Novel isolation and identification methods for *Veillonella* species involved in halitosis

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Title: Novel isolation and identification methods for *Veillonella* species involved in halitosis

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Abstract

The purpose of the present study was to develop a novel selective medium (OVSM) and useful identification method for oral *Veillonella* species, to assess the prevalence of this species using the selective medium, and to investigate whether the monitoring of oral *Veillonella* species levels is useful as a clinical indicator for the diagnosis of halitosis. To examine bacterial populations in the oral cavity, OVSM and multiplex PCR method were developed for isolating and identifying oral *Veillonella* species. OVSM consists of tryptic soy agar, sheep blood, sodium lactate, vancomycin, and oxacillin. Oral *Veillonella* species grew well on OVSM. Oral *Veillonella* species accounted for 5.4 and 27.2%, respectively, of all bacteria in the tongue biofilm samples of healthy and halitosis subjects. Oral *Veillonella* species produced larger amounts of volatile sulfur compounds than other representative oral bacteria except for *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella nigrescens* and *Prevotella intermedia in vitro*. These results indicate that monitoring the levels of these organisms is useful as a clinical indicator for the diagnosis of halitosis.

1. Introduction

The genus *Veillonella* belongs to the family *Veillonellaceae* in the phylum *Firmicutes* [1]. The genus *Veillonella* currently comprises 15 species (https://lpsn.dsmz.de/search?word=Veillonella). These species are members of the oral, genito-urinary, respiratory, and intestinal microbiota of humans and other mammals. The genus *Veillonella* is anaerobic Gram-negative cocci, with cells typically forming pairs or short chains and lacking a flagellum, spore, or capsule [2]. Members of the genus *Veillonella* utilize short-chain organic acids, particularly lactic acid, as an energy source instead of carbohydrates or amino acids, subsequently producing acetic acid and propionic acid [3] [4].

Of the 15 species belonging to the genus *Veillonella*, *Veillonella atypica*, *V. denticariosi*, *V. dispar*, *V. infantium*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis* are representative species isolated from the human oral cavity, such as human saliva or tongue or dental biofilms, and are generally called oral *Veillonella* species. Oral *Veillonella* species are one of the main members of the human oral microbiome [5] [6]. Previous studies identified oral *Veillonella* species as rare causative organisms of meningitis, endocarditis, bacteremia, discitis, vertebral osteomyelitis, and prosthetic joint infection [7]. In addition, *V. tobetsuensis* was also isolated from the intraoperative bronchial fluids of elderly individuals with pulmonary carcinoma [8].

Periasamy and Kolenbrander demonstrated that oral *Veillonella* species were early colonizers during the formation of oral biofilms, along with *Streptococcus* species,

which were identified as the initial colonizers in multispecies communities developing on oral biofilms [9]. Oral *Veillonella* species are associated with severe early childhood caries [10] and intraradicular infections, including abscesses [11], apical root canals [12], and dental tubules [13]. Oral *Veillonella* species were also predominant in the subgingival biofilm samples of patients with chronic periodontitis [14]. Previous findings showed that their distribution and frequency at the species level varied depending on the oral site and oral hygiene status [15] [16] [17] [18] [19]. Therefore, it is important to elucidate the role of oral *Veillonella* species in the formation of oral biofilms in order to improve the prevention and treatment of oral infectious diseases.

Halitosis is caused due by the metabolic products of bacteria in the oral cavity, particularly those on the dorsum of the tongue [20]. Some cases of halitosis have been associated with periodontitis [21] [22], and, thus, various periodontitis-related bacteria species have been detected in tongue coatings. These findings also suggest that the tongue coating plays a role as a reservoir of these bacteria [23]. Most of these bacteria have the ability to produce hydrogen sulfide (H_2S), one of the major components of halitosis [24]. Washio et al. focused on halitosis in patients without oral diseases, such as periodontitis and caries, and found that the predominant H_2S-producing bacteria were not periodontitis-related bacteria, but were mainly indigenous bacteria of the oral cavity, such as *Veillonella* and *Actinomyces* [25]. Moreover, *Veillonella* species, including *V. atypica*, *V. dispar*, and *V. parvula*, were predominant among these microorganisms.

Previous studies [5] [6] [26] [27] [28] that examined the prevalence of oral *Veillonella* species used the well-known selective medium for *Veillonella* species, *Veillonella* agar (VA) [29]. In a pilot study, we attempted to detect oral *Veillonella* species in oral

samples using VA. However, it was impossible to detect some bacterial species among these organisms because VA inhibited their growth. Therefore, previous findings may not have precisely reflected the prevalence of oral *Veillonella* species. A novel suitable selective medium is needed to accurately assess the prevalence of oral *Veillonella* species involved in dental caries, intraradicular infections, and halitosis in the oral cavity.

Veillonella strains are relatively easy to identify at the genus level, but remain difficult to identify at the species level because there are no useful phenotypic or biochemical examinations to distinguish them [30]. A sequence analysis of several target genes is the most reliable method for identifying bacterial species; however, it is expensive, laborious, and time-consuming. Therefore, a simple and more reliable assay for identifying oral *Veillonella* species at the species level, such as a polymerase chain reaction (PCR) amplification method, is needed.

The purpose of the present study was to develop a novel selective medium for the isolation of oral *Veillonella* species, to assess the prevalence of these species in the oral cavity, and to investigate whether the monitoring of *Veillonella* species levels is useful as a clinical indicator for the diagnosis of halitosis. Furthermore, the ability of each oral *Veillonella* species to produce volatile sulfur compounds (VSCs) was investigated *in vitro* and quantitatively compared 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 2-5. Strains of anaerobic bacteria except for *Veillonella* species were maintained by cultivating them on Anaerobic Blood Agar (CDC), that consists of a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with vitamin K_1, hemin, L-cysteine, yeast extract, and sheep blood. *Veillonella* strains were maintained by cultivating them on tryptic soy agar blood (TS-LAC-Blood) composed of tryptic soy agar (TS) (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with 2.1% sodium lactate and 5% sheep blood. 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).

V. atypica isolate (NUM 9110), *V. tobetsuensis* isolate (NUM 9122), *V. rogosae* isolate (NUM 9135), *V. parvula* isolate (NUM 9142), *V. dispar* isolate (NUM 9145) and *V. denticariosi* isolate (NUM 9163) were obtained from the human oral cavity in the present study using a non-selective medium, i.e., TS-LAC-Blood.

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

TS, TS with 2.1% sodium lactate (TS-LAC), TS with 5% sheep blood (TS-blood), and TS-LAC-Blood 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 were cultured at 37°C for 4 days under anaerobic conditions. After cultivation, the number of colony-forming units (CFU)/ml was counted.

2.2.2. Susceptibility tests

Preliminary studies on antibiotic selection were performed using disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA). The microbroth dilution method was employed for susceptibility testing [31].

2.3. Recovery of oral Veillonella *species*

The recoveries of the *Veillonella* reference strains and *Veillonella* isolates were calculated as CFU/ml on selective medium and compared with those on TS-LAC-Blood for total cultivable bacteria, on conventional selective medium (VA) for *Veillonella* species, and on new selective medium in this study. All *Veillonella* strains used in the present study are listed in Table 2.

All bacterial strains were pre-incubated in TS (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with 2.1% sodium lactate at 37°C overnight under anaerobic conditions. 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 were cultured at 37°C for 4 days under anaerobic conditions. After cultivation, the number of CFU/ml was counted.

2.4. Clinical samples

One hundred volunteers (43 men, 57 women; mean age 34 years, range 19-61 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, as previously described [20], according to which participants were divided into two subject groups: healthy subjects without halitosis (n=50) and halitosis subjects (n=50).

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 several types of selective medium plates and CDC plates for total cultivable bacteria. Selective medium plates and CDC plates were cultured at 37ºC for 4 days under anaerobic conditions. CDC plates were also cultured at 37ºC for 4 days in an atmosphere of 5% CO_2 in a CO_2 incubator (NAPCO® Model 5400; Precision

Scientific, Chicago, IL, USA). The selective media used in the present study were a novel selective medium for oral *Veillonella* species, Mitis-salivarius agar for *Streptococcus* species, *Fusobacterium* selective agar for *Fusobacterium* species, and *Bacteroides* selective agar for *Porphyromonas* and *Prevotella* species. After cultivation, CFU/ml was calculated. The number of anaerobic bacteria was calculated by subtracting CFU on CDC in an atmosphere of 5% CO_2 from that under anaerobic conditions. The present study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 18-032).

2.5. Identification of oral Veillonella *species isolated from clinical samples*

Twenty-four out of the approximately 50 colonies that grew on a novel selective medium for oral *Veillonella* species per subject were randomly isolated and subcultured, and their identity was then confirmed by a PCR analysis.

2.6. Identification of bacteria species isolated from clinical samples

Bacterial species, i.e., *Streptococcus*, *Fusobacterium*, oral *Veillonella*, *Porphyromonas*, and *Prevotella* species, except for oral *Veillonella* species on each selective medium, were identified by colony morphology and Gram staining.

2.7. Design of species-specific primers for oral Veillonella *species*

Species-specific primers for six oral *Veillonella* species were designed as previously reported [31]. Briefly, the RNA polymerase B (*rpoB*) gene sequences of *V. atypica* (accession no. EF185159), *V. denticariosi* (EF185162), *V. parvula* (EF185158), *V.*

dispar (EF185161), *V. rogosae* (EF211831), and *V. tobetsuensis* (AB698649), the citrate synthase (*gltA*) gene sequences of *V. atypica* (accession no. KJ580470), *V. denticariosi* (KJ580471), *V. parvula* (KJ580466), *V. dispar* (KJ580469), *V. rogosae* (KJ580467), and *V. tobetsuensis* (KJ580468), and the *dnaK* gene sequences of *V. atypica* (accession no. AF440436), *V. denticariosi* (EF219278), *V. parvula* (AB678219), *V. dispar* (AF440435), *V. rogosae* (AB678220), and *V. tobetsuensis* (AB698650) were obtained from the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/services.html, Mishima, Japan), and a multiple sequence alignment analysis was performed with the CLUSTAL W program; i.e., the *rpoB*, *gltA*, and *dnaK* gene sequences of six oral *Veillonella* species were aligned and analyzed, respectively. Homology among the primers selected for each oral *Veillonella* species and their respective *rpoB*, *gltA*, and *dnaK* gene sequences were confirmed by a BLAST search.

2.8. Development of a multiplex PCR method for identifying oral Veillonella *species using designed primers*

Bacterial cells were cultured in TS supplemented with 2.1% sodium lactate for 24 h, and 1 ml of samples was then collected in microcentrifuge tubes and resuspended at a density of 1.0 McFarland standard (approximately 10^7 CFU/ml) in 1 ml of sterile distilled water. A total of 3.6 µl of the suspension was then used as the PCR template. The detection limit of 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 0.2 µM of each primer, 10 µl of 2 × MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase

(Takara), and 5 μl of the template in a final volume of 20 μl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). PCR conditions included an initial denaturation step at 98°C for 2 min, followed by 30 cycles consisting of 98°C for 30 s, 63°C for 30 s, and 72°C for 30 s. 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. All experiments were performed in triplicate.

2.9. Assessment of VSC-producing abilities

The VSC-producing abilities of oral *Veillonella* species and other representative oral bacteria *in vitro* were assessed as previously reported [32]. 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 TS supplemented with vitamin K_1 (10 μg/ml), hemin (5 μg/ml), 0.5% yeast extract, L-cysteine (25 mM), and L-methionine (25 mM). 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 3.0 McFarland Standard. 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).

2.10. Statistical analysis

The number of total bacteria and oral *Veillonella* species and the proportions of the anaerobes *Streptococcus* species, *Fusobacterium* species, oral *Veillonella* species, and *Porphyromonas* and *Prevotella* species in samples of tongue biofilms from healthy subjects without halitosis and halitosis subjects were compared using the Mann-Whitney U test. P values < 0.05 were considered to be significant.

3. Results

3.1. Development of selective media

3.1.1. Selection of the base medium

A base medium was selected for the growth of oral *Veillonella* species. All of the oral *Veillonella* species grew well on TS-LAC-Blood. The growth of *V. atypica*, *V. denticariosi*, *V. parvula*, and *V. dispar* was enhanced by supplementing with 2.1 % sodium lactate, whereas that of *V. tobetsuensis* and *V. rogosae* was markedly inhibited on TS and TS-LAC free from sheep blood (data not shown). Therefore, TS-LAC-Blood was ultimately selected as the base medium.

3.1.2. Susceptibility to antibiotics

Oral *Veillonella* species were more resistant to vancomycin than oral Gram-positive

bacteria, such as *Streptococcus*, *Actinomyces*, and *Corynebacterium* species. The minimal inhibitory concentration (MIC) of vancomycin for oral *Veillonella* species was 60 μg/ml. Oral Gram-positive bacteria were sensitive to 16 μg/ml of vancomycin. Oral *Veillonella* species were more resistant to oxacillin than oral Gram-negative rods, such as *Fusobacterium*, *Porphyromonas*, and *Prevotella* species. The MIC of oxacillin for oral *Veillonella* species was 8 μg/ml. Oral Gram-negative bacteria were sensitive to 1 μg/ml of oxacillin. *Neisseria* species, which are Gram-negative cocci similar to *Veillonella* species, were also resistant to vancomycin and oxacillin, but were inhibited in an aerobic culture.

3.1.3. Composition of the new selective medium

Table 1 shows the compositions of VA and the new selective medium, designated Oral *Veillonella* selective medium (OVSM). OVSM comprised the following (per liter): 40 g of tryptic soy agar, 21 ml of sodium lactate, 50 ml of sheep blood, 16 mg of vancomycin, and 1 mg of oxacillin. Sheep blood and antibiotics, i.e., vancomycin and oxacillin, were added after the base medium had been sterilized and cooled to 50ºC. VA contained 5 g of tryptone, 5 g of yeast extract, 0.75 g of sodium thioglycolate, 0.002 g of basic fuchsin, 2.1% sodium lactate, and 15 g of agar.

3.2. Establishment of a multiplex PCR method for identifying oral Veillonella *species*

3.2.1. Primer design

Twelve specific primers covering the upstream region of the *rpoB*, *gltA*, and *dnaK* gene sequences of six oral *Veillonella* species were designed in the present study (Figure

1-3). Specific forward primers were designated as VaYF for *V. atypica*, VtYF for *V. tobetsuensis*, VrTF for *V. rogosae*, VpYF for *V. parvula*, VdisYF for *V. dispar*, and VdenYF for *V. denticariosi*, whereas specific reverse primers were designated as VaYR for *V. atypica*, VtYR for *V. tobetsuensis*, VrTR for *V. rogosae*, VpYR for *V. parvula*, VdisYR for *V. dispar*, and VdenYR for *V. denticariosi*. The amplicon sizes of *V. atypica*, *V. tobetsuensis*, *V. rogosae*, *V. parvula*, *V. dispar*, and *V. denticariosi* were 93, 186, 325, 397, 516, and 607 bp, respectively.

3.2.2. Detection limit

Our multiplex PCR method for identifying and detecting six oral *Veillonella* species, i.e., *V. atypica*, *V. tobetsuensis*, *V. rogosae*, *V. parvula*, *V. dispar*, and *V. denticariosi* successfully amplified DNA fragments of the expected size for each species (Figure 4). The detection limit was assessed in the presence of titrated bacterial cells, and the sensitivity of the PCR assay was between 5 × 1 and 5 × 10 CFU per PCR template (5.0 µl) for the *V. atypica*-specific primer set with strain DSM 20739, the *V. tobetsuensis*-specific primer set with strain JCM 17876, the *V. tobetsuensis*-specific primer set with strain JCM 17876, the *V. rogosae*-specific primer set with strain JCM 15642, the *V. parvula*-specific primer set with strain JCM 12972, the *V. dispar*-specific primer set with strain DSM 20735, and the *V. denticariosi*-specific primer set with strain JCM 15641 (data not shown).

3.2.3. Assay of representative oral bacteria

Some *Streptococci*, *Actinomyces*, *Neisseria*, and *Corynebacterium* 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 (Figure 4).

3.3. Recovery of oral Veillonella *species and inhibition of other representative oral bacteria on selective medium*

Table 2 shows the recovery of oral *Veillonella* species on VA and OVSM relative to TS-LAC-Blood. The average growth recoveries of the oral *Veillonella* species reference strains and isolates on VA and OVSM were 14.5 (range: 0 - 29.7) and 98.4% (range: 93.3 - 99.9), respectively. The growths of *V. tobetsuensis* and *V. denticariosi* were markedly inhibited on VA.

3.4. Clinical examination

The detection frequencies of oral *Veillonella* species in tongue biofilm samples from healthy subjects without halitosis (n=50) and halitosis subjects (n=50) are shown in Table 3. The total VSC levels of healthy and halitosis subjects were 78.2 and 812.6 ppb, respectively. The mean numbers of total bacteria in healthy and halitosis subjects were $7.34 \pm 5.08 \times 10^7$ CFU/ml and $9.48 \pm 5.42 \times 10^7$ CFU/ml, respectively. Oral *Veillonella* species were detected in all samples from healthy and halitosis subjects. The mean numbers of oral *Veillonella* species in healthy and halitosis subjects were $3.97 \pm 4.08 \times 10^6$ CFU/ml and $2.58 \pm 1.85 \times 10^7$ CFU/ml, respectively. Oral *Veillonella* species in tongue biofilm samples were significantly higher in halitosis subjects than in healthy subjects ($P < 0.01$).

Figure 5 shows a comparison of the bacteria flora in tongue biofilms between the 2 groups. The proportions of anaerobe and *Veillonella* species were significantly higher in halitosis subjects than in healthy subjects ($P < 0.05$). Increases in anaerobe species in the tongue biofilm samples of halitosis subjects were attributed to elevations in oral *Veillonella* species, which are anaerobic Gram-negative cocci. No significant differences were observed in the proportions of *Fusobacterium*, *Porphyromonas*, and *Prevotella* species between the 2 groups. Comparisons of oral *Veillonella* species in tongue biofilms between the 2 groups are shown in Table 4. The average CFU of each oral *Veillonella* species was higher in halitosis subjects than in healthy subjects. *V. rogosae* was predominant and detected in all subjects, followed by *V. tobetsuensis*, *V. atypica*, and *V. dispar. V. denticariosi* and *V. parvula* were rarely detected in both groups.

In the first isolation, colonies of oral *Veillonella* species on OVSM commonly had a smooth and circular appearance. The colony color and average colony size of oral *Veillonella* species on OVSM were translucent light white and 1.0 mm in diameter, respectively (Figure 6).

3.5. VSC-producing abilities of oral Veillonella *species and other representative oral bacteria* in vitro

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

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 [33]. 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 [33]. 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, particularly 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 [33]. 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 [34].

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 [33]. Bacteria that have been classically associated with halitosis include oral *Veillonella* species, *F. nucleatum*, *P. intermedia*, *P. gingivalis*, and *Treponema denticola* [25] [33].

Most of these bacteria have the ability to produce H_2S, one of the major components of halitosis. Washio et al. [25] focused on oral malodor in patients without oral diseases, such as periodontitis and dental caries, and reported that the predominant H_2S-producing bacteria were not periodontitis-related bacteria, but were mainly indigenous bacteria of the oral cavity, such as the genus *Veillonella*.

VA is used worldwide as the selective medium for *Veillonella* species. In our pilot study, VA inhibited the colony-forming of some *Veillonella* species. The findings obtained also indicated that VA was not appropriate for investigating the prevalence of oral *Veillonella* species. Therefore, a novel suitable selective medium is needed to accurately assess the prevalence of oral *Veillonella* species involved in severe early childhood caries and intraradicular infections as well as halitosis in the oral cavity. In the present study, oral *Veillonella* species grew better on TS-LAC-Blood than on media that did not contain lactic acid and sheep blood. *Veillonella* species utilize lactic acid as carbon and energy sources instead of carbohydrates [3] [4]. Lactic acid and animal blood have been identified as growth factors that promote the growth of these organisms.

Oral *Veillonella* species were more resistant to vancomycin and oxacillin than other representative oral bacteria. The growth of the representative oral bacterial strains used in the present study was inhibited by the addition of 16 mg/L vancomycin and 1 mg/L oxacillin to TS-LAC-Blood. All of the oral *Veillonella* reference strains and isolates tested grew well on the new selective medium, designated as OVSM, while the growth of other bacteria was markedly inhibited (data not shown). Moreover, OVSM allowed the identification of oral *Veillonella* species based on their characteristic colony

morphologies.

Moreover, characteristic colony morphologies on OVSM allowed oral *Veillonella* species to be easily distinguished.

Veillonella strains are relatively easy to identify at the genus level, but remain difficult to identify at the species level because there are currently no useful phenotypic or biochemical examinations to distinguish them [19]. To overcome this issue, Mashima et al. established a novel one-step PCR method with species-specific primer sets based on the variable region of the *rpoB* gene sequences of oral *Veillonella* species [27]. However, the findings of our pilot study showed that the size of each PCR fragment by this method was similar; therefore, it was difficult to accurately identify each oral *Veillonella* species. Moreover, including DNA extraction, it took more than 5 hours to finish the identification. Therefore, a reliable identification method is needed to accurately assess the prevalence of oral *Veillonella* species.

In the present study, we designed species-specific primers with the already mentioned methods for the identification and detection of six *Veillonella* species using a PCR-based method. These primers were able to distinguish each *Veillonella* species and did not display cross-reactivity with representative oral bacteria. Moreover, we developed a one-step multiplex PCR method with the ability to identify and differentiate oral *Veillonella* species (i.e., *V. atypica*, *V. denticariosi*, *V. dispar*, *V. infantium*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis*) using only one PCR tube per sample. Our multiplex PCR method is easy because the use of MightyAmp DNA Polymerase Ver.3 (Takara) means that DNA extraction is not necessary, and species identification and detection using this method only takes approximately 2 hours. Therefore, the method

described herein will allow the prevalence of oral *Veillonella* species and their involvement in various infections to be fully clarified in future studies.

The mean numbers of oral *Veillonella* species in tongue biofilm samples were significantly higher in halitosis subjects than in healthy subjects ($P < 0.01$). The proportions of *Veillonella* species were significantly higher in halitosis subjects than in healthy subjects ($P < 0.05$), compared with those of *Fusobacterium*, *Porphyromonas*, and *Prevotella* species. In addition, six oral *Veillonella* species produced high VSC levels, particularly H_2S, *in vitro*. Higher production levels of VSCs were observed in the presence of cysteine, which is transformed into H_2S, ammonia, and pyruvate by cysteine desulfhydrase [35]. Accordingly, the monitoring of oral *Veillonella* species levels may be useful as a clinical indicator for the diagnosis of genuine halitosis.

5. Conclusion

We developed a selective medium, designated OVSM, to isolate oral *Veillonella* species in the oral cavity of humans. Since OVSM is highly selective for oral *Veillonella* species, it will be useful for assessing the distribution and role of these organisms at various locations in humans. A novel selective medium (OVSM) and our multiplex PCR method as isolation and identification methods, respectively, for oral *Veillonella* species may contribute to the diagnosis of halitosis as well as severe early childhood caries and intraradicular infections. Moreover, the relationship of oral *Veillonella* species and the organoleptic test will be clarified in future studies, using the isolation and identification methods described herein.

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Table 1. Compositions of VA and OVSM.

	VA Conventional selective medium (per L)	OVSM Selective medium in this study (per L)
Tryptone	5 g	
Yeast extract	5g	
Sodium thioglycolate	0.75 g	
Tryptic soy agar		40 g
Sodium lactate	21 ml	21 ml
Sheep blood		50 ml
Agar	15 g	15 g
Basic fuchsin	0.002 g	
Vancomycin	0.0075 g	0.016 g
Oxacillin		0.001 g

Table 2. Recovery of oral *Veillonella* species on VA and OVSM.

Strain		TS-LAC-Blood Tryptic soy agar sheep blood sodium lactate	VA Conventional selective medium	OVSM Selective medium in this study
		CFU/ml, × 10^9	Recovery, %	Recovery, %
Veillonella atypica	DSM 20739	1.8	27.8	99.9
	NUM 9110	2.1	19.3	99.3
Veillonella tobetsuensis	JCM 17876	1.1	0	93.3
	NUM 9122	0.6	0	94.4
Veillonella rogosae	JCM 15642	0.9	25.5	99.3
	NUM 9135	0.8	23.1	98.5
Veillonella parvula	JCM 12972	0.7	23.1	99.1
	NUM 9142	0.6	29.7	99.6
Veillonella dispar	DSM 20735	1.8	11.1	99.8
	NUM 9155	1.6	8.5	99.7
Veillonella denticariosi	JCM 15641	0.7	3.1	99.1
	NUM 9163	0.9	2.9	98.7

Table 3. Detection frequencies of oral *Veillonella* species in tongue biofilm samples from 2 groups.

Table 3. Detection frequencies of oral *Veillonella* species in tongue biofilm samples from 2 groups.

	VSCs (ppb)				No. of total bacteria (CFU)	No. of oral *Veillonella*-positive subjects (%, frequency)	No. of oral *Veillonella* species (CFU)
	Total	H_2S	CH_3SH	$(CH_3)_2S$			
Healthy subjects without halitosis (n=50)	78.2	65.8	8.2	4.2	7.34×10^7 (SD: $\pm$ 5.08 $\times 10^7$)	50 (100)	3.97×10^6 * (SD: $\pm$ 4.08 $\times 10^6$)
Halitosis subjects (n=50)	812.6	772.3	24.6	15.7	9.48×10^7 (SD: $\pm$ 5.42 $\times 10^7$)	50 (100)	2.58×10^7 * (SD: $\pm$ 1.85 $\times 10^7$)

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

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

Table 4. Comparison of oral *Veillonella* species in tongue biofilms between 2 groups.

	Healthy subjects without halitosis (n=50)		Halitosis subjects (n=50)	
	No. of positive subjects (%, frequency)	Average CFU	No. of positive subjects (%, frequency)	Average CFU
Veillonella atypica	42 (84)	7.56×10^5	44 (88)	1.30×10^6
Veillonella denticariosi	0 (0)	0	2 (4)	1.07×10^5
Veillonella dispar	18 (36)	4.20×10^5	23 (46)	1.13×10^6
Veillonella parvula	1 (2)	1.10×10^5	7 (14)	6.20×10^5
Veillonella rogosae	50 (100)	1.93×10^6	50 (100)	1.35×10^7
Veillonella tobetsuensis	47 (94)	1.18×10^6	50 (100)	9.11×10^6

Table 5. VSC-producing ability of *Veillonella* species and other representative oral bacteria *in vitro*.

Ranking	Bacteria species	VSCs (ppb)			
		Total	H_2S	CH_3SH	$(CH_3)_2S$
1	*Fusobacterium nucleatum*	32085.2	17174.7	14910.5	0
2	*Porphyromonas gingivalis*	31708.8	15030.2	16678.6	0
3	*Prevotella nigrescens*	18523.2	17537.1	986.1	0
4	*Prevotella intermedia*	16357.6	14301.2	2056.4	0
5	*Veillonella atypica*	14484.5	12041.7	2442.8	0
6	*Veillonella dentocariosi*	11829.6	11829.6	0	0
7	*Veillonella rogosae*	11860.3	11860.3	0	0
8	*Veillonella dispar*	11679.1	11679.1	0	0
9	*Veillonella parvula*	11594.7	10186.7	1408.0	0
10	*Veillonella tobetsuensis*	10954.1	10133.0	821.1	0
11	*Neisseria sicca*	9673.5	8835.1	838.4	0
12	*A. actinomycetemcomitans*	9588.5	9588.5	0	0
13	*Tannerella forsythia*	6246.5	6246.5	0	0
14	*Streptococcus sanguinis*	4909.3	2792.1	2117.2	0
15	*Actinomyces israelii*	4977.5	4977.5	0	0
16	*Streptococcus salivarius*	3758.7	3206.5	0	0
17	*Rothia aeria*	3303.6	3262.8	40.8	0
18	*Actinomyces oris*	2727.4	440.5	2286.9	0
19	*Streptococcus oralis*	2577.7	2577.7	0	0
20	*Leptotrichia buccalis*	2048.2	1683.7	364.5	0
21	*Corynebacterium durum*	1753.6	541.5	1148.0	64.1
22	*Rothia dentocariosa*	1735.5	729.8	938.5	67.2
23	*C. matruchotii*	1165.5	361.3	804.2	0
24	*Rothia mucilaginosa*	1149.6	39.1	1110.5	0
25	*Actinomyces odontolyticuas*	1118.5	998.4	120.1	0
26	*Actinomyces naeslundii*	891.7	196.2	679.6	15.9

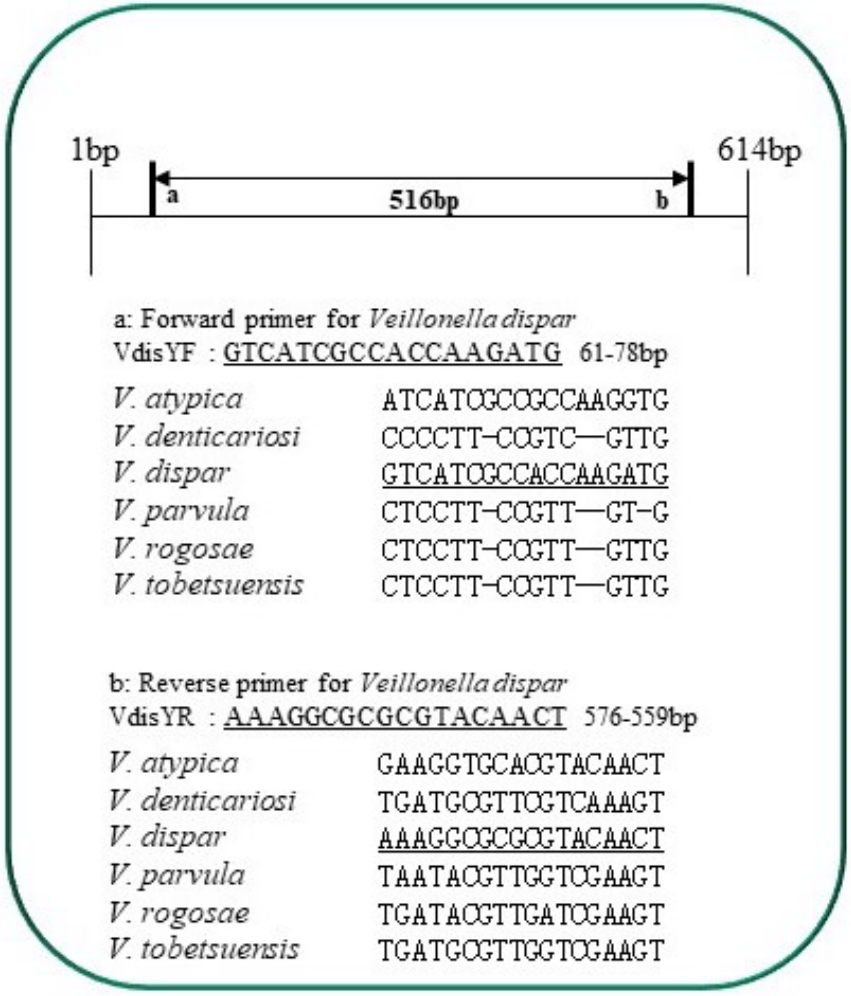

Figure 1. Locations and sequences of species-specific primers for the *dnaK* gene of *V. dispar*.

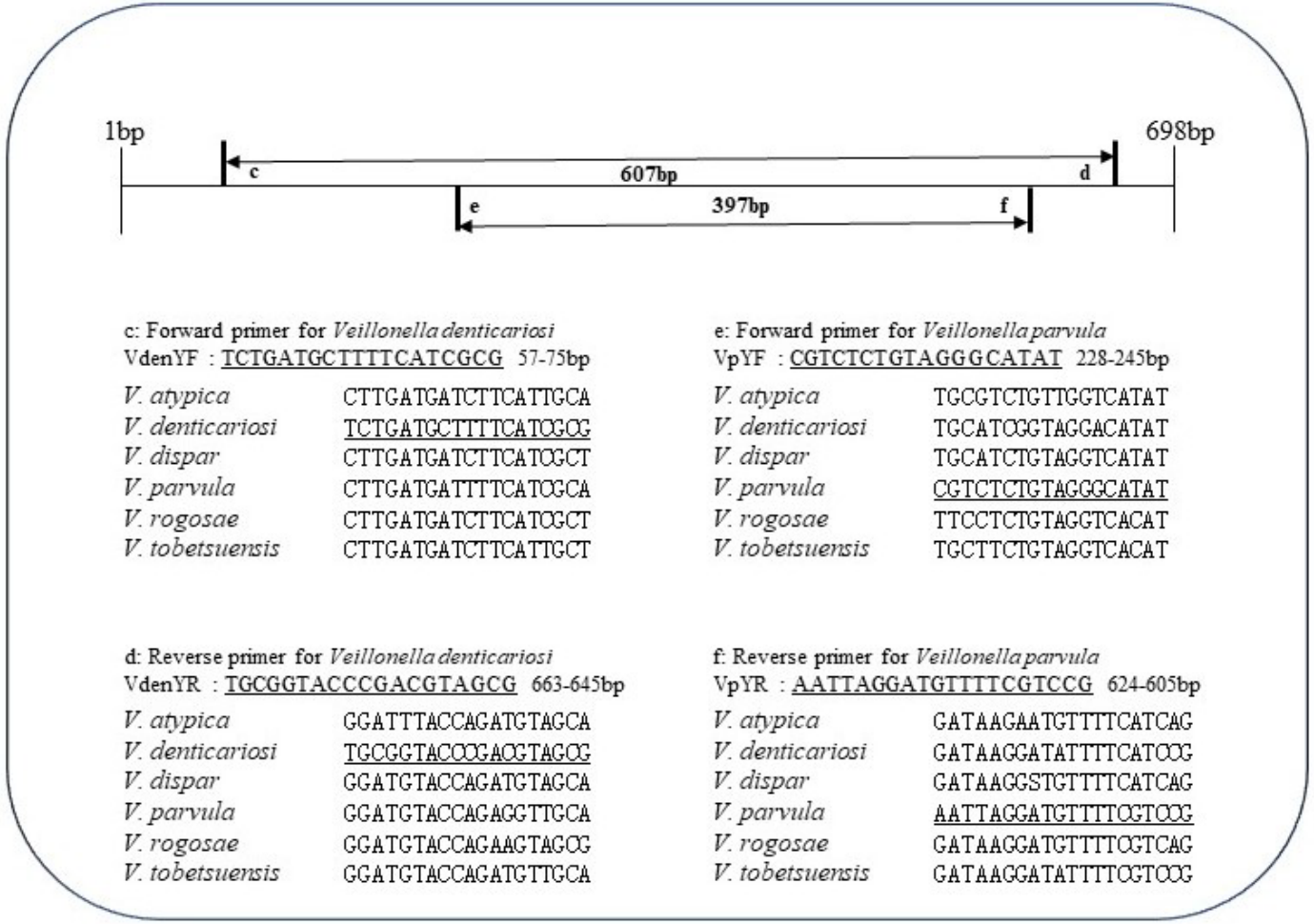

Figure 2. Locations and sequences of species-specific primers for the *gltA* gene of *V. denticariosi and V. parvula*.

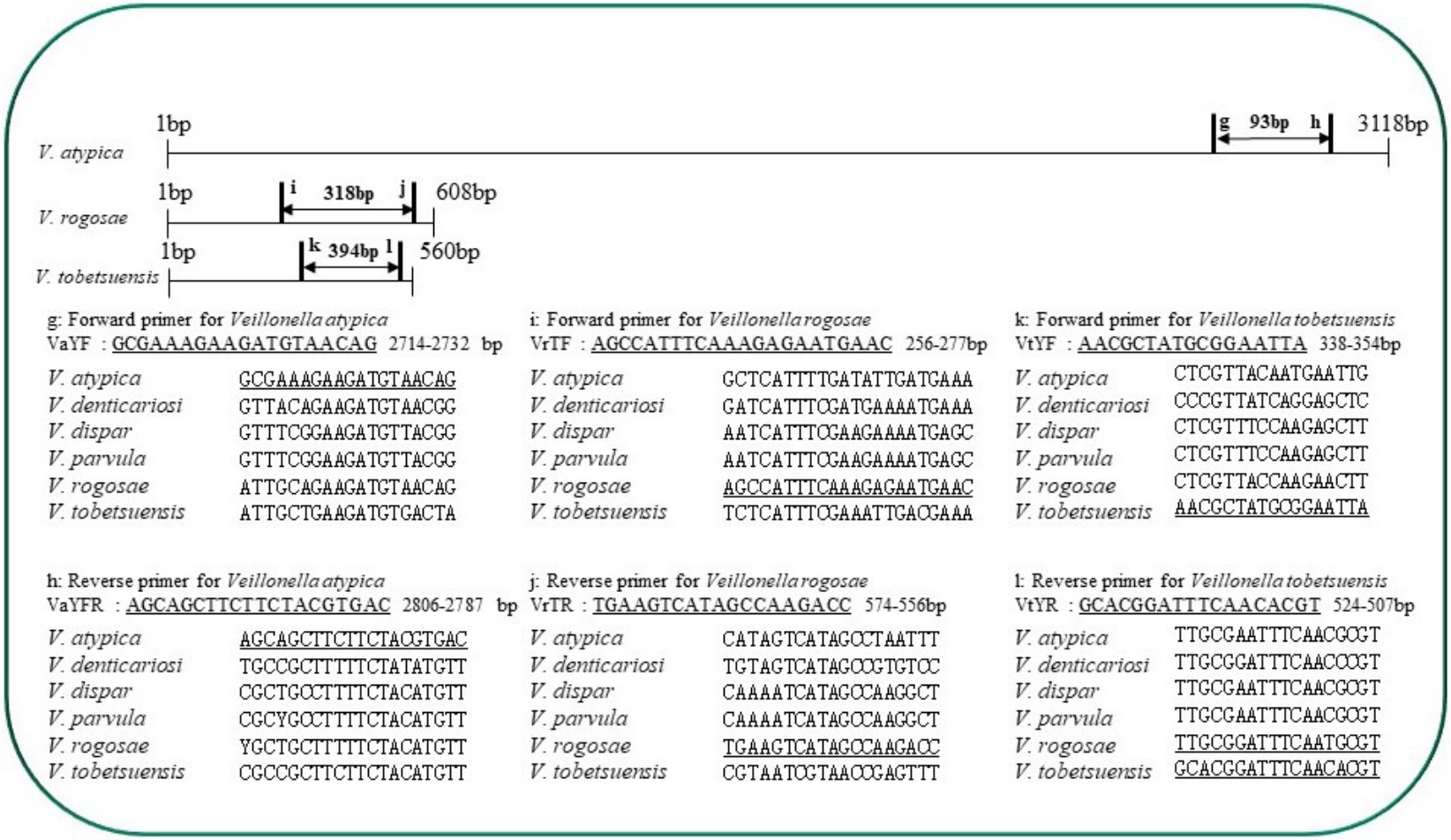

Figure 3. Locations and sequences of species-specific primers for the *rpoB* gene of *V. atypica*, *V. rogosae*, and *V. tobetsuensis*.

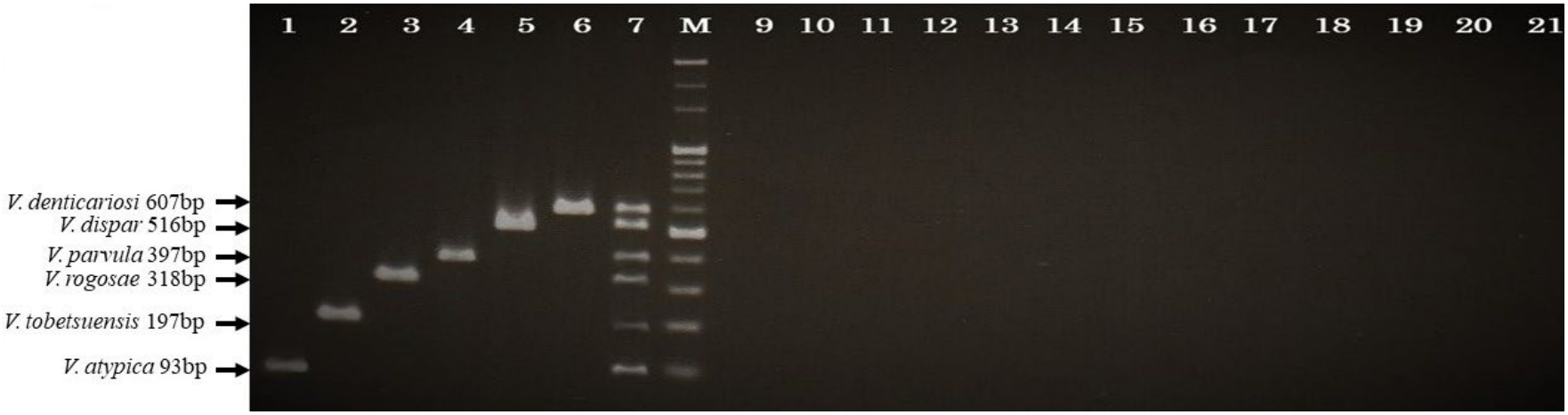

Figure 4. Multiplex PCR assay for identifying *Veillonella* species.

The primer mixture contained VaYF, VaYR, VdenYF, VdenYR, VpYF, VpYR, VdisYF, VdisYR, VrTF, VrTR, VtYF, and VtYR. VdisYF and VdisYR for *V. dispar* were designed on *dnaK* gene. VpYF, VpYR, VdenYF and VdenYR for *V. parvula* and *V. denticariosi* were designed on *gltA* gene. VaYF, VaYR, VrTF, VrTR, VtYF and VtYR for *V. atypica, V. rogosae* and *V. tobetsuensis* were designed on *rpoB* gene.Lanes: 1, *V. atypica* DSM 20739; 2, *V. tobetsuensis* JCM 17876; 3, *V. rogosae* JCM 15642; 4, *V. parvula* JCM 12972; 5, *V. dispar* DSM 20735; 6, *V. denticariosi* JCM 15641; 7, Mixture of *V. atypica, V. tobetsuensis*, *V. rogosae*, *V. parvula, V. dispar*, and *V. denticariosi*; 9, *S. goldonii* ATCC 10558; 10, *S. oralis* ATCC 10557; 11, *S. sanguinis* ATCC 10556; 12, *S. salivarius* JCM 5707; 13, *S. anginosus* ATCC 33397; 14, *S. mutans* NCTC 10449; 15, *Actinomyces naeslundii* ATCC 12104; 16, *A. oris* ATCC 27044; 17, *A. odontolyticus* ATCC17929; 18. *A. israelii* ATCC 12102; 19, *Corynebacterium matruchotii* ATCC 14266; 20, *C. durum* ATCC 33449; 21, *Neisseria sicca* ATCC 29256; M, molecular size marker (100-bp DNA ladder).

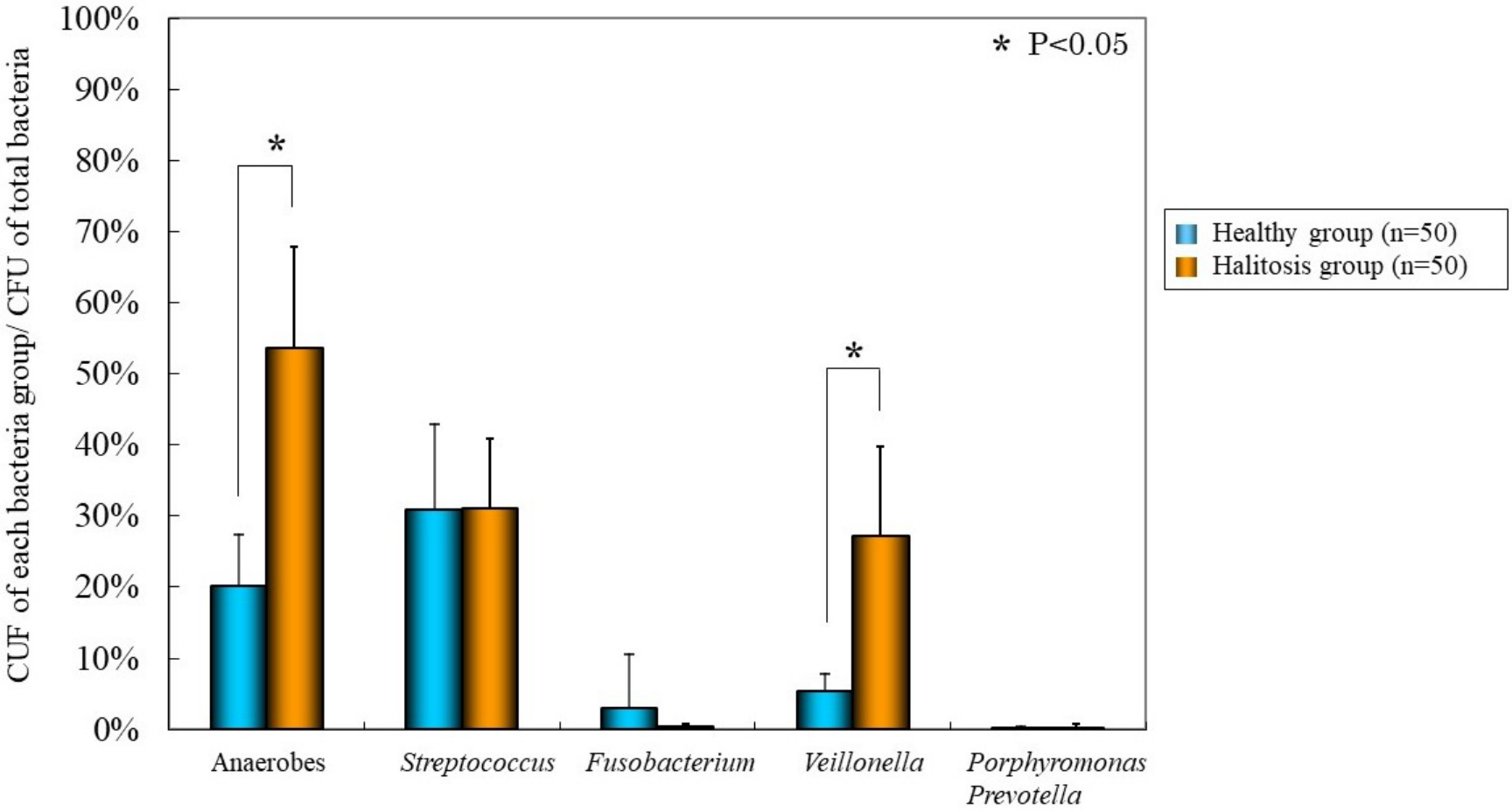

Figure 5. Comparison of the bacteria flora in tongue biofilm samples between 2 groups.

*Mann-Whitney U test; $p < 0.05$.

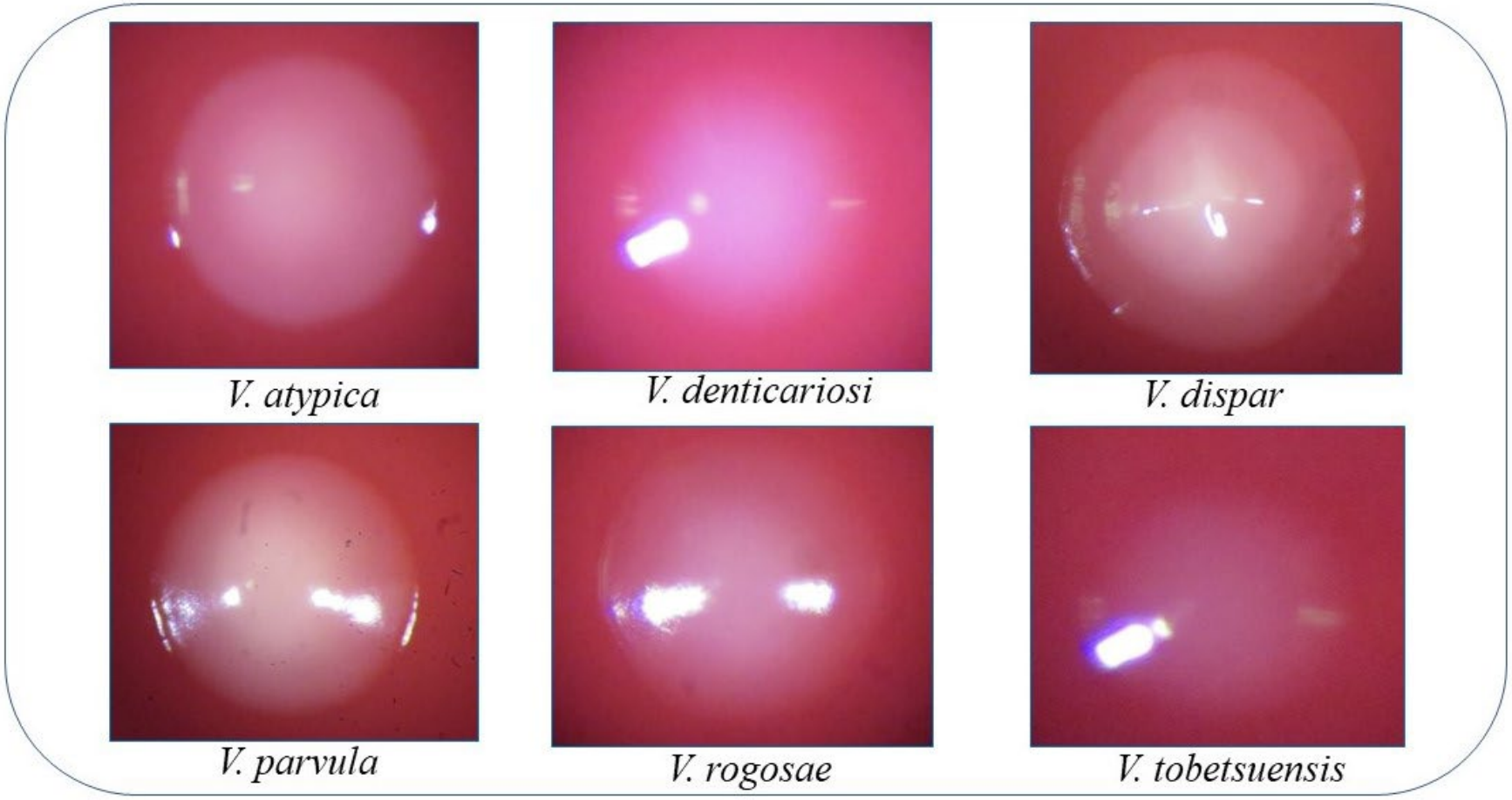

Figure 6. Stereomicroscope images of each oral *Veillonella* species colony on OVSM.