Isolation and identification methods for *Solobacterium moorei* involved in halitosis

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Abstract

The purpose of the present study was to develop selective media (SoloSM) for the isolation of *Solobacterium moorei*, to assess the prevalence of this organism using the selective medium, and to investigate whether the monitoring of *S. moorei* levels is useful as a clinical indicator for the diagnosis of halitosis. *S. moorei* strains grew well on SoloSM. The mean numbers of *S. moorei* in halitosis and healthy subjects were 5.02×10^4 and 2.95×10^6 CFU/ml, respectively. *S. moorei* strains produced higher amounts of volatile sulfur compounds (VSCs) than other representative oral bacteria. These results indicate that monitoring the levels of this organism is useful as a clinical indicator for the diagnosis of halitosis.

1. Introduction

The genus *Solobacterium* comprises only one species, *Solobacterium moorei*. *S. moorei* was isolated from human feces by Kageyama et al. in 2000 [1]. The genus name "*Solobacterium*" was obtained with its nomenclature from Latin terms (so.lo. L.a *solus*, sole, Gr. Dim n. *bacterion* a small rod; M.L. neut. n. *Solusbacterium* sole bacterium). The species name "*moorei*" was adopted in honor of W.E.C. Moore, a contemporary American microbiologist. The genus *Solobacterium* is a member of the *Clostridium* subphylum of Gram-positive bacteria and exhibits the closest phylogenetic association with *Holdemania filiformis*. Kageyama et al. reported a 16S rRNA gene sequence similarity of approximately 87% between *S. moorei* and *H. filiformis* from human feces as well as an approximately 38 mol% G+C content for the DNA base composition of the two species [1]. *S. moorei* is an obligately anaerobic microorganism, its cells are short, straight, or slightly curved, and it is observed as single or pairs of cells with diameters of 0.2 and 0.4-0.7 μm through a microscope [1, 2].

S. moorei has been associated with bacteremia, septicemia, refractory cases of endodontic infections, and localized aggressive periodontitis [3, 4, 5, 6]. Previous studies found large amounts and a more frequent presence of *S. moorei* in patients with oral malodor, but not in control subjects [2]. Vancauwenberghe et al. recently reported a correlation between *S. moorei*, tongue coating, and total volatile sulphur compounds (VSCs: hydrogen sulphide; H_2S, methyl mercaptan; CH_3SH, and dimethyl sulphur; $(CH_3)_2S$) [7]. Stephen et al. demonstrated that when cultured *in vitro*, *S. moorei* produced

H_2S directly from cysteine in quantities that were two- to three-fold higher than those of *Porphyromonas gingivalis* [8]. This relationship may be explained by *S. moorei* being capable of not only producing VSCs, particularly H_2S, but also possessing the enzyme β-galactosidase, which cuts glycoprotein chains from saliva into substrates for Gram-negative microorganisms to produce odorous compounds [2].

However, it currently remains unclear whether this organism is part of the normal oral flora. Thus, a suitable selective medium is needed to assess the prevalence of *S. moorei* involved in halitosis as well as bacteremia, septicemia, and periodontitis, in the oral cavity.

Difficulties are associated with the isolation of *S. moorei* due to the differential exhibition of its phenotypic characteristics. 16S rRNA gene sequences and DNA probe assays have been used to detect strains of *S. moorei* [2, 9]. A sequence analysis of several target genes is the most reliable method for identifying bacterial species; however, it is expensive, laborious, and time-consuming. Thus, a simple and more reliable assay for identifying *S. moorei*, such as a polymerase chain reaction (PCR) amplification method, is needed. The 16S rDNA PCR amplification method is sensitive and useful [10].

The purpose of the present study was to develop selective media for the isolation of *S. moorei*, to assess the prevalence of this organism in the oral cavity, and to investigate whether the monitoring of *S. moorei* levels is useful as a clinical indicator for the diagnosis of halitosis. Furthermore, the ability of *S. moorei* to produce VSCs was investigated *in vitro* and compared quantitatively to other representative oral bacteria using sensor gas chromatography.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All bacterial strains used in the present study are listed in Tables 1. The anaerobic bacteria (i.e., *S. moorei*, *Fusobacterium nucleatum*, *Prevotella nigrescens*, *P. intermedia*, *Veillonella atypica*, *V. dentocariosi*, *V. rogosae*, *V. dispar*, *V. parvula*, *V. tobetsuensis*, *Tannerella forsythia*, *Actinomyces israelii*, and *Leptotrichia buccalis*) used in the present study were maintained by cultivating them on anaerobic blood agar (CDC), which has a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) base supplemented with vitamin K_1 (10 μg/ml), hemin (5 μg/ml), L-cysteine (800 μg/ml), 0.5% yeast extract, and 5% sheep blood. Moreover, N-acetyl muramic acid (Sigma-Aldrich Co., LLC., Tokyo, Japan) disks were placed in the center of CDC plates to promote the growth of *T. forsythia*. These organisms were cultured at 37°C for 48 h in an anaerobic jar with a gas pack system (AnaeroPack®, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). *S. moorei* isolates (NUM-Som 9550, NUM-Som 9555, NUM-Som 9561, and NUM-Som 9564) were obtained with non-selective medium, i.e., CDC, from the human oral cavity in this study. NUM-Som 9550 and NUM-Som 9555 were isolated from two healthy adults without halitosis, and NUM-Som 9561 and NUM-Som 9564 from two adults with halitosis. Halitosis were confirmed by an organoleptic rating of 2 or more.

Strains other than anaerobic bacteria were maintained by cultivating them on Bact™ Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). These organisms were cultured at 37°C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator (NAPCO® Model 5400; Precision Scientific, Chicago, IL, USA).

2.2. Development of the new selective medium

2.2.1. Evaluation of the base medium

BHI supplemented with 1% yeast extract (BHI-Y), BHI-Y supplemented with 5% sheep blood (BHI-Y blood), and CDC were examined as the base medium in the selective medium. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates on which bacteria, except anaerobic bacteria, were inoculated were cultured at 37°C for 72 h in an atmosphere of 5% CO_2 in a CO_2 incubator, and the plates on which anaerobic bacteria were inoculated were cultured at 37°C for 6 days under anaerobic conditions. After cultivation, the number of colony-forming units (CFU)/ml was counted.

2.2.2. Susceptibility tests

Preliminary studies of antibiotic selection were also performed using disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA). The microbroth dilution method was used for susceptibility testing [11].

2.3. Recovery of S. moorei *and other representative oral bacteria*

The recoveries of the *S. moorei* reference strain, *S. moorei* isolates, and other representative oral bacteria were calculated as CFU/ml on selective medium and compared with those on CDC for total cultivable bacteria. All bacterial strains used in the present study are listed in Table 1.

All bacterial strains, except anaerobic bacteria, were pre-incubated in BHI broth at 37°C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator. Anaerobic bacteria were

pre-incubated in Tryptic soy broth (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with vitamin K_1 (10 μg/ml), hemin (5 μg/ml), and 0.5% yeast extract at 37°C overnight under anaerobic conditions. Moreover, N-acetyl muramic acid (Sigma-Aldrich Co. LLC., Tokyo, Japan) was added to Tryptic soy broth to promote the growth of *T. forsythia*. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates on which bacteria, except anaerobic bacteria, were inoculated were cultured at 37°C for 72 h in an atmosphere of 5% CO_2 in a CO_2 incubator, and those on which anaerobic bacteria were inoculated were cultured at 37°C for 5 days under anaerobic conditions. After cultivation, the number of CFU/ml was counted.

2.4. Clinical samples

Forty volunteers (23 men, 17 women; mean age 31 years, range 21-58 years) participated in the present study. They had no systemic disease and received no antibiotic therapy for at least 3 months. A sensor gas chromatograph (ODSA-P3-A, Yamato Scientific Co., Ltd., Tokyo, Japan) was used to measure the concentrations of VSCs. All participants were asked not to brush, rinse, or smoke immediately prior to the assessment and not to eat or drink for at least 2 h beforehand. A gas chromatography analysis was performed in duplicate. After closing the lips for 1 min, 1 ml of mouth air was obtained with a gastight syringe (Termosyringe® SS-02SZ, Termo Co., Tokyo, Japan) and immediately injected into the sensor gas chromatograph. In the present study, the threshold level for genuine halitosis was defined as higher amounts of total VSCs than 200 ppb in mouth air, according to which participants were divided into two

subject groups: healthy subjects without halitosis (n=20), and halitosis subjects (n=20).

In order to collect tongue biofilms, the rear dorsal whole surface of the tongue was firmly scraped 10 times with sterilized cotton swabs. All samples were suspended in a sterile microcentrifuge tube containing 1 ml of Tris-HCl buffer (0.05 M, pH 7.2). Samples were dispersed by sonication for 30 s in an ice bath (50 W, 20 kHz, Astrason® System model XL 2020, NY., USA). Portions (100 μl) of appropriate dilutions of these samples were plated in triplicate on selective medium plates. Selective medium plates were cultured at 37ºC for 5 days under anaerobic conditions. After cultivation, the number of CFU/ml was calculated. This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 18-017).

2.5. Identification of S. moorei *species isolated from clinical samples*

Twenty-four of the approximately 50 colonies that grew on the selective medium plate per subject were randomly isolated and subcultured, and their identity was then confirmed by a PCR analysis.

2.6. Design of species-specific primers for S. moorei

The design of species-specific primers for *S. moorei* was performed as follows. *Bulleidia extructa*, *H. filiformis*, and *Erysipelothrix rhusiopathiae* exhibit the closest phylogenetic association with *S. moorei* [1]. The 16S rRNA sequences of *S. moorei* (accession no. AF101240), *B. extructa* (accession no. AF220064), *H. filiformis* (accession no. Y11466), and *E. rhusiopathiae* (accession no. AB034200) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan), and multiple sequence

alignment analyses were performed using the CLUSTAL W program; i.e., the 16S rRNA sequences of four species were aligned and analyzed. Homologies among the primers selected for *S. moorei* were confirmed by a BLAST search.

2.7. Development of a PCR method for identifying S. moorei *using designed primers*

A PCR method for identifying *S. moorei* using the designed primers was developed as follows. Bacterial cells were cultured in a Trypticase broth (Becton, Dickinson and Co., Sparks, MD, USA) base supplemented with vitamin K_1 (10 μg/ml), hemin (5 μg/ml), L-cysteine (800 μg/ml), and 0.5% yeast extract overnight, and 1 ml of the sample was then collected in a microcentrifuge tube and resuspended at a density of 1.0 McFarland standard (approximately 10^7 CFU in 1 ml of sterile distilled water). A total of 3.6 μl of the suspension was then used as a PCR template. The detection limit for PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 2 μM of each primer, 10 μl of 2 × MightyAmp Buffer Ver.2 (Takara Bio Inc., Shiga, Japan), 0.4 μl of MightyAmp DNA Polymerase (Takara), and 3.6 μl of the template in a final volume of 20 μl. PCR was performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Carlsbad, CA). PCR conditions included an initial denaturation step at 98°C for 2 min, followed by 30 cycles consisting of 98°C for 10 s and 68°C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1 × Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker.

2.8. Assessment of VSC-producing abilities

The VSC-producing abilities of *S. moorei* and other representative oral bacteria *in vitro* were assessed as previously described [12]. In the present study, VSC levels were measured using a sensor gas chromatograph (ODSA-P3-A, Yamato Scientific Co., Ltd., Tokyo, Japan). All bacterial strains were pre-incubated as described above. Each bacteria culture solution was inoculated into 19 × 55 mm sample vials (1880 SV10; AGC Techno Glass Co., Ltd., Shizuoka, Japan) containing Tryptic soy broth supplemented with vitamin K_1 (10 μg/ml), hemin (5 μg/ml), 0.5% yeast extract, L-cysteine (25mM), and L-methionine (25mM). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). After the inoculation, sample vials were loosely sealed with silicon caps and cultured at 37°C under anaerobic conditions until a density of McFarland Standard 3.0. After the incubation, sample vials were immediately sealed tightly with silicon caps. One milliliter of each sample vial headspace air was aspirated by puncturing a silicon cap with the needle of the gastight syringe (Termosyringe® SS-02SZ2232), and immediately injected into the sensor gas chromatograph. The VSC levels of the sample vial headspace were measured using the sensor gas chromatograph (ODSA-P3-A).

2.9. Statistical analysis

The number of *S. moorei* in samples of tongue biofilms from healthy subjects without halitosis and halitosis subjects were compared using the Mann-Whitney U test. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Development of selective medium

3.1.1. Selection of the base medium

The selection of a base medium for the growth of *S. moorei* was performed. *S. moorei* grew well on CDC, but developed extremely small colonies on BHI-Y and BHI-Y blood (data not shown). Therefore, CDC was ultimately selected as the base medium.

3.1.2. Susceptibility to antibiotics

S. moorei was more resistant to colistin than oral Gram-negative cocci, such as *Neisseria* and *Veillonella* species. The minimal inhibitory concentration (MIC) of colistin for *S. moorei* was 1000 μg/ml. Oral Gram-negative cocci were sensitive to 10 μg/ml of colistin. *S. moorei* was more resistant to erythromycin than oral Gram-negative rods, such as *Fusobacterium*, *Porphyromonas*, and *Prevotella* species. The MIC of erythromycin for *S. moorei* was 100 μg/ml. Oral Gram-negative bacteria were sensitive to 3 μg/ml of erythromycin. *S. moorei* was more resistant to kanamycin than oral *streptococcus* species, oral *Corynebacterium* species, and oral *Actinomyces* species. The MIC of kanamycin for *S. moorei* was 400 μg/ml. Oral *streptococcus* species, oral *Corynebacterium* species, and oral *Actinomyces* species were sensitive to 5 μg/ml of kanamycin. *S. moorei* was more resistant to cinoxacin than *Eubacterium*, *Slackia*, and *Leptotrichia* species. The MIC of cinoxacin for *S. moorei* was 500 μg/ml. *Eubacterium*, *Slackia*, and *Leptotrichia* species were sensitive to 20 μg/ml of cinoxacin.

3.1.3. Composition of the new selective medium

The new selective medium, designated *S. moorei* selective medium (SoloSM), was composed of the following (per liter): 40 g of tryptic soy agar, 5 g of yeast extract, 5 mg of hemin, 10 mg of Vitamin K_1, 800 mg of L-cysteine, 50 ml of sheep blood, 50 mg of kanamycin, 100 mg of cinoxacin, 30 mg of erythromycin, and 20 mg of colistin. Sheep blood and antibiotics, i.e., kanamycin, cinoxacin, erythromycin, and colistin, were added after the base medium had been sterilized and cooled to 50ºC.

3.2. PCR method for identifying S. moorei

3.2.1. Primer design

The specific primer set covering the upstream region of the 16S rDNA sequence of *S. moorei* was designed in the present study (Table 2). The amplicon size of *S. moorei* was 452 bp.

3.2.2. Detection limit

A PCR method was used to identify the *S. moorei*-amplified DNA fragment of the expected size for this organism (Fig. 1). The detection limit was assessed in the presence of titrated bacterial cells, and the detection sensitivity of the PCR assay was 50-100 CFU per PCR template (5.6 μl) for the *S. moorei*-specific primer set with the JCM 10645 strain (data not shown).

3.2.3. Assay of S. moorei *and representative oral bacteria*

The PCR method used to identify *S. moorei* produced positive bands from the *S. moorei* reference strain JCM 10645 and *S. moorei* clinical isolate NUM-Som 9550 (Fig.

1). Some *Streptococci*, *Actinomyces*, *Neisseria*, *Corynebacterium*, *Rothia*, *Veillonella*, *Fusobacterium*, *Aggregatibacter*, and *Staphylococcus* species were used as representative oral bacteria in PCR using the designed primer set. No amplicons were produced from any of the representative oral bacteria (Fig. 1).

3.3. Recovery of S. moorei *and inhibition of other representative oral bacteria on selective medium*

Table 1 shows the recovery of the *S. moorei* reference strains and isolates on SoloSM relative to CDC. The growth recoveries of the *S. moorei* reference strain and isolates on SoloSM were between 96.6 and 99.3% (average 98.3%) that on CDC.

Table 1 also shows the inhibition of other representative oral bacteria on SoloSM relative to CDC agar. The growth of other representative oral bacteria was markedly inhibited on the selective medium.

3.4. Clinical examination

The detection frequencies of *S. moorei* in tongue biofilm samples from healthy subjects without halitosis (n=20), and halitosis subjects (n=20) are shown in Table 3. The total VSC levels of healthy and halitosis subjects were 64.8 and 750.9 ppb, respectively. *S. moorei* was detected in all samples from healthy and halitosis subjects. The mean numbers of *S. moorei* in healthy and halitosis subjects were 5.0×10^4 CFU/ml (range: 8.2×10^3 - 7.6×10^4) and 2.9×10^6 CFU/ml (range: 5.9×10^5 - 4.4×10^6), respectively. The mean numbers of *S. moorei* in tongue biofilm samples were significantly higher in halitosis subjects than in healthy subjects ($P < 0.01$).

In the first isolation, *S. moorei* colonies on SomSM commonly had a smooth and circular appearance. The colony color and average colony size of *S. moorei* on SoloSM were translucent light white and 1.0 mm in diameter, respectively (Fig. 2).

3.5. VSC-producing abilities of S. moorei *and other representative oral bacteria* in vitro

The VSC-producing abilities of *S. moorei* reference strains and *S. moorei* clinical isolates are shown in Table 4. No significant differences were observed among the reference strains (JCM 10645, JCM 10646, and JCM 10647), clinical isolates from healthy subjects (NUM-Som 9550 and NUM-Som 9555), and clinical isolates from halitosis subjects (NUM-Som 9561 and NUM-Som 9564).

Comparisons of VSC-producing abilities among *S. moorei* and other representative oral bacteria are shown in Table 5. *S. moorei* produced the highest VSC level, followed by *F. nucleatum* and *P. gingivalis*.

4. Discussion

Halitosis is a relatively common condition in which an individual either has bad breath or perceives oneself to have an offensive mouth odor. Although it has multifactorial origins, bad breath originates from the oral cavity in most cases [12, 13]. The causes of bad breath, except for an oral origin, include nasal and pharyngeal infections, respiratory conditions, gastrointestinal issues, metabolic conditions such as diabetes, and liver diseases [14]. Halitosis originating from the oral cavity is caused by products generated through bacterial metabolic degradation. In individuals with a healthy oral cavity, an important site for the accumulation of bacteria associated with halitosis is the dorsum of

the tongue and, specially, its posterior portion, which provides a suitable environment for the growth of anaerobes because a low oxygen concentration is present in the deep crypts of the tongue [14]. Poor oral hygiene that results in the accumulation of bacterial biofilms in subgingival areas and that may lead to periodontal disease has also been associated with halitosis [15].

VSCs, which include H_2S, CH_3SH, and $(CH_3)_2S$, are considered to be the primary compounds responsible for halitosis originating from the oral cavity, although amines (cadaverine, putrescine, and indole) and short-chain fatty acids (isobutyric and isovaleric acids) also contribute to oral malodor. VSCs are generated through enzymatic modifications to sulfur-containing amino acids (cysteine and methionine), which are made available following the proteolytic degradation of proteins or glycoproteins [16]. Bacteria that have been classically associated with halitosis include *F. nucleatum*, *P. intermedia*, *P. gingivalis*, and *Treponema denticola* [16]. The large array of hydrolytic enzymes produced by these bacteria may act in synergy to produce VSCs [17]. The Gram-positive anaerobic bacterium *S. moorei* (formerly known as *B. moorei*) has recently been specifically associated with oral malodor because it was detected in subjects with halitosis, but not in control subjects [2, 18, 19, 20]. It is important to note that this bacterial species has also been associated with bacteremia, septicemia, refractory cases of endodontic infections and periodontitis, and localized aggressive periodontitis [3, 4, 5, 6].

However, it currently remains unclear whether this organism is part of the normal oral flora. Moreover, difficulties are associated with the isolation of *S. moorei* due to the differential exhibition of phenotypic characteristics. 16S rRNA gene sequences, broad-range PCR, and DNA probe assays have been used to detect *S. moorei* strains [2, 9]. Thus,

a suitable selective medium and reliable identification method are needed in order to assess the prevalence of *S. moorei* involved in halitosis as well as bacteremia, septicemia, and periodontitis in the oral cavity.

In the present study, we designed species-specific primers to identify *S. moorei* using a PCR method. These primers were able to distinguish *S. moorei*, and did not react with representative oral bacteria. Moreover, the PCR method in the present study directly uses bacterial cells with MightyAmp DNA Polymerase Ver.2 (Takara) and is completed within approximately 2 hours.

A useful selective medium for isolating *S. moorei* may contribute to the correct and rapid diagnosis of infectious diseases caused by this organism. However, a selective medium that is useful for the isolation of *S. moorei* currently does not exist. In the present study, *S. moorei* strains were more resistant to kanamycin, cinoxacin, erythromycin, and colistin than other representative oral bacteria. The growth of representative oral bacterial strains used in this study was inhibited by the addition of 50 mg/L kanamycin, 100 mg/L cinoxacin, 30 mg/L erythromycin, and 20 mg/L colistin to CDC agar. All of the *S. moorei* reference strains and isolates tested grew well on the new selective medium, designated as SoloSM, while the growth of other bacteria was markedly inhibited (Table 1). Moreover, SoloSM allowed for the identification of *S. moorei* by its characteristic colony morphology.

The distribution of *S. moorei* in the oral cavity of humans has not yet been reported in detail. In the present study, *S. moorei* was detected in all samples from healthy and halitosis subjects by a culture method using the selective medium, i.e. SoloSM. Haraszthy et al. detected *S. moorei* in 100% of subjects with halitosis, but in only 14% of control

subjects, using a DNA probe assay[2]. A culture method using SoloSM may be more sensitive for detecting *S. moorei* than other methods, including DNA probe assays. Moreover, the present results indicated that *S. moorei* was a part of the normal oral flora. The mean numbers of *S. moorei* in tongue biofilm samples were significantly higher in halitosis subjects than in healthy subjects ($P < 0.01$). In addition, *S. moorei* produced the highest VSC levels *in vitro*. A sentence was deleted. Higher production levels of VSCs were observed in the presence of cysteine, which was transformed into H_2S, ammonia, and pyruvate by cysteine desulfhydrase [9]. Accordingly, the monitoring of *S. moorei* levels may be useful as a clinical indicator for the diagnosis of genuine halitosis.

We developed a selective medium, designated SoloSM, to isolate *S. moorei* in the oral cavity of humans. Since SoloSM is highly selective for *S. moorei*, it will be useful for assessing the distribution and role of this organism at various locations in humans. The selective medium (SoloSM) and our PCR method as isolation and identification methods, respectively, for *S. moorei* may contribute to the diagnosis of halitosis as well as bacteremia, septicemia, endodontic infections, and localized aggressive periodontitis, which are caused by this organism.

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Table 1

Recovery of *S. moorei* and other bacteria on CDC blood and SoloSM

Strain		CDC blood CFU/ml, × 10^8	SomSM CFU/ml, × 10^8	Recovery, %
Solobacterium moorei				
	JCM 10645	2.2 ± 0.3[a]	2.2 ± 0.3	99.3
	JCM 10646	1.9 ± 0.1	1.9 ± 0.2	98.9
	JCM 10647	2.3 ± 0.3	2.2 ± 0.2	99.1
	NUM-Som 9550	1.1 ± 0.2	1.1 ± 0.3	98.0
	NUM-Som 9555	0.9 ± 0.3	0.9 ± 0.2	96.6
	NUM-Som 9561	1.2 ± 0.2	1.2 ± 0.3	96.6
	NUM-Som 9564	0.9 ± 0.2	0.9 ± 0.3	98.1
Streptococcus oralis				
	ATCC 35037	3.4	0	0
Streptococcus salivarius				
	ATCC 10557	1.1	0	0
Streptococcus anginosus				
	ATCC 33397	5.9	0	0
Streptococcus mutans				
	NCTC 10449	3.1	0	0
Actinomyces naeslundii				
	ATCC 12104	0.5	0	0
Actinomyces oris				
	ATCC 27044	0.5	0	0
Actinomyces israelli				
	ATCC 12102	0.3	0	0
Corynebacterium matruchotii				
	ATCC 14266	0.7	0	0
Corynebacterium durum				
	ATCC 33449	1.1	0	0
Rothia dentocariosa				
	JCM 3067	0.9	0	0
Rothia mucilaginosa				
	JCM 10910	0.8	0	0
Rothia aeria				
	JCM 11412	1.1	0	0
Veillonella parvula				
	ATCC10790	1.8	0	0
Fusobacterium nucleatum				
	ATCC22586	1.2	0	0
N. sicca				
	ATCC 29256	0.6	0	0

[a] Ave ± SD.

Table 2

Locations and sequences of species-specific primers for the16S rDNA of *S. moorei.*

Species	Primer	Sequence	Product size (bp)	Position	Accession number
S. moorei	SomF	TCGGAAGGCATCTTCTGGTT	452	178-197	AB31056
	SomR	AAGTGGCTGGATTGGGTTGA		629-610	

Table 3

Detection frequencies of *S. moorei* in tongue biofilm samples from 2 groups.

Group	VSCs(ppb)				No. of S.moorei-Positive subjects (%, frequency)	No. of S.moorei (CFU)	
	Total	H_2S	CH_3SH	$(CH_3)_2S$			
Healthy subjects without halitosis (n=20)	64.8	51.2	3.8	9.4	20(100)	5.02×10^4	*
Halitosis subjects (n-20)	750.9	712.4	18.1	20.5	20(100)	2.95×10^6	

The threshold levels for genuine halitosis were defined as ＞ 200 ppb total VSCs in mouth air.

* Mann-Whitney U test; $p<0.01$.

Table 4

VSC-producing abilities of *S. moorei* reference strains and *S. moorei* clinical isolates.

Bacteria species	Strain	Total VSCs (ppb)
Solobacterium moorei	JCM 10645	35086.8
	JCM 10646	33881.3
	JCM 10647	33569.9
	NUM-Som 9550	34094.0
	NUM-Som 9555	33219.1
	NUM-Som 9561	33721.3
	NUM-Som 9564	34103.3

NUM-Som 9550 and NUM-Som 9555 were isolated from two healthy adults without halitosis, and NUM-Som 9561 and NUM-Som 9564 from two adults with halitosis.

Table 5

Comparison of VSC-producing abilities among *S. moorei* and other representative oral bacteria.

Ranking	Bacteria species	Strain	VSCs (ppb)			
			Total	H_2S	CH_3SH	$(CH_3)_2S$
1	*Solobacterium moorei*	JCM 10645	35086.8	17251.2	17835.6	0
2	*Fusobacterium nucleatum*	ATCC 25586	32085.2	17174.7	14910.5	0
3	*Porphyromonas gingivalis*	ATCC 33277	31708.8	15030.2	16678.6	0
4	*Prevotella nigrescens*	ATCC 33563	18523.2	17537.1	986.1	0
5	*Prevotella intermedia*	ATCC 25611	16357.6	14301.2	2056.4	0
6	*Veillonella atypica*	DSM 20739	14484.5	12041.7	2442.8	0
7	*Veillonella dentocariosi*	JCM 15641	11829.6	11829.6	0	0
8	*Veillonella rogosae*	JCM 15642	11860.3	11860.3	0	0
9	*Veillonella dispar*	DSM 20735	11679.1	11679.1	0	0
10	*Veillonella parvula*	ATCC 10790	11594.7	10186.7	1408	0
11	*Neisseria sicca*	ATCC 29256	10954.1	10133	821.1	0
12	*Aggregatibacter actinomycetemcomitans*	ATCC 33384	9673.5	8835.1	838.4	0
13	*Veillonella tobetsuensis*	JCM 17976	9588.5	9588.5	0	0
14	*Tannerella forzythia*	JCM 10827	6246.5	6246.5	0	0
15	*Streptococcus sanguinis*	ATCC 10556	4909.3	2792.1	2117.2	0
16	*Actinomyces israelii*	ATCC 12102	4977.5	4977.5	0	0
17	*Streptococcus salivarius*	ATCC 7073	3758.7	3206.5	0	0
18	*Rothia aeria*	JCM 11412	3303.6	3262.8	40.8	0
19	*Actinomyces oris*	ATCC 27044	2727.4	440.5	2286.9	0
20	*Leptotrichia buccalis*	ATCC 14201	2048.2	1683.7	364.5	0

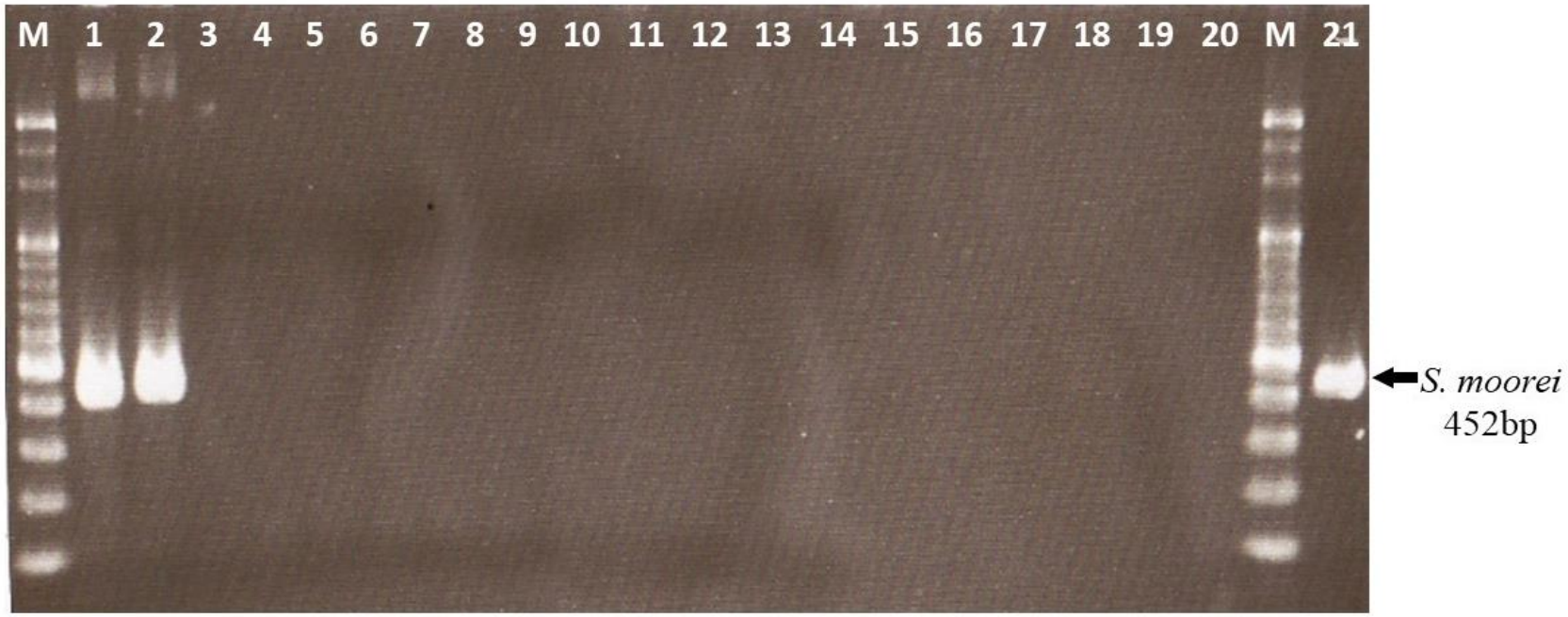

Fig. 1 Primers are a mixture of SomF and SomR. Lanes: 1, *Solobacterium moorei* JCM 10645; 2, *S. moorei* clinical isolate NUM-Som 9550; 3, *Streptococcus oralis* ATCC 10557; 4, *S. sanguinis* ATCC 10556; 5, *S. salivarius* JCM 5707; 6, *S. anginosus* ATCC 33397; 7, *S. mutans* NCTC 10449; 8, *A. naeslundii* ATCC 12104; 9, *A. israelii* ATCC 12102; 10, *A. odontolyticus* ATCC17929; 11, *Rothia dentocariosa* JCM 3067; 12, *R. mucilaginosa* JCM 10910; 13, *R. aeria* JCM 11412; 14, *Corynebacterium matruchotii* ATCC 14266; 15, *C. durum* ATCC 33449; 16, *Neisseria sicca* ATCC 29256; 17, *Veillonella parvula* JCM 12972; 18, *Fusobacterium nucleatum* ATCC 25586; 19, *Aggregatibacter actinomycetemcomitans* ATCC 33384; 20, *Staphylococcus epidermidis* ATCC 14990; 21, *S. moorei*-positive tongue biofilm sample. M, molecular size marker (100-bp DNA ladder).

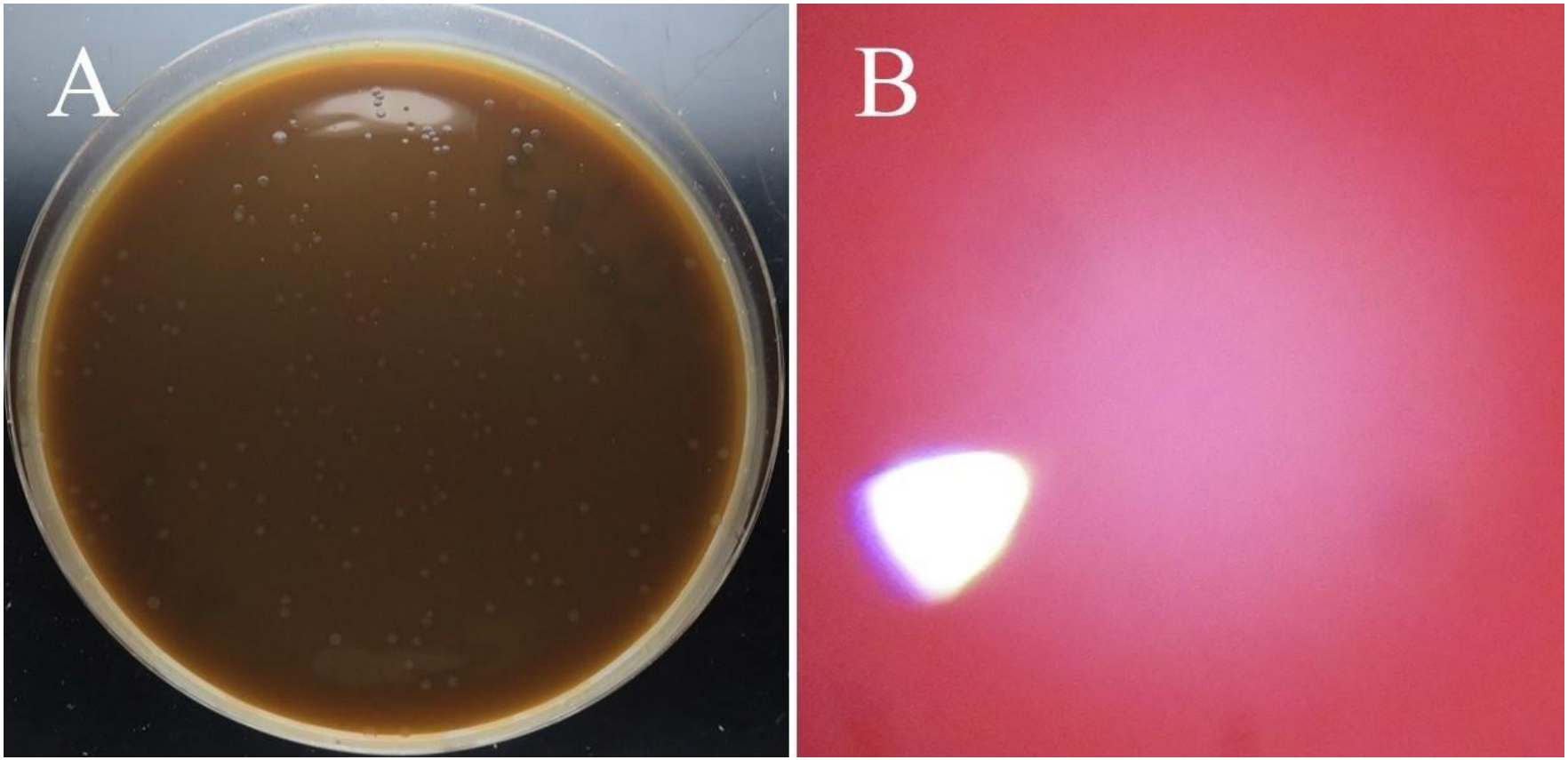

Fig. 2 Appearance of *S. moorei* colonies on SoloSM. A: *S. moorei* colonies on SoloSM inoculated with a tongue biofilm sample.

B: Stereomicroscope image of *S. moorei* colonies on SoloSM.