissolving volumes were used (100 uL, 70 uL, 50 uL). DNA concentrations and purity were assessed using NanoDrop ND-1000 spectrophotometry.

3.2.2.1 Phenol: Chloroform: Isoamyl Alcohol DNA Extraction

DNA isolation was done by using modified phenol-chloroform method (Barker et al., 1998). For the cell lyses step, 100 µL of saliva was mixed with 10 µL of (20 mg/ mL)

proteinase K (Vivantis, MY) and the mixture was incubated at 55 ˚C for two hours. Next, the digested sample was added with the same volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) (Sigma, USA) and centrifuged for 10 minutes at 10,000 rpm. The aqueous layer was transferred to a new 1.5 mL tube and chloroform: isoamyl alcohol (Sigma, USA) was mixed into the solution. After another centrifugation, the step is proceeded with overnight precipitation at -20 ˚C with 2 volumes of absolute ethanol (HmBG, Germany). The DNA pellet obtained after precipitation was then washed with 1 mL of 70% ethanol for salt and organic molecules removal. After air-drying the DNA pellet, 50 uL of double distilled water was used to resuspend the pellet.

3.2.2.2 Chelex-100 Resin DNA Extraction Method

For chelex-100 resin method, a hundred microliters of saliva was mixed with 200 µL of chelex-100 resin (Sigma, USA). Incubation was then done at 56 ˚C for 30 minutes. Subsequently, the mixture was subjected to boiling for 10 minutes followed by centrifugation for 20 minutes at 10, 000 rpm before storage in -20 ˚C.

3.2.2.3 Norgen Saliva DNA Isolation Kit Method

The DNA of the saliva samples was extracted by using the Norgen Saliva DNA Isolation Kit (Norgen Biotek Corp., Canada) according to the manufacturer's instruction. A hundred microliter of saliva was added to a sterile, microcentrifuge tube with 100 µL of Lysis Buffer F. Then, 20 µL of proteinase K was added before vortexing to mix. Incubation was then done at 55°C for 10 minutes. As much as 200 µL of Binding Buffer B was subsequently transferred into the tube before incubation for 5 minutes at 55°C. Then, 720

µL of isopropanol was added and vortexed to mix. The mixture was next transferred to a provided spin column and centrifuge for 1 minute at 6,000 rpm. Wash solution A (500 µL) was added and another centrifugation was done at 8, 00 rpm for a minute. The washing step was repeated once at maximum centrifugation speed for 1 minute. Next, the flowthrough was discarded and the column was spun to dry at maximum speed for 2 minutes. The collection tube was replaced with a new one and Elution Buffer B (50 µL) was added into the new column. It was then incubated at 55°C for 5 minutes followed by 2 minutes spin at 2, 000 rpm. The DNA sample was stored at -20°C.

3.2.3 DNA Concentration and Purity Evaluation

The concentration and purity of DNA of the sample were determined based on UV absorbance, using NanoDrop ND-1000 UV/VIS Spectrophotometer. The absorbance ratio of 260/280 and 260/230 were used.

3.2.4 Bacterial DNA Detection via 16S rRNA PCR Amplification

All the extracted DNA samples were subjected to polymerase chain reaction (PCR) amplification using a pair of universal 16S rRNA primers, 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-ACG GCT ACC TTG TTA CGA CTT-3') to produce amplicons of approximately 1500 bp in sizes (Rôças and Siqueira Jr., 2005). The amplification was performed on a LabCycler System (SensoQuest, Germany). The PCR reaction mixtures used is shown in Table 3.1 whereas Table 3.2 shows the PCR reaction amplification parameters.

Table 3.1: The PCR reagent of 25 µL volume reaction.

PCR reagent	Quantity per reaction (µL)
2X PCR Master Mix (exTEN, Singapore)	12.5
10 pmol/µL of primer 27F (IDT, Singapore)	1.0
10 pmol/µL of primer 1492R (IDT, Singapore)	1.0
Sterile distilled water	7.5
Template DNA (10 ng/µL)	3.0
Total final volume	25.0

Table 3.2: PCR amplification reaction parameters.

Step Cycle	**Temperature/ Time**
Initial denaturation	95 ^{o}C (2 min)
Denaturation	94 ^{o}C (30 sec) ┐
Annealing	52 ^{o}C (30 sec) ├ 35 cycles
Elongation	72 ^{o}C (30 sec) ┘
Final elongation	72 ^{o}C (10 min)

The PCR products were then loaded into 1.5% agarose gel, electrophoresed and visualized onto the UV transilluminator.

3.3 Results and Discussion

In this study, nine saliva samples were collected from two healthy subjects and seven gingivitis patients. A successful PCR amplification indicates the suitable volume to be used for final pellet DNA elution besides as the indicator for the presence of oral bacteria in the DNA extract. The concentration and purity of DNA obtained from each method are as shown in the NanoDrop spectrophotometer readings in Table 3.3. To assess the purity of DNA extract, absorbance ratio 260/280 and 260/30 were used. The absorbance ratio of 260/280 ratio is used to detect for the presence of protein contamination (Mendoza et al., 2016) meanwhile the 260/230 ratio is for the evaluation of salt contamination (Olson & Morrow, 2012). Genomic DNA isolates of good quality ideally falls within the range of 1.8 until 2.0.

Lower readings indicate the possible contamination in the samples used. If a ratio greater than 2.0 is obtained, this indicates the contamination by RNA (Khare et al., 2014). Since the saliva samples are from both healthy and diseased patients, inaccurate quantification can be another cause for concern as a result of the presence of excessive protein and contaminants in the final extracted DNA (Goode et al., 2015).

From Table 3.3, it suggests that the chelex-100 resin method produced the highest DNA concentration on average which is 60.01 ng/ µL. For absorbance ratio of 260/280 used for protein contamination assessment, Norgen kit produced results closer to ideal which is 1.77 followed by PCIA and chelex-100 resin (1.51 and 0.82 respectively). For the salt contamination evaluation (absorbance at 260/230), PCIA produced near to ideal range value of 1.51 whereas chelex-100 resin and Norgen kit with the value of 0.26 and 0.79 respectively.

Table 3.3: Concentration and purity of DNA isolated via three DNA extraction methods.

Sample	**DNA Concentration (ng/ µL)**			**A260/280**			**A260/230**		
	PCIA	**Chelex-100 resin**	**Norgen kit**	**PCIA**	**Chelex-100 resin**	**Norgen kit**	**PCIA**	**Chelex-100 resin**	**Norgen kit**
C1	1.92	76.54	1.37	1.38	0.68	1.44	1.07	0.22	0.74
C2	4.25	97.87	2.68	1.34	0.75	1.47	1.39	0.26	0.86
D1	5.05	80.46	3.82	1.37	0.70	1.52	1.55	0.23	0.67
D2	2.02	72.90	2.30	1.34	0.68	1.57	1.53	0.22	0.64
D3	8.25	73.75	24.13	1.79	1.24	2.09	1.63	0.45	1.91
D4	1.63	31.73	3.63	1.53	0.80	1.89	1.41	0.25	0.90
D5	2.52	33.85	2.85	1.61	0.86	1.85	1.40	0.26	0.34
D6	3.77	34.17	5.87	1.61	0.84	2.09	1.96	0.25	0.54
D7	1.40	38.83	5.07	1.58	0.81	2.04	1.66	0.24	0.47
Mean ±S.D.	3.42 ±2.21	60.01 ±25.19	5.75 ±7.03	1.51 ±0.16	0.82 ±0.17	1.77 ±0.27	1.51 ±0.24	0.26 ±0.07	0.79 ±0.46

In comparison of the three used methods against the absorbance readings, PCIA method produced the closest to ideal quality of DNA with absorbance value 1.51 for both protein and salt contamination evaluation (absorbance at 269/280 and 260/230). However, in the aspect of DNA concentration, the highest yield is shown by chelex-100 resin with 60.01 ±25.19 ng/ µL but failed to be used for 16S rRNA PCR. PCR was performed by targeting the conserved 16S regions using a pair of universal primers 27F and 1492R. The use of PCR based on 16S rRNA gene is because its reliability for phylogenetic analysis due to its ultra-conserves regions and the sequence is commonly found in prokaryotes from diverse environment samples (Claridge, 2004; Tanner et al., 2011; Martinez-Porchas et al., 2017).

Furthermore, DNA isolated from saliva is a mixed of oral bacteria and human DNA detached from epithelial tissues and immune cells in the oral cavity (Nishitani et al., 2018). The amplification method using 16S rRNA gene for identifying and detecting bacteria in a polymicrobial sample increases the chances of successful identification of fastidious microorganisms (Kommedal et al., 2009). This is because the human mouth is host to a variety of microorganisms that includes bacteria, fungi, protozoa, archaea, and viruses (Rosenbaum et al., 2019).

The failure to amplify the target 16S gene sequence of chelex resin DNA extracts is in agreement with Singh et al. (2018) findings. The DNA extract from chelex resin showed low 260/230 absorbance ratio aside from contaminated and pigmented caused by suspended cellular debris (Singh et al., 2018). The successful amplification of a target sequence by PCR

is largely dependent on the amount and quality of the extracted nucleic acids from the collected samples (Bachmann et al., 2008).

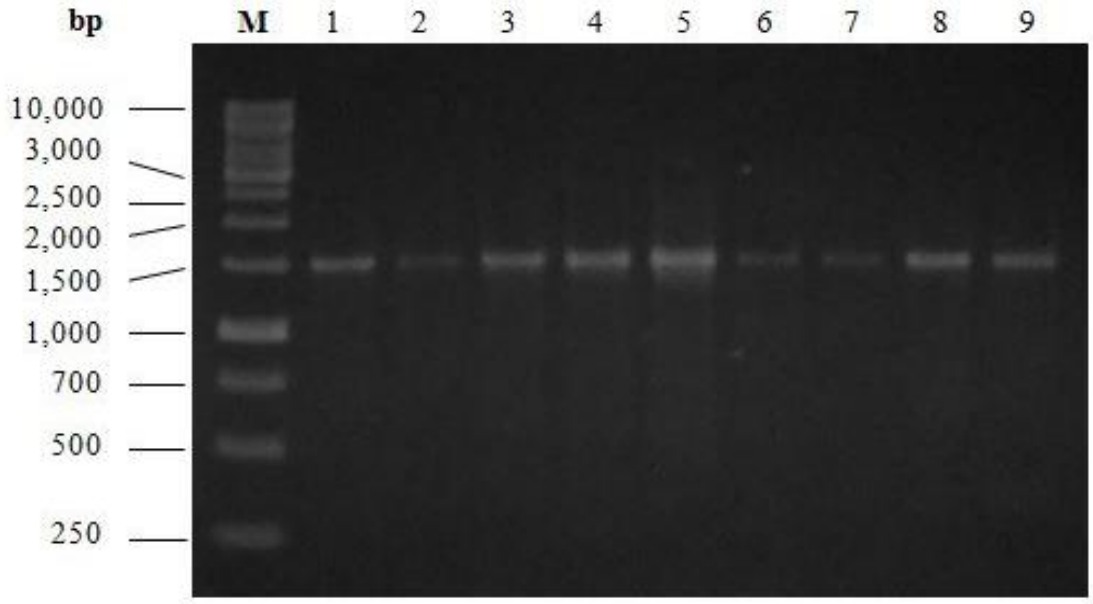

Figure 3.1: PCR products (approximately 1500 bp) of saliva DNA extracted using PCIA method viewed on 1.5% gel. Lane (1) Control 1, (2) Control 2, (3) Diseased 1, (4) Diseased 2, (5) Diseased 3, (6) Diseased 4, (7) Diseased 5, (8) Diseased 6, (9) Diseased 7, (M) 1kb DNA ladder (Thermo Scientific, USA).

Among the three volumes (100 µL, 70 µL, 50 µL) tested, it was found that 50 µL produced a successful 16S rRNA PCR amplification with the expected amplicon size 1500 bp as shown in Figure 3.1 for Norgen kit and Figure 3.2 for PCIA method. However, no amplification was made from the DNA extract of chelex-100 resin method. Larger volume of 70 µL and 100 µL failed to produce the expected 1500 bp product size which could be due to over dilution of the genomic DNA. Furthermore, Sing et al. (2018) demonstrated that occasionally PCR amplification is unsuccessful when the DNA extract used is from Chelex-based method. Despite the fact that saliva is a non-invasive method to collect biological samples, it has been reported that saliva is more vulnerable due to its lytic enzymes

component and inhibitors that compromise the efficiency of PCR amplification yield (Cascella et al., 2015).

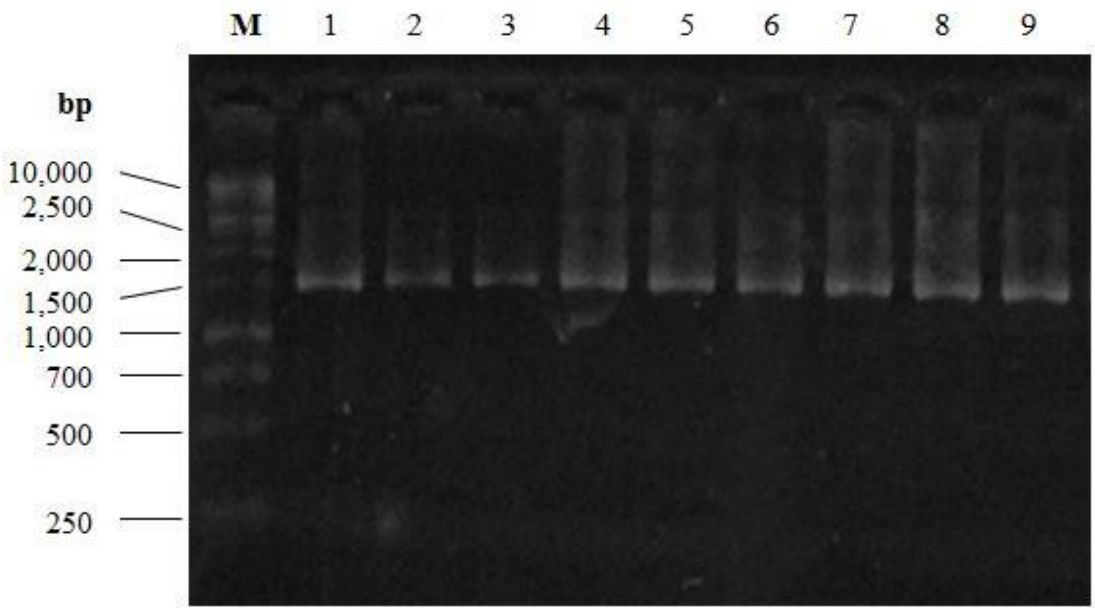

Figure 3.2: PCR products (approximately 1500 bp) of saliva DNA extracted using Norgen kit method viewed on 1.5% gel. Lane (M) - 1kb DNA ladder (Thermo Scientific, USA); Lane (1) - Control 1; Lane (2) - Control 2; Lane (3) - Diseased 1, Lane (4) - Diseased 2; Lane (5) - Diseased 3, Lane (6) - Diseased 4; Lane (7) - Diseased 5; Lane (8) - Diseased 6; Lane (9) - Diseased 7.

From Figure 3.1 and Figure 3.2, PCR products of 1500 bp were successfully amplified from samples of two methods, PCIA and Norgen kits. Even though the correct amplicon size was produced, smearing can still be seen at the electrophoresed bands. Band smearing could be due to the diverse PCR targets in the DNA samples that reflect the richness of the initial 16S rRNA gene population and not to be considered as a non-specific amplification (Zrimec et al., 2013). In general, a successful nucleic acid purification requires the steps nucleic acid purification, cells or tissue breakdown, nucleoprotein complexes denaturation, and nucleases (RNAse and DNAse) inactivation, done in an environment of

reduced exposure to contamination. This is to ensure that the extracted nucleic acid is free of impurities such as protein, lipids, carbohydrate or other nucleic acid. The quality of DNA or RNA isolates is important for further downstream processes as to avoid compromising the subsequent research results (Tan & Yiap, 2009).

3.4 Conclusion

This study has successfully identified the suitable methods for saliva DNA extraction by comparing the quality and yield of DNA obtained from conventional phenol-chloroform isoamyl alcohol (PCIA) method, the alternative chelex-100 resin and the golden standard method, Norgen saliva DNA isolation kit. Among the three methods for saliva DNA isolation, PCIA and Norgen kit were found to be most reliable for use in molecular analyses with minimal PCR inhibition. Future studies can be done to determine and optimize other methods suitable to obtain a pure DNA with higher yield from saliva since saliva collection is preferable over other biological samples because of its simplicity and non-invasive approach.

CHAPTER 4

DETECTION OF *Porphyromonas gingivalis, Tannerella forsythia* AND *Treponema denticola* VIA PCR IN SALIVA OF HEALTHY AND DISEASED YOUNG ADULTS

4.1 Introduction

Saliva composed of many compounds such as organic and inorganic non-protein, polypeptide, hormone and fatty acid derivatives (Chiappin et al., 2007). In terms of collection and storage procedure, saliva has its advantage over plasma although plasma is still widely favoured by the clinicians and researchers (Chiappin et al., 2007). The salivary microbiome carries an important role as an indicator of diseases. A slight alteration in the population of certain relative species may lead to a diseased state of the oral cavity. Thus, the oral microbiota carries a vital influence to the oral and overall health (Lazarevic et al., 2012).

Oral cavity and its microbial community have shown to play an essential role in the initiation and progression of oral and systemic diseases. Since the oral microbiome plays a key role in the body's general health, the complexities of the oral bacterial community is gaining more attention for studies in understanding the mechanisms in health and disease (Zarco et al., 2012). The oral microorganisms that are responsible for the maintenance of oral homeostasis can also be the player in the onset of the diseased state indicating that the normally-found commensal species can also cause disease when there is a shift in its normal ecological state (Zarco et al., 2012). In recent studies of analysis on human microbiome, periodontal disease is considered as a result of dysbiosis rather than bacterial colonization.

Dysbiosis encompasses a shift in the abundance or a single species within a complex microbial community that leads to a disturbance in the homeostatic balance and eventually causing destructive inflammations (Olsen et al., 2017). The increasing evidence in the relationship between the human microbiome and health has led to more research being done to offer a better disease assessment and treatments as well as a more personalized prescriptions of medicines (Sonnenburg & Fischbach, 2011). This is due to the fact that every individual harbours a unique composition of microbiome within the body thus the etiology of the disease and its manifestations may differ according to each person (Rajendhran & Gunasekaran, 2009).

Periodontal disease is known to be caused by a group of causative agents known as red complex bacteria. The bacterial species includes *Treponema denticola, Porphyromonas gingivalis,* and *Tannerella forsythia* (Suzuki et al., 2013) and are commonly linked to advanced periodontal disease state (Mysak et al., 2014). One of the periopathogen that is vital in the progression of periodontal disease is *Porphyromonas gingivalis* (*P. gingivalis*). Classified as catalase-negative microorganism due to its incapability to metabolize carbohydrates, this rod-shaped, non-motile, obligate anaerobic bacterium can be observed as colonies with black pigments when grown on blood agar media (How et al., 2016). Previously, this bacterium was known as *Bacteroides gingivalis* before being changed to a new genus, *Porphyromonas* (Nisengard & Newman, 1994). *P. gingivalis* is found in the dental plaque of humans and is capable of disrupting the host protective mechanisms to maintain a balance ecological environment (Joshi et al., 2016). However, *P. gingivalis* is not unique to diseased state only. It can also be found as a common microorganism in the dental plaque of healthy individuals (Joshi et al., 2016). From several studies done previously,

closely related *P. gingivalis* bacterial species possess similar pathogenic potential but not all strains have equal pathogenicity (Joshi et al., 2016).

T. forsythia is also one of the bacteria in a group of periopathogens called red complex (Wadhwani et al., 2013). This species is an anaerobe, Gram-negative bacteria and was first named as *Bacteroides forsythus* by Tanner et al. (1986), which was then reclassified to *Tannerella forsythensis* by Sakamoto et al. (2002), and was corrected and confirmed to *T. forsythia* after analysing its 16S rRNA phylogenetic data in order to avoid confusion in the species nomenclature (Sharma, 2000; Maiden et al., 2003). *T. forsythia* is often linked in higher numbers with disease such as gingivitis, chronic and advanced periodontitis, as compared to in health (Sharma, 2000).

Among the oral microorganisms, the motile, spiral-shaped *Treponema denticola* is the most commonly isolated and characterized anaerobic oral species (Dashper et al., 2011). *T. denticola* has been reported to be linked to the inflammation and swelling of the gingivae tissue and infection of the root canal during the development of periodontal disease. The pathogenicity of this bacteria is dependent on its cell-surface protein called dentilisin, that can breakdown host proteins and modulate the host resistance to disease (Nieminen et al., 2018). It is an intriguing finding that when grown in a single species biofilm, *T. denticola* loses it spiral morphology. However, in polymicrobial layers and with the presence of *P. gingivalis*, the synergic interaction between these two species resulted in the retention of *T. denticola* spiral morphology (Zhu et al., 2013; Ng et al., 2019).

In this context, saliva genomic DNA was extracted from both healthy and diseased patients using phenol-chloroform isoamyl alcohol protocol. The extracted DNA were then amplified using 16S rRNA polymerase chain reaction (PCR) to identify the presence of bacteria in the sample. Then, the presence of each red complex species in saliva samples were identified using a specific primer set. Therefore, this study aims to investigate the prevalence of red complex species in disease and health found in the saliva of Sarawakian young adults with or without gingivitis, specifically in Kuching area. Since the presence of periodontal pathogen in the oral cavity plays a significant role as an indicator for the presence of systemic disease, its detection could act as a prognostic indicator for health quality.

4.2 Materials and Methods

4.2.1 Saliva Sample Selection and Collection

This study was approved for ethical clearance by medical ethics committee of Faculty of Medicine and Health Sciences UNIMAS under UNIMAS/NC.21.02/03.02 (72). A total of thirty-three (n=33) patients (male=10, female=23; gingivitis-infected=23, and gingivitis-free=10) was recruited to participate in this study. The recruitment of suitable candidates and sample collection were done by a periodontal specialist from Sarawak General Hospital. The inclusion criteria for sampling include male and female with age between 18 until 30 years old. As for the exclusion criteria, those with systemic disease were to be excluded. Gingivitis in patients is indicated by more than 10 percent of bleeding on probing meanwhile intact, healthy gingivae tissue is observed with conditions such as no detection of probing attachment loss, absence of radiographic bone loss, probing pocket depth of less than 3 mm and less than 10 percent bleeding on probing (Chapple et al., 2018).

Before samples were collected, the subjects were briefed on the research and given patient's information sheet and consent form which were prepared following the guidelines by Clinical Research Center Malaysia. A 15-mL Falcon tube was used to collect 2 mL of saliva from each subject and was then mixed with the same volume of buffer [2M Tris-EDTA buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0; 2% SDS)].

4.2.2 Phenol: Chloroform: Isoamyl Alcohol DNA Extraction

DNA isolation was done by using modified phenol-chloroform method (Barkers et al., 1998). For the cell lyses step, 100 µL of saliva was mixed with 10 µL of (20 mg/ mL) proteinase K (Vivantis, MY) and the mixture was incubated at 55 ˚C for two hours. Next, the digested sample was added with the same volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) (Sigma, USA) and centrifuged for 10 minutes at 10,000 rpm. The aqueous layer was transferred to a new 1.5 mL tube and chloroform: isoamyl alcohol (Sigma, USA) was mixed into the solution. After another centrifugation, the step is proceeded with overnight precipitation at -20 ˚C with 2 volumes of absolute ethanol (HmBG, Germany). The DNA pellet obtained after precipitation was then washed with 1 mL of 70% ethanol for salt and organic molecules removal. After air-drying the DNA pellet, 50 uL of double distilled water was used to resuspend the pellet.

4.2.3 Bacterial DNA Detection via 16S rRNA PCR Amplification

For the detection of bacterial DNA in the extracted DNA samples, polymerase chain reaction (PCR) amplification using a pair of universal 16S rRNA primers, 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-ACG GCT ACC TTG TTA CGA CTT-3') was utilized which was performed on a LabCycler System (SensoQuest, Germany). The

primet set was designed according to Rôças and Siqueira Jr. (2005) study with an expected PCR product length of approximately 1500 bp in length.

For the conventional 16S rRNA PCR reaction performed, a 25 µL volume reaction was used. The PCR mixture contained 3 µL of DNA sample, 12.5 µL of 2X PCR Master Mix (exTEN, Singapore), and 10 pmol/µL of each primer (IDT, Singapore). The amplification was done at 35 cycles and the PCR reaction parameters were as followed: initial denaturation at 95 ºC for 2 min, denaturation at 94 ºC for 30 sec, annealing at 52 ºC for 30 sec, elongation at 72 ºC for 30 sec and final elongation at 72 ºC for 10 min. For visualization of the PCR products, samples were loaded into 1.5% agarose gel, and electrophoresed onto the UV transilluminator.

4.2.4 Detection of Red Complex Bacteria via PCR Amplification

Three different pairs of primer sequences specific for each red complex species were constructed as reported in Tamura et al. (2006). For *P. gingivalis*, the forward sequence is PG-F (5'- TGT AGA TGA CTG ATG GTG AAA ACC-3') and the reverse sequence is PG-R (5'-ACG TCA TCC CCA CCT TCC TC -3'). The PCR product size is 197 bp in length.

The forward primer sequence for *T. forsythia* is TF-F (5'- GCG TAT GTA ACC TGC CCG CA -3') and the reverse sequence is TF-R (5'- TCG TTC AGT GTC AGT TAT ACC T -3'). The PCR amplicon size is approximately 641 bp in length.

A set of primer sequence was also designed for *T. denticola* identification in the sample. The *T. denticola* primer sequence is as followed; TD-F (5'- AAG GCG GTA GAG

CCG CCG CTC A -3') and the reverse sequence is TD-R (5'- AGC CGC TGT CGA AAA GCC CA -3') with expected band size to be 311 bp in length.

The thermocycler LabCycler System (SensoQuest, Germany) was used for the amplification. The PCR reaction mixtures used is shown in Table 4.1 meanwhile the PCR reaction parameters are as depicted in Table 4.2.

Table 4.1: The PCR reagent of 25 µL volume reaction.

PCR reagent	Quantity per reaction (µL)
2X PCR Master Mix (exTEN, Singapore)	12.5
10 pmol/µL of primer 27F (IDT, Singapore)	1.0
10 pmol/µL of primer 1492R (IDT, Singapore)	1.0
Sterile distilled water	7.5
Template DNA (10 ng/µL)	3.0
Total final volume	25.0

Table 4.2: PCR amplification reaction parameters for red complex bacteria primer set.

Step Cycle	**Temperature/ Time**	
Initial denaturation	95 ^{o}C (2 min)	
Denaturation	94 ^{o}C (30 sec)	35 cycles
Annealing	*x ^{o}C (1 min)	
Elongation	72 ^{o}C (1 min)	
Final elongation	72 ^{o}C (10 min)	

*x = annealing temperature for each red complex species (*P. gingivalis* = 55°C; *T. forsythia* = 56°C, and *T. denticola* = 65°C).

For visualization of the PCR products, samples were loaded into 1.5% agarose gel, and electrophoresed onto the UV transilluminator.

4.2.5 Statistical Data Analysis

The software GraphPad Prism 8.4.1 was used for analyzing the statistical data. In using the Fisher's exact test, if the P-value is less than 0.05, then the data is considered as statistically significant.

4.3 Results and Discussion

Periodontal disease is not uncommon among adults and the lack of documentation in the oral health studies in Malaysia need to be made a concern. The members of red complex cluster namely *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola,* are commonly associated as the causative agent of periodontal disease (Mohanty et al., 2019). However, these periodontal pathogens that are also considered to be a part of the human normal oral species, are often found at deep subgingival sites especially in severe stage of periodontal disease, or periodontitis patients (Sela, 2001).

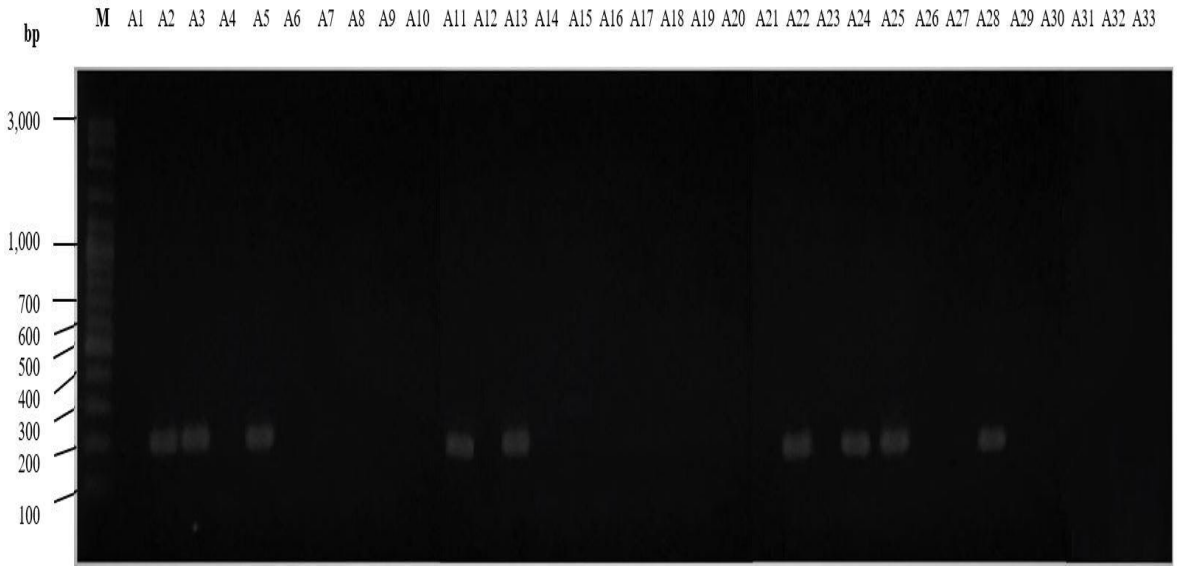

Figure 4.1: PCR products (approximately 197 bp) using *P. gingivalis* specific primers for 33 saliva samples viewed on 1.5% agarose gel. Lane (M) - 100 bp DNA ladder (In Vitro Technologies, Australia); Lane (A1) - (A2): Healthy control ; Lane (A11) - (A33): Diseased.

The molecular method using PCR is a powerful tool and reliable in the detection of microorganisms since the conventional culture method is incapable of reflecting the real microbial population in diverse environmental samples. Findings by researchers globally have shown that non-culturable methods are capable of identifying the species that cause oral infections when cultures failed to produce results. The benefit gained through this method is that more bacterial species that were unidentified previously via culture techniques can be made discoverable (Sanghavi et al., 2014). Based on Figure 4.1, the PCR amplifications of *P. gingivalis* species from the saliva samples of 10 healthy, control patients and 23 diseased patients produced a band of size 197 base pair. A successful *P. gingivalis*-specific PCR amplification of was observed in the healthy sample of patient 2, 3 and 5, and six diseased sample of patient 11, 13, 22, 24, 25 and 28.

Via polymerase chain reaction (PCR) assay, Gomes et al. (2007) successfully identified the occurrence of *P. gingivalis* in almost all of the observed black-pigmented colonies from samples of diseased patients. The result is in agreement with the findings done by Darveau et al. (2012) using mice models. He and his team found that destructive gingival bone loss can be caused by a slight change in the commensal bacteria population done by *P. gingivalis* colonization. The intriguing finding was done via real-time polymerase chain in which *P. gingivalis* population in the total microbiota was less than 0.01% (Darveau et al., 2012). This bacterial species is in fact a normal commensal bacterium in the oral cavity but a disturbed environment can cause the onset of dysbiosis consequently leading to the progression of destructive inflammation of the supporting bone structure (Darveau et al., 2012).

Table 4.3: Fisher's exact test of gender versus the prevalence of *P. gingivalis* in samples.

Table Analyzed	**Gender vs *P. gingivalis***		
P value and statistical significance			
Test	Fisher's exact test		
P value	0.2166		
P value summary	ns		
One- or two-sided	Two-sided		
Statistically significant (P < 0.05)?	No		
Effect size	Value	95% CI	
Odds ratio	4.800	0.5970 to 58.47	
Reciprocal of odds ratio	0.2083	0.01710 to 1.675	
Sensitivity	0.8889	0.5650 to 0.9943	
Specificity	0.3750	0.2116 to 0.5729	
Positive Predictive Value	0.3478	0.1881 to 0.5511	
Negative Predictive Value	0.9000	0.5958 to 0.9949	
Likelihood Ratio	1.422		
Methods used to compute CIs			
Odds ratio	Baptista-Pike		
Sensitivity, specificity, etc.	Wilson-Brown		
Data analyzed	*P. gingivalis* +ve	*P. gingivalis* -ve	Total
Female	8	15	23
Male	1	9	10
Total	9	24	33
Percentage of row total	*P. gingivalis* +ve	*P. gingivalis* -ve	
Female	34.78%	65.22%	
Male	10.00%	90.00%	
Percentage of column total	*P. gingivalis* +ve	*P. gingivalis* -ve	
Female	88.89%	62.50%	
Male	11.11%	37.50%	
Percentage of grand total	*P. gingivalis* +ve	*P. gingivalis* -ve	
Female	24.24%	45.45%	
Male	3.03%	27.27%	

The prevalence of *P. gingivalis* in both male and female patients are analyzed using Fisher's test as shown in Table 4.3. This study included 33 young adults of both healthy and diseased (gingivitis -affected) female and male. A total of 23 females and 10 males participated in this study. From the analysis, it can be observed that there is no significant difference (p-value = 0.2166) between different genders versus the presence of *P. gingivalis*

in their saliva samples. Among females, 34.78% (8 out of 23 females) were found to be positive for the presence of *P. gingivalis* in their saliva samples whereas as much as 10% males (1 out of 10) were affected by this bacterial species. This result is in agreement with the findings made by Nayak et al. (2018) in the plaque samples of chronic periodontitis patients as gender biased is not a concern but rather of age as it does affect the species distribution. However, for females, the fluctuation of hormones may contribute to the exaggerated gingival inflammation. Hormonal changes can affect the individual female's body immune response to irritants such as dental plaque especially in females with elevated levels of hormone (Weinberg & Maloney, 2007).

The prevalence of *P. gingivalis* in both healthy and gingivitis-affected patients are analyzed using Fisher's test as shown in Table 4.4. From the analysis, it can be observed that there is no significant difference (P-value > 0.99) between the gingivitis and healthy patients versus the presence of *P. gingivalis.* From healthy patients, 30% (3 out of 10) of diseased patients were found to be infected with *P. gingivalis* while 26.09% of gingivitis (6 out of 23) were found to harbour this bacteria in their oral cavity. This shows that the periopathogen is not specific to gingivitis-affected patients only. As mentioned by van Winkelhoff et al. (2002), *P. gingivalis* are rarely detected in healthy sites of oral pockets or if present, they are usually found at a low number. However, the detection of its presence may predict the future onset of periodontal disease in a person (van Winkelhoff et al., 2002). In inflamed periondontal sites, *P. gingivalis* can be found almost up to 85% in patients with periodontal disease (Yang et al., 2004).

Table 4.4: Fisher's exact test of periodontal status versus the prevalence of *P. gingivalis* in samples.

Table Analyzed	**Periodontal status vs *P. gingivalis***		
P value and statistical significance			
Test	Fisher's exact test		
P value	>0.9999		
P value summary	ns		
One- or two-sided	Two-sided		
Statistically significant (P < 0.05)?	No		
Effect size	Value	95% CI	
Odds ratio	1.214	0.2709 to 6.511	
Reciprocal of odds ratio	0.8235	0.1536 to 3.691	
Sensitivity	0.3333	0.1206 to 0.6458	
Specificity	0.7083	0.5083 to 0.8509	
Positive Predictive Value	0.3000	0.1078 to 0.6032	
Negative Predictive Value	0.7391	0.5353 to 0.8745	
Likelihood Ratio	1.143		
Methods used to compute CIs			
Odds ratio	Baptista-Pike		
Sensitivity, specificity, etc.	Wilson-Brown		
Data analyzed	*P. gingivalis +ve*	*P. gingivalis* -ve	Total
Healthy	3	7	10
Gingivitis	6	17	23
Total	9	24	33
Percentage of row total	*P. gingivalis +ve*	*P. gingivalis* -ve	
Healthy	30.00%	70.00%	
Gingivitis	26.09%	73.91%	
Percentage of column total	*P. gingivalis +ve*	*P. gingivalis* -ve	
Healthy	33.33%	29.17%	
Gingivitis	66.67%	70.83%	
Percentage of grand total	*P. gingivalis +ve*	*P. gingivalis* -ve	
Healthy	9.09%	21.21%	
Gingivitis	18.18%	51.52%	

Despite the extensive studies done relating *T. forsythia* with oral disease pathogenesis, it is still not studied in depth due to its properties as the only member of the new genus Tannerella (Sharma, 2000). The complex growth requirements to culture this species and the difficulties in performing its genetic manipulations has made *T. forsythia* understudied in its oral community (Sharma, 2000). Inflammation of gingivae tissue is less

seen when colonization is caused by *T. forsythia* monoinfection when compared to infection by multispecies bacteria (Chukkapalli et al., 2015).

The current study demostrated that the prevalence of *T. forsythia* is common in both control (gingivitis-free) and gingivitis saliva samples using the polymerase chain reaction (PCR) technique that amplified the specific gene for *T. forsythia* species. Positive bands of approximately 641 bp were succesfully amplified as shown in Figure 4.2 in 33 saliva samples; ten from healthy-control and 23 from gingivitis patients' saliva samples. PCR has higher detection capability than culture technique. This is proved by the findings made by Bankur et al. (2014) who showed that *T. forsythia* requires strict conditions to grow and is not easy to culture. On the other hand, PCR demonstrated higher sensitivity than culture method (Bankue et al., 2014).

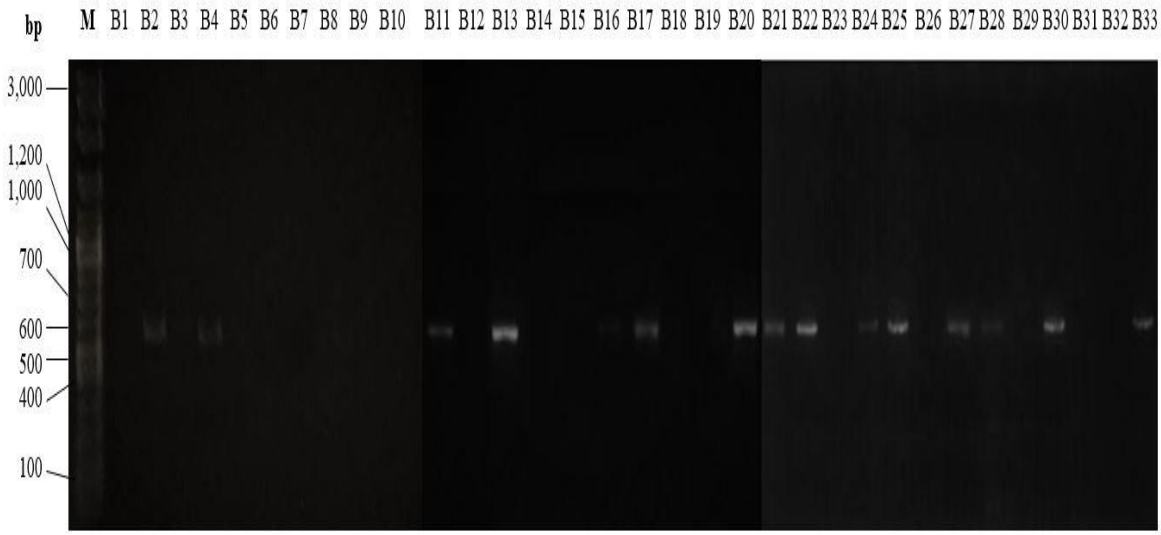

Figure 4.2: PCR products (approximately 641 bp) using *T. forsythia* specific primers for 33 saliva samples viewed on 1.5% agarose gel. Lane (M) - 100 bp DNA ladder (In Vitro Technologies, Australia); Lane (B1) - (B2): Control samples; Lane (B11) - (B33): Gingivitis samples.

Out of the 33 samples in total, 20.0% of healthy patients (2 out of 10 control) were found to have *T. forsythia* detected in their saliva samples. As for the gingivitis samples, 56.52% (13 out of 23) were found to be positive for the presence of *T. forsythia.* Despite having a higher number of positive detections among gingivitis-affected individuals, it is inadequate to cause a significant difference (p-value = 0.0696) as seen in the data tabulated in Table 4.5.

Table 4.5: Fisher's exact test of periodontal status versus the prevalence of *T. forsythia* in samples.

Table Analyzed	**Periodontal status vs *T. forsythia***		
P value and statistical significance			
Test	Fisher's exact test		
P value	0.0696		
P value summary	ns		
One- or two-sided	Two-sided		
Statistically significant (P < 0.05)?	No		
Effect size	Value	95% CI	
Odds ratio	0.1923	0.03679 to 0.9736	
Reciprocal of odds ratio	5.200	1.027 to 27.18	
Sensitivity	0.1333	0.02369 to 0.3788	
Specificity	0.5556	0.3372 to 0.7544	
Positive Predictive Value	0.2000	0.03554 to 0.5098	
Negative Predictive Value	0.4348	0.2563 to 0.6319	
Likelihood Ratio	0.3000		
Methods used to compute CIs			
Odds ratio	Baptista-Pike		
Sensitivity, specificity, etc.	Wilson-Brown		
Data analyzed	*T. forsythia* +ve	*T. forsythia* -ve	Total
Healthy	2	8	10
Gingivitis	13	10	23
Total	15	18	33
Percentage of row total	*T. forsythia* +ve	*T. forsythia* -ve	
Healthy	20.00%	80.00%	
Gingivitis	56.52%	43.48%	
Percentage of column total	*T. forsythia* +ve	*T. forsythia* -ve	
Healthy	13.33%	44.44%	
Gingivitis	86.67%	55.56%	
Percentage of grand total	*T. forsythia* +ve	*T. forsythia* -ve	
Healthy	6.06%	24.24%	
Gingivitis	39.39%	30.30%	

In reference to Table 4.6, there is no significant difference (P-value = 0.2828) in the comparison of gender against the prevalence of *T. forsythia* in saliva samples. From the total of 33 samples analyzed, up to 52.17% (12 out of 23) females are found to be positive of having *T. forsythia* in their saliva whereas for male, 30% (3 out of 10) of them had *T. forsythia* detected in their saliva. There is no gender biased for this as both genders showed common occurrence for *T. forsythia*. Although more females are linked to periodontal disease in this study, there has been reports documenting the awareness of women in taking care of their oral health through their higher dental visit frequency than men (Bonfim et al., 2013). Furthermore, it is highly likely to find periodontal bacteria in the saliva of adults with at least one per six adults on ratio. When periopathogens are detected in combinations, it reflects the risk for the presence of disease (Kononen et al., 2007).

Table 4.6: Fisher's exact test of gender versus the prevalence of *T. forsythia* in samples.

Table Analyzed	**Gender vs *T. forsythia***		
P value and statistical significance			
Test	Fisher's exact test		
P value	0.2828		
P value summary	ns		
One- or two-sided	Two-sided		
Statistically significant (P < 0.05)?	No		
Effect size	Value	95% CI	
Odds ratio	2.545	0.5841 to 10.54	
Reciprocal of odds ratio	0.3929	0.09488 to 1.712	
Sensitivity	0.8000	0.5481 to 0.9295	
Specificity	0.3889	0.2031 to 0.6138	
Positive Predictive Value	0.5217	0.3296 to 0.7076	
Negative Predictive Value	0.7000	0.3968 to 0.8922	
Likelihood Ratio	1.309		
Methods used to compute CIs			
Odds ratio	Baptista-Pike		
Sensitivity, specificity, etc.	Wilson-Brown		
Data analyzed	*T. forsythia* +ve	*T. forsythia* -ve	Total
Female	12	11	23
Male	3	7	10
Total	15	18	33

Table 4.6 continued

Percentage of row total	*T. forsythia* +ve	*T. forsythia* -ve
Female	52.17%	47.83%
Male	30.00%	70.00%
Percentage of column total	*T. forsythia* +ve	*T. forsythia* -ve
Female	80.00%	61.11%
Male	20.00%	38.89%
Percentage of grand total	*T. forsythia* +ve	*T. forsythia* -ve
Female	36.36%	33.33%
Male	9.09%	21.21%

Treponema denticola, is one of the members in red complex cluster and is commonly associated as the causative agent of periodontal disease (Mohanty et al., 2019). However, this periodontal pathogen is also considered to be a part of the human normal oral species that is often found at deep subgingival sites (Sela, 2001). Even though *T. denticola* is still not as well-studied as compared to the other members of red complex, this spirochete is rarely detected in healthy sites (Mohanty et al., 2019).

Figure 4.3: PCR products (approximately 311 bp) using *T. denticola* specific primers for 33 saliva samples viewed on 1.5% agarose gel. Lane (M) - 100 bp DNA ladder (In Vitro Technologies, Australia); Lane (C1) - (C10): Healthy control; Lane (C11) - (C33): Gingivitis-affected samples.

For the prevalence of *T. denticola*, species identification from each saliva samples was done via PCR assay and the outcome is shown as in Figure 4.3. Based on Tamura et al. (2003), the successful amplification of species-specific PCR for *T. denticola* would result in an amplicon size of approximately 311 bp. The detection rate of *T. denticola* was very low in the 33 saliva samples in which detection were only observed among diseased samples (3 out of 23 diseased samples). The band produced were not of a high intensity (Figure 4.3). The appearance of weak band is probably due to the sample containing low numbers of the target bacteria, that is near to the detection threshold (Kononen et al., 2007). The reason might be because of the anaerobic nature of the species that are commonly found colonizing the subgingival area (Sela, 2001) and rarely in salivary carriage.

A comparison between periodontal health condition (healthy versus gingivitis) and the prevalence of *T. denticola* in samples was made with binary logistic regression analysis (Fisher's exact test) as shown in Table 4.7. There is no significant difference ($p > 0.99$) found between the two groups. Generally, as shown in Table 4.7, the evidence of having only 3 positive detection from gingivitis patient (13.04% from total) is not strong enough to associate the impact of health condition to the *T. denticola* prevalence in saliva samples. This outcome is in agreement to the findings made by Martinez-Pabon et al. (2007) showing that higher occurrence of *T. denticola* is associated to the advanced stage of periodontal disease (chronic periodontitis) and rarely detected in healthy saliva samples.

Table 4.7: Fisher's exact test of periodontal status versus the prevalence of *T. denticola* in samples.

Table Analyzed	**Periodontal status vs *T. denticola***		
P value and statistical significance			
Test	Fisher's exact test		
P value	0.5363		
P value summary	ns		
One- or two-sided	Two-sided		
Statistically significant (P < 0.05)?	No		
Effect size	Value	95% CI	
Odds ratio	0.000	0.000 to 2.625	
Reciprocal of odds ratio	Infinity	0.3810 to Infinity	
Sensitivity	0.000	0.000 to 0.5615	
Specificity	0.6667	0.4878 to 0.8077	
Positive Predictive Value	0.000	0.000 to 0.2775	
Negative Predictive Value	0.8696	0.6787 to 0.9546	
Likelihood Ratio	0.000		
Methods used to compute CIs			
Odds ratio	Baptista-Pike		
Sensitivity, specificity, etc.	Wilson-Brown		
Data analyzed	*T. denticola +ve*	*T. denticola* -ve	Total
Healthy	0	10	10
Gingivitis	3	20	23
Total	3	30	33
Percentage of row total	*T. denticola +ve*	*T. denticola* -ve	
Healthy	0.00%	100.00%	
Gingivitis	13.04%	86.96%	
Percentage of column total	*T. denticola +ve*	*T. denticola* -ve	
Healthy	0.00%	33.33%	
Gingivitis	100.00%	66.67%	
Percentage of grand total	*T. denticola +ve*	*T. denticola* -ve	
Healthy	0.00%	30.30%	
Gingivitis	9.09%	60.61%	

Other than that, statistical analysis on gender against the prevalence of *T. denticola* was also done as shown in Table 4.8. In the current study, gender demonstrated to have very little influence on the presence of *T. denticola* in saliva. Although the findings in this study (Table 6.7) showed that female was more likely to have *T. denticola* than male, the differences was insufficient to reach statistical significance (p-value was greater than 0.99). It was found that 13.04% female (3 out of 23 total) were positive for *T. denticola* and none were detected in male subject. Individuals having at least one of the red complex bacteria

(*P. gingivalis, T. forsythia, T. denticola*) may pose a higher susceptibility for the initiation of severe periodontal disease (Naka et al., 2009).

Table 4.8: Fisher's exact test of gender versus the prevalence of *T. denticola* in samples.

Table Analyzed	**Gender vs *T. denticola***		
P value and statistical significance			
Test	Fisher's exact test		
P value	0.5363		
P value summary	ns		
One- or two-sided	Two-sided		
Statistically significant (P < 0.05)?	No		
Effect size	Value	95% CI	
Odds ratio	Infinity	0.3810 to Infinity	
Reciprocal of odds ratio	0.000	0.000 to 2.625	
Sensitivity	1.000	0.4385 to 1.000	
Specificity	0.3333	0.1923 to 0.5122	
Positive Predictive Value	0.1304	0.04538 to 0.3213	
Negative Predictive Value	1.000	0.7225 to 1.000	
Likelihood Ratio	1.500		
Methods used to compute CIs			
Odds ratio	Baptista-Pike		
Sensitivity, specificity, etc.	Wilson-Brown		
Data analyzed	*T. denticola* +ve	*T. denticola* -ve	Total
Female	3	20	23
Male	0	10	10
Total	3	30	33
Percentage of row total	*T. denticola* +ve	*T. denticola* -ve	
Female	13.04%	86.96%	
Male	0.00%	100.00%	
Percentage of column total	*T. denticola* +ve	*T. denticola* -ve	
Female	100.00%	66.67%	
Male	0.00%	33.33%	
Percentage of grand total	*T. denticola* +ve	*T. denticola* -ve	
Female	9.09%	60.61%	
Male	0.00%	30.30%	

Overall, by using the culture-independent PCR method to identify the red complex bacterial species in comparison against two categories (gender and periodontal status), differences can be observed in the probability of finding at least one of the member species

in the saliva of subjects (Table 4.9). In healthy individuals, the prevalence of red complex member species in the saliva samples are as follows; *P. gingivalis* (30.0%), *T. forsythia* (20.0%), and no detection (0.0%) for *T. denticola* and red complex group. Out of 23 gingivitis subjects, *T. forsythia* recorded the highest detection of 56.5%, followed by *P. gingivalis* (26.1%), and a similar detection frequency for *T. denticola* (13.0%) and red complex (13.0%). This shows that the periopathogen is not specific to gingivitis-affected patients only. As mentioned by van Winkelhoff et al. (2002), *P. gingivalis* are rarely detected in healthy sites of oral pockets or if present, they are usually found at a low number. However, the detection of its presence may predict the future onset of periodontal disease in a person (van Winkelhoff et al., 2002). In inflamed periondontal sites, *P. gingivalis* can be found almost up to 85% in patients with periodontal disease (Yang et al., 2004).

Table 4.9: Presence of red complex species in Kuching young adults with or without gingivitis.

	Periodontal status		**Gender**	
Bacteria	**Healthy (n=10)**	**Gingivitis (n=23)**	**Male (n=10)**	**Female (n=23)**
P. gingivalis	3 (30.0%)	6 (26.1%)	1 (10.0%)	8 (34.8%)
T. forsythia	2 (20.0%)	13 (56.5%)	3 (30.0%)	12 (52.2%)
T. denticola	0 (0.0%)	3 (13.0%)	0 (0.0%)	3 (13.0%)
Red complex*	0 (0.0%)	3 (13.0%)	0 (0.0%)	3 (13.0%)

* Includes all the red complex members (*P. gingivalis, T. forsythia, T. denticola*)

In reference to Table 4.9, when comparing gender group and red complex member species detection, the species occurrence in female saliva from the most prevalent to the least are as follows: 52.2% (*T. forsythia*), 34.8% (*P. gingivalis),* and 13.0% (both *T. denticola* and

red complex). In male saliva samples, only *P. gingivalis* and *T. forsythia* was detected for as much as 10.0% (*P. gingivalis*) and 30.0% (*T. forsythia*) detection, respectively. Most of the patients detected positive for the presence of at least one of the periopathogens are females. In general, *T. forsythia* was detected most frequently in both healthy and gingivitis category as well as in gender group.

4.4 Conclusion

In a conclusion, the findings from this study has shown that the distribution of red complex is not limited to diseased state only. The presence of red complex species that is commonly known as the periodontal pathogens can be detected in the saliva of both healthy and gingivitis patients. This indicate that members of red complex species are a part of the normal microbiota in the oral cavity, in which the increased presence could pose a future risk for progression to a periodontally diseased state and indicator for other systemic diseases. Further work should be done to target different age groups and a more advanced molecular technique could be useful to explore the prevalence of this periopathogen in the oral cavity of Malaysian community since the study is still limited in this country. This study may be useful as a reference point for further study that may help to explain aspects involved in initiation and progression of periodontal disease.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

This study provides an overview of the differences in the prevalence of the member species of red complex which are *P. gingivalis, T. forsythia* and *T. denticola* in saliva samples. Red complex bacteria comprise of the key pathogens in adult periodontal disease progression. Although many studies have been done worldwide on these bacteria in oral samples, there is still inadequate reports from our Malaysian local community. All the three periodontal pathogens *(P. gingivalis, T. forsythia* and *T. denticola)* was successfully isolated and detected from the saliva of healthy and gingivitis-affected young adult subjects (age 18 to 30 years old) in Kuching, Sarawak.

For the different methods used to isolate bacterial DNA from saliva, phenol-chloroform isoamyl alcohol (PCIA) method and Norgen saliva DNA isolation kit, both were found to be suitable for molecular analysis especially for PCR amplification. The yield and the quality of the DNA extracted by both methods were found to have minimal PCR inhibitions. Polymerase Chain Reaction (PCR) was used to identify the presence of oral bacteria from the saliva sample by targeting the 16S rRNA gene which a conserved region for prokaryotes. Since saliva consists of different microorganisms such as bacteria, fungi, protozoa, archaea, and viruses it is necessary for the 16S rRNA to be done.

There was no statistical difference observed between the presence of each red complex bacteria against the health status and gender group despite the varying detection number. Out of the 33 saliva samples (10 control and 23 gingivitis) tested, *T. forsythia*

recorded the highest positive detection in gingivitis sample (56.5%), followed by *P. gingivalis* (26.1%) and a similar detection frequency for *T. denticola* (13.0%), and red complex (13.0%) respectively. Up to 30.0% of the healthy samples were found positive for both *P. gingivalis*, followed by *T. forsythia* (20.0%) no detection for *T. denticola* and red complex. This is relevant to some studies done that have also detected low counts of red complex species in oral samples of healthy subjects. On the other hand, all three members of red complex was detected in 3 gingivitis saliva sample. This might imply the progression of the disease severity as red complex, being the late colonizers, are often linked to chronic stage of periodontal disease.

In comparing the gender group to positive detection of red complex bacteria, the species occurrence in female saliva are as follows: 52.2% (*T. forsythia*), 34.8% (*P. gingivalis*), and 13.0% (*T. denticola* and red complex). In male saliva samples, no *T. denticola* was detected except for *T. forsythia* (30.0%) and *P. gingivalis* (10.0%), respectively. A majority of positive detection for the presence of at least one of the periopathogens are among female patients. The outcome for comparison between periodontal health status and gender group versus the red complex species was *T. forsythia* species being the most prevalent in both groups.

In summary, the findings of this study suggest that red complex bacteria are prevalence in both health and disease state regardless of gender among Malaysia young adults, particularly Sarawak people. Despite the gingivitis condition, the presence of red complex is limited to the natural microbiome of individual's oral cavity. The detection may be low in numbers and considered harmless to the host. This is due to the fact that some

periopathogens are natural inhabitants in the oral cavity and only become virulent when the homeostasis environment is disturbed. Furthermore, red complex species are late colonizers and seldom found in less severe periodontal cases like gingivitis. The detection of at least one of the red complex species, does increase the risk of a person in getting periodontal disease or even mirrors the progression of the disease. Such detection may become an important component in prognosis of periodontal disease and its severity. As a recommendation, future studies should include a larger-scale study in a wider region and a varying stage of periodontal disease from health, gingivitis to periodontitis to compare the bacterial composition and how their dynamic interactions affect the severity of the disease.

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APPENDICES

Appendix 1

PARTICIPANT INFORMATION SHEET (Page 1 of 3)

1. **Title of study**: A pilot study of bacterial diversity related to periodontal disease among Sarawakian

2. **Name of researcher(s) :** Dr. Elexson Nillian
   Dr. Azham Zulkharnain
   Dr. Tan Cheng Siang
   Ms. Grace Bebey

3. **Name of institution**: Universiti Malaysia Sarawak (UNIMAS)

4. **Invitation**
   You are invited to participate in this study. Before you decide to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like to have more information (see our details below). Thank you for reading this.

5. **Introduction and purpose of the study**
   Periodontal disease in children and adults are often neglected in the Malaysian healthcare scenario. Severe dental health deterioration may lead to lower quality of life apart from being risk factors for other serious diseases. Periodontal disease caused by bacteria is assessed by dental healthcare practitioners. This research aims to investigate and develop a new test as an adjunctive tool to assess the severity of periodontal disease by identifying bacterial species that could be responsible for the disease progression.

6. **What are the criteria for participants' recruitment?**
   Inclusion criteria:
   - Age 18-30 years old (male and female).

   Exclusion criteria:
   - Those with systemic disease are excluded.

7. **What will you be asked to do?**
   Swab from dental plaque surface, and saliva (2 mL) will be obtained from young adults of age 18 to 30 years old (diseased and periodontal disease-free subjects). Before sampling, participants will need to rinse their mouth with plain water and fast for at least 30 minutes from foods and drinks.

8. **What are the risks?**
   The risks are minimal as the procedure is slightly invasive. There might be discomfort due to swabbing at the infected area.

9. **Will my participation in this study be kept confidential?**
   You will be given a unique participant ID to anonymize your sample. The sample will only be identified by this number during the study. All information obtained from the sample will only be used only for the purpose of this study and the data reported anonymously. The information on the consent form is recorded and stored for research governance purposes only.

PARTICIPANT CONSENT FORM (Page 3 of 3) – Continue

Title of Study: *A pilot study of bacterial diversity related to periodontal disease among Sarawakian*

I, _____ NRIC No. _____

agree to voluntary enroll myself to participate in the study above. I was given a thorough explanation regarding this research in the terms of background, objectives, methodology, risks and possible complications. I understand that I have the right to withdraw from this study at any time without giving any reason. I understand that all personal information collected will be kept secret and will only be used for research governance only.

Participant

Signature: Contact no.:

Date:

Age: _____ years old Gender : Male/ Female

Race:

Witness:

Signature: I/C number:

Name: Date:

Researcher:

Signature: I/C number:

Name: Date:

Appendix 2

Journal Publications

1. Elexson, N., Grace, B., Fatin Nabilah, N., Nur Diyana, Amirah, Z., Eddy, B., & Melvin Chung, H. L. (2021). Detection of *Tannerella forsythia* from Oral Saliva Samples in Different Ethnic Majority Groups in Sarawak. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, *29*(1), 52-59.
2. Elexson, N., Nur Shafiqah, H. R., & Grace, B. (2018). The Effect of Mouthwash on the DNA Yield and Quality of Oral Bacteria. *Malaysian Journal of Microbiology*, *14*, 407-412.

Appendix 3

ASIA PACIFIC JOURNAL OF MOLECULAR BIOLOGY & BIOTECHNOLOGY

ASIA-PACIFIC JOURNAL OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY
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