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The effect of mouthwash on the DNA yield and quality of oral bacteria

Elexson Nillian*, Nur Shafiqah Hanim Rakwi, Grace Bebey

Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia.
Email: nelexson@frst.unimas.my

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ABSTRACT

Aims: The application of mouthwash is one of the oral hygiene treatments that commonly use after tooth brushing to control the bacterial colonization from overgrowth. This research is focused on investigating the effect of mouthwash on oral microbiome by analyzing the quality and yield of DNA obtained before and after using mouthwash and also to compare the bacterial abundance via 16S rRNA PCR detection.
Methodology and results: The DNA was extracted from the saliva samples before and after using mouthwash using Phenol-Chloroform extraction method. The DNA extract was then evaluated using Nano Drop ND-1000 UV/VIS Spectrophotometer to determine the DNA quality and DNA yield. After that, the 16S rRNA gene was amplified via PCR for bacterial detection in the saliva using 27 F and 1492 R primers set, and the PCR products were observed on 1.5% gel electrophoresis. Statistical analysis was performed by using Graphpad Prism 7.03 software. For DNA yield, there was significantly higher yield observed after mouthwash usage with 80% of the samples was found to yield more DNA. To assess DNA quality, absorbance ratio of A260/A280 and A260/A230 were used. The DNA quality was seen to be similar for both A260/A280 and A260/A230 absorbance ratio even after the usage of mouthwash. The amplification of 16S rRNA gene was successful and 1500 bp expected band size was observed.
Conclusion, significance and impact of study: This study demonstrated the usage of mouthwash is useful to increase the DNA yield as compared to without using mouthwash. However in terms of quality, no difference is seen. This result can be used to provide insight on mouthwash usage for saliva sampling in a non-invasive manner.

Keywords: Saliva, mouthwash, DNA yield, DNA quality, 16S rRNA gene

INTRODUCTION

Every part of the human body consists of bacteria that colonize to be symbiont to the host subject. The mouth is the second part of the body that comes after gut that has the highest bacterial communities (Killian *et al*., 2016). About 500 to 700 bacterial species were detected for the oral microbiome and most of them interact with each other to complement the host. The bacteria from the saliva are from intraoral surface of the mouth shaded and also from the environment or anything that being consume or in contact with mouth (Takeshita *et al*., 2016). The application of mouthwash is one of the oral hygiene treatments that is commonly used after tooth brushing to control the bacterial colonization from overgrowth.

Its ability to reduce bacterial colonization in mouth had been approved in many studies including the studies done by Wade (2013) and Killian *et al*. (2016) based on targeted ingredient. The mouthwashes in the market today have added features such as antimicrobial effects for the purpose of improvement of oral health maintenance (Mat Ludin and Md Radzi, 2014).

The identification of oral bacteria may be useful as a prognostic tool for early treatment of oral disease and any related disease. Not all the bacteria present in the oral cavity are harmful to the host since some are co-exist without causing health risk. This includes *Porphyromonas gingivalis and Tannerella forsythensis* from Bacteroidetes phylum whereas *Treponema denticola* from the Spirochaete phylum have been associated with periodontal disease and considered as pathogenic (Tamura *et al.,* 2006; Kang *et al*., 2009). The bacteria is not the only component of the saliva (Chartier and Birnboim, 2005), there is need of specific identification to distinguish the bacterial DNA from another. The use of PCR based on 16S rRNA gene commonly use in study of bacteria because this gene are commonly found in bacteria and it is reliable for phylogenetic analysis due to its ultra-conserves regions (Zayats *et al.,* 2009; Tanner *et al*., 2011).

This study aims to compare the quality and yield of DNA obtained before and after using mouthwash. Another objective is to identify the difference in bacterial

***Corresponding author**

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abundance before and after using mouthwash using 16S rRNA PCR detection.

MATERIALS AND METHODS

Sampling

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 study area is at University Malaysia Sarawak, where 10 random people were asked to give their saliva samples. Pre-sampling, individual subject was instructed to rinse their mouth vigorously with tap water. After one minute, sampling was done by collecting 2 mL of saliva into a sterile 15-mL tube. Sampling was repeated with mouthwash to replace the tap water. Twenty samples were collected from 10 individuals, in which 2 samples were obtained for comparison of before and after using mouthwash. Once collected, all the samples were stored at −4 °C before DNA extraction were performed.

DNA extraction

This procedure was performed by referring to modified Barker (1998) protocol. For tissue digestion procedures, 100 µL of saliva was mixed with lysis buffer buffer (1 M Tris, pH 8, 5 M NaCl, 0.5 M EDTA, 10% SDS) and 10 µL of proteinase K (20 mg/mL) was added into 1.5 mL microcentrifuge tube. The sample was then incubated for 2 h at 55 °C in waterbath. To start the extraction and ethanol precipitation of DNA, an equal volume of phenol: chloroform: isoamyl alcohol solution (25:24:1) was added into the tube to digest the DNA and the solution was gently mixed for 5 min. Next, the sample was centrifuged for 10 min with 10,000 rpm, and the upper aqueous layer was transferred to a new tube. The PCIA step was repeated and followed by the addition of equal volume of chloroform: isoamyl alcohol (24:1) solution. After centrifugation for 10 min at 10,000 rpm, the aqueous layer was mixed with double volume of ice-cold 100% ethanol. The solution was stored in −20 °C for overnight. The following day after centrifugation, the pellet obtained was washed with 100 µL of 70% ethanol before drying the pellet and dissolved in 50 µL double distilled water. Storage was done at −20 °C.

DNA yield and purity evaluation

The yield and purity of DNA of the sample was determined based on UV absorbance (A260/A280 and A260/A230 ratio), measured with NanoDrop ND-1000 UV/VIS Spectrophotometer. The application was based on manufacturer's instruction. The absorbance was the determined using ND-1000 V3.5 software.

Polymerase Chain Reaction (PCR)

DNA extracted from each method was subjected to PCR analysis. PCR analysis involved the use of universal primer 16S rRNA set which were the 27F (5'- AGA GTT TGA TCM TGG CTC AG -3') and 1492R (5'- TAC GGY TAC CTT ACGACTT -3'). Three microliter of extracted DNA was added to a reaction mixture (final volume 25 µL) containing 2.5 µL of 10x EasyTaq buffer (Transgen Biotech, Beijing, CN), 1.5 mM of $MgCl_2$, 10 pmol of each primer, 0.2 mM of dNTPs (Promega, USA) and 0.25 U of GoTaq Flexi DNA polymerase (Promega, USA).

The samples were preheated at 95 °C for 2 min, followed by amplification under the following conditions: denaturation at 94 °C for 30 sec, annealing at 94 °C for 30 sec, and elongation at 50 °C for 30 sec. A total of 30 cycles were performed with a final elongation step at 72 °C for 10 min. The results of the PCR amplification were examined by electrophoresis using 1.5 % agarose gel.

Statistical analysis

The paired t-test was performed on the results of DNA concentration and DNA quality by using Graphpad Prism 7.03 software. Statistical significance level was set at 0.05 and *p* value less than 0.05 was considered as significantly statistically different.

RESULTS

DNA concentration

From Figure 1, out of ten saliva samples collected, 8 out of 10 which is equivalent to 80% of the samples showed increment in DNA concentration after using mouthwash except for sample E and G. The greatest DNA concentration was provided by individual with sample labeled F with DNA concentration before and after using mouthwash at 21.6 ng/µL and 36.3 ng/µL respectively.

From Table 1, there was a significant increase ($p < 0.05$) in the DNA concentration obtained after the usage of mouthwash as compared to before using it. It shows that the usage of mouthwash could help to increase the collection of oral microbiome for sampling.

DNA Quality Evaluation via A260/A280 Ratio and A260/A230 Ratio

The A260/280 ratio is used to calculate the protein contamination present in sample (Mendoza *et al.*, 2016). Ideal range for ratios A260/A280 and A260/A230 is within 1.8 to 2.0 (Olson and Morrow, 2012). From Figure 2, it can be seen that after using mouthwash the DNA extracted have varied ratio of absorbance with most of them fall below the ideal range but closer to ideal 1.8 ratio.

Referring to Table 2, there was no significant difference ($p > 0.05$) observed in the purity of the DNA extract before and after using mouthwash for absorbance ratio at A260/A280. This indicates that there is no improvement seen in protein contamination after using mouthwash as substitute to using pure saliva in DNA extraction.

Absorbance ratio (A260/A230) is used to detect for the presence of other salt contamination (Olson and Morrow,

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2012). From Figure 3, most of the extract provided values that are lesser than the ideal ratio of highly purified nucleic acid that should be between 1.8 until 2.0.

There was no significant difference (p > 0.05) observed in the purity of the DNA extract before and after using mouthwash for absorbance ratio at A260/A230 (Table 3). This could be due to the choice of method used for DNA extraction which is phenol-chloroform method.

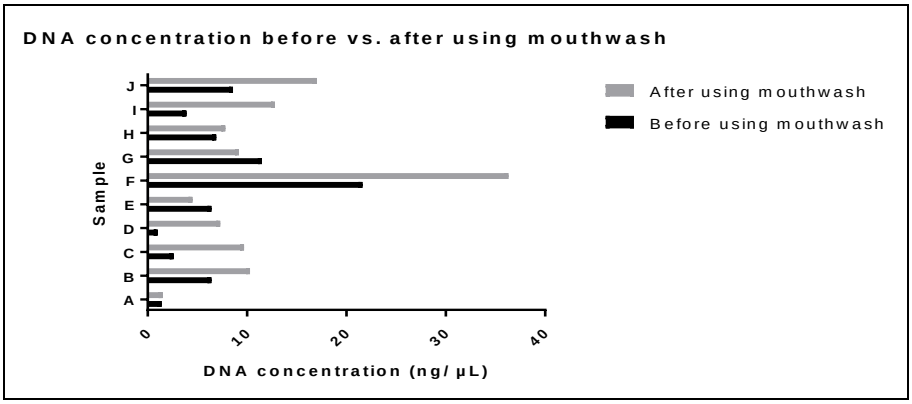

Figure 1: Comparison of DNA concentration (ng/ µL) in 10 samples before and after using mouthwash.

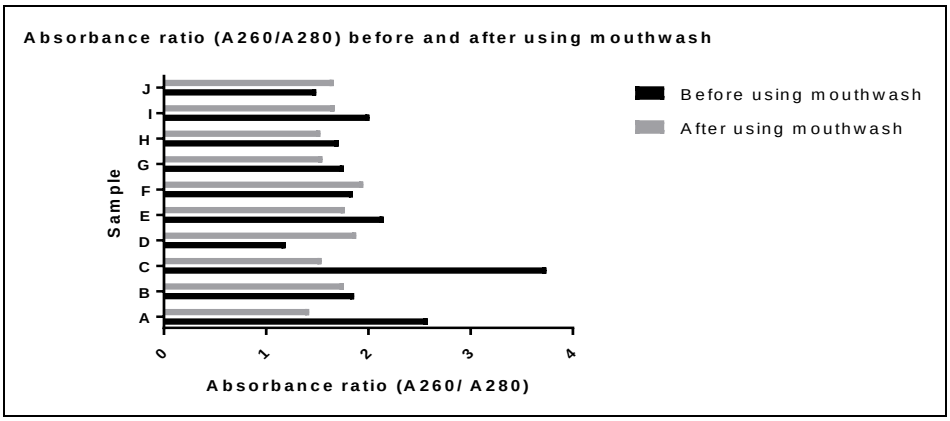

Figure 2: Comparison of absorbance ratio (A260/A280) before and after using mouthwash of 10 samples.

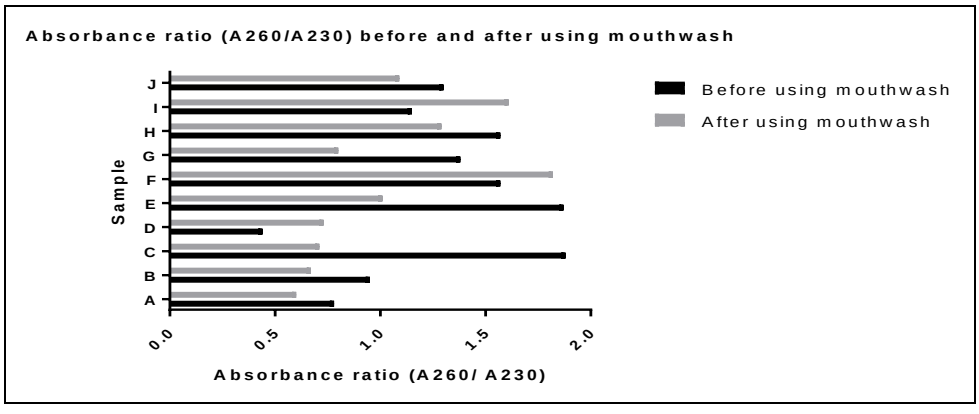

Figure 3: Comparison of absorbance ratio (A260/A230) before and after using mouthwash of 10 samples.

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Table 1: T-test result for comparison of DNA concentration (ng/µl) before and after using mouthwash of 10 samples.

Paired t test	
P value	0.0256
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.67 df=9
Number of pairs	10
How big is the difference?	
Mean of differences	4.61
SD of differences	5.461
SEM of differences	1.727
95% confidence interval	0.7037 to 8.516
R squared (partial eta squared)	0.4419
How effective was the pairing?	
Correlation coefficient (r)	0.8539
P value (one tailed)	0.0008
P value summary	***
Was the pairing significantly effective?	Yes

Table 2: T-test result for comparison in absorbance ratio (A260/A280) before and after using mouthwash of 10 samples.

Paired t test	
P value	0.1868
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.429 df=9
Number of pairs	10
How big is the difference?	
Mean of differences	-0.361
SD of differences	0.7989
SEM of differences	0.2526
95% confidence interval	-0.9325 to 0.2105
R squared (partial eta squared)	0.1849
How effective was the pairing?	
Correlation coefficient (r)	-0.4759
P value (one tailed)	0.0822
P value summary	ns
Was the pairing significantly effective?	No

Table 3: T-test result for comparison in absorbance ratio (A260/A230) before and after using mouthwash of 10 samples.

Paired t test	
P value	0.1500
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.574 df=9
Number of pairs	10
How big is the difference?	
Mean of differences	-0.256
SD of differences	0.5144
SEM of differences	0.1627
95% confidence interval	-0.6239 to 0.1119
R squared (partial eta squared)	0.2158
How effective was the pairing?	
Correlation coefficient (r)	0.3329
P value (one tailed)	0.1737
P value summary	ns
Was the pairing significantly effective?	No

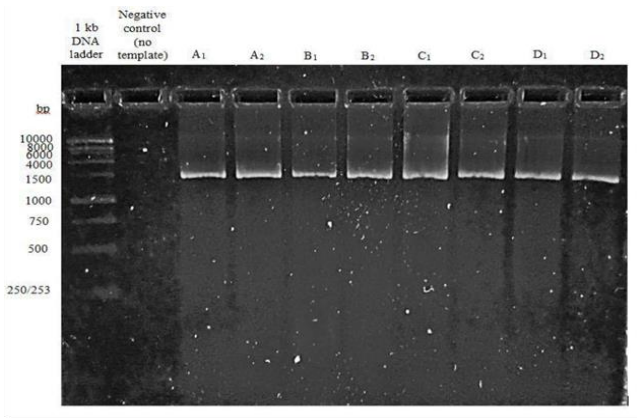

Figure 4: PCR product viewed using 1.5% gel electrophoresis. Negative control; distilled water. A1, B1, C1, D1; saliva sample from individual before using mouthwash. A2, B2, C2, D2; saliva sample from individual after using mouthwash.

16S rRNA gene amplification

The PCR products are generated from the saliva samples taken from all eight individuals (Figures 4 and 5). The A1 is the saliva sample from individual A before using mouthwash (mouth rinsing using tap water) and the A2 is the saliva sample from individual A after using mouthwash, the same for other samples. All the bands are observed at the 1500 bp DNA ladder. The 16S rRNA genes were successfully amplified at 1500 bp as expected. The 16S rRNA sequence is the most common marker to detect the presences of bacteria (Glazer and Nikaido, 2007).

DISCUSSION

DNA concentration

The DNA yield measurement is questioned as they are influenced by the absorbance reading shown by the Nano Drop. The absorbance could be interrupted by other component within the sample extract including the RNA itself. To prevent this, DNA purification are required to reduce the potential of false result. Even the yield of the DNA in the extract is quite low for most of the samples, but it is enough to be used for downstream application. According to Khare *et al.* (2014) in their study, which support this outcome, where the DNA from saliva sample

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can be utilized for PCR process, one of downstream applications.

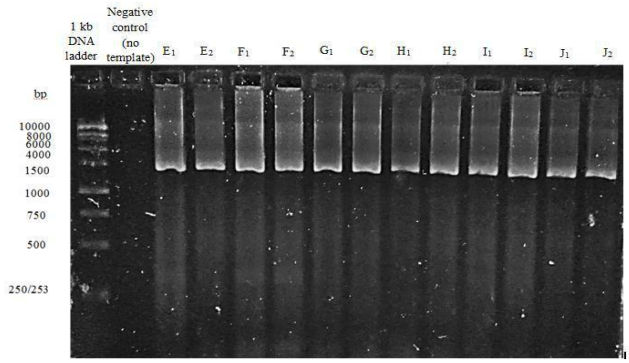

Figure 5: PCR product viewed using 1.5% gel electrophoresis. Negative control; distilled water. E1, F1, G1, H1, I1, J2; saliva sample from individual before using mouthwash. E2, F2, G2, H2, I2, J2; saliva sample from individual after using mouthwash.

DNA quality

Having no improvement in the DNA quality obtained for A260/280 reading indicates that the DNA sample has an acceptably low protein and other contaminant. This could be due to ccontamination by phenol, which is commonly used in nucleic acid purification which can significantly throw off quantification estimates. Phenol absorbs with a peak at 270 nm and at A260/280 of 1.2. Nucleic acid preparations free from phenol contamination should have reading at A260/280 of about 2.0 (Sambrook and Russell, 2001). Also in the study by Khare and his colleagues (2014), the ratio that is greater than 2.0 may indicate the contamination by the RNA which can be observed in DNA extracted before using mouthwash (4 out of 10). The application of RNase can be used to reduce the amount of RNA. It is also will be useful to check the DNA quality by using the agarose gel.

For the A260/A230 ratio, it is expected to observe lower value from the samples extracted since phenol-chloroform extraction method was used for DNA extraction. Other contaminant such as polysaccharide can also interrupt the result. However, this did not inhibit the PCR amplification of the 16S rRNA gene. The DNA was extracted from the saliva and the bacterial DNA is not the only component in the saliva (Chartier and Birnboim, 2005). Other component such as the enzyme and human buccal cells can also be detected in the saliva. There are also certain factors that can influence the accuracy of the ratios including; the pH solution (Olson and Morrow, 2012), where the acidic solution have potential to lower the ratio by 0.2 until 0.3 and the basic solution have potential to increase the ratio by 0.2 until 0.3. Improvement in handling and isolation method can be done to increase both the DNA yields and also the quality of the sample interested (Khare *et al*., 2014).

16s rRNA gene amplification

The PCR amplification of this 16S rRNA gene was done to confirm for the presence of bacteria in the samples extracted since the saliva samples contained different contaminants from intraoral sites aside from bacteria. This gene is present in all bacteria and archae (Srinivasan *et al*., 2015). The specific identities of the bacterial DNA extracted are still unknown and the true effectiveness of this study is limited. The quantity of the bacterial DNA cannot be determined by this method, hence further DNA sequencing can be done to identify and quantify the bacteria.

CONCLUSION

The usage of mouthwash in general does help to increase the DNA yield as compared to without using mouthwash. However, in terms of quality and purity of DNA it does not contribute as much significance to the result as even after mouthwash usage, since almost similar result was obtained. For bacterial abundance comparison before and after using mouthwash via 16S rRNA PCR detection, similar results are obtained as both managed to show same intensity of DNA band. Thus, mouthwash can be used as an alternative for non-invasive saliva sampling method which can be used for further downstream processing.

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