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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Key Words

Oral malodor • Anaerobes • Halimeter • Oral Chroma

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-cystine 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. The concentrations of methyl mercaptan recorded by the Halimeter were lower compared to those assessed by the Oral Chroma. **Conclusion:** The results suggest that the Oral Chroma may produce a more comprehensive assessment of VSC production by oral microflora than the Halimeter.

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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 halito-

sis [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 μ g/ml of gentamicin, 1 μ g/ml hemin and 5 μ g/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- μ g disks. A bat-

Table 1. Production of VSCs by anaerobic oral bacteria using L-cysteine as substrate and measured by the Halimeter and the Oral Chroma

	Halimeter, ppb	Oral Chroma, ppb			
		H ₂ S	CH ₃ SH	(CH ₃) ₂ S	total, ppb
<i>Campylobacter ureolyticus</i>	1,357	1,402	0	0	1,402
<i>Porphyromonas gingivalis</i>	1,259	1,321	53	21	1,395
<i>Tannerella forsythia</i>	1,028	1,240	0	0	1,240
<i>Prevotella intermedia</i>	637	823	0	0	823
<i>Aggregatibacter actinomycetemcomitans</i>	567	751	24	0	775
<i>Gemella morbillorum</i>	175	182	5	0	187
<i>Fusobacterium nucleatum</i>	114	152	0	0	152
<i>Peptostreptococcus asaccharolyticus</i>	102	139	5	2	146
<i>Porphyromonas asaccharolytica</i>	85	118	20	2	140
<i>Veillonella parvula</i>	19	47	5	0	52
<i>Bacteroides eggerthii</i>	15	6	12	13	31
<i>Fusobacterium necrophorum</i>	10	5	4	4	13
<i>Propionibacterium acnes</i>	9	9	0	2	11
<i>Prevotella oralis</i>	5	4	2	2	8

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.

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 2. Production of VSCs by anaerobic oral bacteria using L-methionine as substrate and measured by the Halimeter and the Oral Chroma

Organisms	Halimeter, ppb	Oral Chroma, ppb			
		H ₂ S	CH ₃ SH	(CH ₃) ₂ S	total
<i>Campylobacter ureolyticus</i>	293	11	1,419	0	1,430
<i>Porphyromonas gingivalis</i>	257	150	1,020	9	1,179
<i>Fusobacterium nucleatum</i>	137	13	1,155	0	1,168
<i>Prevotella intermedia</i>	42	109	1,006	2	1,117
<i>Bacteroides eggerthii</i>	36	11	284	0	295
<i>Tannerella forsythia</i>	13	50	130	25	205
<i>Propionibacterium acnes</i>	13	2	180	0	182
<i>Gemella morbillorum</i>	11	13	160	2	175
<i>Veillonella parvula</i>	8	2	147	0	149
<i>Porphyromonas asaccharolytica</i>	6	13	14	2	29
<i>Peptostreptococcus asaccharolyticus</i>	4	11	12	0	23
<i>Fusobacterium necrophorum</i>	3	2	7	2	11
<i>Prevotella oralis</i>	2	1	0	0	1
<i>Aggregatibacter actinomycetemcomitans</i>	0	0	0	0	0

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, methyl mercaptan and dimethyl sulfide. The Halimeter has been shown to be more sensitive to H₂S than to methyl mercaptan and almost insensitive to dimethyl sulfide, whereas the Oral Chroma measures all 3 gases [4] with equally high sensitivities. These results were confirmed in the present study. With L-methionine as substrate, the results showed much higher values for methyl mercaptan when using the Oral Chroma. With cysteine, both instruments recorded high values for H₂S.

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

- 1 Kazor CE, Mitchell PM, Lee AM, Stokes LN, Loesche WJ, Dewhirst FE, Paster BJ: Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. *J Clin Microbiol* 2003;41:558–563.
- 2 Doran A, Kneist S, Verran J: Ecological control: in vitro inhibition of anaerobic bacteria by oral streptococci. *Microb Ecol Health Dis* 2004;16:23–27.
- 3 Takahashi N: Microbial ecosystem in the oral cavity: metabolic diversity in an ecological niche and its relationship with oral diseases. *Int Congr Ser* 2005;1284:103–112.
- 4 Tangerman A, Winkel EG: The portable gas chromatograph Oral Chroma™: a method of choice to detect oral and extra-oral halitosis. *J. Breath Res* 2008;2:1–6.
- 5 Yaegaki K, Coil JM: Genuine halitosis, pseudo-halitosis and halitophobia: classification, diagnosis and treatment. *Compend Contin Educ Dent* 2000;21:880–889.
- 6 Goldberg S, Kozlovsky A, Gordon D, Gelernter I, Sintov A, Rosenberg M: Cadaverine as a putative component of oral malodor. *J Dent Res* 1994;73:1168–1172.
- 7 Tangerman A: Halitosis in medicine: a review. *Int Dent J* 2002;52:201–206.
- 8 Tonzetich J: Production and origin of oral malodor: a review of mechanisms and methods of analysis. *J Periodontol* 1977;48:13–20.
- 9 Tangerman A, Winkel EG: Intra- and extra-oral halitosis: findings of a new form of extra-oral blood-borne halitosis caused by dimethyl sulfide. *J Clin Periodontol* 2007;34:748–755.
- 10 Van den Velde S, van Steenberghe D, van Hee P, Quirynen M: Detection of odorous compounds in breath. *J Dent Res* 2009;88:285–289.
- 11 Ayers MSK, Colquhoun NKA: Halitosis: causes, diagnosis and treatment. *NZ Dent J* 1998;94:156–160.
- 12 Loesche WJ, Kazor C: Microbiology and treatment of halitosis. *Periodontol* 2000 2002;28:256–279.
- 13 Al-Ansari JM, Boodi H, Al-Sumait N, Al-Khabbaz AK, Al-Shammari K, Salako NO: Factors associated with self-reported halitosis in Kuwaiti patients. *J Dent* 2005;34:444–449.
- 14 Kozlovsky A, Gordon D, Gelernter I, Loesche WJ, Rosenberg M: Correlation between the BANA test and oral malodor parameters. *J Dent Res* 1994;73:1036–1042.
- 15 Rosenberg M, Kulkarni GV, Bosy A, McCulloch CA: Reproducibility and sensitivity of oral malodor measurements with a portable sulfide monitor. *J Dent Res* 1991;11:1436–1440.
- 16 Furne J, Majerus G, Lenton P, Springfield J, Levitt DG, Levitt MD: Comparison of volatile sulfur compound concentrations measured with a sulfide detector vs gas chromatography. *J Dent Res* 2002;81:140–143.
- 17 Hanada M, Koda H, Onaga K, Tanaka K, Okabayashi T, Itoh T, Miyazaki H: Portable oral malodor analyzer using highly sensitive In_2O_3 gas sensor combined with a simple gas chromatography. *Anal Chim Acta* 2003;475:27–35.
- 18 Yoshimura M, Nakano Y, Yamashita Y, Oho T, Saito T, Koga T: Formation of methyl mercaptan from L-methionine by *Porphyromonas gingivalis*. *Infect Immun* 2000;68:6912–6916.
- 19 Quirynen M, Eldere JV, Bollen ML, Steenberghe DV: In vitro sulfur compound production of oral bacteria in different culture media. *Quintessence Int* 1999;30:351–356.
- 20 Oral Chroma instruction manual: measurement procedure and saving measurement data. Osaka, Abilit Corp, 2007, pp 16–21.
- 21 Sanz M, Roldan S, Herrera D: Fundamentals of breath malodor. *J Contemp Dent Pract* 2001;2:1–17.
- 22 De Boever EH, Loesche WJ: Assessing the contribution of anaerobic microflora of the tongue to oral malodor. *J Am Dent Assoc* 1995;126:1384–1393.
- 23 Kleinberg I, Codipilly DM: Cysteine challenge testing: a powerful tool for examining oral malodor processes and treatments in vivo. *Int Dent J* 2002;52:221–228.
- 24 Nakano Y, Yoshimura M, Koga T: Methyl mercaptan production by periodontal bacteria. *Int Dent J* 2002;52:217–220.
- 25 Persson S, Edlund MB, Claesson R, Carlsson J: The formation of hydrogen sulphide and methyl mercaptan by oral bacteria. *Oral Microbial Immunol* 1990;5:195–201.