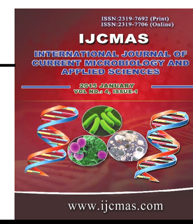
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 3 (2015) pp. 150-160**
http://www.ijcmas.com



Original Research Article

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

Izumi Mashima[1], Mari Fujita[1], Yuko Nakatsuka[2], Takashi Kado[2], Yasushi Furuichi[2], Sulistyani Herastuti[1] and Futoshi Nakazawa[1]*

[1]Department of Oral Microbiology, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido Japan
[2]Department of Periodontology and Endodontology, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan
**Corresponding author*

A B S T R A C T

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* (Saravanan and Kolenbrander, 2009; Saravanan 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_2, 10% CO_2, and 10% H_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™ 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_2:CO_2:H_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′-GAAAGAAGCGCGCACCGACAGT-3′) for *V. denticariosi*, PARF (5′-GAAGCATTGGAAGCGAAAGTTTCG-3′) for *V. parvula*, ROGF (5′-ATTGCAGAAGATGTAACATGAAGC-3′) for *V. rogosae*, ATYF (5′-TCTCTTTGGGAAGAATTAGAACGC-3′) for *V. atypica*, and DISF (5′-AACGCGTTGAAATTCGTCATGAAC-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′-CTCTCAACGTCAAGCAACAAAAGATG C-3′) and reverse VTR (5′-GATAAGG TAGTTCATGATGCGTTGG-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_2O, 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) $\times 10^6$ with a median of 2.3 $\times 10^6$ in periodontal pockets and 1.3 (± 0.09) $\times 10^6$ with a median of 0.2 $\times 10^6$ 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) $\times 10^5$ with median of 0.6 $\times 10^5$ in periodontal pockets and 1.3 (± 0.1) $\times 10^5$ with a median of 0.01 $\times 10^5$ 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 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).

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.

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

Subject	Sex	Total number All bacteria CFU/mL (× 10^6^)	*Veillonella* spp. CFU/mL (× 10^5^)	Isolated *Veillonella* spp. Total number (100%)	*V. atypica* (%)	*V. denticariosi* (%)	*V. dispar* (%)	*V. parvula* (%)	*V. rogosae* (%)	*V. tobetsuensis* (%)	Unknown (%)
1	M	2.6	10.0	10	0.0	0.0	0.0	100.0	0.0	0.0	0.0
2	F	0.9	1.3	13	15.4	0.0	15.4	30.8	0.0	38.5	0.0
3	M	3.8	1.7	17	0.0	0.0	0.0	100.0	0.0	0.0	0.0
4	M	1.5	0.14	14	0.0	0.0	0.0	50.0	7.1	42.9	0.0
5	F	1.0	0.05	5	0.0	0.0	0.0	100.0	0.0	0.0	0.0
6	M	3.8	0.02	20	30.0	0.0	10.0	50.0	0.0	0.0	10.0
7	F	5.2	0.018	18	61.1	0.0	16.7	0.0	0.0	16.7	5.6
8	F	0.04	0.019	19	57.9	0.0	15.8	21.1	5.3	0.0	0.0
9	F	28.3	0.0013	13	7.7	0.0	69.2	7.7	7.7	7.7	0.0
10	F	26.5	1.0	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0
11	F	0.09	1.5	15	13.3	0.0	0.0	66.7	13.3	6.7	0.0
12	F	0.08	0.0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13	M	0.1	0.04	4	25.0	0.0	50.0	25.0	0.0	0.0	0.0
14	M	1.9	1.3	13	0.0	0.0	0.0	100.0	0.0	0.0	0.0
15	M	6.3	11.0	11	0.0	0.0	9.1	81.8	9.1	0.0	0.0
16	M	6.6	18.0	18	0.0	0.0	11.1	44.4	22.2	22.2	0.0
17	F	8.8	14.0	14	0.0	0.0	0.0	85.7	0.0	14.3	0.0
18	M	1.5	0.0001	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0

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

Subject	Sex	Total number All bacteria CFU/mL (× 10^6^)	*Veillonella* spp. CFU/mL (× 10^5^)	Isolated *Veillonella* spp. Total number (100%)	*V. atypica* (%)	*V. denticariosi* (%)	*V. dispar* (%)	*V. parvula* (%)	*V. rogosae* (%)	*V. tobetsuensis* (%)	Unknown (%)
1	M	0.1	0.5	5	0.0	0.0	0.0	100.0	0.0	0.0	0.0
2	F	0.06	0.0015	15	0.0	0.0	0.0	0.0	0.0	100.0	0.0
3	M	0.5	0.001	10	20.0	0.0	60.0	20.0	0.0	0.0	0.0
4	M	0.07	0.018	18	0.0	0.0	11.1	0.0	11.1	77.8	0.0
5	F	1.0	1.4	14	35.7	0.0	0.0	50.0	14.3	0.0	0.0
6	M	0.1	0.02	20	10.0	0.0	15.0	20.0	0.0	0.0	55.0
7	F	0.2	0.0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	F	0.02	0.002	20	60.0	0.0	40.0	0.0	0.0	0.0	0.0
9	F	0.02	0.002	11	0.0	0.0	0.0	90.9	9.1	0.0	0.0
10	F	0.3	0.031	31	48.4	0.0	51.6	0.0	0.0	0.0	0.0
11	F	0.004	0.0011	11	27.3	0.0	0.0	63.6	0.0	9.1	0.0
12	F	0.0002	0.0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13	M	9.0	8.0	8	12.5	0.0	0.0	87.5	0.0	0.0	0.0
14	M	8.4	12.0	12	0.0	0.0	0.0	100.0	0.0	0.0	0.0
15	M	0.7	0.14	14	7.1	0.0	14.3	0.0	0.0	0.0	78.6
16	M	0.04	0.002	20	0.0	0.0	40.0	20.0	25.0	5.0	10.0
17	F	1.0	0.7	7	0.0	0.0	28.6	14.3	0.0	57.1	0.0
18	M	1.0	0.002	20	5.0	0.0	0.0	0.0	15.0	0.0	80.0

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

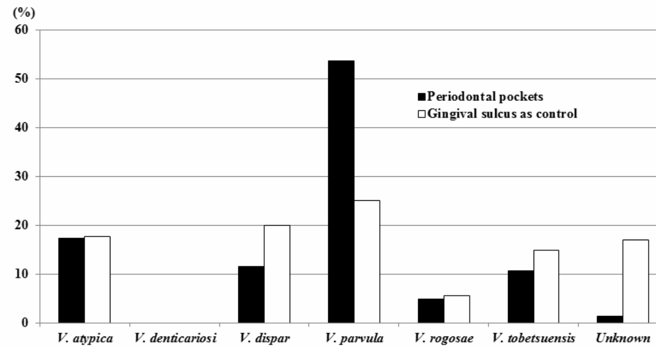
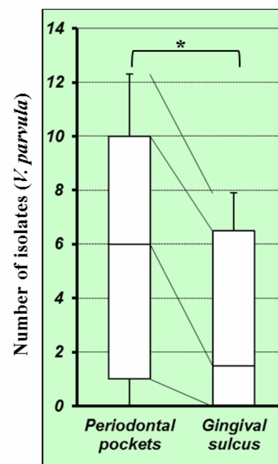


Fig.2 Significant difference in number of V. parvula between periodontal pockets and gingival sulcus * P < 0.05



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.

References

Aas, J.A., Paster, B.J., Stokes, L.N., Olsen, I., Dewhirst, F.E. 2005. Defining the normal bacteria flora of the oral cavity. *J. Clin. Microbiol.,* 43: 5721–5732.

Arif, N., Do, T., Byun, R., Sheehy, E., Clark, D., Gilbert, S.C., Beighton, D. 2008. *Veillonella rogosae* sp. nov., an anaerobic, Gram-negative coccus isolated from dental plaque. *Int. J. Syst. Evol. Microbiol.,* 58: 581–584.

Baumgartener, J.C., Falkler, W.A. Jr. 1991. Bacteria in the apical 5 mm of infected root canals. *J. Endod.,* 17: 272–282.

Beighton, D., Clark, D., Hanakura, B., Gilbert, S., Do, T. 2008. The predominant cultivable *Veillonella* spp. of the tongue of healthy adults identified using *rpoB* sequencing. *Oral. Microbiol. Immunol.,* 23: 344–347.

Bjarnsholt, T., Alhede, M., Alhede, M., Eickhardt-Sørensen, R.S., Moser, C., Kühl, M., Jensen. Ø.P., Høiby, N. 2013. The *in vivo* biofilm. *Trend. Microbiol.,* 21: 466–474.

Byun, R., Carlier, J.P., Jacques, N.A., Marchandin, H., hunter, N. 2007. *Veillonella denticariosi* sp. nov., isolated from human carious dentine. *Int. J. Syst. Evol. Microbiol.,* 57: 2844–2848.

Costerton, J.W., Stewart, P.S., Greenberg, E.P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science,* 284: 1318–1322.

Delwiche, E.A., Pestka, J.J., Tortorello, M.L. 1985. The Veillonellae: gram negative cocci with unique physiology. *Annu. Rev. Microbiol.,* 39: 175–193.

Diaz, P.I., Chalmers, N.I., Rickard, A.H., Kong, C., Milburn, R.J., Palmer, Jr., Kolenbrander, P.E. 2006. Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Appl. Environ. Microbiol.,* 72: 2837–2848.

Faveri, M., Feres, M., Shibli, J.A., Hayacibara, R.F., Hayacibara, M.M., de Figueiredo, L.C. 2006. Microbiota of the dorsum of the tongue after plaque accumulation: an experimental study in humans. *J. Periodontol.,* 77: 1539–1546.

Fisher, G.R., Denison, R.M. 1996. *Veillonella parvula* Bacteremia without an Underlying source. *J. Clin. Microbiol.,* 34: 3235–3236.

Frank, O.M. 2000. *Veillonella parvula* Meningitis: Case Report and Review of *Veillonella* Infections. *Clin. Infect. Dis.,* 31: 839–840.

Haraszthy, V.I., Zambon. J.J., Sreenivasan, P.K., Zambon, M.M., Gerber, D., Rego, R., Parker, C. 2007. Identification of oral bacterial species associated with halitosis. *J. Am. Dent. Assoc.,* 138: 1113–1120.

Heller, D., Silva-Boghossian, C.M., do Souto, R.M., Colombo, A.P. 2012. Subgingival microbial profiles of generalized aggressive and chronic periodontal diseases. *Arch. Oral. Biol.,* 57: 973–980.

Hojo, K., Nagaoka, S., Murata, S., Taketomo, N., Ohshima, T., Maeda, N.

2007. Reduction of vitamin K Concentration by salivary *Bifidobacterium* strains and their possible nutritional competition with *Porphyromonas gingivalis*. *J. Appl. Microbiol.,* 103: 1964–1974.

Hughes, C.V., Kolenbrander, P.E., Andersen, R.F., Moore, L.V. 1988. Coaggregation properties of human oral *Veillonella* spp.: relationship to colonization site and oral ecology. *Appl. Environ. Microbiol.,* 54: 1957–1963.

Igarashi, E., Kamaguchi, A., Fujita, M., Miyakawa, H., Nakazawa, F. 2009. Identification of oral species of the genus *Veillonella* by polymerase chain reaction. *Oral. Microbiol. Immunol.,* 24: 1–4.

Kanasi, E., Dewhirst, F.E., Chalmers, N.I., Kent, R. Jr., Moore, A., Hughes, C.V., Pradhan, N., Loo, C.Y., Tanner, A.C. 2010. Clonal analysis of the microbiota of severe early childhood caries. *Caries Res.,* 44: 485–497.

Keijser, B.J.F., Zaura, E., Huse, S.M., Van der Vossen, J.M.B.M., Schuren, F.H.J., Montijn, R.C., Ten Cate, J.M., Crielaard, W. 2008. Pyrosequencing analysis of the oral microflora of healthy adults. *J. Dent. Res.,* 87: 1016–1020.

Khemaleelakul, S., Baumgartner, J.C., Pruksakorn, S. 2002. Identification of bacteria in acute endodontic infections and their antimicrobial susceptibility. *Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod.,* 94: 746–755.

Kolenbrander, P.E., Moore, L.V.H. 1992. The genus *Veillonella*. In: Balows, H.G., Trüper, M., Dworkin, W., Harder, W., Schleifer, K.H. (Eds). The prokaryotes, 2nd edn. Springer, New York. Pp. 2034–2047.

Lindhe, J., Karing, T., Lang, N.P. 2003. Clinical periodontology and implant dentistry, 4th edn. Blackwell Munksgard, Oxford, United Kingdom. 1448 Pp.

Maddi, A., Scannapieco, F.A. 2013. Oral biofilms, oral and periodontal infections, and systemic disease. *Am. J. Dent.,* 26: 249–254.

Marcotte, H., Lavoie, M.C. 1998. Oral microbial ecology and the role of salivary immunoglobulin A. *Microbial. Mol. Biol. Rev.,* 62: 71–109.

Marger, D.L., Ximenez-Fyvie, L.A., Haffajee, A.D., Socransky, S.S. 2003. Distribution of selected bacterial species on intraoral surface. *J. Clin. Periodontol.,* 30: 644–654.

Mashima, I., Kamaguchi, A., Miyakawa, H., Nakazawa, F. 2013. *Veillonella tobetsuensis* sp. nov., an anaerobic, Gram-negative coccus isolated from human tongue biofilms. *Int. J. Syst. Evol. Microbiol.,* 63: 1443–1449.

Mashima, I., Kamaguchi, A., Nakazawa, F. 2011. The distribution and frequency of oral *Veillonella* spp. in the tongue biofilm of healthy young adults. *Curr. Microbiol.,* 63: 403–407.

Mashima, I., Nakazawa, F. 2013. Identification of *Veillonella tobetsuensis* in tongue biofilm by using a species-specific primer pair. *Anaerobe,* 22: 77–81.

Mashima, I., Nakazawa, F. 2014. The influence of oral *Veilloenlla* species on biofilms formed by *Streptococcus* species. *Anaerobe,* 28: 54–61.

Mays, T.D., Holdeman, L.V., Moore, W.E.C., Rogosa, M., Johnson, J.L. 1982. Taxnomy of the genus *Veillonella* Prèvot. *Int. J. Syst. Bacteriol.,* 32: 28–36.

Meng, C., Qingsong, Y., Hongmin, S. 2013. Novel strategies for the prevention and treatment of biofilm related infections. *Int. J. Mol. Sci.,* 14: 18488–18501.

Mohangi, G.U., Singh-Rambirich, S.,

Volchansky, A. 2013. Periodontal disease: Mechanisms of infection and inflammation and possible impact on miscellaneous systemic diseases and conditions. *SADJ,* 68: 462–467.

Nyvad, B., Kilian, M. 1987. Microbiology of the early colonization of human enamel and root surfaces in vivo, Scand. *J. Dent. Res.,* 95: 369–380.

Okuda, K., Kato, T., Ishihara, K. 2004. Involvement of periodontopathic biofilm in vascular diseases. *Oral Dis.,* 10: 5–12.

Oppermann, V.R., Weidlich, P., Musskopf, L.M. 2012. Periodontal disease and systemic complications. *Braz. Oral. Res.,* 26: 39–47.

Pereira, C.V., Stipp, R.N., Fonseca, D.C., Pereira, L.J., Höfling, J.F. 2011. Detection and clonal analysis of anaerobic bacteria associated to endodontic-periodontal lesions. *J. Periodontol.,* 82: 1767–75.

Peters, L.B., Wesselink, P.R., Buijs, J.F., van Winkelhoff, A.J. 2001. Viable bacteria in root dentinal tubules of teeth with apical periodontitis. *J. Endod.,* 27: 76–81.

Rogosa, M. 1984. Anaerobic Gram-negative cocci. 1984. In: Bergey's manual of systematic bacteriology, Vol. 1. Krieg, N.R., Holt, J.G. (Eds), Williams and Wilkins, Baltimore. Pp. 680–685.

Rogosa, M., Fitzgerald, R.J., Mackintosh, M.E., Beaman, A.J. 1958. Improved medium for selective isolation of *Veillonella*. *J. Bacteriol.,* 76: 455–456.

Said, S.H., Suda, W., Nakagome, S., Chinen, H., Oshima, K., Kim, S., Kimura, R., Ihara, A., Ishida, H., Fujita, J., Mano, S., Morita, H., Dohi, T., Ootta, H., Hattori, M. 2014. Dysbiosis of salivary microbiota in inflammatory bowel disease and its association with oral immunological biomarkers. *DNA Res.,* 21: 15–25.

Saravanan, P., Kolenbrander, P. E. 2009. Mutualistic biofilm communities develop with *Porphyromonas gingivalis* and initial, early, and late colonizers of enamel. *J. Bacteriol.,* 191: 6804–6811.

Saravanan, P., Kolenbrander, P.E. 2010. Central role of the early colonizer *Veillonella* sp. in establishing multispecies biofilm communities with initial, middle, and late colonizers of enamel. *J. Bacteriol.,* 192: 2965–2972.

Savage, A., Eaton, K.A., Moles, D.R., Neeleman, I. 2009. A systematic review of definitions of periodontits and methods that have been used to identify this disease. *J. Clin. Periodontol.,* 36: 458–467.

Silva-Boghossian, C.M., Neves, A.B., Resende, F.A., Colombo, A.P. 2013. Suppuration associated bacteria in subjects with chronic and aggressive periodontitis. *J. Periodontol.,* 84: e9–e16.

Sundqvist, G. 1992. Associations between microbial species in dental root canal infections. *Oral. Microbiol. Immunol.,* 23: 47–51.

Takeshita, T., Nakano, Y., Kumagai, T., Yasui, M., Kamio, N., Shibata, Y., Shiota, S., Yamashita, Y. 2009. The ecological proportion of indigenous bacterial populations in saliva is correlated with oral health status. *ISME J.,* 3: 65–78.

Washio, J., Sato, T., Koseki, T., Takahashi, N. 2005. Hydrogen sulfide-producing bacteria in tongue biofilm and their relationship with oral malodour. *J. Med. Microbiol.,* 54: 889–895.

Wittgow, W.C. Jr., Sabiston, C.B. Jr. 1975. Microorganisms from pulpal chambers of intact teeth with necrotic pulps. *J. Endo.,* 1: 168–171.