Comparison of the Use of the Halimeter and the Oral Chroma™ in the Assessment of the Ability of Common Cultivable Oral Anaerobic Bacteria to Produce Malodorous Volatile Sulfur Compounds from Cysteine and Methionine

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Abstract

Objective: To compare the use of the Halimeter and the Oral Chroma™ to assess the ability of common oral anaerobic bacteria isolated from the Kuwaiti population to produce volatile sulfur compounds (VSCs).

Materials and Methods: Broth cultures of common anaerobes isolated from supragingival plaque were centrifuged and pellets resuspended in phosphate buffer (pH 7.7) with an optical density OD550 of 0.3. 100 μl of this suspension and 870 μl of buffer were added in 2 sterile 15-ml head space vials. Reaction was initiated by addition of 30 μl of 33 mM L-methionine and L-cysteine, respectively, in each vial and incubation at 37°C for 90 min. 500 μl of 3 M phosphoric acid was added to tubes and was kept aside for 10 min. Production of VSCs was measured using the Halimeter and the Oral Chroma.

Results: The major VSC producers identified by both Halimeter and Oral Chroma with L-cysteine as substrate were Campylobacter ureolyticus, Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia, Aggregatibacter actinomycetemcomitans and Gemella morbillorum. The concentrations of hydrogen sulfide recorded by both Halimeter and Oral Chroma were essentially identical. With L-methionine as substrate, both Halimeter and Oral Chroma identified different complements of anaerobes with C. ureolyticus, P. gingivalis, Fusobacterium nucleatum and P. intermedia as major VSC producers.

Conclusion: The results suggest that the Oral Chroma may produce a more comprehensive assessment of VSC production by oral microflora than the Halimeter.

Key Words
Oral malodor • Anaerobes • Halimeter • Oral Chroma

Introduction

Halitosis, or oral malodor, is a common complaint of up to one third of the general population and a large concern for the many individuals whom it affects [1]. Oral production of malodorous substances is most commonly associated with the by-products of bacterial metabolic degradation and occurs on oral surfaces, in periodontal pockets and especially on the dorsal tongue surface. These products result from microbial fermentation of proteins, peptides and mucins found in saliva, blood, gin-
gival crevicular fluid, lysed neutrophils, desquamated epithelial cells and any residual food retained on the oral surfaces [2]. The most conspicuous malodorous compounds are termed volatile sulfur compounds (VSCs), with hydrogen sulfide, methyl mercaptan and dimethyl sulfide [1, 3–5] accounting for roughly 90% of the VSCs [3]. Other gases such as indole, skatole, putrescine, cadaverine and the foul-smelling fatty acids (propionic acid, butyric acid) have also been postulated to contribute to halitosis [1, 6], although this was disputed by others [7–9]. It was recently shown that the breath concentrations of these latter compounds in patients with halitosis were too low to be detected organoleptically (below the threshold level) [10]. The VSCs appear to be the major contributors to halitosis whereas the roles of other compounds like amines and organic acids seem insignificant.

Gram-negative anaerobic bacteria are the main culprits, capable of producing VSCs from blood and serum products and debris. In particular, Treponema denticola, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia and Fusobacterium spp. can produce significant amounts of hydrogen sulfide and methyl mercaptan from serum proteins, cysteine and methionine [11].

Many cultivable anaerobic bacteria associated with the subgingival plaque produce both VSCs and foul-smelling fatty acids [12]. Three species often associated with periodontal disease, T. denticola, P. and T. forsythia [12, 13], produce both VSCs and volatile fatty acids such as butyrate and propionate, and their detection in plaque and/or tongue samples might provide additional information concerning factors contributing to malodor [12]. These organisms can be detected in the plaque or in tongue coating samples by the presence of enzyme(s) that degrades benzoyl-DL-arginine-α-naphthylamide, a synthetic trypsin substrate, forming a colored compound [12, 14]. This test can be carried out by the use of a commercially available kit, the so-called benzoyl-DL-arginine-α-naphthylamide test [14].

Malodor measurement is an important aspect of determining the magnitude of the problem in individual patients as this impacts on the type of management that each may receive. However, this task might be quite cumbersome, as it is influenced by many parameters which include the sampling procedure, the composition of the different malodorous gaseous substances to be determined and the types of equipment available for such measurements.

Organoleptic measurement is recognized as one of the most reliable and practical diagnostic measures of halitosis [5]. This method requires the training of odor judges who are calibrated against a standard range of malodorous substances. The gold standard of measurement of oral malodor is the gas chromatography (GC) [4, 5, 7]. By using a sulfur detector, GC can specifically detect VSCs such as hydrogen sulfide, methyl mercaptan and dimethyl sulfide, the major components of oral malodor. However, a gas chromatograph is an expensive apparatus, and GC is a laboratory-based method which does not lend itself to field or clinical use. In addition, it requires trained personnel.

Most breath clinics make use of the Halimeter, a portable sulfide monitor, to measure VSCs and to detect oral malodor [15]. However, the Halimeter cannot differentiate between the 3 VSCs, and it is more sensitive to hydrogen sulfide than to methyl mercaptan and is almost insensitive to dimethyl sulfide [16]. A portable gas chromatograph named Oral Chroma™ (Abilit Corp., Osaka, Japan) has been introduced in the market to detect VSCs [17]. This is a very inexpensive instrument and can be used everywhere. Unlike standard GC, it does not need a special carrier gas like nitrogen or helium. It uses room air as the carrier gas for the chromatographic column [4].

The aims of the present study were to assess the ability of common oral anaerobic bacteria isolated from the Kuwaiti population to produce VSCs and to compare the VSC-measuring devices Oral Chroma and Halimeter for measuring them.

**Materials and Methods**

**Specimen Collection**

Supragingival plaque samples were collected from 25 children aged 5–12 years attending for dental treatment at Bneid Al Gar Specialist Dental Center and the School Oral Health Program in Salmiya. The plaque sample was immediately dispersed into a tube containing 0.3 ml of prereduced transport fluid and was taken to the laboratory for analysis and transported to the laboratory for processing within 24 h.

**Sample Processing and Bacterial Isolation**

Serially diluted samples were inoculated onto brain heart infusion agar (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) sheep blood, 75 µg/ml of gentamicin, 1 µg/ml hemin and 5 µg/ml L-cysteine HCl and incubated at 37°C in an anaerobic environment (10% H2, 10% CO2 and 80% N2) for 3–4 days. After incubation, the total anaerobic count was taken. Representative colonies of isolates were identified.

**Bacterial Identification**

Each of the representative colonies were identified by Gram staining and colony morphology. They were also initially tested for their susceptibility to metronidazole using 5-µg disks. A bat-
tery of biochemical tests was carried out using commercially available API 20A test kits (Bio-Mérieux, Marcy-l’Etoile, France) to obtain species level identification of each of the isolates.

Analysis of VSC Production by the Oral Isolates

The anaerobes, isolated from the oral cavity, were inoculated into tryptic soy broth (Sigma-Aldrich, St. Louis, Mo., USA) and incubated at 37 °C in an anaerobic environment (10% H2, 10% CO2 and 80% N2) for 3–4 days so as to obtain good growth. After incubation, the broth cultures were centrifuged at 1,000 rpm for 10 min. The pellets were washed with the phosphate buffer (pH 7.7) and were resuspended in the buffer to achieve an OD550 of 0.3.

100 µl of the above cell suspension and 870 µl of phosphate buffer solution were added in 2 sterile 15-ml head space vials. The reaction was initiated by the addition of 30 µl of 33 mM L-methionine and L-cysteine, respectively, in each vial. The reaction mixtures were incubated at 37°C for 90 min. After incubation, the reaction was stopped by the addition of 500 µl of 3 M phosphoric acid and was kept aside for 10 min [18].

The VSC production was measured using the Halimeter and the Oral Chroma according to the manufacturers’ instructions. The sampling tube of the Halimeter was introduced into the tube containing the culture suspension by means of a needle, which was pushed through the rubber septum of the vials, and the readings were recorded [19]. For the Oral Chroma, 1.0 ml of the air sample was drawn from the headspace vial, 0.5 ml was expelled out, the remaining 0.5 ml was injected into the Oral Chroma and the readings were recorded [20].

### Results

The production of VSCs by various oral anaerobes, using L-cysteine and L-methionine as substrates, is given in tables 1 and 2. The major VSC producers identified by both Halimeter and Oral Chroma, namely Campylobacter ureolyticus, P. gingivalis, T. forsythia, P. intermedia, Aggregatibacter actinomycetemcomitans and Gemella morbillorum, are shown in table 1. The concentrations of H2S recorded by both Halimeter and Oral Chroma were essentially identical.

When L-methionine was used as substrate instead of L-cysteine, the bacteria that produced the greatest amount of VSCs were C. ureolyticus, P. gingivalis, Fusobacterium nucleatum and P. intermedia (table 2). The concentrations of methyl mercaptan recorded by the Halimeter were lower compared to values obtained with the Oral Chroma.

### Discussion

Halitosis is a major concern to the general public and the source of a multimillion-dollar industry worldwide [7]. The information regarding the prevalence of breath malodor is scarce due to the difficulty in determining the

<table>
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<tr>
<th>Table 1. Production of VSCs by anaerobic oral bacteria using L-cysteine as substrate and measured by the Halimeter and the Oral Chroma</th>
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<tbody>
<tr>
<td><strong>Halimeter, ppb</strong></td>
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<tr>
<td>Campylobacter ureolyticus</td>
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<td>Porphyromonas gingivalis</td>
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<td>Tannerella forsythia</td>
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<td>Prevotella intermedia</td>
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<td>Aggregatibacter actinomycetemcomitans</td>
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<td>Gemella morbillorum</td>
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<td>Fusobacterium nucleatum</td>
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<td>Peptostreptococcus asaccharolyticus</td>
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<td>Porphyromonas asaccharolytica</td>
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<td>Veillonella parvula</td>
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<td>Bacteroides eggerthii</td>
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<td>Fusobacterium necrophorum</td>
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<td>Propionibacterium acnes</td>
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<td>Prevotella oralis</td>
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The Halimeter is more sensitive to H2S than CH3SH and almost insensitive to (CH3)2S; hence total VSCs were grouped for the Halimeter. H2S = Hydrogen sulfide; CH3SH = methyl mercaptan; (CH3)2S = dimethyl sulfide.
exact number or percentage of the population suffering from oral malodor [21]. Oral halitosis is commonly the result of microbial action mainly from the tongue coating and plaque [1, 22]. The microbial proteolysis of proteins to peptides, amino acids and substrates with free thiol groups gives rise to VSCs such as hydrogen sulfide (H₂S) and methyl mercaptan (CH₃SH) [11, 20].

The VSCs are produced from the sulfur-containing amino acids like cysteine and methionine. These two amino acids were used in our study to test the ability of different anaerobic bacterial species to produce VSCs as previously described [15, 16]. Earlier studies have successfully used either the Halimeter (portable sulfide monitor) or GC to measure the VSCs produced by the bacteria [15–17]. In this study, a portable GC equipment, the Oral Chroma [4], was tested against the Halimeter. The 3 most common VSCs implicated in oral malodor are hydrogen sulfide (H₂S) and methyl mercaptan (CH₃SH) [11, 20].

Kleinberg and Codipilly [23] showed that a cysteine challenge is a good model for initiating odor production in vivo and in vitro. While this may be true for H₂S production, our results clearly showed that for evaluation of mercaptan production, methionine is a better substrate and therefore provides a more comprehensive assay of VSCs in the system.

Many studies have evaluated the production of H₂S and methyl mercaptan by common oral bacterial species and reported that P. gingivalis, P. intermedia, F. nucleatum, T. denticola and Veillonella alcalescens produce H₂S, while P. gingivalis, P. intermedia and F. nucleatum produce methyl mercaptan [24]. In addition, Persson et al. [25] observed that the bacteria producing the largest amount of H₂S from L-cysteine belonged to the genera Peptostreptococcus, Eubacterium, Selenomonas, Centipeda, Bacteroides and Fusobacterium. With L-methionine, P. gingivalis and F. nucleatum produced large amounts of methyl mercaptan. In addition, they reported no methyl mercaptan production by A. actinomycetemcomitans and Streptococcus species with L-methionine as substrate. These findings were also supported by the present study.
Conclusion

The Oral Chroma may produce a more comprehensive assessment of VSC production by oral microflora than the Halimeter. The use of the Halimeter might give erroneous results, especially for methyl mercaptan or dimethyl sulfide, because of a decreased sensitivity of this instrument for these two VSCs.

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References