The Anaerobic Microflora: The Accused in Oral Malodour?
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Abstract
Background: Oral malodour is foul-smelling breath from the oral cavity and is due to metabolic products of bacteria in the oral cavity but can also be caused by many other systemic diseases. Oral malodour is believed to originate from foul-smelling gases, such as hydrogen sulfide produced by oral bacteria. Aim: To identify hydrogen sulfide producing bacteria from tongue biofilm and to investigate the relationship between bacterial flora and hydrogen sulphide levels in the oral cavity. Materials and Methods: Oral malodour levels in 26 subjects (aged 16-69 years) were assessed by portable volatile sulfide monitor Halimeter and gas chromatography. They were accordingly divided into two groups: an odour group and a no/low odour group. Tongue coatings were sampled and organisms were categorized. Results: Tongue coatings were spread onto sheep blood agar (anaerobic) containing Casein enzymic hydrolase, Papain digest of soyabean meal, yeast extract, sodium chloride, L-cysteine, Hemin, and Agar at final pH (at 25°C) 7.4±0.2. The plates were incubated at 37°C for 48-72hrs aerobically and anaerobically in an anaerobic chamber with mixed gas supply. Bacteria forming black or grey colonies were selected as hydrogen sulphide-producing phenotypes. The numbers of total bacteria (P < 0.05) and hydrogen sulphide-producing bacteria (P < 0.05) in the odour group were significantly larger than those in the no/low odour group. Species of Veillonella, Prevotella, Bacteroids and Peptostreptococcus were the predominant hydrogen sulphide-producing bacteria in odour group and low in no/low odour group. Conclusion: These results suggest that an increase in the number of hydrogen sulphide-producing bacteria in the tongue biofilm is responsible for oral malodour, although the bacterial composition of tongue biofilm did not differ between the two groups.

Keywords: Oral malodour; Halimeter; Hydrogen sulphide; Oral malodor measurement; Volatile sulphur compounds; Tongue coating; Tongue biofilm; Microorganisms.

Introduction
Oral malodour is an unpleasant or offensive odour emanating from the breath. Even though majority of oral malodour is of oral origin, there are multiple other systemic causes that have to be addressed while we diagnose and treat this condition. Halitosis has a multifactorial aetiology, but its main cause is the decomposition of the organic material by microorganisms of the oral cavity. The principal components of oral malodour are volatile sulphur compounds, which are primarily hydrogen sulphide (H2S), methyl mercaplan (CH3SH) and Dimethyl sulphide, produced through the putrefaction of proteins containing methionine or cysteine by oral anaerobic gram-negative microorganisms.

Proteolytic activity in the mouth is an important factor in the development of oral malodour. Putrefaction of proteins, mucins and peptides by microorganisms that reside on the tongue and in dental plaque results in the formation of volatile sulphur compounds, which are thought to be responsible for offensive breath. The quantities of these molecules in the breath can be measured at chair side with a portable sulphide monitor. The organoleptic assessment is a subjective measurement. It is a very good qualitative method, however not very precise concerning to quantity. In vitro studies have demonstrated that gram-negative anaerobic bacteria are capable of producing volatile sulphur compounds from blood products. In particular Treponema denticola, Porphyromonas gingivalis, Prevotella intermedia, Bacteroides forsythus and Fusobacterium can produce significant amounts of hydrogen sulfide and methylmercaptan from serum proteins, cysteine and methionine. Recent studies have indicated that the dorsal surface of the tongue may be the primary source of microbial putrefaction in the mouth. Tongue coating is an important factor in the formation of oral malodour in both periodontally diseased and healthy people. However, it is not known which bacterial species in the tongue coating are...
Materials and Methods
Twenty six subjects complaining of halitosis, was selected for this study. Informed consent was obtained from each subject. Patients with any systemic cause of halitosis were excluded. All patients confirmed that they were not suffering from any disease and did not receive medical treatment (especially no antibiotics and/or corticosteroids) within three months before measurements. Patients with signs of pharyngitis or acute/chronic tonsillitis were also excluded. All patients received a letter with instructions before the examinations.

Two days before their appointment, they had to avoid the intake of garlic, onions and spicy food. Twelve hours before the measurements, they also had to refrain from alcohol or coffee, and from smoking. On the morning of the appointment, it was forbidden to use chewing gums, mints, drops, scents and mouth rinses. On the other hand, they could perform normal oral hygiene (tooth brushing) and have breakfast. All measurements were recorded between 8:30 and 11:30 hours (before lunch) and at least two hours after eating or drinking and tooth brushing. On the first visit, an assessment of oral malodour and observable tongue coating, a clinical oral examination and sampling of tongue biofilm, was performed as described below.

Odor Assessment: We made quantitative measurements of volatile sulfur compounds using a portable sulfide monitor set at 1 part per million full scale (Interscan Model RH17-B Halimeter, Interscan Corporation). We set the monitor to zero on ambient air before each measurement. The test consists on asking the patient to breathe deeply inspiring the air by nostrils and expiring by mouth, while the examiner sniffs the odour at a distance of 20 cm, considering it unpleasant or not in a scale of 0 to 5. A teflon tube connected to a flexible drinking straw was attached to the air inlet of the monitor. For each measurement, the straw was inserted three centimetres into the mouth until the lips touched a stent attached to the straw. The participant lightly closed his or her lips over the straw and the monitor collected a sample of the ambient air from the mouth. We took three separate readings of peak volatile sulphur compound levels, instructing the patient to close his/her mouth for 30-60 seconds before inserting the straw. We calculated a mean value for all the readings and used this value in the statistical analysis. Based on the halimeter reading we categorised them in to two groups, the odour group (n=14) and the non-odour group (n=12). Subjects with low halimeter reading less than 100 ppb were considered as non-odour group/control group. Subjects with halimeter reading more than 300 ppb were considered as odour group/study group.

The oral cavity was examined, paying attention to caries, the level of oral hygiene (plaque accumulation, gingival inflammation), periodontal pockets (using a manual periodontal probe), removable appliances, dry mouth and tongue coating. Thickness and extent of tongue coating was estimated by the naked eye according to the method of Nara. Both thickness and extent of tongue coating were scored as 0, 1, 2 or 3, and then the thickness score and the extent score were multiplied.

Sampling of Tongue Biofilm: In order to collect tongue biofilm, an area of 1 cm², predetermined by a window made of sterilized plain paper on the rear dorsal surface of the tongue, was firmly scraped with sterilized toothpicks. All samples were immediately introduced into an anaerobic chamber suspended in 1 ml of distilled 40 mm potassium phosphate buffer (PPB, pH 7.0) solution.

Culture Conditions: Specimen collected in RCM broth was incubated at 37°C for 48 hours. Subcultures were done on sheep blood agar (anaerobic) containing Casein enzymic hydrolyase (15gms/litre), Papaic digest of soyabean meal (5gms/litre), yeast extract (5gms/litre), sodium chloride (5gms/litre), L-cysteine (0.5gms/litre), Hemin (0.005gms/litre), Agar (13.5gms/litre), final pH (at 25°C) 7.4±0.2. The plates were incubated at 37°C for 48-72hrs aerobically and anaerobically in anaerobic chamber with mixed gas supply (hydrogen and nitrogen-Coy laboratories) (Figure 6 and 7). The plates for anaerobic incubation also had a
disc of metronidazole applied in the centre of primary streak. Various biochemical reactions and microbiological tests that are used for identification of microorganism are given in table 1.

**Statistical Analysis:** The data was coded and analysed using SPSS version 11.5. The level of statistical significance was kept at p value of 0.05. An unpaired t-test was used for analysis significance.

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Table 1: Biochemical reactions and Microbiological tests that are used for identification of microorganisms

**Results**

Our study subjects were within the age range of 16 to 69 years with mean being 31 years. The 14 individuals were included in odour group of which 10 (71.4%) were males and 4 (28.6%) were females. Twelve individuals were included in non-odour group of which three (25%) were males and nine (75%) were females. (Figure 1)

Bacteria forming black or grey colonies were selected as H2S-producing phenotypes. The numbers of total bacteria (P < 0.05) and H2S-producing bacteria (P < 0.05) in the odour group were significantly larger than those in the no/low odour group. Species of Veillonella, Prevotella, Bacteriods, and Peptostreptococcus were the predominant H2S-producing bacteria in odour group and microorganisms like streptococcus mutans and E.coli were low in odour group. (Figure 2) In addition, the number of black or grey colonies in the odour group was significantly higher than that in the no/low odour group (P < 0.05) The predominant microorganisms that were cultured in the non-odour group were streptococcus mitis, streptococcus mutans, and staphylococcus aureus and these microorganisms were significantly higher in odour group when compared with the odour group.(p<0.05) (Figure 3)

Tongue biofilm samples were obtained from the same part of the tongue using a standardized method, and there was significant differences were noted in observable tongue coating.(p < 0.05) (Figure 4) In the odour group (n=14) tongue coating was present in 13 (93%) tongue coating was present in 2 (13%) patients and in the non-odour group tongue coating was present in 2 (13%) of the patients. This indicates that the amounts of observable tongue coating were higher among the subjects in the odour group when compared with non-odour group. On correlation with the tongue coating and the microorganisms, the predominant microorganisms that were present in the individuals with high tongue coating are Peptostreptococcus, Prevotella, Veillonella, Bacteriods.(Figure 5)

**Discussion**

The present study was conducted with the aim to identify hydrogen sulphide (H2S)-producing bacteria among tongue biofilm microflora and to investigate the relationship between bacterial flora and H2S levels in the oral cavity. Oral malodour levels in 26 subjects were assessed by halimeter and organoleptic scores. Based on these assessments, subjects were divided into two groups: an odour group and a no/low odour group. Age and gender matching was done between the study group and the control group. In terms of clinical parameters, there were no significant differences in number of present teeth, number of teeth with untreated caries, number of teeth with probing depth more than 4 mm or oral hygiene status between the two groups. (Figure 1) There was a significant difference in tongue coating score between the two groups and individuals with coated tongues had higher malodour scores than those with non-coated tongues. In the present study fourteen individuals were in the odour group and tongue coating was present in 13 (93%) patients and in the non-odour group tongue coating was present in 2 (13%) of the patients. Similar results were observed in a study done by Mager et al., (2003)13 states that Veillonella species was one of the prominent bacteria in the tongue biofilm.

**Identification of H2S-producing Bacteria in Tongue Biofilm:**

The H2S-producing bacteria isolated in this study were identified using molecular biological methods. Peptostreptococcus micros, Veillonella parvula and Prevotella intermedia species were the predominant H2S-producing bacteria, followed by Streptococcus sangius species, in the odour and no/low odour groups (Figure 2, 3). Veillonella is par accounted for over 15% of total (H2S)-producing bacteria in each sample. However, there were no significant
differences in the profiles of H$_2$S-producing bacteria between the two groups. Tyrell et al., (2003)$^{12}$ in his study also found that the most common microorganisms isolated were Peptostreptococcus, Eubacterium group, Actinomyces species, Eikenella corrodens, Veillonella species, Pigmented Prevotella species. Eh De Boever et al., (1995)$^8$ in their study stated that the two known volatile-sulfur compound producing organisms were Fusobacterium species and Prevotella intermedia.

Figure 1: Gender variation among the study and control group.

Figure 2: The different microorganisms involved in the study group.

Figure 3: The different microorganisms involved in the control group.

Figure 4: The correlation between the tongue coating and malodour scores.

Figure 5: The correlation between the tongue coating and microbial load.

Figure 6: The culture media used in the study.

Figure 7: The anaerobic chamber used for culture.
Hartley et al., (1996)\textsuperscript{15} also frequently identified these bacterial species in both odour and no/low odour groups and Donaldson et al., (2005)\textsuperscript{18} reported that Veillonella, Prevotella and Fusobacterium species were found in both odour and no/low odour groups, and that Vibrio species and unidentifiable Gram-negative and Gram-positive anaerobes were more commonly found in the odour group. Loesche and Kazor (2002)\textsuperscript{17} reported that 74\% of total cultivable bacteria of the tongue biofilm could be Veillonella parvula, Actinomyces odontolyticus, Streptococcus intermedius and Clostridium. However, in all these studies, the H\textsubscript{2}S-productivity of the bacteria was not assessed. Thus, our study is the first report to show that Veillonella, Bacterioids and Prevotella are predominant as H\textsubscript{2}S-producing bacteria in tongue biofilm and are responsible for oral malodour when they increase in number.

**Tongue Coating and Oral Malodour**

In the present study, Out of 26 individuals who are complaining of halitosis, 48\% (12 individuals) were because of tongue coating. Quirynen et al., (1998)\textsuperscript{14} in their study reported that individuals with coated tongue showed significantly higher malodour scores than individuals with non-coated tongue. Microbial putrefaction on the tongue is an important factor responsible for oral malodour. Few studies reported correlations between the degree of oral malodour and the amount of observable tongue coating. They also suggested that periodontal disease can induce observable tongue coating accumulation. The tongue biofilm comprises not only micro-organisms but also epithelial cells released from the oral mucosa and leukocytes from periodontal pockets. This indicates that the amounts of observable tongue coating bear little relationship to the microbial population density on the tongue coating and it is only the latter (microbial density) that relates to hydrogen sulfide levels or oral malodour.

De Boever and Loesche (1998)\textsuperscript{6} in their study observed tongue with deep fissures to have higher number of bacterial colony as compared to patients with smooth tongue surfaces. Delanghe et al., (1998)\textsuperscript{18} found that in 87\% of the patients with oral malodour, the cause was of oral nature. Of these oral causes, 51\% were because of tongue coating, 17\% a result of gingivitis, 15\% a result of periodontitis and 17\% a result of combinations. In a study by Oho et al., (2001)\textsuperscript{19} it was stated that the amount of tongue coating in patients complaining of halitosis was significantly greater in the halitosis-positive group compared to the halitosis-negative group. Morita and Wang (2001)\textsuperscript{20} investigated the relationship between sulcular sulphide level and oral malodour in subjects with periodontal disease. The volume of tongue coating and the percentile of sites with bleeding upon probing were significantly associated with oral malodour.

**Conclusion**

Microbial putrefaction on the tongue is an important factor in the development of bad breath. Volatile sulfur compounds, tongue coating and deep fissures in the tongue are all associated with oral malodour and, therefore, appear to be important factors in the halitosis process. The predominant H\textsubscript{2}S-producing bacteria are mainly commensal species of the oral cavity, such as Prevotella intermedia, Veillonella and Actinomyces species. The numbers of both H\textsubscript{2}S-producing bacteria and total bacteria in the tongue biofilm were higher in the odour group, suggesting that for subjects with low to intermediate levels of malodour an increase in bacterial density in the tongue biofilm is associated with oral malodour.

**References**


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