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Full Length Research Paper

Application of polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis for analysis of microbiota on the tongue dorsa of subjects with halitosis

Xiaoyu Li[1,2,3], Fanxing Xu[1], He Liu[1], Bing Liang[1], Yongping Xu[1,2] and Liji Jin[1,2]*

[1]School of Life Science and Technology, Dalian University of Technology, Dalian, 116024, China.
[2]Ministry of Education Center for Food Safety of Animal Origin, Dalian, 116620, China.
[3]Dalian SEM Bio-Engineering Technology Co. Ltd., Dalian, 116620, China.

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The purpose of this study was to compare the microbial profiles on the tongue dorsa of healthy subjects and subjects with halitosis using polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE). Tongue dorsum scrapings from five healthy subjects and six subjects with halitosis were analyzed by PCR-DGGE using primers specific for the V6 to V8 region of the eubacterial 16S rRNA gene. A dendrogram was derived from the PCR-DGGE fingerprints and the prominent DGGE bands of interest were identified through DNA sequencing. The similarity of the PCR-DGGE fingerprints was determined using the unweighted pair group method with an arithmetic mean dendrogram derived using Dice's Coefficient of Similarity. The sequence of PCR amplicons indicated that the microbial species most associated with halitosis were *Haemophilus parainfluenzae* and a phylotype of *Lachnospiraceae* (*Lachnospiraceae* genomosp. C1), whereas *Streptococcus salivarius* subsp. *salivarius*, *Neisseria mucosa* and *Neisseria cinerea* were species that did not appear to be associated with halitosis and are likely part of the healthy tongue flora. These results suggest that the presence of *H. parainfluenzae* and *Lachnospiraceae* genomosp. C1 may be associated with a shift in the balance of oral microbes in subjects with halitosis.

Key words: Polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE), 16S rRNA, halitosis, microbial profiles.

INTRODUCTION

Halitosis, also known as oral malodor, is a common condition that affects more than 20% of the general population throughout the world (Delanghe et al., 1999; Liu et al., 2006; Miyazaki et al., 1995). Although halitosis is a multifactorial condition and may involve both oral and non-oral conditions, approximately 80 to 90% of all cases originate from oral bacterial infection. These bacteria reside in various locations within the oral cavity (for example, tongue dorsum, interdental space, periodontal pockets), and they break down proteins in the oral cavity into free amino acids (Allaker et al., 2008).

Some of these amino acids, such as methionine and cysteine, are further metabolized yielding malodorous volatile sulfide compounds such as hydrogen sulfide and methylmercaptan (Yaegaki and Sanada, 1992). The tongue dorsum, especially its posterior region, is considered as the primary location for this process because the large surface area of the tongue and its papillary structure retains considerable food debris and

*Corresponding author. E-mail: xythird@gmail.com. Tel: +86-411-8470-6359. Fax: +86-411-8470-6359.

has a large microbial population (Loesche and Kazor, 2002).

Tongue microbiota have been shown to be an important factor that accelerates the production of volatile sulfide compounds in patients with periodontitis as well as in orally healthy individuals (Messadi and Younai, 2003). Comprehensive analyses of tongue biofilm microflora and identification of the predominant bacteria using culture methods or molecular biological methods have been reported (Haraszthy et al., 2007; Kazor et al., 2003). Studies of tongue microbiota using bacterial culture techniques are limited by the difficulties of *in vitro* growth techniques, the low total recovery of organisms and problems with microbial identification. As opposed to bacterial culture, direct amplification of 16S rRNA can identify both cultivable and non-cultivable bacteria. However, due to its complexity and the high cost of DNA sequencing, it is difficult to acquire a complete assessment of the overall bacterial composition of the tongue dorsum.

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has been shown to be a rapid, inexpensive and simple tool for studying complex bacterial communities in a variety of habitats, including environmental biofilms (Bonin et al., 2002; Peixoto et al., 2002), food fermentation processes (Meroth et al., 2003), gastrointestinal tract infection (Walter et al., 2000), saliva (Li et al., 2005), dental plaque (Li et al., 2006) and the subgingival pocket (Zijnge et al., 2003). Without requiring previous knowledge of the species composition of the microbial population, the technique can provide species profiles by differentiating 16S rRNA gene segments PCR-amplified from a complex bacterial sample without culturing. Compared with other molecular techniques such as microarray approach used for profiling, PCR-DGGE involves lower costs but provides only a limited resolution (Rytkönen et al., 2012). The purpose of the present study was to apply a PCR-DGGE approach to determine the bacterial diversity of the tongue dorsa of subjects with and without halitosis and to identify the strains that are likely associated with this oral problem.

MATERIALS AND METHODS

Subjects

Eleven adults (five females and six males; 32.5 ± 10.7 years; range: 23 to 58 years) were selected for this study. Informed consent was obtained from each subject. Six of the subjects were patients who visited the Dalian Dental Hospital (Dalian, China) complaining of halitosis, and exhibited an organoleptic score ≥ 2.0 and total volatile sulfide compounds ≥ 200 ppb, while five were healthy subjects without evidence of halitosis displaying an organoleptic score < 2.0 and total volatile sulfide compounds < 200 ppb. Each subject had at least 20 natural teeth, including at least one molar and one premolar in each quadrant. They had no systematic disease, no smoking history, and had received no antibiotic therapy in the last three months. The subjects were asked to refrain from any kind of oral activity such as eating, drinking, brushing, oral rinsing or gum chewing within two hours of testing.

Halitosis assessment

Organoleptic assessment

One judge from the Dalian Stomatological Hospital with training and experience in calibration tests was in charge of the organoleptic assessment. Subjects were instructed to exhale briefly through the mouth, at a distance approximately 10 cm from the nose of the judge. Organoleptic scores were assessed on a scale of 0 to 5 (0, no odor; 1, barely noticeable odor; 2, slight but clearly noticeable odor; 3, moderate odor; 4, strong odor; 5, extremely strong odor).

Sulfide monitor

A portable sulfide detector (Halimeter, Interscan Company, Chatsworth, CA) was used to measure volatile sulfide levels found in the air inside the mouth of the subjects as previously described (Rosenberg et al., 1991). Subjects were asked to refrain from talking for 5 min prior to measurement and breathe through their noses during the measurement. Results were recorded as peak ppb hydrogen sulfide equivalents.

Observable tongue coating assessment

The thickness and extent of tongue coating were determined by the naked eye according to the method of Gross et al. (1975). Both thickness and extent of tongue coating were scored from 0 (no observable coating) to 3 (heavy coating).

Sampling collection and DNA extraction

Samples were collected by firmly scraping an area (approximately 1 cm^2) of the posterior dorsal tongue surface near the midline with a sterile cotton swab and the samples were subsequently placed in 1 ml of sterilized TE Buffer (Tris-hydrochloride buffer, pH 8.0, containing 1.0 mM EDTA). Total genomic DNAs of the bacteria extracted from the tongue scrapings were isolated with a TIANamp Swab DNA Kit (Tiangen, Beijing, China) following treatment with lysozyme (final concentration of 20 mg ml^{-1}) for 30 min.

Polymerase chain reaction (PCR) amplification

Bacterial 16S rRNA genes were amplified by a thermo cycler (TaKaRa, Dalian, China) using a universal primer set (F -968-GC, 5'GCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGGAACGCGAAGAACCTTA-3'; and R -1401, 5'-CGGTGTGTACAAGA CCC-3') to generate an amplicon approximately 430-bp long for eubacteria (Nubel et al., 1996). Primers were synthesized by TaKaRa. A standardized DNA template (80 ng) was added to a PCR mixture containing 5 µl of 10 × buffer, 10 pmol of each primer, 40 nmol of dNTPs, and 2.5 U of Ex Taq polymerase (TaKaRa) in a total volume of 50 µl. To increase primer specificity, a touchdown PCR was used. The PCR conditions were as follows: initial denaturation at 94°C for 5 min and 2 cycles consisting of 30 s at 94°C, 1 min at 63°C, and 1 min at 72°C, then 10 cycles of touchdown PCR (94°C for 30 s, 63°C for 1 min, with a 0.8°C per cycle decrement, and 72°C for 1 min), followed by 23 cycles of PCR (94°C for 30 s, 55°C for 1 min, and 72°C for 1 min), and a final extension step at 72°C for 10 min. The products were evaluated by electrophoresis in 1.0% agarose gels run at 100 V for 45 min.

Denaturing gradient gel electrophoresis (DGGE)

A standardized 10 µl sample of each PCR-amplified product was loaded into the DGGE gel and separated with the Bio-Rad DCode™ System (Bio-Rad, Hercules, CA). A 45 to 60% linear DNA denaturing gradient (100% denaturant is equivalent to 7 mol l^{-1} urea and 40% deionized formamide) was formed in 6% (w/v) polyacrylamide gels. PCR products were directly loaded into the gel. Electrophoresis was performed at 80 V and 60°C for 15 hours in 1 × Tris-acetate-EDTA (TAE) buffer (pH 8.5). After electrophoresis, the gels were rinsed and stained for 20 min in 1 × TAE buffer containing 0.01% SYBR Green I (Biovision, Xiamen, China).

Analysis of denaturing gradient gel electrophoresis (DGGE) profiles

DGGE profiles were digitally captured and recorded with the Bio-Rad Gel Doc™ 2000 gel documentation system (Bio-Rad, Hercules, CA). Levels of similarity between fingerprints were analyzed using Quantity One Software (Bio-Rad) and calculated according to the Dice Coefficient. A dendrogram was constructed from the average matrix using the unweighted pair group method with an arithmetic mean (Zhang et al., 2008). Differences in the microbial composition of the tongue dorsa among healthy subjects and those with halitosis were assessed by comparing the DGGE profiles of PCR-amplified 16S rRNA fragments.

Excision and sequence analysis of products

Bands of interest were excised from the gels under ultraviolet light and were each incubated in 30 µl of sterilized double distilled water at 4°C overnight to release the DNA from the gel. Aliquots (1 µl) of the water containing the released DNA were used as templates in a universal DGGE-PCR reaction with F986GC not containing the GC-clamp and R1401 primer set.

The PCR products were cloned into pMD19-T using a DNA ligation kit (TaKaRa) according to the manufacturer's instructions. Plasmid DNA was isolated from the transformed cells using a MiniBEST plasmid purification kit (Version 2.0; TaKaRa) and then sequenced by TaKaRa.

Phylogenetic analysis

The DNA sequences obtained from the bands of interest were compared to the 16S rRNA sequences using SeqMatch in a Web-based program, RDP 10 (Ribosomal Database Project). 16S rRNA sequences with > 98.5% identity were considered as a single phylotype (Paster and Dewhirst, 2009).

RESULTS

Analysis of denaturing gradient gel electrophoresis (DGGE) profile

A total of 11 samples from 5 healthy subjects and 6 subjects with halitosis were analyzed using DGGE banding patterns. DGGE profiles from the 11 subjects contained 39 bands. There was little difference in the number of distinct bands between the two groups with 33 distinct bands detected in the healthy subjects compared with 36 distinct bands detected in halitosis subjects (Figure 1). The microbial ecology of the tongue dorsa shown by the top three severe cases of halitosis (M2, M3 and M4) reflected a high degree of similarity in electrophoresis profiles, while the microbial ecology of the tongue dorsa of healthy subjects showing the lowest concentration of volatile sulfur compounds (H2 and H4) also had similar DGGE profiles (Figure 2). However, overall, there was no clear and consistent difference between the DGGE fingerprints of healthy subjects compared with those with halitosis.

Sequence analysis of selected denaturing gradient gel electrophoresis (DGGE) amplicons

For each band represented by A to N in Figure 1, the corresponding bands from each subject were selected for sequencing to confirm their identities (data not shown). The results obtained from the sequencing matched with the sequences of bands A to N. The prevalence of each phylotype in the two groups is shown in Table 1. A phylotype of *Lachnospiraceae* (*Lachnospiraceae* genomosp. C1) and *Haemophilus parainfluenzae* were the species that were found to be most associated with halitosis, and these microbes were found in 83.3% of the halitosis group but only 20.0% of the healthy group. In contrast, *Streptococcus salivarius* subsp. *salivarius*, *Neisseria mucosa* and *Neisseria cinerea* were species that did not appear to be associated with halitosis. *S. salivarius* subsp. *salivarius* was more prevalent in healthy subjects (4 out of 5 or 80.0%) than in subjects with halitosis (3 out of 6 or 50%) Similarly, *N. mucosa* and *N. cinerea* were also more prevalent in healthy subjects (both 100%) than in subjects with halitosis (66.7%). Phylotypes with no overt difference in prevalence in both groups were *Eubacterium* sp. oral clone DO016, *Veillonella* sp. oral clone VeillF12, *Veilonella atypical, Actinomyces odontolyticus,* and *Selenomonas* sp. oral clone DO042.

DISCUSSION

In this study, the microbial profiles of the tongue dorsa of subjects with and without halitosis were compared using PCR-DGGE. Overall, there appeared to be no particular band pattern associated with either healthy subjects or those with halitosis as seen from the dendrogram (Figure 2).

The sequences of the fragments extracted from the gel were obtained to identify the phylotypes and their prevalence in healthy and halitosis subjects. It is worth noting that *H. parainfluenzae* and a phylotype of *Lachnospiraceae* (*Lachnospiraceae* genomosp. C1) were the bacteria most commonly associated with halitosis. Takeshita et al. (2010) found that two phylotypes of *Lachnospiraceae* were most often detected in the cluster

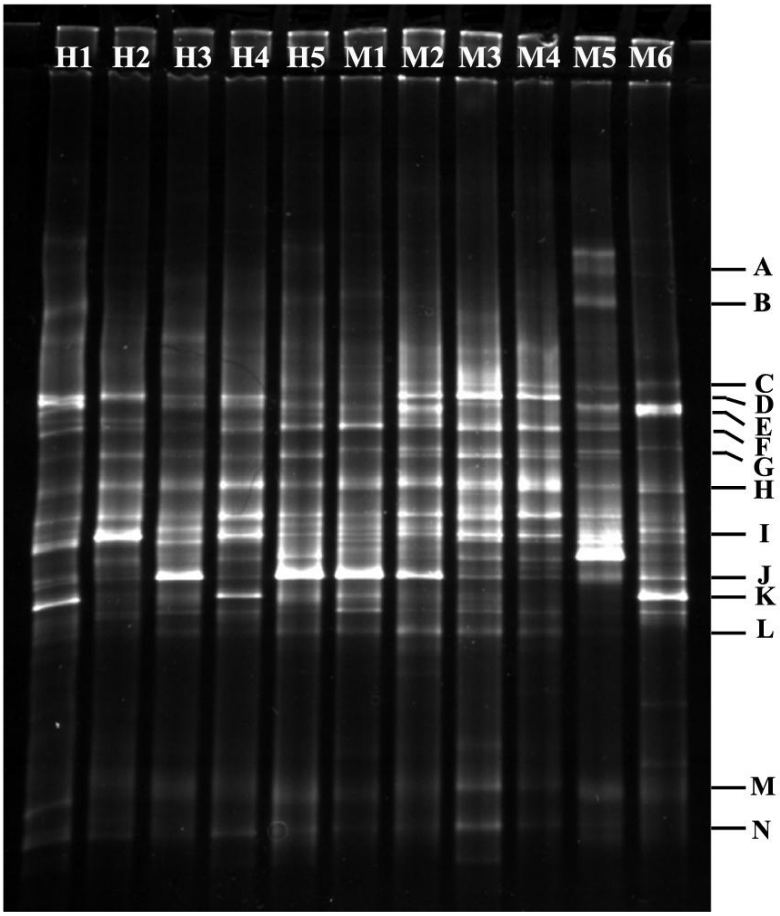

Figure 1. PCR-DGGE fingerprint representing the bacterial diversity in healthy and halitosis subjects. Lanes labeled H, healthy subjects; M, subjects with oral malodor. Bands of interest (A-N) were cloned and sequenced. The identified bacterial species from the sequence data are shown in Table 2.

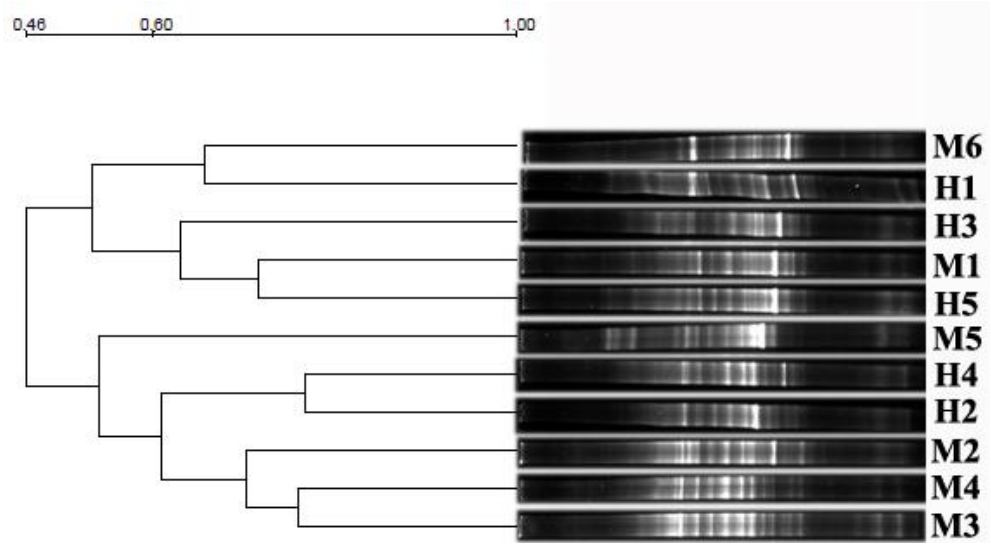

Figure 2. Dendrogram derived from PCR-DGGE fingerprint. H, healthy subjects; M, oral malodor subjects.

Table 1. Clinical assessment of the test subjects.

Group	Subject number	Sex	Age (years)	Organoleptic score [a]	Volatile sulfur compound concentration (ppb) [b]	Tongue coating [c]
Malodor	M1	Female	26	4	388	2
	M2	Male	39	4	462	2
	M3	Male	40	3	585	2
	M4	Male	23	5	426	2
	M5	Male	40	4	298	2
	M6	Female	58	3	351	1
Healthy	H1	Male	28	0	110	1
	H2	Female	23	0	54	1
	H3	Female	24	0	94	1
	H4	Female	28	0	59	0
	H5	Male	29	1	109	1

[a] 0 = no odor to 5 = extremely foul odor. [b] Volatile sulfur compound concentration was measured by Halimeter (Interscan Co., Chatsworth, CA). [c] 0 = no observable coating to 3 = heavy coating.

that represented relatively severe halitosis. However, it is still unclear whether these phylotypes have a direct relationship with halitosis. The most frequently described infections associated with *H. parainfluenza* are meningitis and subacute bacterial endocarditis (Black et al., 1988), but *H. parainfluenza* is mainly detected in refractory periodontitis sites, and is not detected in healthy subjects (Paster et al., 2001).

As reported by Kazor et al. (2003), *S. salivarius* is the species that is found to be most associated with healthy individuals. In a study conducted by Burton et al. (2006), subjects with halitosis who used a lozenge containing probiotic *S. salivarius* K12 had substantially reduced volatile sulfide compounds in their mouth one week after the initiation of treatment. This preliminary study of the effect of this probiotic on oral malodor parameters revealed a link between *S. salivarius and* healthy breath scores. Our data also support this as seen from the higher prevalence of this bacteria in healthy subjects compared with halitosis subjects (80% versus 50%). Similarly, *N. mucosa* and *N. cinerea* were also found to have a higher prevalence in healthy subjects in this study, whereas no significant difference in the prevalence of *N. mucosa* between healthy and halitosis subjects was observed according to a previous study by Paster et al. (2001). This may be due to the use of different subjects from different countries who likely had different food habits that may result in different oral microbiota. This discrepancy may also be due to different sampling areas in the mouth cavity which could have different anaerobic and nutrient conditions.

Veillonella and *Actinomyces* species were bacteria that were highly prevalent in all samples (healthy and halitosis groups). These species are the predominant H_2S-producing bacteria in both subjects with oral malodor and those with no or low oral malodor (Washio et al., 2005). *Eubacterium* and *Selenomonas* species, which have been proven to have the ability to form significant amounts of H_2S from L-cysteine (Persson et al., 1990), were also found in the majority of the samples that we analyzed (Table 2). However, it is still difficult to speculate about the contribution to oral malodor by these species, which form volatile sulfide compounds *in vitro*, because there are no reports on the connection between the prevalence of the bacteria and their ability to produce malodorous compounds.

Newer methods such as broad-range 16S rRNA gene PCR and microarrays of oligonucleotides have been conducted to enumerate the microbes that are difficult to culture in some microbial ecosystems (Relman, 2002). However, the features of high cost and finite detection of the number of phylotypes have limited the utilization of these methods. As shown in this study, PCR-DGGE offers a great opportunity to study shifts in the microbial composition at a population level, including the identification of shifting species. However, as with any other method, PCR-DGGE has its own limitations such as the fact that species that make up less than about 1000 cells in the population may not be enumerated due to the test's low limit of detection (Cocolin et al., 2000). In addition, PCR-DGGE only generates semi-quantitative results of the relative abundance of bacterial species within individual flora, because of some technical factors like the efficiency of DNA extraction and PCR amplification biases, and the different 16S rRNA gene copy number of bacteria present in the flora (Tabatabaei, 2009; Fogel et al., 1999). Despite these limitations, this approach has been shown to be reliable, reproducible, rapid, and inexpensive.

In conclusion, although the number of subjects per group in this study was not large, our findings suggest that this method can be a useful tool in the analysis of

Table 2. Sequence of PCR amplicons derived from DGGE.

Level	Closest relative (GenBank accession number)	Sequence similarity (%)	Sequence length (bp)	Prevalence (%)	
				Healthy (n=5)	Malodor (n=6)
A	*Actinomyces turicensis* APL11 (X78721)	99.5	435	0.0	16.7
B	*Eubacterium* sp. oral clone EI074 (AF385573)	99.5	433	40.0	33.3
C	*Lachnospiraceae* genomosp. C1 (AY278618)	99.3	436	20.0	83.3
D	*Streptococcus salivarius* subsp. *salivarius* (AF459433)	99.8	433	80.0	50.0
E	*Gemella sanguinis* 2045-94 (Y13364)	100	440	20.0	33.3
F	*Eubacterium* sp. oral clone DO016 (AF385510)	99.5	435	100.0	83.3
G	*Haemophilus parainfluenzae* CIP 102513 (EU083530)	99.5	434	20.0	83.3
H	*Veillonella* sp. oral clone VeillF12 (AY995757)	99.8	435	100.0	100.0
I	*Actinomyces odontolyticus* CCUG 28084 (AJ234041)	99.1	435	80.0	83.3
J	*Neisseria subflava* U37 (AJ239291)	99.8	433	100.0	100.0
K	*Neisseria mucosa* M5 (AJ239290)	99.8	433	100.0	66.7
L	*Neisseria cinerea* F1 (AJ239299)	99.3	433	100.0	66.7
M	*Veilonella atypical*; ATCC 17744 (AF439641)	99.1	436	100.0	100.0
N	*Selenomonas* sp. oral clone DO042 (AF385514)	99.3	435	100.0	100.0

bacterial diversity of the tongue dorsa of subjects with and without halitosis. Further studies such as multiplex PCR assay should be conducted to ascertain the presence of interesting species that have a low percentage in the bacterial communities. Future studies should focus on a combination of molecular and cultural methods in analyzing the bacteria that contribute to halitosis.

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