Journal of Advances in Biology & Biotechnology

22(3): 1-7, 2019; Article no.JABB.51847
ISSN: 2394-1081

Quantitative Comparison of Oral Site-specific DNA Isolates Reveals Differential Outcomes

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AA and KD were involved in data collection and sample preparation. Authors GC and KK were responsible for overall study design and data analysis. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2019/v22i330116
Editor(s):
(1) Dr. Sahib Gul Afridi, Assistant Professor, Department of Biochemistry, Garden Campus, Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa, Pakistan.
Reviewers:
(1) Francesca Gorini, National Research Council, Italy.
(2) Alicia Noemí Kohli Bordino, Italian University Institute of Rosario, Argentina.
Complete Peer review History: https://sdiarticle4.com/review-history/51847

Original Research Article

Received 03 August 2019
Accepted 10 October 2019
Published 22 October 2019

ABSTRACT

Introduction: More and more evidence has accumulated that suggests salivary sampling may provide direct analysis of oral conditions and microbial constituents, but may also be useful in the diagnosis and early detection of other chronic diseases. Although multiple methods of oral sampling currently exist, some methods are prohibitively expensive or based upon technologies not ubiquitously available at public health centers or state-funded colleges. This study provides a comparative analysis of DNA concentrations and quality from five specific oral sites derived using sterile paper points, including the gingival crevice between the upper central incisors, biofilm of the upper first molar, lingual incisor, and the dorsum of the tongue for comparison with unstimulated saliva collection.
Methods: This study analyzed previously collected unstimulated saliva and paper point samples. In brief, DNA was isolated from each using TRIzol (phenol: Chloroform) extraction and DNA quantification and quality was measured using a NanoDrop spectrophotometer at 260 and 280 nm.

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Results: Analysis of Paper Point (PP) biofilm sampling sites from upper first molar, lower incisor, and dorsum of the tongue revealed similar average DNA concentrations, ranging between 14,342 ng/uL and 14,402 ng/uL (p=0.9851). Although variations were observed between different patients, samples from different oral sites within the same patient were strikingly similar, R=0.8355. Comparison of DNA isolated from fluids, gingival crevicular fluid (GCF) and unstimulated saliva revealed average DNA concentrations that were similar to the biofilm sampling sites (14,686 ng/uL and 13,743 ng/uL, respectively), which were not significantly different from one another (p=0.7893). DNA concentrations ranged considerably between patients (low = 4,410 ng/uL; high = 48,783 ng/uL), but were most similar with different samples (GCF, saliva) from the same patient (Pearson's R=0.6979). In addition, DNA purity measured by A260:A280 nm absorbance did not reveal any significant difference among sampling sites (range 1.62 – 1.70; p=0.427).
Discussion: Although many methods are available to provide oral sampling, simple and low-cost methods such as paper point sampling, unstimulated saliva collection and buccal swabs may represent tools that provide sufficient DNA quality and quantity for molecular screening. In addition, although heterogeneity between patient samples will always be present – samples from various oral sites within the same patient may provide roughly equivalent DNA samples for further screening and molecular analysis.

Keywords: Saliva sampling; paper points; DNA concentration; DNA purity.

ABBREVIATIONS

Deoxyribonucleic acid (DNA); fluorescence in situ hybridization (FISH); Point of Care (PoC); Office for the Protection of Research Subjects (OPRS); Institutional Review Board (IRB); University of Nevada, Las Vegas (UNLV); School of Dental Medicine (SDM); polymerase chain reaction (PCR); phosphate buffered saline (PBS); relative centrifugal force (RCF); paper points (PP); gingival crevicular fluid (GCF).

1. INTRODUCTION

More and more evidence has accumulated that suggests salivary sampling may provide direct analysis of oral conditions and microbial constituents, but may also be useful in the diagnosis and early detection of other chronic diseases [1,2]. For example, new studies have demonstrated that significant detectable changes in the subgingival microbial flora in patients with periodontitis may not only predict prognosis and treatment success, but may also correlate with and predict systemic changes to type 2 diabetes mellitus or cancer [3-5]. Despite these advances, there have been relative few studies comparing site-specific oral sampling with bacterial DNA yields and other microbial screening outcomes [6].

Although multiple methods of oral sampling currently exist, some methods such as fluorescence in situ hybridization (FISH) "lab-on-a-chip" or point-of-care (PoC) immunoflow assays are prohibitively expensive or based upon technologies not ubiquitously available to oral health researchers at public health centers or state-funded colleges [6,7]. The remaining low-cost and easily accessible methods for microbial detection (including unstimulated saliva collection, sterile paper point sampling) have relatively few studies providing both qualitative and quantitative DNA analysis [8,9]. Quantitative and qualitative comparisons of DNA isolated using these low-cost and ubiquitous sampling methods may provide valuable analysis to determine if these methods result in widely varying measures and outcomes [10].

The objective of the current study is to provide a comparative analysis of DNA concentrations and quality from five specific oral sites derived using sterile paper points, including the gingival crevice between the upper central incisors, biofilm of the upper first molar and lingual incisor, as well as the dorsum of the tongue. In addition, comparisons can be made with unstimulated saliva, which was also concurrently collected from each patient at the time of the original sample collection. This analysis may provide significant insights into the comparative heterogeneity and sampling outcomes associated with site-specific oral sampling methods.

2. METHODOLOGY

2.1 Study Approval

The Office for the Protection of Research Subjects (OPRS) and the Institutional Review

Board (IRB) of the University of Nevada, Las Vegas (UNLV) reviewed and approved the original protocol for collection of saliva and oral samples under "The Prevalence of Oral Microbes in Saliva from the UNLV – School of Dental Medicine pediatric and adult clinical population (#1502-506M)." In brief, patients (and parents or guardians if under 18 years of age) were asked for voluntary participation. All patients that declined participation were excluded. Any patient (with consent of parent or guardian if needed) that volunteered to participate was asked to provide Informed Consent and/or Pediatric Assent for those under 18 years of age. No remuneration was given to any subject.

2.2 Sample Collection

In brief, all patients were given a sterile saliva collection tube and subsequently asked to provide up to 5 mL of unstimulated saliva. In addition, sterile paper points were used to acquire samples from the dorsum of the tongue, buccal surface of the maxillary first molar (tooth #3), lingual surface of the mandibular central incisor (tooth #25) and the buccal gingival crevice of the maxillary central incisor (tooth #9), which were each placed in individual sterile collection tubes. All samples were stored on ice and transferred to a biomedical biosafety level 2 (BSL-2) laboratory for long-term storage and processing.

2.3 DNA Isolation

As previously described, DNA isolation from each of the saliva samples was performed using the Invitrogen TRIzol reagent, which involves a sequential precipitation of DNA from a single sample suitable for obtaining polymerase chain reaction (PCR) quality DNA [11,12]. In brief, 100 uL of sterile filtered 1X phosphate buffered saline (PBS) was added to each of the paper point containing collection tubes and vortexed for 20 seconds to elute any attached bacteria [13]. 100 uL of saliva or the 1X PBS-eluted samples was added to 300 uL of TRIzol reagent and triturated prior to incubation for five minutes at room temperature. To this mixture 200 uL of chloroform was added and mixed and then incubated for an additional two to three minutes.

The samples were then centrifuged at 4C at 12,000 g or relative centrifugal force (RCF) for 15 minutes. The DNA-containing interphase was transferred to a new sterile microcentrifuge tube with the addition of 300 uL of 100% ethanol, which was mixed by inverting each sample prior to incubation for two to three minutes at room temperature. Each sample was then centrifuged for an additional five minutes at 2,000 g or RCF to pellet the DNA. The ethanol was aspirated and each DNA pellet was resuspended in 100 uL of sterile DNA rehydration solution for analysis and comparison.

2.4 DNA Analysis

The quality and quantity of DNA was assessed by spectrophotometric absorbance readings at 260 and 280 nm (A260:A280) using a NanoDrop spectrophotometer from Thermo Fisher. DNA concentration is generally estimated by this method by measuring A260 nm absorbance, adjusting this measurement for turbidity at A320 nm) and the dilution factor. High-quality DNA will have an A260:A280 ratio of approximately 1.7 – 2.0.

2.5 Statistical Analysis

Statistical differences between DNA concentrations (ng/uL) were measured using two-tailed Students t-tests, which are appropriate for parametric data [14]. Analysis of DNA concentrations within the same patient were assessed using Pearson's correlation or R, which will reveal the association between different sites within the same patient and are also appropriate for this type of parametric data.

3. RESULTS

A total of n=105 patient samples were available for DNA analysis and comparison in this study. Analysis of the samples collected using paper points (PP) revealed average DNA concentrations at all three biofilm sampling sites were similar; maxillary first molar (buccal), mandibular central incisor (lingual), and dorsum of tongue (14,324 ng/uL, 14,402 ng/uL, 14,341 ng/uL, respectively; p=0.9851). Although the DNA concentration ranged quite significantly between patients (low = 4,065 ng/uL; high = 48, 676 ng/uL), these were most similar among different oral sampling sites within the same patient (Pearson's R=0.8355).

Analysis of the samples collected using liquid or aqueous components revealed average DNA concentrations that were slightly higher among the paper point (PP) samples of gingival crevicular fluid (GCF) than unstimulated saliva (14,686 ng/uL and 13,743 ng/uL, respectively),

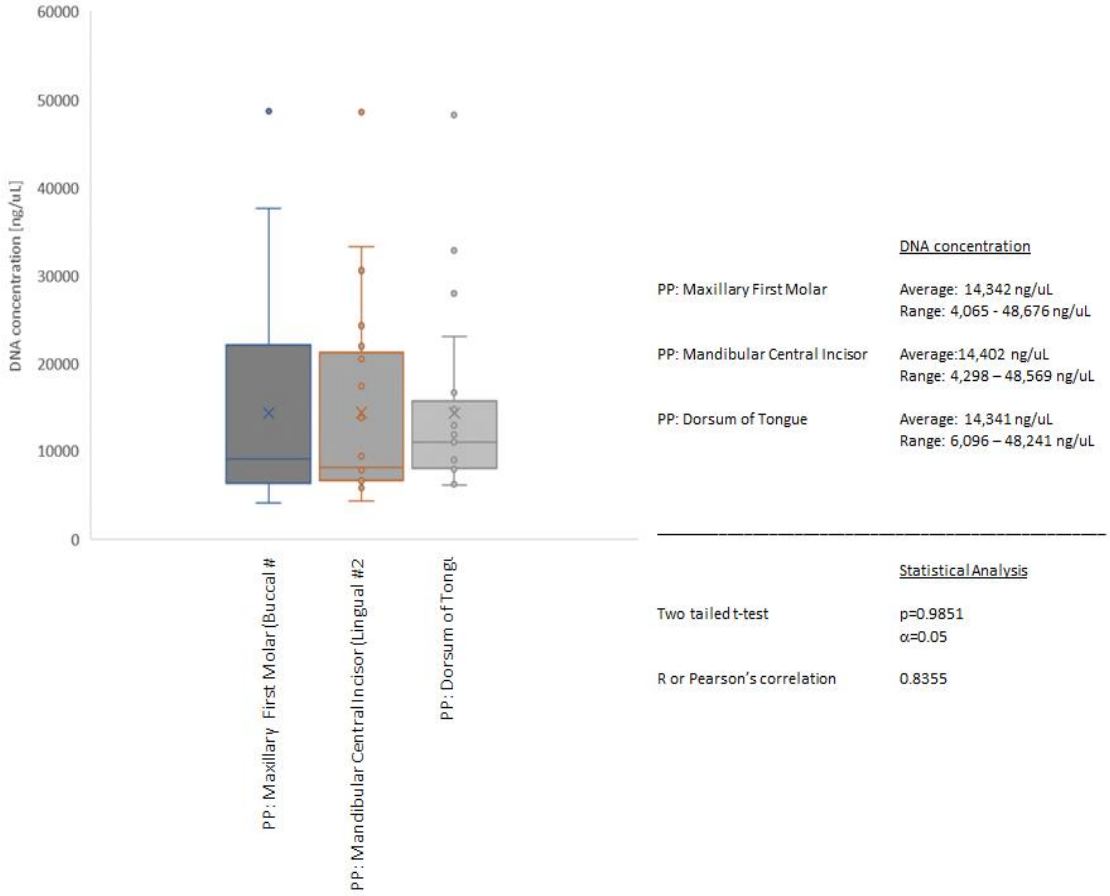

Fig. 1. Analysis of paper point (PP) biofilm sampling sites. Comparison of DNA isolated from maxillary first molar (buccal), mandibular central incisor (lingual) and dorsum of the tongue revealed similar DNA concentrations, ranging between 14,342 ng/uL and 14,402 ng/uL (p=0.9851). Although variations were primarily observed between different patients, samples from different oral sites within the same patient were strikingly similar (R=0.8355)

although this was not statistically significant (*p*=0.7893) (Fig. 2). Although DNA concentrations ranged quite significantly between patients (low = 4,410 ng/uL; high = 48,783 ng/uL), these were most similar with different samples (GCF, saliva) from the same patient (Pearson's R=0.6979).

To determine if the overall quantity of DNA isolated from any given oral sampling site was correlated with the overall quality of DNA, absorbance readings at 260 and 280 nm were taken to provide an estimate of DNA purity (Fig. 3). These data clearly indicate that no statistically significant relationship between DNA concentration and DNA purity were observed (R=0.2175). Although a small subset of samples at the very lowest concentrations were found to have slightly higher DNA purity, the vast majority of samples did not vary significantly in DNA purity, with average DNA concentrations ranging between 1.62 and 1.70 (*p*=0.427).

4. DISCUSSION

The objective of the current study was to provide a comparative analysis of DNA concentrations and quality from five specific oral sites derived using sterile paper points, including the gingival crevice between the upper central incisors, biofilm of the maxillary first molar and mandibular central incisor, as well as the dorsum of the tongue and unstimulated saliva. The results of this analysis demonstrated that paper point sampling of biofilm directly from the tooth or tongue surface revealed strikingly similar average DNA concentrations. This may be among the first studies to specifically assess

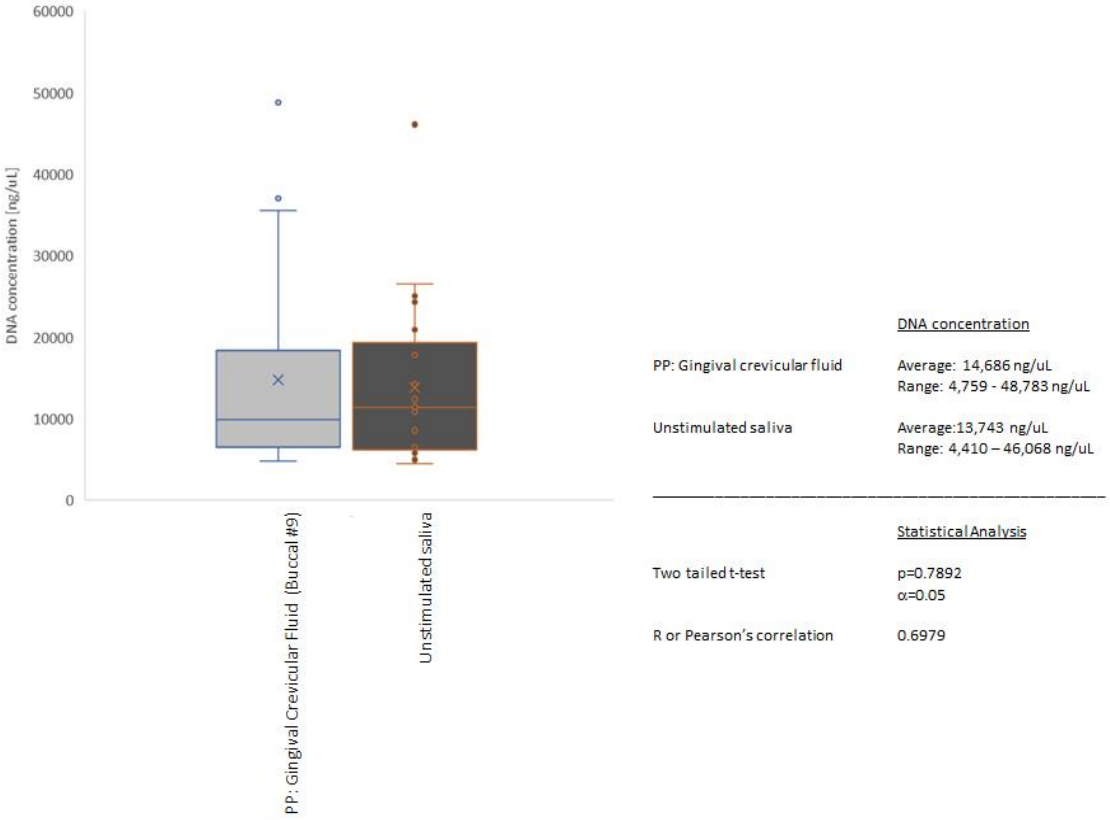

Fig. 2. Analysis of gingival crevicular fluid (GCF) and unstimulated saliva sampling. Comparison of DNA isolated from GCF at the maxillary central incisor (buccal) using PP and unstimulated saliva revealed similar DNA concentrations (14,686 ng/uL and 13,743 ng/uL, respectively), which were not statistically significant (p=0.7893). DNA concentrations ranged significantly between patients (low = 4,410 ng/uL; high = 48,783 ng/uL), but were most similar with different samples (GCF, saliva) from the same patient (Pearson's R=0.6979)

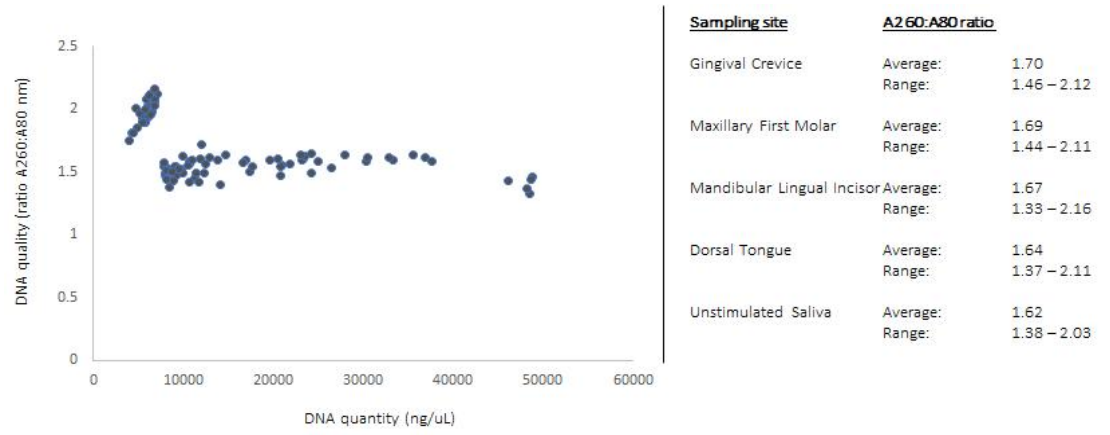

Fig. 3. Analysis of DNA quality (A260:A80 nm) compared with DNA quantity (ng/uL).The comparison of DNA quantity with DNA quality did not reveal any significant association (R=0.2175). The DNA concentration averages for each oral sampling site were comparable and not significantly different from one another, ranging between 1.62 and 1.70 (p=0.427)

these parameters, although some previous work has compared DNA quantity with various acquisition methods (buccal swab, unstimulated saliva) [15,16].

In addition, these data demonstrated that no significant or specific relationships appeared to exist between the overall quantity of DNA obtained and the assessment of DNA quality. This may be another significant finding, as few previous studies have specifically assessed these parameters when evaluating DNA recovery from various sites within the oral cavity [17,18]. This may represent an important clinical finding, as many institutions and public health facilities may not have access to both salivary collection tubes and site-specific sampling tools.

This study does have some inherent limitations, which must also be considered when evaluating these results. First, there were financial and time constraints on the number of samples that could be analyzed and screened. This may be a common limitation to many clinical and epidemiologic studies, but it is hoped that the larger sample size in this study (n=105) may reduce any bias that could be evident in smaller samples [14]. In addition, not all samples were collected or processed on the same day – therefore, it is always possible that other factors not directly associated with the parameters measured may have influenced the outcomes. This is also an inherent risk in any type of biomedical study and every effort was made to ensure that samples were measured in duplicate or triplicate and all results were averaged to minimize any potential bias.

5. CONCLUSIONS

Although many methods are available to provide oral sampling, simple and low-cost methods such as paper point sampling, unstimulated saliva collection and buccal swabs may represent tools that provide sufficient DNA quality and quantity for molecular screening. In addition, although heterogeneity between patient samples will always be present – samples from various oral sites within the same patient may provide roughly equivalent DNA samples for further screening and molecular analysis.

CONSENT

Any patient (with consent of parent or guardian if needed) that volunteered to participate was asked to provide Informed Consent and/or Pediatric Assent for those under 18 years of age.

ETHICAL APPROVAL

The Office for the Protection of Research Subjects (OPRS) and the Institutional Review Board (IRB) of the University of Nevada, Las Vegas (UNLV) reviewed and approved the original protocol for collection of saliva and oral samples.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. James Mah and the Department of Advanced Education, Orthodontic program as well as Dr. Jeffrey Ebersole and the Office of Research at the University of Nevada, Las Vegas – School of Dental Medicine for the funding and support to complete this project.

COMPETING INTERESTS

The authors have declared that no bias or conflicts of interest exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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