Original Research Article

The Distribution and Frequency of Oral Veillonella spp. Associated with Chronic Periodontitis

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ABSTRACT

Six species of oral Veillonella, V. atypica, V. denticariosi, V. dispar, V. parvula, V. rogosae, and V. tobetsuensis, have been suggested to facilitate succession of species in developing oral biofilms in vivo. However, the pathogenicity and distribution of oral Veillonella species in periodontal pockets have not been clarified. In this study, the distribution and frequency of oral Veillonella in periodontal pockets were examined at species level. Subgingival plaques were collected from eighteen patients who presented with chronic periodontitis. Samples were cultured under anaerobic condition after homogenization, dilution, and inoculation. Genomic DNA was extracted from each isolate for polymerase chain reaction analysis after CFUs were counted. To identify the Veillonella species present in the isolates, the species-specific primers for six oral Veillonella were used. V. parvula was the predominant species in the periodontal pockets. V. atypica, V. dispar, and V. tobetsuensis were the next most abundant species. This is the first report indicating the distribution and frequency of oral Veillonella spp. in the subgingival biofilm from periodontal pockets. As conclusion, V. parvula was frequently isolated from periodontal pockets as the predominant species. Therefore, V. parvula associated with the state of chronic periodontitis.

Keywords
Oral Veillonella; V. parvula; Periodontal pockets; Polymerase chain reaction; Species-specific primers

Introduction

Bacteria growing in natural settings often form biofilms. A biofilm is a community of bacteria attached to a substratum or surface. The bacteria in the biofilm are embedded in an extracellular polymeric matrix that they produce. Bacteria develop biofilms on submerged surfaces such as natural aquatic systems, water pipes, living tissues, tooth surfaces, in-dwelling medical devices, and implants (Meng et al., 2013). Such biofilms are not easily eliminated by immune responses and are resistant to antimicrobial agents (Bjarnsholt et al., 2013; Costerton et al., 1999).
Human dental plaque is a well-recognized example of a natural, oral biofilm. The composition of oral biofilms is different from health to disease. Dental plaque is the source of microorganisms that cause oral infections, including dental caries and periodontal disease (Maddi and Scannapieco, 2013). In addition, in the past 20 years, oral biofilms have increasingly been reported to be involved in systemic diseases (Maddi and Scannapieco, 2013; Mohangi et al., 2013; Okuda et al., 2004; Oppermann et al., 2012).

It was reported that the human oral cavity contains more than 19,000 microbial phylotypes (Keijser et al., 2008). Dental plaque is a multispecies biofilm, whose development is initiated by adherence of pioneer species to the salivary proteins and glycoproteins adsorbed onto the tooth enamel. Biofilms are not formed by random, simultaneous colonization by these species, but rather by selective, reproducible, and sequential colonization (Diaz et al., 2006; Nyvad and Kilian, 1987). Oral species of Veillonella have been reported to play a central role as early colonizers in the multispecies community formation involved in biofilm formation, and to facilitate species succession in development of oral biofilms in vivo (Saravananan and Kolenbrander, 2009; Saravananan and Kolenbrander, 2010). The genus Veillonella consists of small, strictly anaerobic, gram-negative cocci that lack flagella, spores, or a capsule. At the present time, 6 species, V. atypica, V. denticariosi, V. dispar, V. parvula, V. rogosae, and V. tobetsuensis, have been isolated from human oral cavities (Byun et al., 2007; Arif et al., 2008; Kolenbrander and Moore, 1992; Mays et al., 1982; Rogosa, 1984; Mashima et al., 2013). The primary habitats of these oral Veillonella spp. are dental plaque biofilms of the tongue and the buccal mucosa (Mays et al., 1982; Rogosa, 1984; Aas et al., 2005; Beighton et al., 2008; Mashima et al., 2013). V. parvula and other oral Veillonella spp. have also often been identified in cases of severe early childhood caries (Kanasi et al., 2010), in intraradicular infections (Sundqvist, 1992; Wittgow and Sabiston, 1975; Baumgartener and Falkler, 1991) including abscesses (Khemaleelakul et al., 2002), and in dentinal tubules in this region (Peters et al., 2001). They are volatile sulfur-compound-producing bacteria that are responsible for oral malodor (Aas et al., 2005; Faveri et al., 2006; Haraszthy et al., 2007; Hughes et al., 1988; Marger et al., 2003; Washio et al., 2005). However, the pathogenic roles of Veillonella spp. in oral infections have not yet been fully clarified, nor have the distribution and frequency of these 6 species been clarified in subgingival biofilm from patients who present with chronic periodontitis.

Previously, Igarashi et al. (Igarashi et al., 2009) reported the successful design of species-specific primer sets for 5 species of oral Veillonella (V. atypica, V. denticariosi, V. dispar, V. parvula, and V. rogosae) based on a highly variable region (positions 2500–3100) of the rpoB gene. In addition, Mashima and Nakazawa (2013) designed a species-specific primer pair for V. tobetsuensis based on a region (positions 424–1048) of the dnaK gene.

In a previous study, we investigated the distribution and frequency of oral Veillonella species in tongue biofilms of healthy adults using these species-specific primers (Mashima et al., 2011; Mashima and Nakazawa, 2013). These species-specific primers could be useful to clarify the distribution and frequency of oral Veillonella species, towards an investigation of their relationship with oral infections and systemic infectious diseases (Mashima et al., 2011; Mashima and Nakazawa, 2013; Igarashi et al., 2009).
Therefore, the aim of this study was to determine the distribution and frequency of oral Veillonella species in subgingival biofilms from patients presenting with chronic periodontitis using these species-specific primers. This is the first report indicating the distribution and frequency of oral Veillonella at the species level in biofilms from pathogenic sites of human oral cavities.

Materials and Methods

Subjects

This study received an approval from the Ethics Committee, Health Sciences University of Hokkaido, Hokkaido, Japan, under process number 084/2013, and data were collected over a period between 2013 and 2014. The participants were made aware of the objectives and procedures of the study and agreed to participate by providing written, informed consent.

Eighteen teeth were selected from 9 males and 9 females (age range: 26 to 80 years) with periodontal lesions diagnosed through clinical and radiographic examinations at the dental clinic of the Health Sciences University of Hokkaido. None of the patients was a smoker or was using any medication (including contraceptive pills for females).

The inclusion criteria included chronic periodontitis and the absence of systemic diseases. For the diagnosis of chronic periodontitis, the following clinical-radiographic factors were considered: clinical attachment loss, the presence of inflammation, probing depth $\geq 4$ mm and bone loss (Lindhe et al., 2003; Savage et al., 2009; Pereira et al., 2011). Exclusion criteria were the use of antibiotic therapy (within 6 months before sample collection).

Sampling of Subgingival Biofilm

Subgingival biofilm samples were collected, using sterile paper points, from the periodontal pockets. Samples were also collected from the gingival sulcus of the contra lateral site, as a control, for comparison of the distribution and frequency of Veillonella species in regions that did not exhibit periodontal disease.

Samples in reduced transport fluids were transported in an anaerobic box containing 80% N\textsubscript{2}, 10% CO\textsubscript{2}, and 10% H\textsubscript{2} to the Laboratory of Oral Microbiology, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan, in $<1$ hour from the time of collection. The samples were immediately placed in 1 mL of sterile saline. Samples were homogenized 100 times to disperse the biofilm and serially diluted 10-fold with sterile saline from $10^3$–$10^8$.

Culture Conditions

Aliquots of serial 10-fold dilutions (100 µL) were used to inoculate Bacto\textsuperscript{TM} Brain Heart Infusion (Difco Laboratories, BD) supplemented with 5% (volume/volume) defibrinated sheep blood (BHI agar), and the selective medium, Veillonella agar (Rogosa et al., 1958). After inoculation, all samples were incubated under anaerobic conditions (N\textsubscript{2}:CO\textsubscript{2}:H\textsubscript{2} = 80%:10%:10%) at 37°C; samples on Veillonella agar were incubated for 5 days and those on BHI agar were incubated for 7 days. The total number of bacteria in the samples was determined by counting the total number of colonies on the BHI agar, and the number of Veillonella was determined by counting the total number of typical Veillonella colonies on the Veillonella agar. Bacterial cells of typical colonies were confirmed using light microscopy after gram staining.
DNA extraction

Genomic DNA was extracted from individual bacterial cells using the InstaGene Matrix Kit (Bio-Rad) according to the manufacturer’s instructions. DNA concentrations were determined by measuring their absorption at OD_{260}.

Identification of Veillonella spp.

For identification of oral Veillonella at the species level, we used species-specific forward primers, DENF (5'-GAAAGAAGGCCGCACGCAGT-3') for *V. denticariosi*, PARF (5'-GAAGCATTGGAAGCGAAAGTTTCG-3') for *V. parvula*, ROGF (5'-ATTGCAGAAGATGTAACATGAGC-3') for *V. rogosae*, ATYF (5'-TCTCTTTGGGAAGAATTAGAACGC-3') for *V. atypica*, and DISF (5'-AACCGTGGAAATTGTCATGAAC-3') for *V. dispar* (Igarashi et al., 2009). In addition, a single reverse primer, VR (5'-GTGTAACAAGGGAGTACGGACC-3'), was used for all five oral Veillonella species. These primers were designed based on the sequence of a conserved region of the rpoB gene (Igarashi et al., 2009). The species-specific primer pair, forward VTF (5'-CTCTCAAACGTCAAGCACAACAAAAAGATGC-3') and reverse VTR (5'-GATAAGG TAGTTCATGATGCGTGGG-3'), were used for *V. tobetsuensis*; it was designed based on the sequence of a conserved region of the dnaK gene (Mashima et al., 2013). For identification of oral Veillonella at the genus level, we used a single primer set: Veill-rpoBF (5'-GTAACAAAGGTGTCGTTTC TCG-3') and Veill-rpoBR (5'-GCACCRTC AAATACAGG TGTAGC-3') (Arif et al., 2008; Mashima et al., 2013).

PCR Protocol

PCR was performed using 1 µL of template, 2.5 µL of each primer (10 pmol/mL), 19 µL of H_{2}O, and 25 µL of AmpliTaq Gold® 360 Master Mix (Applied Biosystems). These mixtures were subjected to preheating at 94°C for 15 min followed by 20 cycles of 92°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. When using VTF and VTR, the reactions were subjected to preheating at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, annealing at 61.5°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were applied to 1.5% agarose gels. Following electrophoresis, gels were stained with SYBR® Safe DNA gel stain.

Data Analysis

Statistical significance was determined using Wilcoxon t-test with ystat 2008 software. A *p*-value <0.05 was considered statistically significant.

Result and Discussion

The subgingival biofilms yielded high numbers of bacterial colonies on BHI agar. Mean (± SE) colony-forming units (CFU/mL) per paper point were 5.5 (± 0.3) × 10^6 with a median of 2.3 × 10^6 in periodontal pockets and 1.3 (± 0.09) × 10^6 with a median of 0.2 × 10^6 in the gingival sulcus, as a control (Table 1, 2). In addition, when the number of *Veillonella* spp. present in the samples from the 16 subjects was counted using selective medium (Table 1, 2), the mean (± SE) colony-forming units (CFU/mL) of *Veillonella* spp. per subject were 3.3 (± 0.2) × 10^5 with median of 0.6 × 10^5 in periodontal pockets and 1.3 (± 0.1) × 10^5 with a median of 0.01 × 10^5 at control sites. Typical *Veillonella* colonies on the selective medium were 2–4 mm in diameter, regular and slightly domed in shape with an
entire edge, opaque, and grayish white; they were composed of small, gram-negative coccal cells, mainly growing as single cells but with some short chains visible. The detection limit was dependent on the number of bacteria in the sample, but was <0.1% of the total colony count.

From the 18 periodontal pockets and 18 gingival sulcus sites, a total of 206 and 236 isolates, respectively, were identified as members of genus _Veillonella_ using the genus-specific primer set, Veill-rpoBF and Veill-rpoBR. Using species-specific primers, 399 of the 442 total isolates from both sites were identified as either _V. atypica_, _V. denticariosi_, _V. dispar_, _V. parvula_, _V. rogosae_, or _V. tobetsuensis_. Of the 206 isolates from periodontal pockets, 36, 24, 111, 10, and 22 isolates were identified as _V. atypica_, _V. dispar_, _V. parvula_, _V. rogosae_, and _V. tobetsuensis_, respectively. Of the 236 control isolates, 42, 47, 59, 13, and 35 isolates were classified as _V. atypica_, _V. dispar_, _V. parvula_, _V. rogosae_, and _V. tobetsuensis_, respectively.

The ratio of the number of isolates of each species to the total number of _Veillonella_ isolates from each subject is shown in table 1, 2. Figure 1 shows the ratio of the total number of isolates of each species to the total number of _Veillonella_ isolates, from both periodontal pockets and the gingival sulcus, respectively.

_V. parvula_ was found to be the predominant oral _Veillonella_ species, and _V. rogosae_ was detected at low frequency in both the periodontal pockets and the gingival sulcus in several subjects (Fig. 1). In our previous study, _V. rogosae_ was found to be the predominant species in tongue biofilms, but several isolates from that region were identified as _V. parvula_ (Mashima et al., 2011). Although there is no clear explanation at present, this result may indicate differential habitat selection between _V. parvula_ and _V. rogosae_ in human oral cavities.

_V. denticariosi_ has been established as the species most closely related to _V. rodentium_, according to 16S rRNA and _dnaK_ gene sequence analyses published in 2007 (Byun et al., 2007). To date, only 2 strains of _V. denticariosi_ have been isolated from human carious dentine (Byun et al., 2007) and 1 strain from human tongue biofilm (Mashima et al., 2011). In this study, no strain of _V. denticariosi_ was isolated from either periodontal pockets, or the gingival sulcus. This result might indicate that _V. denticariosi_ occupied different habitats in human oral cavity from other oral _Veillonella_ species.

_V. parvula_ was found to be the predominant oral species in both the periodontal pockets and the gingival sulcus (Fig. 1). In periodontal pockets, _V. parvula_ was detected in 14 out of 18 subjects. Furthermore, in subjects 1, 3, 5, and 14, only _V. parvula_ was isolated from periodontal pockets (Table 1). _V. atypica_, _V. dispar_, and _V. tobetsuensis_ were detected in both periodontal pockets and the gingival sulcus, at comparable levels (Fig. 1). However, _V. rogosae_ was detected at a low frequency, and _V. denticariosi_ was not detected in any of the subjects (Fig. 1).

Of the 442 strains isolated in this study, no PCR products were detected using the species specific primers with DNA from 43 isolates from 2 subjects from periodontal pockets (3 isolates) and in 3 subjects from gingival sulcus as control (40 isolates).

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_Hellar et al._ (2012) and _Silva-Boghossian et al._ (2013) identified _Veillonella_ species in subgingival biofilm samples from patients presenting with chronic periodontitis.
Similarly, Takeshita et al. (2009) demonstrated that oral Veillonella species were more often predominant in saliva from patients with chronic periodontitis than in healthy people. However, these reports did not identify the Veillonella isolates to the species level.

In this study, Veillonella species-specific primer sets were used for identification of 442 isolates identified as member of genus Veillonella. Consequently, five of the six known species of oral Veillonella were identified, with the exception of V. denticariosi, in these subjects collected from the periodontal pockets and the gingival sulcus. V. parvula was frequently detected as the predominant species in periodontal pockets and the gingival sulcus as control. Compared with subjects from the gingival sulcus as control, the number of isolates of V. parvula was significantly high level in the subjects from periodontal pockets (Fig. 2). These findings indicate that frequent isolation of V. parvula might be an index for the state of chronic periodontitis.

Many types of infections have been reported to be caused by V. parvula, such as meningitis and V. parvula bacteremia (Frank et al., 2000; Fisher and Denison, 1996). Recently, it was reported that dysbiosis of salivary microbiota, such as Veillonella species, is associated with inflammatory responses in patients with inflammatory bowel disease (Said et al., 2014). In addition, oral biofilms have increasingly been reported to be involved in systemic diseases (Maddi and Scannapieco, 2013; Mohangi et al., 2013; Okuda et al., 2004; Oppermann et al., 2012).

These reports and our findings in this study suggest that patients who present with chronic periodontitis are likely to be affected with other systemic diseases caused by Veillonella species, especially V. parvula.

Several reports have demonstrated that Veillonella produces vitamin K, which is thought to stimulate the growth of the typical periodontal pathogen Porphyromonas gingivalis (Marcotte and Lavoie, 1998; Hojo et al., 2007). In a summary of the implications of Veillonella in periodontal diseases, Delwiche et al. (Delwiche et al., 1985) stated that: (a) Veillonella makes up part of the microbial community of biofilms and becomes more prominent as the biofilm develops; (b) Veillonella produces highly lipopolysaccharides (LPS); (c) Veillonella can form associations with other oral microbes that facilitate its establishment in the oral microbial ecosystem. Thus, although Veillonella species have so far been recognized as early colonizers in oral biofilms (Saravanan and Kolenbrander, 2009; Saravanan and Kolenbrander, 2010; Mashima and Nakazawa, 2014), it was suggested in this study that they might also contribute to form the biofilm at late stage as the one of periodontal pathogens.

In this study, 43 isolates could not be identified as belonging to the 6 oral Veillonella species reported to date, using species-specific primer sets for PCR. However, these 43 strains were confirmed as members of the genus Veillonella, using Veillonella genus-specific primers. Although only six species of oral Veillonella have previously been reported, these results suggest that other Veillonella species may also inhabit the human oral cavity. In addition, these 43 isolates may be members of one or more novel Veillonella species.
Table.1 The ratio of the number of isolates of each species to the total number of Veillonella isolates from periodontal pockets

<table>
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<tr>
<th>Subject</th>
<th>Sex</th>
<th>Total number</th>
<th>All bacteria CFU/mL (×10^3)</th>
<th>Veillonella spp CFU/mL (×10^3)</th>
<th>Isolated Veillonella spp Total number (100%)</th>
<th>V. dispar (%)</th>
<th>V. parvula (%)</th>
<th>V. rogosae (%)</th>
<th>V. tobetsuensis (%)</th>
<th>Unknown (%)</th>
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The total colony counts for anaerobic bacteria on BHI agar, the total colony counts for Veillonella species on Veillonella agar, and the total number of isolates identified using the Veillonella genus-specific primer set. CFU: colony forming unit; detection limit <0.1% of the total count. Individual species as a percentage of the number of isolates identified using the species-specific primer sets for each subject (n = 18) from the periodontal pockets.

Table.2 The ratio of the number of isolates of each species to the total number of Veillonella isolates from the gingival sulcus, as a control

<table>
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<tr>
<th>Subject</th>
<th>Sex</th>
<th>Total number</th>
<th>All bacteria CFU/mL (×10^3)</th>
<th>Veillonella spp CFU/mL (×10^3)</th>
<th>Isolated Veillonella spp Total number (100%)</th>
<th>V. dispar (%)</th>
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<th>V. rogosae (%)</th>
<th>V. tobetsuensis (%)</th>
<th>Unknown (%)</th>
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Total colony counts of anaerobic bacteria on BHI agar, total colony counts of Veillonella species on Veillonella agar, and total number of isolates identified using the Veillonella genus-specific primer set. CFU: colony forming unit; detection limit <0.1% of the total count. Individual species as a percentage of the number of isolates identified using the species-specific primer sets for each subject (n = 18) from the gingival sulcus, as a control.
Fig.1 The percentage of the six oral *Veillonella* species (including unknown strains belonging to the genus *Veillonella*). Total isolated number of each *Veillonella* species were expressed in percent of total isolated number as *Veillonella* in samples (n = 18) from the periodontal pockets and in samples (n = 18) the gingival sulcus, as control.

Fig.2 Significant difference in number of V. *parvula* between periodontal pockets and gingival sulcus * P < 0.05

In conclusion, to the best of our knowledge, this is the first report indicating the distribution and frequency of oral *Veillonella* species in the subgingival biofilm from periodontal pockets, as determined using species-specific PCR primers. The results of this study demonstrate that the distribution and frequency of oral *Veillonella* differed in different oral sites. The results also suggested that several factors are likely to influence their colonization of human oral cavities. In addition, the present study showed that the frequency of *V. parvula* was significantly higher in the periodontal pockets than that in the gingival sulcus when the frequency was compared at species level among oral *Veillonella*. Therefore, *V. parvula* associated with the state of chronic periodontitis. Further studies are needed to reveal the pathogenic roles of oral *Veillonella* species in oral infections, especially chronic periodontitis.

Acknowledgments

We are grateful to Dr. Arihide Kamaguchi...
and Hiroshi Miyakawa of the Department of Oral Microbiology, School of Dentistry, Health Sciences University of Hokkaido, for technical assistance. This study was supported in part by a JSPS KAKENHI Grant Number 26462793, a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology, a Grant-in-Aid for the 2014-2015 Research Project of the Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, and an Iwadare Scholarship from the Iwadare Scholarship Foundation.

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