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SHORT COMMUNICATION

A preliminary study on detection of periodontal pathogens from saliva samples of selected Sarawakian

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ABSTRACT

Aims: The oral cavity has the most complex microbiota after the stomach. A disturbed oral equilibrium can lead to the onset and development of periodontal disease. The known causative agents are the red complex bacteria (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*). This study was carried out to provide insights on the prevalence of periodontal pathogens in Sarawakian oral cavity since the data at present is still lacking.
Methodology and results: A total of two millilitres (2 mL) of saliva samples were collected from twenty-seven (n=27) individuals (21 gingivitis, 6 healthy) between aged 18 until 30 years old, from Sarawak General Hospital. DNA extraction for the saliva samples was done by using phenol-chloroform method. Then, 16S rRNA PCR was performed followed by species-specific PCR for red complex bacteria detection. Statistical data was analysed using GraphPad Prism 8.4.1 software. As a result, 14% of gingivitis-affected female subjects were found with all the member of red complex species. Co-occurrence of red complex species was observed but no significant difference was found. An alarming presence of red complex bacteria particularly *T. forsythia* was detected in 57% of gingivitis subject as compared to the other red complex species.
Conclusion, significance and impact of study: The risk of acquiring periodontal disease increases by having at least one of the red complex species in the oral environment. Therefore, the rapid molecular detection of red complex bacteria in this study is useful for risk assessment of periodontal disease and proper species-targeted treatment to patients especially Sarawakian in general as the result has shed lights to the fairly poor oral status of volunteers.

Keywords: Saliva, red complex bacteria, periodontal disease, polymerase chain reaction (PCR)

INTRODUCTION

The oral cavity comes after gut as the part of the body with the highest and complex bacterial communities (Kilian *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 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

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originates from cells that shed from the intraoral surface and leukocytes (Looi *et al*., 2012).

The microbiota in the oral cavity are divided into different complexes (Sbordone and Bortolaia, 2003). 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*., 2013). 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 (Mohanty *et al*., 2019). 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).

In comparison to the oral microbiology findings documented worldwide, Malaysia is still lacking in its oral data contribution. Therefore, the findings can provide enlightenment on of the prevalence of periodontal pathogens in Malaysian oral cavity. Besides, this study will also establish a rapid molecular detection of red complex bacteria among periodontal disease patients before the disease progresses to a chronic stage.

MATERIALS AND METHODS

Sample collection

In collaboration with Sarawak General Hospital, saliva samples were collected from 27 patients (gingivitis-infected and gingivitis-free) of aged from 18 until 30 years. Out of a total of 27 individuals studied, 21 were gingivitis-affected and 6 were healthy (control). There were 6 males and 21 females that participated in this study. 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. A 15 mL Falcon tube was used to collect 2 mL of saliva from each subject. 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. Medical ethical clearance was approved by Faculty of Medicine and Health Sciences UNIMAS under UNIMAS/NC.21.02/03.02 (72).

DNA extraction

The DNA extraction procedure was based on phenol: chloroform: isoamyl alcohol (PCIA) method by Barker *et al*. (1998). A hundred microliter of saliva was mixed with 10 µL of (20 mg/ mL) proteinase K (Vivantis, Malaysia) before incubated for a minimum of 2 h at 55 °C. After incubation, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma, USA) was added to the digested solution and the mixture was centrifuged at 10, 000 rpm for 10 min. Subsequently, the supernatant was extracted with an equal volume of chloroform: isoamyl alcohol (Sigma, USA) and centrifuged for 10 min at 10, 000 rpm. The collected supernatant was then mixed with 2 volumes of absolute ethanol (HmBG, Germany) followed by an overnight precipitation at –20 °C. After washing of the DNA pellet with 1 mL of 70% ethanol on the following day, the pellet was dried and dissolved in double distilled water (50 uL).

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 (Rôças and Siqueira Jr., 2005). The amplification was performed on a LabCycler System (SensoQuest, Germany).

The PCR was run in a 25 µL final volume reaction containing 3 µL of DNA template, 12.5 µL of 2× PCR Master Mix (exTEN, Singapore) and 10 pmol/µL of each primer. The amplification was performed over 35 cycles on a thermocycler according to the following conditions: denaturation for 30 sec at 94 °C, annealing for 30 sec at 52 °C, and elongation for 30 sec at 72 °C. The PCR products were then loaded into 1.5% agarose gel, electrophoresed, and visualized onto the UV transilluminator.

Detection of red complex bacteria via PCR amplification

The primer sequences of red complex bacteria (*P. gingivalis, T. forsythia*, *T. denticola*) were synthesized based on Tamura *et al*. (2006) and shown in Table 1. The PCR reagent mixture used was the same as for 16S PCR amplification. The PCR amplification was performed for 35 cycles with 30 sec denaturation step at 94 °C, annealing for 1 min at varying temperature for each bacterium: *P. gingivalis* (55 °C), *T. forsythia* (56 °C) and *T. denticola* (65 °C). The elongation step was done for 1 min at 72 °C.

Data analysis

The analysis of data was done by using GraphPad Prism 8.4.1 software. Fisher's exact test which is a 2 × 2 contingency table analysis, is considered as statistically different if the *p*-value is less than 0.05. Odd ratio (OR) is used to estimate the risk of an outcome, where OR = 1 means exposure is not associated to any risk, OR < 1 means lower risk and OR > 1 means higher risk of acquiring infection or disease.

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Table 1: PCR primer sets for red complex bacterial species identification.

Bacteria	Forward sequence (5'-3')	Reverse sequence (5'-3')	Product size (bp)
P. gingivalis	5'- TGT AGA TGA CTG ATG GTG AAA ACC-3'	5'-ACG TCA TCC CCA CCT TCC TC -3'	197
T. forsythia	5'- GCG TAT GTA ACC TGC CCG CA -3'	5'- TCG TTC AGT GTC AGT TAT ACC T -3'	641
T. denticola	5'- AAG GCG GTA GAG CCG CCG CTC A -3'	5'- AGC CGC TGT CGA AAA GCC CA -3'	311

RESULTS

A total of 27 individuals were studied in this research (21 gingivitis and 6 healthy control) with different age of between 18 until 30 years old. Overall, there were 6 males and 21 females that took part in this study. As observed in (Figure 1), the 16S rRNA targeted gene of 1500 bp size was successfully amplified from all the 27 samples extracted using PCIA method as viewed on 1.5% gel electrophoresis. PCR amplification of targeted gene for each bacterial species can be observed as in Figures 2, 3 and 4. In terms of gender comparison, as observed in Table 2, the detection rate for *T. forsythia* was the highest in both females (57%) and males (33%), respectively. Only *T. forsythia* species was detected in the gingivitis-affected male respondent as opposed to female subjects that were found to have all the three species individually detected in their oral cavity. All the three members of red complex was detected in female participants with 14% detection rate (3 out of 21 females) and there was no detection of red complex in male.

The detection rate of red complex species also can be analysed from the periodontal status (healthy versus gingivitis) of the participating subjects as tabulated in Table 2. The occurrence of red complex bacteria in healthy individuals was about 33% for both *T. forsythia* and *P. gingivalis*, respectively. The prevalence of these periodontal pathogens was higher in gingivitis subjects in which the highest detection was *T. forsythia* (57%), followed by *P. gingivalis* (28%) and the lowest, *T. denticola* (14%). The prevalence of red complex is approximately 14% (3 out of 21) among gingivitis patients.

Table 2: Detection and frequency of red complex member species in terms of gender and periodontal status of the patients.

Bacteria	Gender		Periodontal status	
	Male (n=6)	Female (n=21)	Healthy (n=6)	Gingivitis (n=21)
P. gingivalis	0 (0%)	8 (38%)	2 (33%)	6 (28%)
T. forsythia	2 (33%)	12 (57%)	2 (33%)	12 (57%)
T. denticola	0 (0%)	3 (14%)	0 (0%)	3 (14%)
Red complex**	0 (0%)	3 (14%)	0 (0%)	3 (14%)

**Red complex: having all three member species of red complex in the sample

Fisher's exact test and OR analysis was done to compare the probability of detecting co-existence of red complex species in gingivitis subjects against periodontally healthy individuals (Table 3). A similar positive relationship was found between *P. gingivalis*, *T. forsythia*, and *T. denticola* group (OR = 0.167) and *P. gingivalis*, and *T. forsythia* group (OR = 0.167), but it is a weak association as there is no statistical significance found (*p*-value > 0. 999). A total of 9 gingivitis subjects were found to have no detection for red complex species (no detection of at least one red complex member species).

DISCUSSION

Generally, the red complex bacteria including *P. gingivalis*, *T. forsythia*, and *T. denticola* have been classified as Gram-negative anaerobic bacteria that is responsible for causing periodontal disease (Tamura *et al*., 2006). These bacteria have been associated with late colonization of dental plaque and increased pocket depth during progression of the later stage of periodontal disease, which is periodontitis (Mohanty *et al*., 2019). In the current study, highest detection of red complex species was found in female subject with every species found in varied frequency. The species *T. forsythia* was the most prevalent in female participants with 57% detection frequency. The hormonal changes that occur throughout a female's lifetime can modify the periodontal health status of females (Lopez-Marcos *et al*., 2005). Female in reproductive age are affected by fluctuating conditions of oral microbiome and is prone to periodontal disease (Kessler *et al*., 2017). The limitation to this study was the recruitment of male subject that was also observed in the study done by Ardila *et al*. (2014) and Lanza *et al.* (2016) as the number of male participants was below 50%. The active involvement of female subjects' in this study shows that female has better

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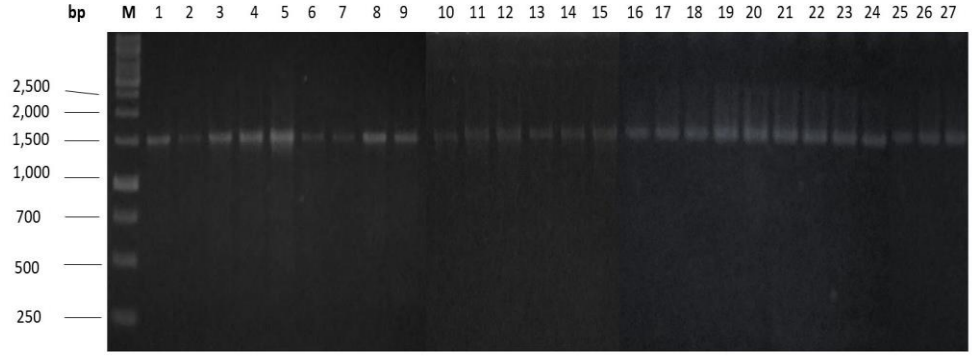

Figure 1: PCR products (approximately 1500 bp) of bacterial DNA from saliva extracted using PCIA method viewed on 1.5% gel. Lanes 1-6 are the healthy control samples; lanes 7-27 are the gingivitis samples; lane M: 1 kb DNA ladder (Thermo Scientific, USA).

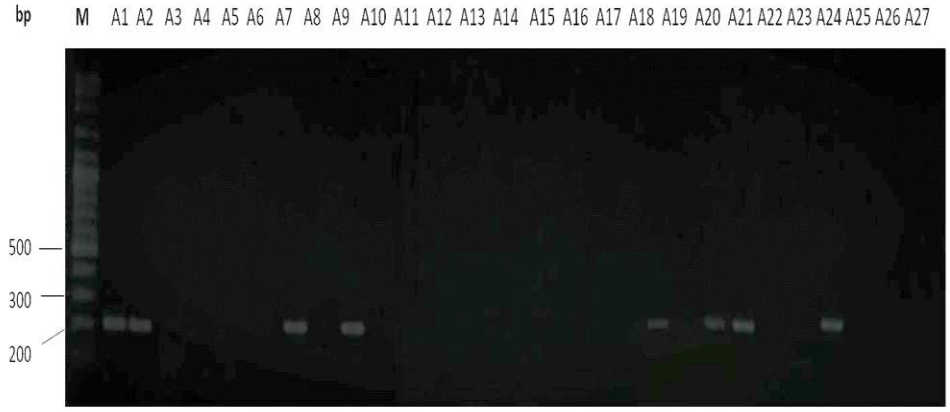

Figure 2: PCR products of size approximately 197 bp for the detection of *P. gingivalis* in saliva samples (Lanes A1-A6: healthy; Lanes A7-A27: gingivitis). Lane M: 100 bp DNA ladder (In Vitro Technologies, Australia). Successful amplifications were observed in lane A1, A2, A7, A9, A18, A20, A21 and A24.

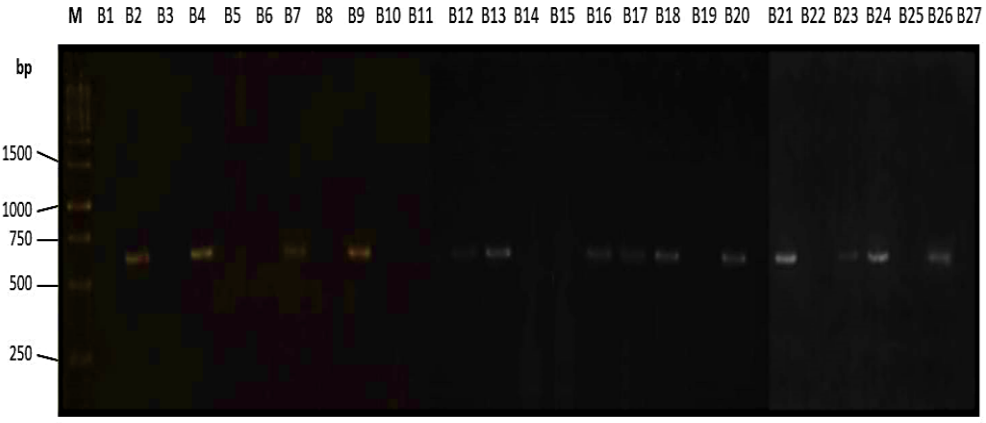

Figure 3: PCR products (approximately 641 bp) for the detection of *T. forsythia* in saliva samples (Lanes B1-B6: healthy; Lanes B7-B27: gingivitis). Lane M: GeneRuler^{TM} 1 kb DNA ladder (Thermo Fisher Scientific, USA). Amplicons of size 641 bp were observed in lanes B2, B4, B7, B9, B12, B13, B16, B17, B18, B20, B21, B23, B24 and B26.

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Table 3: Evaluation of red complex species coexistence in gingivitis patients.

Bacterial species	Frequency	Detection (%)	OR[b] (95% CI)	*p*-value
P. gingivalis, *T. forsythia*, and *T. denticola*	3	14.3	0.167 (0.009-0.976)	> 0.999
P. gingivalis and *T. forsythia*	3	14.3	0.167 (0.039-0.492)	> 0.999
None[a]	9	42.0	0.750 (0.306-1.772)	> 0.999

[a]None: No detection of at least one single member of red complex species.
[b]OR: Odd ratio.

Figure 4: The PCR products (approximately 311 bp) for the detection of *T. denticola* in saliva samples (Lanes C1-C6: healthy; Lanes C7-C27: gingivitis). Lane M: GeneRuler™ 1 kb DNA ladder (Thermo Fisher Scientific, USA). Target gene for *T. denticola* was successfully amplified in lanes C9, C20 and C21.

awareness on oral health and dental visits besides the incompatibility to be in the study after criteria screening. A high detection rate of *T. forsythia* in periodontal disease sample was in agreement to the study by Mineoka *et al.* (2008). His finding suggests that in biofilm development, *T. forsythia* colonizes the dental biofilm before the other red complex member species.

Red complex species was also detected in periodontally healthy patients with its prevalence as much as 33% for both *T. forsythia* and *P. gingivalis*, respectively. The detection of at least one red complex species in healthy oral sample is also similar to the finding by Faghri *et al*. (2007) that detected *P. gingivalis* in about 40% of the healthy subjects. It is not uncommon to detect lower densities of Gram-negative, pathogenic species in the healthy gingival sulcus (Hashim *et al*., 2018). Red complex species are normally detected in higher frequency among individuals that suffer from periodontitis (Wara-aswapati *et al*., 2009). In the study done by Wara-aspati *et al*. (2009), individuals with moderate until severe chronic periodontitis were found to have higher detection frequency of the red complex species.

The biofilm layer that forms on dental surfaces are composed of different interacting microorganisms. The findings in this study has shown that there is co-existence of red complex species in different periodontal status (healthy vs. gingivitis) despite the insignificant difference (*p*-value > 0.999) in the statistical analysis (Table 3) and close to zero OR value. In the gingivitis saliva samples, *P. gingivalis*, *T. forsythia* and *T. denticola* co-existence were as much as 14.3% which is similar to the *P. gingivalis,* and *T. forsythia* pair (14.3%) co-occurrence detection frequency. It is common to find red complex species co-exist in diseased patients as in agreement with Ardila *et al.* (2014) has found that the three species of red complex are most frequently detected together among the chronic periodontitis subjects. On the other hand, Sanghavi *et al.* (2014) observed the highest detection of *P. gingivalis* and *T. denticola* pair in patients with periodontal tissue abscesses. The interaction among these three red complex species is primarily due to their metabolic dependence to each other for survival (Nayak *et al*., 2018). Having 9 gingivitis individuals with no detection of any red complex species may be due to the fact that red complex bacteria are known to be late colonizers and are more prone to be detected in the advanced stage of periodontitis (Mohanty *et al*., 2019). 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).

CONCLUSION

The outcome of this study shows that the detection count of red complex bacteria especially *T. forsythia* is higher among gingivitis-affected Sarawakian young adults. The establishment of molecular approach in this study will introduce a rapid detection of red complex bacteria in periodontal disease patients as opposed to commonly practiced method of prognosis based on general screening and observation, thus aid in the proper species-targeted oral treatment administration. In addition, the data from this preliminary study has shed some lights on

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the periodontal status of the locals in Sarawak which can be used as additional reference when conducting a future larger-scale, longitudinal study to understand the progression of periodontal disease caused by interspecies interaction of red complex bacteria.

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