



Oral malodor in children and volatile sulfur compound-producing bacteria in saliva: preliminary microbiological investigation

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Abstract

Purpose: This study examined and compared levels of salivary bacteria which produced volatile sulfur compounds (VSC) in young children with and without oral malodor.

Methods: Clinic populations of children aged two to seven years, whose parents presented with an unsolicited major complaint of oral malodor in their child (OM+), or aged-matched controls in whom oral malodor was not detected by parents (OM-), were investigated. Saliva specimens were cultured anaerobically on media that differentiated VSC+ bacteria. These were quantified and identified. Levels in OM+ and OM- children were compared and statistically analyzed.

Results: OM+ children harbored significantly higher levels of VSC+ isolates in saliva than OM- children (OM+=44% of total viable counts, TVC; OM-=24% of TVC; $P=0.0083$). Types of recovered bacterial species did not differ in the two groups, but levels of *Prevotella oralis* were significantly higher in OM+ children ($P=0.0001$). *Veillonella* species followed by *P. oralis* were the predominant VSC+ isolates recovered in both populations.

Conclusions: VSC+ salivary bacteria may differ both in type and quantity in young children with and without parent-perceived oral malodor. (*Pediatr Dent* 21:320-324, 1999)

Oral malodor or halitosis is a common condition affecting people of all ages. The term oral malodor is a generic descriptor for foul smells emanating from the mouth which encompasses ozostomia, stomatodysodia, halitosis (both pathological and physiological), and fetor oris or fetor ex ore. The latter terms denote local or systemic production of malodor.¹

Experimental evidence strongly suggests that putrefaction of sulfur-containing proteinaceous substrates by predominantly gram-negative oral microorganisms, such as *Fusobacterium* species and *Bacteroides* species, is a primary cause of oral malodor.^{2,3} These volatile sulfur compounds (VSC) are hydrogen sulfide, methyl mercaptan, and dimethyl sulfide.¹ Hydrogen sulfide and other volatile sulfur compounds are considered to be very toxic,⁴ increase the permeability of oral mucosa,⁵ and facilitate the access of toxic metabolites into the underlying connective tissue, thereby contributing to collagen degradation.⁶

Two aspects of halitosis research that have not been adequately addressed are 1) prevalence in specific populations and

2) identification of oral bacteria responsible for halitosis. The objectives of this investigation were to determine and quantify specific organisms associated with halitosis in a pediatric population.

Methods and Materials

Patient Selection

Ten subjects between the ages of two and seven years, in the primary dentition or early mixed dentition stage, with the major unsolicited complaint of "mouth odor", not associated with dental caries, as subjectively assessed by parents or family dentists, were selected from the clinic population of the Department of Pediatric Dentistry at the University of Maryland Dental School in Baltimore, Maryland. Thorough medical histories were obtained in an attempt to account for predisposing factors leading to oral malodor, but non-microbiological measures to verify the condition clinically were not taken. A control population of ten subjects meeting the same age and dentition criteria, who did not exhibit oral malodor as reported by parents when specifically asked, were also selected. All subjects had not received antibiotics or non-steroidal anti-inflammatory drugs at least one month prior to the experiment. Subjects also refrained from use of mouth rinses and tooth brushing the night before and the morning of the sampling procedure.

Sampling Procedure

On the first visit, the child received a complete oral examination and his/her medical history was recorded. A baseline saliva sample was obtained by placing a sterile cotton swab in the patient's mouth for 60 seconds. The tip of the swab was then placed in 1.0 ml of reduced transport fluid (RTF)⁷ for culturing.

Microbiological Procedure

The microbial specimens were introduced into a vinyl anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI), dispersed by sonication for 10 seconds, serially diluted in RTF, and plated in duplicate on the following agar media:

- The control media was comprised of Columbia Blood Agar Base (Difco, Detroit, MI), hemin (0.05%), glutathione (1.0g/l), and menadione (0.1%). This was designated as a control medium for oral H₂S+ organisms (OHO-C).
- The test OHO medium, which differentiated VSC-positive bacteria, contained in addition to the same formulation as OHO-C, lead acetate (0.2 g/l). On this medium, VSC-positive isolates appeared as black colonies, or contained black inclusions within colonies.⁸
- Peptostreptococcus micros medium (PMM)⁹ which was originally formulated to differentiate *Peptostreptococcus micros*, an oral pathogen and odorigen, but also recovered other VSC+ species. It was comprised of Columbia CNA agar (Difco) supplemented with hemin, glutathione (1.2 g/l), and lead acetate (0.2 g/l). *P. micros* colonies appeared tan colored or white, convex and raised and showed black precipitation around the colonies in the agar. Other VSC+ colonies on this medium appeared dark or contained dark inclusions.

OHO and PMM were employed for recovery and quantification of VSC+ bacteria in saliva. OHO-C was used to control for spontaneous emergence of black colonies, and the inhibitory effect of lead acetate on total recovery. All media were incubated anaerobically at 37° C for seven days after which total colony forming units (total viable counts, TVC), and total VSC+ bacteria (black colonies, BLK) were enumerated on each agar plate. Predominate VSC+ colonies on OHO and PMM were subcultured on TSA (Trypticase Soy Agar; TSA) for purification and identification. The AN-IDENT system (bioMerieux Vitek, Inc., Hazlewood, MO) was used for identification of the isolates. The AN-IDENT system is a rapid, standardized micro-method for the identification of anaerobes.

Data Analysis

Microbial data consisted of TVC, BLK, and percent BLK relative to TVC (%BLK) values from each agar medium and identification data of predominate isolates expressed as %BLK. A data set consisted of each category of microbial counts for the OM+ and OM- populations on each agar medium. Raw data were entered on Microsoft Excel 5.0 (Microsoft Corp.

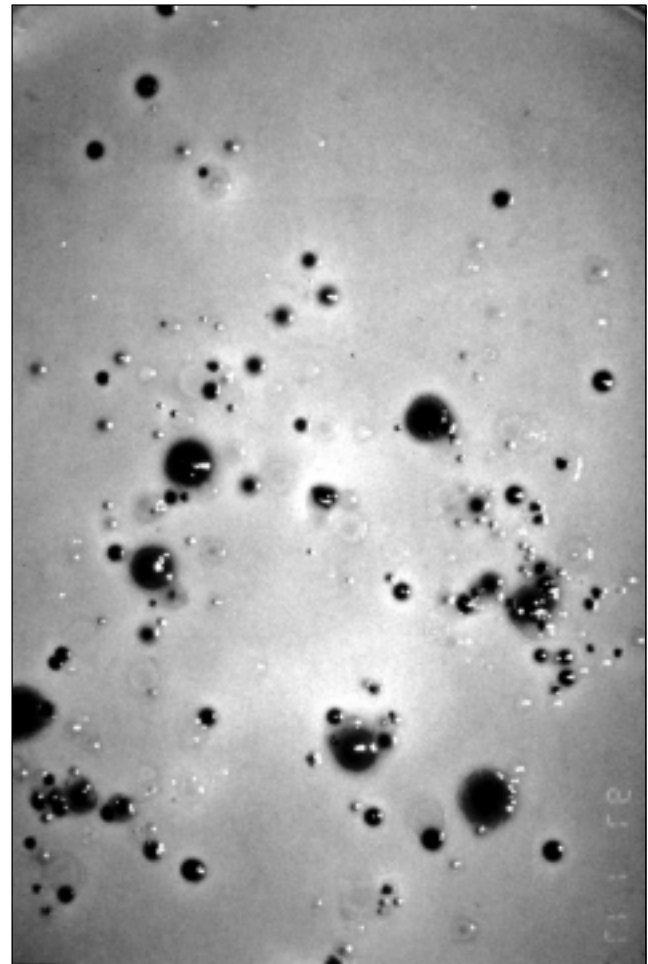


Fig 1. VSC (+) and (-) colonies on OHO.

Redmond, WA) in order to transform absolute counts to Log(10) values and compute %BLK data. Processed data were copied to Sigma Stat (Jandel Scientific, San Rafael, CA) for statistical analysis using the Mann-Whitney Rank Sum test. The identification data were compared in both populations as %BLK and statistically analyzed by the Signed Rank test.

Results

Patient History

Patients with the major complaint of mouth odor and a control population without oral malodor were selected. Baseline information, which included medical history, dental history, date of birth, number of teeth present, gingival health, presence of decay, or existing restorations was recorded for each subject. Patients were all between ages of two and seven years with the mean age of 4.1 years. Patients had different racial and ethnic backgrounds. All had fair to excellent oral hygiene practices with mild localized gingivitis to healthy gingival tissue. They were all in primary or early mixed dentition with only a few permanent molars and permanent incisors erupted. None of the OM+ patients exhibited carious lesions or existing restorations. Oral malodor negative (OM-) patients exhibited a moderate to high caries activity. All patients were in good health, and were not taking medications. If patients received antibiotics for medical reasons, the sampling proce-

Table 1. Salivary Cultures of 20 Children With or Without Oral Malodor (OM)

Measurements/ Groups	Microbial counts x 10 ⁶ ±SD*		
	OHO**	PMM**	OHO-C†
TVC†			
OM+	94.3±39.7	58.2±40.6	66.5±94.7
OM-	26.7±32.3	28.2±32.3	34.2±46.7
BLK§			
OM+	27.2±31.9	6.3±6.9	
OM-	5.7±6.8	0.8±1.4	
%BLK§§			
OM+	44.4±16.2	17.3±14.6	
OM-	24.0±14.6	2.5±2.7	

*Standard deviation. **Differential agar media for VSC+isolates.

†Control agar medium. ‡Total viable counts. §VSC+isolates.

§§BLK/TVC x 100.

Table 2. Mean Values of Predominant VSC Positive Isolates on Differential Agar Media From 20 Children With and Without Oral Malodor (OM)

Isolate/Medium	OM+	Frequency N=10	OM-	Frequency N=10
OHO*				
Fuso**	1.2±2.1†	3	0.4±0.8	2
Po	9.8±8.8	7	0	0
Pmg	5.1±5.4	7	1.7±4.3	3
VL-	8.4±9.7	6	5.8±8.4	6
VL+	6.8±9.5	6	6.0±13.0	6
TotVL	15.9±12.5	8	14.3±12.5	6
Gray	7.8±10.2	5	0.4±1.3	1
Sf	0	0	1.9±3.0	4
UnID	2.9±3.5	5	4.0±8.5	3
PMM*				
Fuso	0.6±1.7	2	0	0
Po	14.4±13.8	10	1.0±2.1	3
Sf	1.0±2.4	2	0.7±2.0	2
Pm	0	0	0.1±0.3	1
UnID	1.3±2.6	3	0.6±0.8	5

*Differential agar media for VSC+ isolates. **Fuso=*Fusobacterium nucleatum* Po=*Prevotella oralis*,mg=*Peptostreptococcus magnus*, VL=catalase negative *Veillonella* species, VL+=catalase positive *Veillonella* species, Gray=unidentified Gm- rod, Sf=*Streptococcus faecalis*, UnID=unidentified, Pm=*Peptostreptococcus micros*.
†Values expressed as mean % of total viable count±standard deviation.

procedure was delayed for a minimum of three weeks. No subject had a history of tonsillitis, sinusitis, current respiratory infections, nasal polyps, episodes of otitis media, placement of pressure equalization tubes for otitis media, or exclusive mouth breathing, as reported by the parents. All patients tolerated the sampling procedures well, and no problems were reported. Patients were rewarded and compensated for their cooperation.

Comparison Of Microbial Values

TVC values on all media were higher for OM+ children, but differences were not statistically significant (Table 1, Fig 2). BLK values were also higher for the OM+ population on OHO and PMM, but differences were significant only on PMM ($P=0.009$). VSC+ colonies are illustrated in Figure 1. The %BLK values were significantly higher in OM+ subjects on OHO ($P=0.0083$) and PMM ($P=0.0001$). VSC+ bacteria represented 44% of TVC on OHO and 17% on PMM versus 24% and 3% for OM- on OHO and PMM, respectively (Table 1, Figure 3).

Identification of the VSC+ bacteria (colonies representing greater than 5% of BLK) was conducted on OHO and PMM and expressed as percent of TVC (Table 2, Figures 4 & 5). Predominate isolates or categories of isolates in OM+ children on OHO in decreasing order were total *Veillonella* species (tot VL), *Prevotella oralis* (Po) [Note: The AN-IDENT system could not distinguish *P. oralis* from *P. melaninogenia*. Since no dark colonies appeared on OHO-C, which did distinguish pigmented *Prevotella* species, the isolate was presumptively identified as *P. oralis*], catalase negative *Veillonella* species (VL-), an unidentified gram negative isolate with a characteristic gray colony

(gray), catalase positive *Veillonella* species (VL+), *Peptostreptococcus magnus* (Pmg), other unidentified isolates (unID) and *Fusobacterium nucleatum* (Fn). Predominate isolates in OM- on OHO in decreasing order were tot VL, VL+, VL-, unID, *Streptococcus faecalis* (Sf), Pmg and Fn. Sf was not recovered in OM+ and Po was not recovered in OM-.

Predominate isolates on PMM in OM+ subjects in decreasing order were Po, unID, Sf, and Fn. In OM- in decreasing order were Po, Sf, unID, and *Peptostreptococcus micros* (Pm). On PMM, Pm was not recovered in OM+ and Fn was not recovered in OM-. On OHO and PMM, Po levels were significantly higher in OM+ on differential media (OHO, $P=0.0087$; PMM, $P=0.0001$).

Discussion

Oral malodor can be evaluated or measured by a variety of techniques.^{10,3} These include subjective assessments by human judges (organoleptic evaluations), industrial sulfide monitoring instruments which analyze exhaled air or microbial specimens removed from the mouth and gas chromatography of exhaled air, or bacterial specimens removed from the mouth.¹¹ A technique developed by Loesche^{12,13} is based on the detection of certain anaerobes which can hydrolyze a synthetic trypsin substrate, N-benzoyl-DL-arginine-Z-naphthyl-amine (BANA). BANA-positive tests correlated with VSC levels detected with portable sulfide monitors.^{13,14} In the present investigation, measurement of odor potential was by plate counts of VSC+ bacteria in saliva specimens.

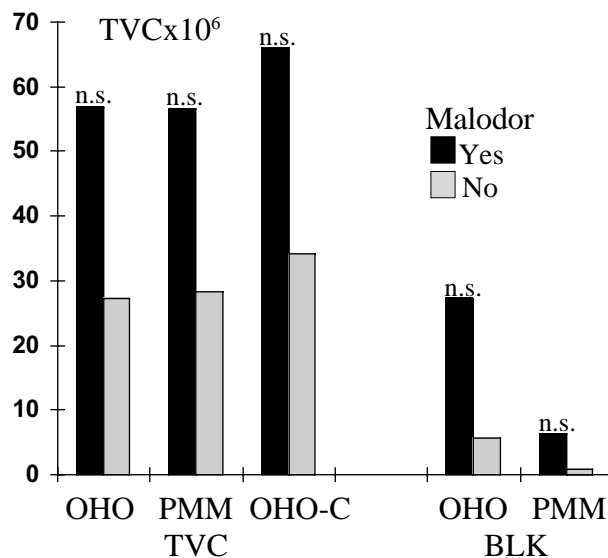


Fig 2. Total viable counts (TVC) and VSC+ bacteria (BLK) in saliva of children with and without oral malodor. n.s.=not significant.

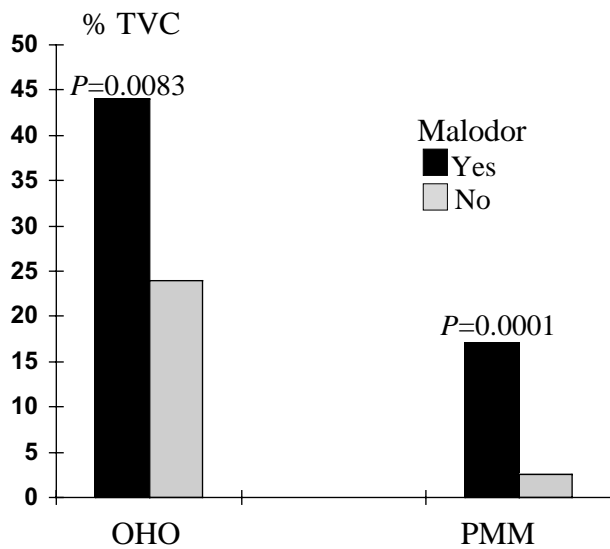


Fig 3. Percent VSC+ bacteria in saliva of children with and without oral malodor.

A noteworthy finding of the present study was the high quantity of VSC+ bacteria in saliva from child subjects (44% of TVC in OM+ and 24% in OM-, on OHO). Although no measurements of clinical halitosis were employed other than subjective assessments by parents, the large difference in levels of odorigens in the two populations may account, in part, for the parents' perceptions. This conclusion is supported by recent data from this laboratory showing highly significant positive correlations between counts of VSC+ bacteria in saliva of adults and VSC measurements of the same saliva specimens using an industrial sulfide monitor (Halimeter, Interscan Corp., Chatsworth CA).¹⁵

A primary objective of the present investigation was to quantify and characterize predominant odor forming bacteria in saliva of young children. This has not been reported previously with either child or adult populations other than the study noted above.¹⁵ *Veillonella* species (VL) and *P. oralis* were the predominate odorigens in the child population. VL are anaerobic gram negative cocci which appear in oral sites where dietary sugars are frequently present. This genus requires lactic acid as a nutrient, a by product formed by oral streptococci and other bacteria during sugar metabolism. All VL isolates were non-reactive in the AN-IDENT system, but could be differentiated by catalase activity. Most were catalase negative.

P. oralis is a gram negative anaerobic bacillus which has not received much attention in the oral microbiology literature. It is not a major pathogen, and its presence in young children has not been examined. Many researchers have associated oral gram negative anaerobic bacilli with halitosis, but have not implicated *P. oralis* nor *Veillonella*. Present findings suggest the profile of odorigenic bacteria may be even wider and include gram positive cocci such as *Peptostreptococcus* species and *Streptococcus fecalis*.

The microbiological comparison of OM+ and OM- revealed higher quantities and levels of odorigens in the former population, but also indicated that the types of bacteria in both were

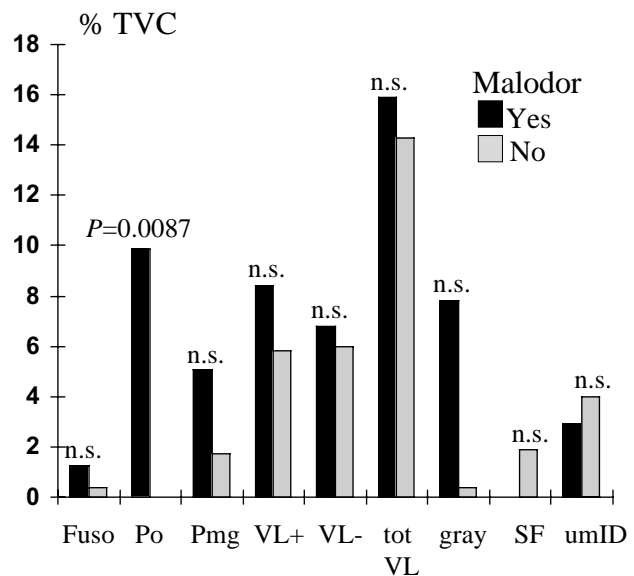


Fig 4. Predominant VSC+ bacteria in children with and without oral malodor on OHO. Fuso=*Fusobacterium nucleatum*; Po=*Prevotella oralis*; Pmg=*Peptostreptococcus magnus*; VL=catalase negative *Veillonella*; VL+catalase positive *Veillonella*; tot VL=total *Veillonella*; gray=*Gm-bacillus* with gray colony; Sf=*Streptococcus fecalis*; unID=unidentified; n.s.=not significant.

generally similar (Fig. 4). *P. oralis* which showed considerably higher %BLK values on OHO and PMM in OM+ children (Figs. 2 and 3) and appeared more frequently in this population (Table 2), was the only species which differed significantly between the two groups. *P. oralis* may not serve as a single diagnostic indicator of malodor, however, since three OM-subjects also harbored this species. In an adult halitosis study,¹⁵ specific salivary isolates which showed statistically significant positive correlation with oral malodor (including *P. oralis*), may not be adequate diagnostic indicators since they did not appear in every OM+ sample, or were also present in OM-subjects. In the adult and present studies, the most consistent difference between OM+ and OM- volunteers appeared to be higher concentrations of total VSC+ bacteria in salivary specimens of individuals with oral malodor.

A long-range goal is to develop microbiological techniques that can facilitate clinical diagnosis of oral malodor. Present results demonstrated that both OHO and PMM differentiated odorigens effectively in the child populations. OHO was the more non-selective and may be useful in characterization of the microbiota associated with oral malodor, but PMM, despite its lower recovery of BLK or diversity of isolates compared to OHO, was more effective in differentiating OM+ and OM-populations. This medium may be beneficial in comparative studies, such as trials of anti-microbial agents, or as a diagnostic aid. A major limitation of microbial assessments in general, however, is absence of clinical differentiation of the subjects. Measures should be adopted in future studies to correlate presence, quantities, and possibly types of odorigens with levels of VSC present in the mouth. In addition, comprehensive medical and dietary histories should be obtained in an effort to reveal specific etiologies.

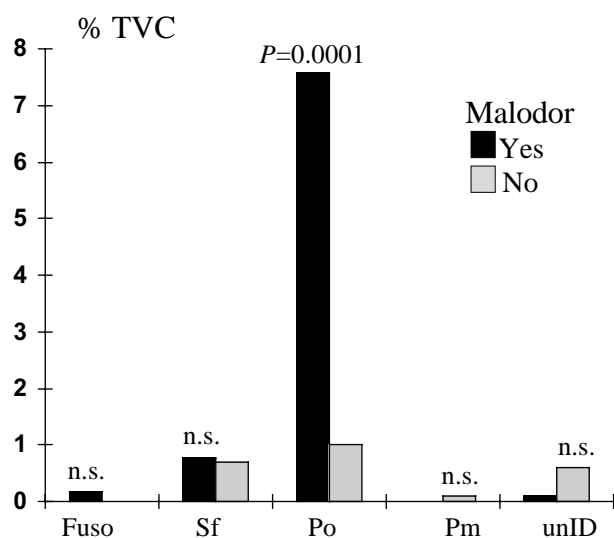


Fig 5. Predominant VSC+ bacteria in children with and without oral malodor on PMM. Fuso=*Fuso-bacterium nucleatum*, SF=*Streptococcus fecalis*, Po=*Prevotella oralis*, Pm=*Peptostreptococcus micros*, unID=unidentified; n.s.=non significant.

Conclusions

1. Children with parent perceived oral malodor exhibited significantly higher concentrations of odorogenic bacteria in saliva than children without parent perceived malodor.
2. *Veillonella* species and *Prevotella oralis* were the predominant VSC+ isolates recovered.
3. Levels of *P. oralis* were significantly higher in OM+ versus OM- children.

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