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).

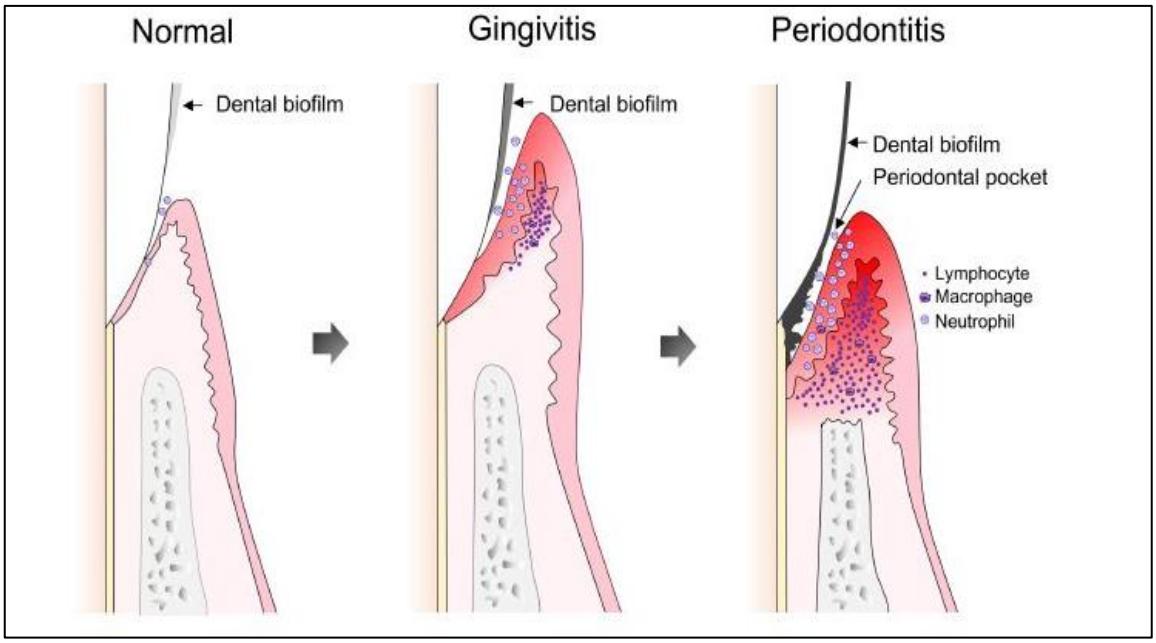

Figure 2.1: The progression of gingivitis and periodontitis following the formation of biofilm on tooth surface (Kriebel et al., 2018).

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