**Saliva collection methods for DNA biomarker analysis in oral cancer patients**

**Abstract**

Patients with head and neck cancers are predisposed to local recurrence and second primaries due to the phenomenon of field cancerisation. Clinical detection of recurrence remains challenging, but DNA biomarkers in saliva may prove adjunctive to current diagnostic methods. However, primary site irradiation often leads to xerostomia.  We assessed the ability of three saliva collection methods to generate DNA of sufficient quantity and quality for use in biomarker assays. Paired saliva samples were collected in OrageneTM vials and as saline mouthwash from non-irradiated (n=21) patients with oral squamous cell carcinoma (OSCC) and with OrageneTM sponge kits and as mouthwash from irradiated (n=24) patients with OSCC. Quantitative polymerase chain reaction demonstrated that OrageneTM vials contained DNA in significantly greater amounts (median: 122g) than mouthwash (median: 17g) (p=0.0001) in non-irradiated patients, while OrageneTM sponge kits (median: 4g) and mouthwash (median: 5.5g) generated comparable DNA concentrations from the irradiated cohort. All90 samples contained DNA of sufficient quantity and quality for p16 promoter quantitative methylation specific PCR (qMSP).   WhileOrageneTM vials contained the greatest amounts of DNA in this study, all three methods yielded sufficient DNA for the detection of DNA biomarkers using qMSP and the method of collection should be selected on the basis of patient compliance and oral competency.

Running title: saliva collection methods in oral cancer

Keywords: Saliva, Collection method, Mouthwash, Biomarker, Oral cancer, DNA, PCR

**Introduction**

Oral squamous cell carcinoma (OSCC) is a molecularly heterogeneous disease1 which develops in areas of field change with a propensity for recurrence and second primary tumours.2 Molecular biomarkers associated with OSCC have been detected in saliva3-5 despite evidence that more than 70% of DNA contained in saliva samples from normal individuals is from bacteria6 and that the human genomic DNA component is derived as much from immune as epithelial cells.7 Genomic DNA is a stable macromolecule which has been extracted by a variety of methods in normal participants8-10 and has been successfully obtained from saliva returned by postal services and stored at room temperature without preservatives.11,12 These robust features lend it to translation into the clinical setting as a source of biomarkers

It is accepted that early diagnosis of OSCC can significantly improve clinical outcome.13 The main clinical value of saliva biomarkers in this disease would be a role in early detection and disease surveillance where multiple, chronological samples are required and recurrent biopsy impractical.   Saliva collection is non-invasive and known to be more acceptable to patients than venepuncture.14 Saliva bathes the whole oral cavity and is thus more likely to be representative of the entire exposed field when compared to invasive, localised tissue biopsy, but issues of sensitivity in relation to bacterial and immune cell contamination remain unanswered.

Post-surgical radiotherapy in the treatment of OSCC identifies patients at risk of recurrence with a need for close follow-up,15 but the clinical detection of early recurrence or second primaries in the surgically altered, irradiated field can be challenging. Xerostomia, a known sequela of radiotherapy, could make the collection of saliva for adjunctive molecular methods of detection difficult.

In this study, we aim to establish the quality and quantity of DNA extracted from saliva of oral cancer patients, inclusive of a cohort of post-radiotherapy patients, using three different collection methods, including a new sponge collection technique developed for patients for whom conventional methods of saliva collection are difficult.

**Methods**

Two, temporally separated, cohorts of 21 and 24 patients (45 in total) were recruited from consecutive patients attending Aintree University Hospital Maxillofacial Unit for treatment of OSCC.  The study received ethical approval (EC 47.01) and all patients consented to participate.

The first cohort comprised 10 preoperative and 11 postoperative patients, four of whom had received radiotherapy prior to saliva collection. Two saliva samples were collected from each patient as follows:  25ml 0.9% normal saline was swilled in the mouth for 30 seconds and deposited into a tube.4 Ten minutes later, patients deposited whole saliva directly into an OrageneTM vial (DNA Genotek, Inc. Ottawa, Canada) until the 2ml fill line was reached. Mouthwash samples were immediately stored at 4oC for a maximum of three hours before being centrifugated at 1,200g for five minutes and the cell pellet stored at -80oC. OrageneTM vials required no processing prior to storage.

The second cohort comprised 24 patients who had received surgery and radiotherapy for OSCC. Paired saliva samples were collected from each patient as follows:  five individual sponges (DNA Genotek, Inc. Ottawa, Canada) were placed into pools of whole saliva in the patient’s mouth (Figure 1a), then removed and placed into a standard OrageneTM vial (Figure 1b).. Mouthwash collection and sample storage were carried out as before.

DNA was extracted from the mouthwash cell pellets according by a spin column protocol (DNeasy Blood & Tissue Kit: Qiagen, Crawley, UK). Briefly, phosphate buffered saline was added to each cell pellet to make a final volume of 500l. 25 l proteinase K (20mg/ml) was added to a 180l aliquot of this and separation continued using spin columns with elution in 200l of AE buffer.

DNA extraction from the OrageneTM vials was performed using the manufacturer’s protocol for 0.5ml of sample. In brief, vials were incubated at 50oC for one hour to lyse cells and digest nuclear proteins. 20 l OrageneTM purifier was added to 0.5ml of sample before ethanol precipitation with final resuspension in 200l 0.1 x TE buffer.

DNA extraction from the OrageneTM sponges was undertaken using the manufacturer’s protocol. The vials were incubated in a 50°C water bath for 1 hour as above and recovery of liquid from the sponges was effected by centrifugation in a 5ml plastic syringe placed into a 15ml centrifuge tube. The manufacturer’s OrageneTM/Saliva 4ml protocol was followed for the entire liquid volume of the sample.DNA was re-suspended in200l 0.1 x TE buffer.

To accurately quantify the total human DNA concentration, a quantitative PCR (qPCR) using the human RNAse P gene16 was carried out using the AB7500 Fast system on the standard curve programme according to the manufacturer’s guidelines (Applied Biosystems, Foster City, CA). Controls of known DNA concentration were diluted from a stock of human lymphocyte DNA (500ng/l). Samples were run in triplicate. The cycle threshold (CT) was plotted against a log2 scale for the standard dilutions to create a linear model of their relationship and used to calculate DNA concentration of the samples based on CT value.

500ng of each DNA sample was bisulphite treated as per the manufacturer’s protocol using Zymo EZ-96 DNA Methylation-Gold kit (Zymo Research Corporation, Orange, CA), eluted in 30l of M-elution buffer. 100ng/l of lymphocyte DNA, methylated *in vitro* with Sss-I methylase (Zymo Research), was diluted to 0.1, 0.5, 1.0 and 5.0% methylation to act as reference samples and bisulphite treated at the same time as the sample DNA.

To assess the quality of DNA for downstream application, Taqman p16 methylation qMSP assays17 were performed on the bisulphite modified DNA with beta actin as an internal control gene (probe details on request). The ΔΔCT method was used to correct for amount of starting DNA and samples were deemed to be positive for p16 promoter methylation if the mean dCT was ≥ that obtained for control DNA methylated to a level of 0.5%. This value was determined from data on levels of p16 methylation in saliva from a larger cohort of normal individuals with similar smoking histories (data not shown).

Results were analysed for statistical significance using the Wilcoxon signed rank test in SPSS software package.

**Results**

A total of 45 patients participated in this study and provided 90 saliva samples. The second cohort of 24 patients targeted post-radiotherapy patients following observations of saliva collection difficulties in the first cohort of both pre- and post-operative patients.

All 90 samples contained human DNA, as measured by the RNAse P qPCR assay and both cohorts demonstrated a large variation in the final DNA concentrations. In study 1, the DNA concentration from the OrageneTM whole saliva samples (range 10-929ng/l; median 306ng/l; SD 291ng/l) was significantly greater (p=0.001) than that of the matched mouthwash samples (range 7-657ng/l; median 62ng/l; SD 169ng/l) (Figure 2). In the post-radiotherapy cohort of study 2, lower median DNA concentrations were observed than for study 1, and there was no statistically significant difference in DNA concentrations obtained using OrageneTM sponge (range 0.3-306ng/l; median 22ng/l; SD 86 ng/l) and matched mouthwash (range 1.6-267ng/l; median 20ng/l; SD 77ng/l) collection methods (Figure 2).

The total DNA yield for each sample was calculated and reflected the DNA concentration data, with the total amount of DNA in whole saliva collected using OrageneTM (range 4-379g; median 122g; SD 116g) being significantly greater than mouthwash (range 2-194g; median 17g; SD 47g) in study 1 (p=0.0001 Wilcoxon signed rank test). There was again no significant difference between DNA yield from the OrageneTM sponge and mouthwash methods in study 2 (range 0.1-61g; median 4g; SD 17g and range 0.1-75g; median 5.5g; SD 21g, respectively)

All 90 of the bisulphite-treated saliva DNAs amplified within 37 cycles using the beta actin probe (range 24-37; mean/median 27 to 2 SD).  Four patients had p16 methylation in matched OrageneTM and mouthwash samples: 3/4 of these had their samples collected pre-operatively.  Three other positive results were obtained from single samples from three post-operative radiotherapy patients; two sponge samples and one mouthwash sample.

**Discussion**

Oral cancer displays genetic and biological heterogeneity which is not always evident at the histological level.1 Clinical behaviour can be unpredictable in some individual tumours and implies a role for molecular profiling in diagnosis and prognostication in conjunction with clinical observation and screening. OSCC-specific DNA biomarkers have been detected in saliva2,3,18,19 and may have prognostic value.4

The main strength of this current study is that it is the first to compare three different methods of saliva collection in OSCC patients, in particular post-radiotherapy patients in whom xerostomia could potentially limit saliva collection and post-surgery patients whose oral competence may be compromised making saliva collection problematic. To date it is also the first use of OrageneTM sponges in this application. For improved accuracy in DNA concentration estimation, we used an RNAse P qPCR assay16 which allowed the differentiation of human from non-human DNA, while spectrophotometry can be influenced by particulate matter, protein contamination, or the presence of non-human DNA.

The median DNA yields determined in this study are broadly comparable with those published in current literature for predominantly healthy participants.8,10,12 However, the median DNA yields obtained from the radiotherapy cohort in study 2 are less than that from the cohort in study 1, including when the data from the 4 postradiotherapy patients in cohort 1 were excluded (data not shown). The range of DNA concentrations in the samples is large across all three methods and has previously been observed with the OrageneTM method of collection.10 All three methods are subject to variation due to sampling technique, individual variation of the number of oral epithelial cells in saliva7,20 or the availability of saliva. While the sponge method may also be affected by the site of collection, all our samples were taken from the floor of mouth and a recent study has shown that sampling at different locations in the mouth give similar quantity and quality of DNA.21 Objectively, the collection of saliva from patients with compromised oral competence is easier to undertake in the clinical setting using sponges rather than mouthwash and requires less pre-storage processing. However, we did not collect any subjective patient opinion on preferred method of collection.

One of the concerns with the use of surrogate samples for biomarker analysis is the relatively small amounts of DNA for use in downstream applications. The DNA from every sample in our series retained sufficient integrity post-bisulphite modification to amplify in beta actin qPCR methylation assays and produced enough DNA for more than 50 such assays in each case. The observation that 3/10 preoperative patients demonstrated p16 promoter methylation in their paired saliva samples is comparable to our previous tumour data, where 28% of tumours demonstrate p16 methylation.22 It should be noted that matched tumour methylation data for this series of patients was not collected, but concordance between saliva and tumour tissue has previously been shown4,18 and was not a primary aim of this study. The detection of p16 promoter gene methylation in 3 specimens, but not in their paired samples, is probably indicative of levels of methylation at the limits of sensitivity of the test rather than a function of the amount or integrity of DNA collected or non-concordance between methods of collection, but the sample size is too small to comment. The clinical relevance of these results is part of a larger study.

A considerable body of evidence now exists to support saliva as an adequate source of DNA suitable for use in PCR9,10 to detect potential biomarkers.4,5,23 The advantage of this study is that it shows the feasibility of these collection techniques in a working clinical setting with a patient group in whom oral competence and saliva production is challenged. The ‘ideal’ conditions for sample collection are not always possible outside of the laboratory where sampling is much more time-consuming, less well tolerated and often produces ‘invalid’ samples.24 One acknowledged limitation of this study is the ten minute interval between sample collections imposed by the clinical setting and a swill time of 30 seconds for the mouthwash samples. An hour between sample collections would be preferable as a chance for saliva to replenish and other authors have described swilling mouth wash for periods of up to 3 minutes,3 but we found this was not tolerated by patients whose oral competence was compromised by surgery.

This paper demonstrates that sufficient DNA can be isolated from saliva collected from oral cancer patients at various stages of treatment, including those who have undergone radiotherapy, for use in testing a substantial panel of biomarkers. Whilecollection using OrageneTM vials provided the greatest amounts of DNA in this study, all three methods yielded sufficient DNA for the detection of biomarkers using qMSP. Thus, the method of collection for any given situation should be selected on the basis of patient compliance and oral competency. Saliva could, therefore, have a role as a tumour surrogate in longitudinal clinical biomarker testing applications. Further work is currently underway to establish a panel of DNA methylation biomarkers that can be reliably used to detect and monitor OSCC disease.

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**Conflict of Interest**

DNA Genotek provided 25 OrageneTM sponge collection kits free of charge.

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**Figure Legends**

**Figure 1**

a. Collection of saliva using OrageneTM sponge kit

b. Sponge tip, loaded with saliva, in the OrageneTM collection pot

**Figure 2**

DNA concentration range (ng/l) using qPCR in cohort 1 comparing mouthwash to OrageneTM; \*p=0.001 (Wilcoxon signed rank test) and cohort 2, the post-radiotherapy cohort, comparing mouthwash to OrageneTM sponge kits

\* and o signify samples that are outliers.

**Figure 1**

**Figure 2**