

A novel method for sampling the microbiota from the oral mucosa

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Abstract The purpose of this study was to develop a site-specific sampling method that could give representative and quantitative results for defined areas of the oral mucosa and would be easy to use. Two site-specific sampling methods (swab and filter paper imprint) were compared. The filter paper sampling method was developed for this study. Samples were collected from 14 volunteers. All samples were cultured under aerobic and anaerobic conditions. The number of viable bacteria and yeasts was determined and expressed per unit area. The filter paper recovered a significantly higher number of colony types of bacteria compared to the swab sample. Both collected a large number and variety of different oral microbes. The filter paper sampling method could be the optimal technique for quantitative site-specific oral mucosal samples and is highly suitable for both culture-based and non-culture-based identification of oral microbes.

Keywords Oral bacteria · Sampling · Oral mucosa

Introduction

Oral microorganisms may be the primary cause of oral mucosal lesions or secondary invaders in an already established non-infectious inflammatory mucosal lesion. It is well-recognized that oral microorganisms impact on the development of mucosal lesions. The microflora has been shown to differ both in intensity and spectrum compared to adjacent healthy mucosa, in for example, oral cancer lesions [2, 4], aphthous ulcers [3], and in chronic mucosal oral diseases [5]. Material for microbiological analysis of oral lesions should be collected from a site representative of the active disease process by appropriate methods.

Many site-specific and non-site-specific sampling methods are used to sample the oral cavity. Saliva and mouth rinse samples are often used for general sampling but do not represent the infection process at a specific lesion or site. A sterile swab is the most commonly used method for site-specific sampling and has generated a wealth of useful epidemiological data. However, although the swab samples can provide a quantitative estimate of the colonization status of the area sampled the technique is very difficult to standardize. The only concomitantly quantitative and representative method for site-specific sampling is the foam pad imprint method but it is not suitable for routine practice due to the unavailability of a commercial product [1, 7]. The aim of this study was to develop an easy-to-use method that would give representative and quantitative results for samples of specific unit areas from the oral mucosa.

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Materials and methods

Subjects

A total of 14 healthy volunteers, seven male and seven female, were enrolled in the study. The age of the participants ranged from 27 to 50 years (mean age 36 years, ± 11.3). The subjects were not receiving topical or systemic antimicrobial treatment at the time of sampling or during the previous 3 months. All participants had good oral health and did not suffer from acute or chronic diseases of the oral mucosa. The subjects were asked not to consume any food or clean their teeth for 1 h prior to the sampling. This study was approved by the Helsinki University Central Hospital Ethical Committee.

Sampling methods

Two site-specific non-invasive sampling methods for microbiological analyses of the oral mucosa were compared. The samples were taken with a swab and a filter paper from adjacent buccal mucosa sites using a standardized procedure as far as possible. Samples from adjacent areas for each subject were collected consecutively in the following order, i.e. swab sample and filter paper imprint sample. For the swab sample an area of diameter approximately 13 mm, estimated using a template, was rubbed with a dry cotton wool tipped sterile swab applicator (Copan Diagnostics, Corona, USA). The filter paper sampling method was developed for this study. A hydrophilic mixed cellulose ester MF-Millipore Membrane filter (GSWP01300; Millipore inc., MA, USA, pore size 0.22 μm , diameter of 13 mm) was placed gently on the buccal mucosa for 30 s, with the glossy side of the filter paper placed against the mucosa. The sampling time for the filter paper was chosen based on a pilot study.

Culture

The swab and the filter paper were placed into sterile test tubes with 5 ml of sterile saline immediately after sampling and cultured in a similar manner within 30 min. The swabs were agitated in the saline for 30 s and then the suspension was diluted (10^{-1}). The filter papers were respectively agitated in the saline for 30 s with four 3-mm glass beads and then the suspension was diluted (10^{-1}). Of each dilution 100 μl was cultured on selective and non-selective media under anaerobic and aerobic conditions. The mixing time and method for the filter paper was based on a pilot study. Fastidious anaerobe agar (FAA; Fastidious Anaerobe agar (LAB-M LAB 90) supplemented with 5% horse blood) was used to

enumerate the total cultivable bacteria. Lysed blood agar (BA; Trypticase soy agar (BBL 211047) and Mueller Hinton agar (BBL 212257) supplemented with 5% horse blood) was used for enumeration of total aerobic bacteria. Neomycin-vancomycin blood agar (NV; blood agar and neomycin sulphate (Sigma N-1876) supplemented with vancomycin (7.5 $\mu\text{g/ml}$), menadion (0.5 $\mu\text{g/ml}$) and sheep blood 5%) was used to enumerate anaerobic gram-negative bacteria. Cysteine-, lactose- and electrolyte-deficient agar (CLED; C.L.E.D medium (BBL 212218)) was used to select aerobic gram-negative fermentative rods. To detect yeasts Sabouraud Dextrose agar (SP; Sabouraud Dextrose Agar (Lab M), Bacto Agar (Difco) supplemented with penicillin (100,000 iu/ml) and streptomycin) was used. The BA, CLED and SP plates were incubated at 37°C for 48 h and the FAA and NV plates were incubated under anaerobic conditions at 37°C for 7 days. After incubation the numbers of bacteria and yeasts and different bacterial colony morphology types were enumerated (colony forming units, CFU). The analyses were performed by three independent observers without knowledge of the sample type or interpretation by others. In cases of discrepancies a consensus was reached by re-evaluation of the culture plates together. Gram stain was performed on all different colony morphology types from CLED and NV agars and the number of gram-negative colonies was recorded. The ratio of gram-negative to gram-positive bacteria and the ratio of aerobic to anaerobic bacteria were determined.

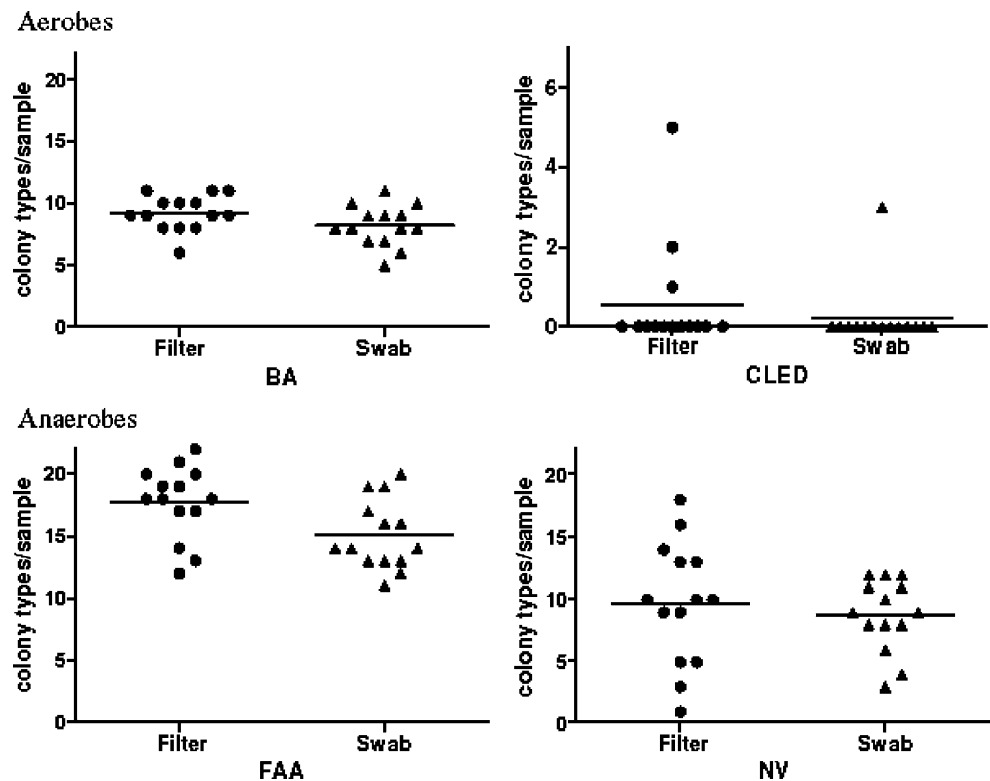
Statistical analysis

Data was analyzed by using GraphPad Prism version 5.00 (GraphPad Inc. San Diego, CA, USA). The Wilcoxon's signed rank test was used for analysis. Data is presented as mean and standard deviation in groups with a normal distribution and as median in groups with a skew distribution. *P* values of less than 0.05 were considered statistically significant.

Results and discussion

The novel filter paper imprint sample was compared with a conventional site-specific sampling method: a swab sample that was taken and processed following previously published protocols. The filter paper imprint sampling method detected a higher number of colony morphology types of aerobic and anaerobic bacteria compared to the swab (Fig. 1). The mean of the total number of morphology types per sample recovered from FAA was 17.7 (SD \pm 2.95) using the filter paper and 15.1 (SD \pm 2.8) using the swab. These values equate to 0.13 (SD \pm 0.02)

Fig. 1 The number of colony morphology types recovered by the site-specific filter paper imprint and swab sampling methods cultured on two aerobic culture media (BA and CLED) and two anaerobic culture media (FAA and NV). Each data point represents results from each subject ($n=14$). The filter paper imprint sample detected a significantly higher number of colony morphology types on FAA compared to the swab sample ($P=0.0094$). On the BA, CLED and NV culture media the difference were not significant ($P=NS$)



and 0.10 ($SD\pm 0.02$) colony morphology types of bacteria per square mm of mucosa, respectively. The difference was statistically significant on FAA ($P=0.0094$). The filter paper did not significantly differ from the swab in the gram-positive/gram-negative ratio (median: filter 25.9; swab 62.3) or for the aerobic/anaerobic ratio (median: filter 2.3; swab 3.5). The mean of the total number of CFUs was 1.4×10^5 ($SD\pm 1.7\times 10^5$) per swab sample and 0.4×10^5 ($SD\pm 0.5\times 10^5$) per filter paper imprint sample, the difference being statistically significant ($P=0.0001$). The filter paper imprint sample and the swab sample did not differ in their sensitivity in detecting yeast colonization. The filter paper and swab detected yeast from one subject only.

This study revealed that the novel filter paper imprint sample method is comparable to the swab sample method for site-specific microbial sampling of the buccal mucosa. Both methods recovered a large number and variety of different oral microbes and were found to be easy to use. The filter paper method recovered a higher number of colony morphology types of bacteria when compared to the swab. In contrast to the swab, the filter paper method recovers microbes from a defined unit area and the density of microbial colonization can be calculated. It was apparent in this study that sampling using a swab is influenced by a number of variables, including the area sampled, the force applied to the mucosa, and retention of the sample in the swab matrix when diluted prior to

culture. Both the filter paper and the swab revealed a wide deviation in total microbial counts due to the variability of colonization density between subjects. The level of oral hygiene in the volunteer group was not standardized in order to evaluate the flexibility of the methods in different patient groups.

The difference in the number of colony types between the two methods was mainly due to the filter paper recovering proportionally more gram-negative bacteria. It is possible that the capillary suction created by the filter paper pores is especially efficient in capturing these from the mucosa or that they became released from the filter paper to a greater extent than from the cotton wool matrix of the swab. Most microbes are larger than the pores within the filter paper and accumulate primarily on the surface and can be easily washed off for culture whereas the matrix of the cotton tips have large cavities where microorganisms may become entrapped.

The filter paper used in this study was selected mainly based on its pore size allowing capillary flow of saliva into the filter paper creating a gentle suction and thereby releasing adherent microorganisms without rubbing. The microbes larger than the $0.22\ \mu\text{m}$ pores within the filter paper are segregated from the saliva, accumulating primarily on the surface of the filter paper. A hydrophilic filter paper is essential for this process.

In the past decade much of the understanding of oral microbiology has resulted from improvement in the

techniques used in identification of oral microbes, whereas only little attention has been given to the first steps in diagnostics *i.e.* sampling methods. Interpretation of the findings of various clinical studies is difficult due to the different methodologies used. The infection and the site to be studied dictate selection of the optimal sampling method [6].

In conclusion, the filter paper sampling method was found to be an ideal technique for obtaining quantitative data from defined areas of the oral mucosa and is highly suitable for both culture-based and non-culture-based identification of oral microbes. The method is robust and easy to perform in a routine setting.

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The authors declare that they have no conflict of interest.

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