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Saliva Sampling of Alcoholic Participants using Three Saliva Collection Methods

(Persampelan Air Liur menggunakan Tiga Kaedah Pengumpulan Air Liur daripada Peserta yang Mengamalkan Minuman Beralkohol)

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ABSTRACT

*The potential of using saliva as a diagnostic fluid is well documented. The aim of this study was to assess the quality and quantity of saliva DNA of alcoholic and non-alcoholic participants using three saliva collection methods; DNA-Sal™ (Oasis Diagnostics, USA), Oragene-DNA (DNA Genotek Inc, Ontario, Canada) and whole saliva collection method. Saliva DNA of non-alcoholic (*n*=30) and alcoholic participants (*n*=10) age between 25 and 35 years was assessed qualitatively and quantitatively using spectrophotometry. Saliva DNA quantity was the highest for all participants when using the DNA-Sal™ saliva collection kit (*p*<0.05). The use of a mechanical scraper provided only in the DNA-Sal ™ kit may have contributed to the highest DNA yield for all participants. The quantity of saliva DNA when assessed using spectrophotometer was found to be significantly lower (*p*<0.05) for the alcoholic (16±3.57 ng/μL) than non-alcoholic participants (19.92±6.18 ng/μL). To determine the integrity of the DNA samples, PCR amplification of the* Alcohol Dehydrogenase *gene,* ADH1B *was carried out and the PCR was found to be successful. For all participants, the DNA quality of the saliva collected using the three saliva collection methods was found to be in the acceptable range considered as pure DNA. The DNA quality and quantity of saliva collected from the three saliva collection methods were considered suitable for research purposes.*

Keywords: Alcohol; PCR; saliva collection methods

ABSTRAK

*Potensi menggunakan air liur sebagai alat diagnostik telah pun mendapat pendedahan yang meluas. Tujuan kajian ini adalah untuk mengkaji kualiti dan kuantiti DNA menggunakan sampel air liur yang diperoleh daripada peserta yang mengamalkan minuman beralkohol. Sampel air liur diperoleh daripada semua peserta menggunakan 3 kaedah pengumpulan air liur; DNA-Sal™ (Oasis Diagnostics, USA), Oragene-DNA (DNA Genotek Inc, Ontario, Canada) dan pengumpulan air liur secara langsung daripada kaviti mulut. Persampelan air liur melibatkan peserta yang tidak mengamalkan minuman beralkohol (*n*=30) dan yang mengamalkan minuman beralkohol (*n*=10) serta berumur antara 25 dan 35 tahun. Kualiti dan kuantiti DNA air liur daripada semua peserta dikaji menggunakan spektrofotometer. Kuantiti DNA air liur bagi semua peserta adalah paling tinggi apabila menggunakan kaedah pengumpulan air liur DNA-Sal™ (*p*<0.05). Penggunaan alat mengikis yang dibekalkan hanya untuk kaedah pengumpulan air liur DNA-Sal™ didapati berkemungkinan menyumbang terhadap kuantiti DNA yang paling tinggi. Walau bagaimanapun, pemeriksaan spektrofotometer mendapati bahawa kuantiti DNA air liur bagi peserta yang mengamalkan alkohol (16±3.57 ng/μL) adalah lebih rendah (*p*<0.05) berbanding dengan peserta yang tidak mengamalkan alkohol (19.92±6.18 ng/μL). Untuk memastikan integriti DNA air liur, DNA yang diperoleh daripada air liur digunakan untuk amplifikasi PCR. Amplifikasi PCR didapati telah berjaya bagi salah satu gen kumpulan* Alkohol Dehidrogenase, ADH1B. *Kualiti DNA air liur yang dikumpul menggunakan ketiga-tiga kaedah pengumpulan air liur bagi semua jenis peserta didapati berada dalam lingkungan yang dianggap sebagai DNA tulen. Kualiti dan kuantiti DNA bagi air liur yang dikumpul menggunakan ketiga-tiga kaedah pengumpulan air liur adalah dianggap sesuai bagi kegunaan penyelidikan.*

Kata kunci: Air liur; alkohol; kaedah pengumpulan air liur; PCR

INTRODUCTION

Over the last decade, the use of saliva as a potential diagnostic tool have been gaining interest due to its ease and non-invasive accessibility along with its abundance of biomarkers such as genetic material and proteins. Recent advances in the use of salivary biomarkers to diagnose diseases such as autoimmune diseases (human immunodeficiency virus infection, Sjogren's syndrome, cystic fibrosis), cardiovascular diseases, diabetes, oral cancer, caries and periodontal diseases have been reviewed (Javaid et al. 2016; Osman et al. 2012). The potential use of genetic material such as DNA, RNA and protein molecules for screening of disease causing genes have also been reported (Ahn et al. 2014 ; Chai et al. 2016 ; Fabryova & Celec 2014).

Compared to using DNA obtained from blood, not much is known about the potential use of saliva DNA for genotyping studies involving alcohol metabolism (Crabb

et al. 1989; Philibert et al. 2008a). To obtain DNA of high quality from saliva samples, it is crucial that saliva sampling is carried out appropriately. Before the invention of saliva collection kits, saliva sampling mainly involved collection of whole saliva or saliva from a particular salivary gland (Dawes 1987). The whole saliva collection method has been reported to be successful in producing DNA of higher yields with lengthier DNA fragments (Feigelson et al. 2001; Heath et al. 2001). However, when compared to the use of saliva collection kits that are available commercially, the quantity of DNA extracted from whole saliva samples were often found to be lower (Koni et al. 2011; Nemoda et al. 2011; Pulford et al. 2013a).

Oragene-DNA is one of the widely used saliva collection kits that requires the patient to expectorate their saliva into a collection tube. The saliva is then mixed with a buffer that stabilizes the saliva DNA and the saliva samples have been found to be stable at room temperature for as long as 8 months (Nunes et al. 2012). In 2012, the Food and Drug Administration (FDA) approved the Oragene-DNA device as a saliva collection tool and since then, the Oragene-DNA device has been widely used in saliva-related research (Abraham et al. 2012; Pulford et al. 2013b; Smith et al. 2015). Another device that has been introduced in the market is the DNA-Sal™ kit where a mechanical scraper is included in the kit for scraping the inner surface of the cheek (buccal cells). Not much is known about this saliva collection kit but it is anticipated that the action of scraping the buccal cells would help to increase the DNA yield.

This aim of this study was to assess the quantity and quality of saliva DNA extracted in saliva samples obtained using three different collection procedures; whole saliva, DNA-Sal™ and Oragene-DNA collection methods of alcoholic and non-alcoholic participants of matching age and sex. DNA samples were assessed for its suitability for use in PCR amplifications of an alcohol dehydrogenase gene involved in the ethanol metabolism, *ADH1B*.

Materials and Methods

Participants and Samples

Human saliva samples from 30 non-alcoholics and 10 alcoholic male Malaysian participants were collected in accordance with the ethical code DFOB1503/0064(L). Participants included in this research were between the ages of 25 and 35 years and were in good health with no history of surgery, major illness, hospital admission and were not on any medication. None of the participants were malnourished or showed any visible symptoms of nutritional deficiencies. The participants had satisfactory oral hygiene with no clinical signs of gingivitis or any other compromised dental conditions. The alcoholic participants (n=10) were chosen based on their alcohol consumption where they must have been consuming alcohol for at least one year and the amount consumed were between 2 and 3 glasses of alcohol on a daily basis.

Saliva Collection

Saliva collection was carried out in the morning and participants were told to avoid taking food or alcoholic drinks for at least 60 min prior to giving saliva. Dental treatment was also prohibited for at least 24 h before sample collection. All participants were asked to thoroughly rinse their mouths with water for 15 s. To avoid sample dilution, the participants were asked to wait for another 10 min after rinsing their mouths with water. For each of the participants, saliva was obtained using three saliva collection methods; DNA-Sal™ (Oasis Diagnostics, USA), Oragene-DNA (DNA Genotek Inc, Ontario, Canada) saliva collection kits and whole saliva collection method. All participants were informed about the procedures involved with the three saliva collection methods and a visual demonstration was given for each of the method. Saliva was first collected using the whole saliva collection method and this was followed by the Oragene-DNA and lastly by the DNA-Sal™ saliva collection kit. This sampling order was observed because saliva obtained from the DNA-Sal™ kit requires the use of a mechanical scraper as opposed to the first two methods where patients were required to expectorate their saliva directly into the respective collection tubes. A wash period of 30 min was allowed between each collection method.

Quantitative and Qualitative Assessments of Saliva DNA Samples

Extraction of genomic DNA from saliva samples was performed using the DNA extraction kit by QIAGEN (QlAamp DNA mini kit, Germany). Following DNA extraction, the DNA samples were spectrophotometrically assessed using the Nano Drop technologies, Nano Drop2000 (Thermo Scientific, USA).

Qualitative analysis was determined by taking the ratios of OD260:OD280 wavelength. Value of ratio above 2.0 would indicate contamination of samples by RNA, while a low ratio value would indicate contamination by protein or phenol. Quantitative analysis of the DNA extract was based on spectrophotometry readings. 1 OD (optical density) reading at 260 nm wavelength corresponds to 50 µg/mL of double stranded DNA. To ensure the reliability of the data obtained for each saliva sample, this procedure was repeated three times.

PCR Amplifications

Prior to using the DNA samples for PCR, the DNA concentration of all the samples was standardized. PCRs were undertaken in a 25 µL volume containing 12.5 µL of Top Taq PCR master mix (containing 1.25 units TopTaq DNA Polymerase, 1 × of the manufacturer's buffer with 1.5 mM $MgCl_2$ and 200 µM of each dNTP), 2 µL of each of the forward and reverse primers (0.2 µmol), 5 µL RNase-free distilled water, 2.5 µL 1 × corraload concentrate and 1 µg DNA template (1 µg/µL). Thermal cycling for PCR was performed using a Mastercycler Gradient instrument

(Eppendorf AG, Hamburg, Germany). The primers used for PCR amplification are listed in Table 1. The PCR cycling conditions consisted of an initial denaturation step at 96°C for 10 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension of 72°C for 10 min.

TABLE 1. Oligonucleotide primers used for PCR

Primers:	Sequence 5' to 3':
ADH1B reverse primer	GAAGGGGGGTTTTCTCTT
ADH1B forward primer	AATCTTTTCTGAATCTGAACAG
18S rRNA reverse primer	CCATCCAATCGGTAGTAGCG
18S rRNA forward primer	GTAACCCGTTGAACCCCATT

STATISTICAL ANALYSIS

The quantity (concentration) and quality (ratio of A260/280) of DNA yield obtained using three saliva collection methods from non-alcoholic participants were compared using Kruskal-Wallis One Way Analysis of Variance (ANOVA) on Ranks followed by Tukey test. The quantity and quality of DNA yield between alcoholic and non-alcoholic groups were compared using t-test/Mann-Whitney Rank Sum Test.

RESULTS

QUANTITATIVE AND QUALITATIVE ASSESSMENTS OF SALIVA DNA FROM NON-ALCOHOLIC PARTICIPANTS USING THREE SALIVA COLLECTION METHODS

Among the three saliva collection methods, the highest concentration of DNA was significantly achieved ($p<0.05$) by the DNA-Sal™ kit method (19.92±6.18 ng/μL) followed by Oragene-DNA (14.05±3.62 ng/μL) and the lowest DNA concentration obtained using the whole saliva collection method (8.42±3.06 ng/μL) (Figure 1(A)). As shown in Figure 1(B), the spectrophotometer readings of OD260:OD280 indicated that there was no significant difference in the quality of DNA between the three saliva collection methods. The A260/A280 ratios were found to be in the acceptable range (1.74-1.80) that is generally considered as pure DNA.

QUANTITATIVE AND QUALITATIVE ASSESSMENTS OF SALIVA DNA FROM ALCOHOLIC PARTICIPANTS USING THREE SALIVA COLLECTION METHODS

Similar to the non-alcoholic saliva samples, the saliva DNA of alcoholic participants using the DNA-Sal™ collection method showed significantly ($p<0.05$) higher amounts of DNA (16±3.57 ng/μL) when compared with Oragene-DNA (10.85±1.95 ng/μL) or whole saliva collection method (6.11±2.15 ng/μL) (Figure 1(B)). Qualitatively, the A260/A280 ratios for all three saliva collection methods were also in the range (1.74-1.80) that is generally considered as pure DNA (Figure 1(B)).

PCR ANALYSIS OF SALIVA DNA FROM ALCOHOLIC AND NON-ALCOHOLIC PARTICIPANTS

Figure 2 shows that the PCR product for both genes; *ADH1B* and 18S rRNA, were successfully amplified. In addition, there was no visible smearing of the DNA fragments for all samples indicating no qualitative degradation of the saliva DNA samples.

DISCUSSION

In this study, the DNA concentration was measured using the NanoDrop method that is based on UV absorption for measuring DNA, RNA and proteins. Although the NanoDrop method is a very common method used for measuring DNA concentration, there is some limitation to the results obtained in this study as it have been reported that this method is less sensitive when compared to other methods such as those involving the use of intercalating dyes (e.g. SYBR Green) or 5′ hydrolysis probes (e.g. Taqman®) coupled with real-time quantitative PCR (qPCR) (Robin et al. 2016; Sedlackova et al. 2013). Further evaluation of the results obtained in this study could be attempted in the future with the use of more sensitive methods as accurate measurement of DNA concentration can be especially crucial for downstream analysis of the DNA sample, especially for next generation sequencing (NGS) or high throughput genotyping studies.

Between the three saliva collection method; DNA-Sal™, Oragene-DNA saliva collection kits and whole saliva collection method, the highest quantity of DNA extracted from saliva samples was obtained using the DNA-Sal™ kit

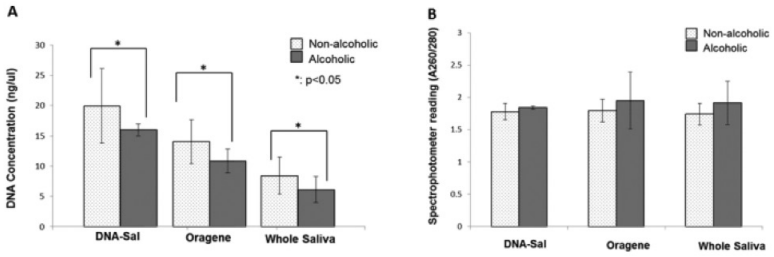

FIGURE 1. Quantitative (A) and qualitative (B) assessments of extracted DNA from saliva samples of non-alcoholic and alcoholic participants obtained using three saliva collection methods. *: $p<0.05$, t-test/Manan-Whitney Rank Sum Test

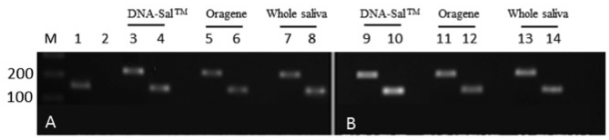

FIGURE 2. PCR analysis of DNA samples obtained from saliva samples of non-alcoholic (A) and alcoholic (B) participants. The saliva samples were obtained using three saliva collection kits. Lane 1- Positive control PCR reaction. Lane 2 - Negative control PCR reaction. Detection of *ADH1B* (lanes 3, 5, 7, 9, 11 and 13) and 185 rRNA (lanes 4, 6, 8, 10, 12 and 14). Lane M: DNA molecular size markers (bp)

while the lowest quantity was obtained using the whole saliva collection method. The use of a mechanical tool in the former procedure to scrape off the buccal cells could have contributed to this high DNA content. Such step was not required when using whole saliva or the Oragene-DNA method. Similar observations have been reported whereby the buccal scraping procedure can produce high yield of DNA (Cozier et al. 2004; King et al. 2002). In this study, although a specific tool was not provided in the Oragene-DNA saliva collection kit, patients were instructed to thoroughly rub their tongue against the oral mucosa for 15 s before saliva collection was carried out. This procedure too, could have contributed to a considerable desquamation of the oral mucosa, although the dislodging effect may not be equal to that produced when a mechanical scraper was used.

The low quantity of DNA extracted from the whole saliva collection method may have been caused by some form of DNA degradation although preventive steps were taken to minimize the degrading effect such as performing DNA extractions on the sampling day itself. Such findings are in line with other reported studies whereby the DNA yield extracted using whole saliva collection method was in the range of 8–30 µg from 1-2 mL of saliva (Koni et al. 2011; Nemoda et al. 2011; Ng et al. 2006). Compared with the whole saliva collection method, the Oragene-DNA saliva collection kit was more efficient as the method yielded much higher DNA (Hansen et al. 2007; Philibert et al. 2008b; Pulford et al. 2013a). According to Nunes et al. (2012), 52% more DNA was obtained using the Oragene-DNA saliva collection method, which actually exceeded the manufacturer's estimation of 110 µg DNA from 2 mL of saliva (Birnboim 2004).

Observations made in this study also suggested that there were no significant differences in the quality of DNA extracted from saliva samples using all three saliva collection methods. With the mean values (OD260:OD280) recorded for all saliva samples were within the range (1.74-1.80) that is generally accepted as pure DNA, this would indicate that any of the three saliva collection methods would enable the extraction of high quality saliva DNA for use in further analysis.

The three saliva collection methods were then used for saliva collection from alcoholic participants. With regards to DNA quantification, the results obtained for the alcoholic participants were similar to that of the non-alcoholic participants, where the quantity of saliva DNA was the highest with the DNA-Sal™ followed by Oragene-DNA saliva collection kit and whole saliva collection method. This finding could strengthened the use of the mechanical scraper for increasing the DNA yield. However, the quantity of saliva DNA was significantly higher with the non-alcoholic group of participants. In this study, collection of saliva from the all participants was carried out in the morning and although the time between saliva collection and the consumption of an alcoholic drink could have been more that 60 min, it could be that the saliva DNA yield of alcoholic participants was effected by some components of the alcoholic drinks that were consumed on a daily basis. Abaz et al. (2002) had measured the DNA yield of saliva recovered from common drinking containers and carried out DNA profiling on alcoholic and non-alcoholic drinks. The presence of alcohol in alcoholic drinks was found to effect the overall DNA yield and as a result, the amount of DNA template had to be increased for PCR before DNA profiling was carried out. It was suggested that the components of the alcoholic drinks could have had some inhibitory effects on the PCR analysis which have then carried on to effect the outcomes of the profiling process. As the DNA yield was lower with the alcoholic participants, this could also mean that increasing the amount of DNA template may be required in future molecular biology techniques.

To determine the integrity of saliva DNA of alcoholic and non-alcoholic participants obtained using three saliva collection methods, PCR amplifications were carried out for amplification of one of the human alcohol dehydrogenase gene, *ADH1B*, along with the housekeeping gene, 18S rRNA. ADH activity from the human oral mucosa or microorganisms of the oral cavity are capable of oxiding ethanol to acetaldehyde, a known carcinogen (Bakri et al. 2015, 2014). PCR amplifications of both genes were successful and when the PCR products for both genes were run on agarose gel, it was observed that there was a clear band for both genes, *ADH1B* and 18s rRNA and as there was no smearing of the bands, this would indicate that there was no DNA degradation. The results obtained would suggest that the DNA obtained from alcoholic and non-alcoholic participants could be used for genotyping and mutational screening of *ADH1B* gene and other disease causing genes. However, the results obtained should be analyzed with caution due to the limited number of samples available for this study. There were some difficulty in obtaining saliva samples from alcoholic participants of matching

age and sex to that of the non-alcoholic participants. It was also acknowledge that the PCR method used in this study to determine the integrity of the DNA samples may not be very sensitive. As the number of samples are small, other method such as quantitative PCR could be employed to further differentiate the sensitivity of saliva sampling between alcoholic and non-alcoholic participants. Additionally, the findings of this study can be strengthened if more sensitive and high-end techniques such as microarray, mass spectrometry, high resolution melting (HRM) PCR or restriction fragment length polymorphism (RFLP) PCR could employed in further studies (Nunes et al. 2012).

Saliva, like blood, contains an abundance of proteins, nucleic acid molecules, antibodies, hormones, enzymes, growth factors and antimicrobial constituents (de Almeida Pdel et al. 2008; Humphrey & Williamson 2001). Based on the preliminary findings of this study, saliva has the potential to be used as a bio diagnostic tool for research purposes as well as for high throughput genotyping studies (Abraham et al. 2012; Gudiseva et al. 2016; Pulford et al. 2013b).

CONCLUSION

It is suggested that the addition of the mechanical scraper as supplied in the DNA-Sal™ saliva collection kit, contributed to the highest quantity of DNA extracted from saliva samples when compared with Oragene-DNA or whole saliva collection method. The DNA yield was significantly higher in the non-alcoholic saliva samples. PCR amplification of the *ADH1B* gene was successful and the DNA quality of all saliva samples of alcoholic and non-alcoholic participants was of the acceptable range. Saliva DNA obtained from alcoholic participants could be considered for future work involving genotyping and mutational screening of *ADH1B* gene and other disease causing genes.

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