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〔原著〕

Phylogenetic Study of Oral *Veillonella* Isolates from Saliva of Children in Hokkaido

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Key words : Oral *Veillonella* species ; Saliva ; Children ; Oral hygiene status ; One−step PCR ; Hokkaido

Abstract

The objective of this study is to analyze the distribution and frequency of oral *Veillonella* species isolated from saliva of the children with different oral hygiene status in Hokkaido, also the phylogenetic study oral *Veillonella* species. The 18 children consisted of 10 males and 8 females, ranging from 4 to 13 years of children in age were divided into two groups. The first group (good oral hygiene) was composed of 9 children (3 males and 6 females) with the Simplified Oral Hygiene Index (OHI－S) scores of 0－1.2. The second group (moderate oral hygiene) was composed of 9 children (7 males and 2 females) with OHI−S scores of 1.3 –3.0. *Veillonella* isolates were identified at the species level using one−step PCR with species−specific primer sets. In addition, phylogenetic analysis based on 16S rRNA, *rpoB* and *dnaK* gene sequences for the unknown strains were used in this study. Our results indicated that *V. rogosae* and *V. atypica* were higher in good oral hygiene group. Meanwhile, *V. dispar* had been higher significantly as the oral hygiene status decreased. In contrast, the detection rates of *V. parvula* lower significantly in good oral hygiene group as the oral hygiene status decreased. Furthermore, phylogenetic analysis of 16S rRNA, *rpoB* and *dnaK* gene sequences of unknown strains from the saliva samples suggested novel species of the genus *Veillonella* in the oral cavity of children. In conclusion, the results of this study indicated that changes in number of some oral *Veillonella* species in the saliva of Hokkaido children could serve as an index for deteriorating oral hygiene status. In addition, the present study indicated the possibility of a number of novel *Veillonella* species in the oral cavity of the children.

Introduction

The most common childhood oral infectious disease is dental caries caused by biofilm on the teeth and gums surface. It is estimated to occur five times more often than the second most common childhood illness, asthma (Peterson et al., 2013). Therefore, it is essential to understand more fully the mechanisms of cariogenic strain activity based on the biofilm formed by the microbial, which may be applied in the prevention and early diagnostics of dental caries in children.

Oral biofilm have three−dimensional structures, consisting of bacterial species anchored to solid surfaces such as tooth enamel, tooth roots or dental implants (Zijnge et al., 2010). These biofilm also consist of proteins from the saliva (Huang et al., 2011). Saliva is recognized as pool of biological markers that consist of biochemical molecules, such as DNA, RNA and proteins, also the structure of microbiota (Zhang et al., 2016). Furthermore, saliva plays an important role in preventing the microbial imbalance and maintaining

health in oral cavity (Mandel, 1989 ; Dawes et al., 2015).

It was reported that *Veillonella* species has a central role in oral biofilm formation as an early colonizer with *Streptococcus* species reported as initial colonizer for establishing multispecies oral biofilm communities (Periasamy & Kolenbrander 2010 ; Li et al., 2004 ; Kreth et al., 2005). Crielaard et al. (2011) demonstrated that the relative abundance of *Veillonella* species in saliva increased from deciduous to permanent dentition. Additionally, in the case of dental caries, oral *Veillonella* species has been found in the entire oral cavity, comprise as much as 10% of the bacterial community initially colonizing the enamel (Chalmers et al., 2008).

The genus *Veillonella* consists of small, strictly anaerobic, gram−negative cocci lacking flagella, spores, and capsules, and they are characterized by their ability to obtain energy from short−chain organic acids (Carlier, 2015 ; Delwiche et al., 1985). Currently, 14 species have been established in the genus *Veillonella*. From these species, only 7 species (*V. atypica, V. denticariosi, V. dispar, V. parvula, V. rogosae, V. tobetsuensis and V. infantium*) have been isolated from human oral cavities (Mashima et al., 2016 ; 2018). The main habitats of the oral *Veillonella* are tongue biofilm, dental biofilm, buccal mucosa, and saliva (Arif et al., 2008 ; Hughes et al., 1988 ; Mashima et al., 2016). Oral *Veillonella* has been found in severe early childhood caries (Kanasi et al., 2010), apical root canals (Baumgartener & Falkler, 1991), and dental tubules (Peters et al., 2001). Additionally, oral *Veillonella* are also predominantly found in saliva (Takeshita et al., 2009) and subgingival biofilm specimens (Heller et al., 2012) from patients with chronic periodontitis.

Many reports indicated that age, oral hygiene habits, diet and potentially socioeconomic status might influence the oral microbiota community structures (Haffajee et al., 2006 ; Tanwir et al., 2009 ; Belstrøm et al., 2014 ; Theodorea et al., 2017). In addition, the possibility of having country−, community−, or family−specific distribution and frequency of bacteria in oral biofilm associated with life style has been considered to affect the oral bacterial composition (Splieth et al., 2016 ; Belstrom et al., 2014). Hence, an understanding of the composition and ecological events that drive changes in biofilm composition, from good to poor oral hygiene status, is required to develop novel preventive strategies to promote oral health in children.

In this study, therefore, we analyzed the distribution and frequency of oral *Veillonella* species isolated from saliva of the children with different oral hygiene status in Hokkaido. Furthermore, this study seeks to report the phylogenetic position and diversity of the unknown *Veillonella* strains by phylogenetic analysis based on the 16S rRNA, *rpoB*, and *dnaK* gene sequences.

Materials and Methods

Ethics Statement

The saliva samples used in this study was collected in Hospital of Health Sciences University of Hokkaido, Ainosato, Hokkaido, Japan conducted between 2016 and 2017. Prior to the experiment, the present study was submitted for evaluation and approved by the Ethics Committee of Health Sciences University of Hokkaido under process number 2016−015. Informed consent was taken from all the parents before the start of the study.

Subjects and Clinical Oral Examination

The 18 children consisted 10 males and 8 females, ranging in age from 4 to 13 years who came to the Department of Pediatric Dentistry and Preventive Dentistry, Hospital, Health Sciences University of Hokkaido, Ainosato, Japan were participant in this study. Children with a history of immunosuppression or systemic diseases (e.g. diabetes and HIV), or had any condition that would require antibiotics for monitoring or treatment procedures (e.g. heart conditions, joint replacements), or had mucosal lesions, previous chemotherapy, radiation therapy or medications that reduce saliva flow, and those under treatment with antimicrobials in the previous three months, were excluded from the study.

The subjects were evaluated by the Simplified Oral Hygiene Index (OHI−S) according to the criteria of Greene & Vermillion (1964) and divided into two groups. The first group (good oral hygiene) was composed of 9 children (3 males and 6 females) with OHI−S scores of 0–1.2. The second group (moderate oral hygiene) was composed of 9 children (7 males and 2 females) with OHI−S scores of 1.3–3.0.

Sample Collection and Culture Condition

Stimulated saliva were collected by paraffin chewing for about 1 min. Subjects were asked to refrain from eating or having tooth cleansing for at least 2 hours before. These samples were obtained by collecting whole saliva in a sterile tube and were transported in an anaerobic box (HIRASAWA WORKS Inc., Tokyo, Japan), containing 80% N_2, 10% CO_2,

and 10% H_2. Samples were homogenized for 1 min with a BioMasher®II (Nippi, Incorporated Protein Engineering Office, Tokyo, Japan) to disperse the biofilm and were serially diluted 10−fold with sterile saline from 10^{-3} to 10^{-8}.

Aliquots of serial 10−fold dilutions (100 μL) were inoculated in two media agar : Bacto™ Brain Heart Infusion Agar (BHI, Difco Laboratories, MD, USA) supplemented with 5% (v/v) defibrinated sheep blood, hemin (10 μg/mL), menadione (5 μg/mL) (BHI Agar), and the selective medium, *Veillonella* agar (Rogosa et al., 1958). After inoculation, all media were incubated under anaerobic conditions, with 80% N_2, 10% CO_2, and 10% H_2, at 37 ℃ ; *Veillonella* agar was incubated for 5 days, while BHI agar was incubated for 7 days.

The total number of bacteria in the samples was determined by counting the total number of colonies on BHI agar, while the number of *Veillonella* was determined by counting the total number of typical *Veillonella* colonies on *Veillonella* agar. Bacterial cells of typical *Veillonella* colonies were confirmed under a light microscopy after Gram staining.

Bacterial Strains and DNA Extraction

Genomic DNA extracted from *V. atypica* ATCC 17744^T, *V. denticariosi* JCM 15641^T, *V. dispar* ATCC 17748^T, *V. parvula* ATCC 10790^T, *V. rogosae* JCM 15642^T, and *V. tobetsuensis* ATCC BAA−2400^T were used as positive control in PCR.

Genomic DNA was extracted from individual bacterial cells using an InstaGene Matrix Kit (Bio−Rad Laboratories, CA, USA). The DNA concentration was determined based on fluorescence using a Qubit® 3.0 Fluorometer (Invitrogen life technologies, MA, USA), according to the manufacturer's instructions.

Protocol and Primers for PCR

For genus−level identification, the PCR protocol was based on the protocols described by Arif et al. (2008) and Beighton et al. (2008). The PCR products were subjected to electrophoresis in a 1.5% agarose gel. For species−level identification, the PCR protocol described by Mashima et al. (2016) was used. The PCR products were subjected to electrophoresis in a 3.0% agarose gel. After electrophoresis, the gels were stained with SYBR® Safe DNA gel stain (Invitrogen life technologies, MA, USA).

DNA Sequencing and Phylogenetic Analysis

We isolated unknown strains, which showed PCR products with genus−specific primers, but not with the species−specific primers. Nine of these strains (S9−1, S13−5, S18−5, S19−4, S24−1, S25−4, S26−18, S28−9, and S31−2 were chosen to be representatives of all the unknown strains. Genomic DNA was extracted from individual bacterial cells isolated from the *Veillonella* agar using the InstaGene Matrix Kit (Bio−Rad Laboratories, California, USA), according to the manufacturer's instructions. Specific primers for the genus *Veillonella* (Kolenbrander & Moore, 1992 ; Beighton et al., 2008) were used for amplification and partial sequence analysis of *rpoB* and *dnaK*. The PCR products were sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystem, CA, USA). The nucleotide sequences were aligned with each other and analyzed using SEQMAN II of the LASERGENE program (DNASTAR ver. 15.2, WI, USA). The MEGALIGN programs, including CLUSTAL W and NJPlot, were used to compare sequences and to reconstruct the evolutionary tree using the neighbor−joining method. The sequence similarity values were also determined with MEGALIGN in the LASERGENE program. The 16S rRNA, *rpoB* and *dnaK* partial sequences of the representative 9 strains were aligned against the sequences of the 14 established species retrieved from GenBank.

Statistical Analysis

Statistical analysis was examined using non−parametric Mann–Whitney U test with Bonferroni correction using IBM SPSS Statistic V.24.0. $p < 0.05$ was considered to evaluate the correlation between the number of oral *Veillonella* species and oral hygiene status.

Results

The saliva samples yielded a high number of bacterial colonies on the BHI agar. The average number of colony−forming units (CFU/mL) (±SE) per sample was 9.7 (±4.11) × 10^7 with a median of 5.7 × 10^7 in the good oral hygiene group (Table 1), and 5.03 (±2.75) × 10^8 with a median of 1.6 × 10^8 in the moderate oral hygiene group (Table 2).

Typical *Veillonella* colonies in the saliva sample were also enumerated on the *Veillonella* agar. These colonies were opaque, grayish−white colonies of 2−4 mm in diameter, regular and slightly domed in shape with an entire edge. They were small, Gram−negative coccal cells, mainly exist-

Table 1. The CFU of all anaerobic bacteria on the BHI agar and *Veillonella* strains on *Veillonella* agar (detection limit < 0.1% of the total count). The total of *Veillonella* isolates identified by the *Veillonella* genus−specific PCR primer. Each species was expressed as a percentage of the number of isolates identified by one−step PCR with the species−specific primer sets for each subject (n = 9) from saliva of the good oral hygiene group.

Good oral hygiene with OHIs 0 − 1.2												
Subject			Total number			Isolated *Veillonella* species						
Name	Age	Sex	All bacteria CFU/mL	*Veillonella* spp CFU/mL	Total number (100%)	*V. atypica* (%)	*V. denticariosi* (%)	*V. dispar* (%)	*V. parvula* (%)	*V. rogosae* (%)	*V. tobetsuensis* (%)	Unknown (%)
G1	10	F	4.80E+07	1.50E+05	15	0	0	0	9 (60.0)	5 (33.3)	0	1 (6.7)
G2	13	F	7.60E+07	3.00E+05	3	0	0	0	1 (33.3)	2 (66.7)	0	0
G3	6	M	2.04E+08	5.00E+06	5	2 (40.0)	0	0	0	1 (20.0)	0	2 (40.0)
G4	6	F	3.52E+08	1.20E+06	12	0	0	4 (33.3)	0	8 (66.7)	0	0
G5	12	F	5.44E+07	7.00E+05	7	2 (28.6)	0	1 (14.3)	0	0	0	4 (57.1)
G6	7	M	1.96E+07	1.70E+05	17	0	0	10 (58.8)	0	4 (23.5)	0	3 (17.7)
G7	5	F	6.00E+07	1.80E+05	18	0	0	0	3 (16.7)	4 (22.2)	2 (11.1)	9 (50.0)
G8	6	F	2.70E+07	2.00E+06	20	2 (10.0)	0	3 (15.0)	4 (20.0)	9 (45.0)	0	2 (10.0)
G9	8	M	3.20E+07	1.70E+02	17	3 (17.6)	0	4 (23.5)	1 (5.9)	8 (47.1)	0	1 (5.9)

Table 2. The CFU of all anaerobic bacteria on the BHI agar and *Veillonella* strains on *Veillonella* agar (detection limit < 0.1% of the total count). The total of *Veillonella* isolates identified by the *Veillonella* genus−specific PCR primer. Each species was expressed as a percentage of the number of isolates identified by one−step PCR with the species−specific primer sets for each subject (n = 9) from saliva of the moderate oral hygiene group.

Moderate oral hygiene with OHIs 1.3 − 3.0												
Subject			Total number			Isolated *Veillonella* species						
Name	Age	Sex	All bacteria CFU/mL	*Veillonella* spp CFU/mL	Total number (100%)	*V. atypica* (%)	*V. denticariosi* (%)	*V. dispar* (%)	*V. parvula* (%)	*V. rogosae* (%)	*V. tobetsuensis* (%)	Unknown (%)
M1	4	M	7.40E+08	1.60E+06	16	0	0	0	12 (75.0)	3 (18.8)	0	1 (6.3)
M2	8	M	7.92E+08	1.30E+06	13	0	0	0	9 (69.2)	1 (7.7)	1 (7.7)	2 (15.4)
M3	10	F	2.48E+08	6.00E+07	6	0	0	4 (66.7)	0	2 (33.3)	0	0
M4	8	M	2.08E+08	2.00E+07	20	0	0	0	10 (50.0)	0	0	10 (50.0)
M5	8	M	4.40E+07	1.60E+07	16	1 (6.3)	0	0	12 (75.0)	0	0	3 (18.8)
M6	5	M	8.00E+07	1.50E+07	15	0	0	0	4 (26.7)	11 (73.3)	0	0
M7	5	M	1.20E+06	1.90E+04	19	0	0	0	0	11 (57.9)	0	8 (42.1)
M8	6	M	1.10E+08	2.00E+05	20	2 (10.0)	0	1 (5.0)	5 (25.0)	10 (50.0)	0	2 (10.0)
M9	8	F	2.30E+09	1.50E+07	15	5 (33.3)	0	1 (6.7)	1 (6.7)	7 (46.7)	0	1 (6.7)

ing as single cells, although some short chains were visible. The detection limit was < 0.1% of the total colony count. Oral *Veillonella* species were detected in all subjects from all oral hygiene groups (Tables 1 and 2).

The average number of colony−forming units (CFU/mL) (±SE) of *Veillonella* species per subject was 1.1 (±0.60) × 10^6 with a median of 5.0 × 10^5 in the good oral hygiene group (Table 1), and 1.4 (±1.00) × 10^7 with a median of 6.9 × 10^6 in the moderate oral hygiene group (Table 2).

From the good (9 subjects) and moderate (9 subjects) oral hygiene groups, 114 and 140 isolates, respectively, were identified as *Veillonella* species using PCR with the genus−specific primer set (254 isolates in total). Using the one−step PCR method with species−specific primer sets, 205 out of 254 isolates were identified as either *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, or *V. tobetsuensis* (Tables 1 and 2). Of the 114 isolates from the good oral hygiene group, 9, 22, 18, 41, and 2 isolates were identified as *V. atypica*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis*, respectively. In addition, 8, 6, 53, 45, and 1 isolates from the moderate oral hygiene group were identified as *V. atypica*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis*, respectively. However, *V. denticariosi* was not detected in the both oral hygiene groups.

Figure 1 shows the ratio between the total number of each *Veillonella* species and the total number of *Veillonella* isolates in the good and moderate oral hygiene groups. *V. rogosae* was the predominant species detected in all groups. In addition, the number of *V. rogosae* higher in good oral hygiene groups than in moderate oral hygiene group, but not significant ; its detection rates were 36.0% and 32.1% in the good and moderate oral hygiene groups, respectively (Fig. 1). Similarly, the detection rates of *V. atypica* was higher in good oral hygiene group than in moderate oral hygiene

group, but no significant difference ; its detection rates were 7.9% and 5.7% in the good and moderate oral hygiene groups, respectively (Fig. 1). Except, the ratio of *V. dispar* was higher significantly in good oral hygiene group than in moderate oral hygiene group as the oral hygiene status decreased ; its detection rates were 19.3% and 4.3% in the good and moderate oral hygiene groups, respectively (Fig. 1). In contrast, the detection rates of *V. parvula* lower significantly in good oral hygiene group than in moderate oral hygiene group as the oral hygiene status decreased ; its detection rates were 15.8% and 37.9% in the good and moderate oral hygiene groups, respectively (Fig. 1). *V. tobetsuensis* was detected in only two subjects of the good oral hygiene group, and in eight subjects of the moderate oral hygiene group (Fig. 1).

Among the 254 strains isolated in this study, 22 isolates from 7 subjects in the good oral hygiene group and 27 isolates from 7 subjects in the moderate oral hygiene group were not assigned to any oral *Veillonella* species (49 isolates in total). The number of unknown *Veillonella* isolates in the good and moderate oral hygiene groups were similar.

In the phylogenetic tree constructed using 16S rRNA gene sequences, the representative of 9 unknown strains formed distinct taxa with a robust bootstrap support (71.9%) against the 14 established *Veillonella* species (Fig. 2). The sequence similarity among these representative strains was 91.2－99.5%, and they were closely related to *V. dispar*.

In the phylogenetic tree constructed using the partial sequence of *rpoB* gene, the representative of 9 unknown strains formed distinct taxa with a robust bootstrap support (93.1%) against the 14 established *Veillonella* species (Fig. 3). Among the 9 unknown strains, strains S13−5 and S19−4 were found to be closely related to *V. infantium*, which has been suggested as a novel species in a previous study (Mashima et al., 2018)

In the phylogenetic tree constructed using the partial se-

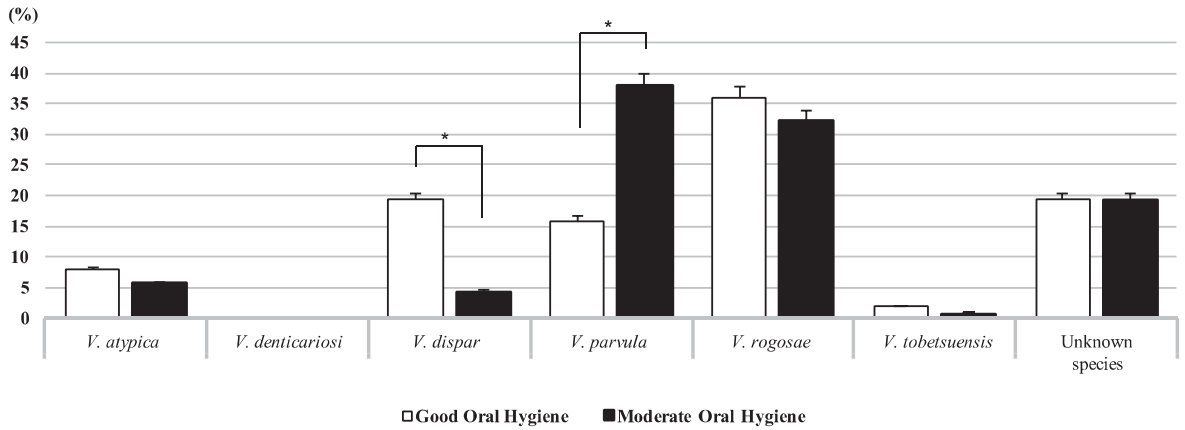

Figure 1. Mean and standard deviation of percentages of *Veillonella* species isolated from all samples (n = 9) in the good oral hygiene group and moderate oral hygiene group (n = 9). Significant difference in detection rates of *V. parvula* and *V. dispar* isolates based on oral hygiene status (using the Kruskal−Wallis H−test post hoc Mann–Whitney U test with Bonferroni correction * $p < 0.05$).

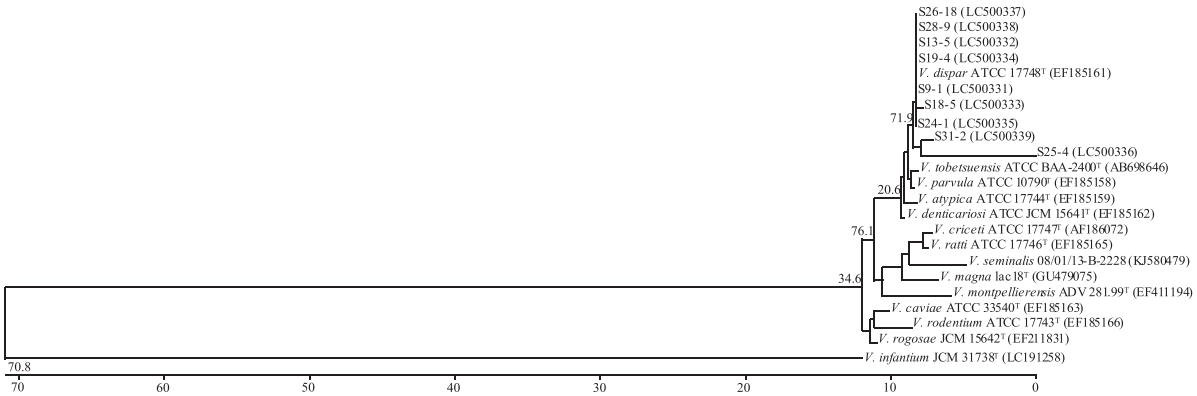

Figure 2. Neighbor−joining tree based on 16S rRNA gene sequences (1501–1562 nt) showing the relationship between the 9 unknown *Veillonella* strains and the type strains of the recognized members of the genus *Veillonella*. GenBank/EMBL/DDBJ accession numbers for 16S rRNA gene sequences are given for each strain. Bootstrap values are indicated at corresponding nodes.

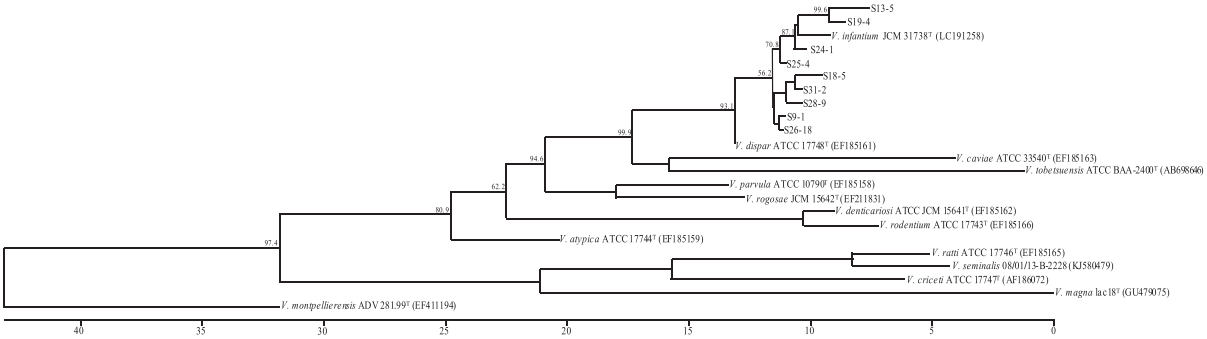

Figure 3. Neighbor−joining tree based on *rpoB* gene sequences (604−657 nt) showing the relationship between the 9 unknown *Veillonella* strains and the type strains of the recognized members of the genus *Veillonella*. Bootstrap values are indicated at corresponding nodes.

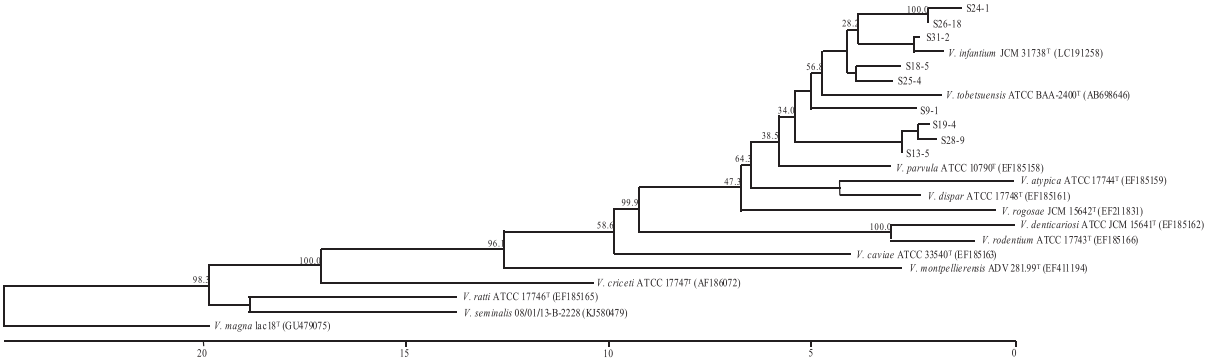

Figure 4. Neighbor−joining tree based on *dnaK* gene sequences (548−640 nt) showing the relationship between the 9 unknown *Veillonella* strains and the type strains of the recognized members of the genus *Veillonella*. Bootstrap values are indicated at corresponding nodes.

quence of *dnaK* gene, the representative of 9 unknown strains formed distinct taxa with a robust bootstrap support (56.8%) against the 14 established *Veillonella* species (Fig. 4). Among the 9 unknown strains, strains S31−2 was found to be closely related to *V. infantium* with 98.4% of sequence similarity.

Discussion

As shown in Tables 1 and 2, the CFU count of all the bacteria, including the oral *Veillonella*, in saliva was associated with oral hygiene status. In fact, oral *Veillonella* species were twice more likely to be detected in a subject with moderate oral hygiene than in one with good oral hygiene.

In the previous study (Theodorea et al., 2017), we had investigated the distribution and frequency of oral *Veillonella* at the species level in saliva of children (107 Thai children ; 51 males and 56 females ; aged 7−15 years) based on oral hygiene status. We had detected *Veillonella* species in 101 out of the 107 subjects in the three oral hygiene groups ; and the total number of *Veillonella* isolates was 1609 from the 101 subjects (Theodorea et al., 2017). Similar with the present study, *Veillonella* isolates in the saliva samples were detected in almost all subjects from the good and moderate oral hygiene groups, and the total number of isolates was 254 from 18 subjects. However, Mashima et al. (2016) reported that, when oral *Veillonella* species were isolated from tongue biofilm, only 10 out of the 89 subjects (11.2 %) detected as *Veillonella* species. Thus, oral *Veillonella* species were easier to detect in the salivary specimen than that in tongue biofilm specimen. It has been suggested that the majority of salivary bacteria were washed off the tongue surface (Gibbons et al., 1964).

This study showed that *V. rogosae* was one of the predominant species in saliva samples from all oral hygiene groups of Japanese children. Similar with previous study, that *V. rogosae* was the predominant *Veillonella* species isolated from saliva of Thai children (Theodorea et al., 2017). Beighton et al. (2008) had investigated the predominant cultivable *Veillonella* species in tongue biofilm of healthy adults in the UK (11 subjects ; gender and age were not reported), and found *V. rogosae* as one of the predominant species. Based on the results of previous studies and the present one, *V. rogosae* is the predominant species of oral *Veillonella* in the saliva and tongue microbiome. In this study,

V. denticariosi was not isolated from the saliva samples of all oral hygiene groups. Mashima et al. (2016) and Beighton et al. (2008) also showed that *V. denticariosi* did not detect in any of the tongue biofilm specimens. Therefore, *V. denticariosi* might be the least common oral *Veillonella* species in the saliva and tongue microbiome. These observations revealed that the oral *Veillonella* species composition in the saliva were closely resembled that in the tongue biofilm.

The present study investigated the distribution and frequency of six oral *Veillonella* species in saliva samples stratified by oral hygiene status. Interestingly, we found that the number of *V. rogosae* in the saliva was higher in good oral hygiene group than in moderate oral hygiene group with the oral hygiene status decreased, but not significantly. Similarly, Arif et al. (2008) had detected *V. rogosae* only in the carious-free lesions of dental plaques. These data indicated that an oral cavity with good hygiene status might be a suitable environment for *V. rogosae*.

In addition, the number of *V. atypica* and *V. dispar* in the saliva were higher in good oral hygiene group than in moderate oral hygiene group as the oral hygiene status decreased. However, in our previous study (Theodorea et al., 2017) there was no significant difference between detection rates of *V. atypica* and *V. dispar* isolated from saliva of Thai children. Meanwhile, *V. tobetsuensis* was detected in only 2 subjects (Table 1 and 2). *V. tobetsuensis* was detected in one out of nine subjects (1.8%) of the good oral hygiene group and in one out of nine subjects (0.7%) of the moderate oral hygiene group (Tables 1 and 2). The ratio between the number of subjects with *V. tobetsuensis* was higher in good oral hygiene group as the oral hygiene status decreased. However, in our previous study (Theodorea et al., 2017), the ratio between the number of subjects with *V. tobetsuensis* and the total number of subjects of each group increased slightly with decreasing oral hygiene quality of Thai children. We therefore suggested that these differences between the studies were likely due to differences in geographical location, age, diet, lifestyle, and socioeconomic status, all of which might have roles in defining the composition of the oral *Veillonella* community. Therefore, further studies are needed to investigate the distribution and frequency of oral *Veillonella* species in the saliva of children in other countries.

The number of *V. parvula* in the saliva was lower significantly in good oral hygiene group than in moderate oral hygiene group as the oral hygiene status decreased. Interestingly, this data is consistent with our previous study (Theodorea et al., 2017), also *V. parvula* was frequently detected in active occlusal carious-lesions (Arif et al., 2008) and periodontal pockets (Mashima et al., 2015). These data suggested that a suitable environment for *V. parvula* was oral cavities with poor hygiene status. However, there are limited report about the roles of *V. parvula* in infectious oral diseases especially dental caries and periodontitis. Thus, it would be investigated in the future.

Out of the 254 isolates, 49 (19.3%) could not be assigned to any of the six known *Veillonella* species through one-step PCR with the species-specific primers, although they were confirmed as *Veillonella* species. Theodorea et al. (2017) had reported that 167 (10.3%) of the 1609 *Veillonella* isolates from saliva of the children could not be identified as any of the six known oral *Veillonella* species. These results indicated the presence of unknown *Veillonella* species in the human oral cavity.

In the phylogenetic analysis with 16S rRNA, *rpoB* and *dnaK* gene sequences, the 9 representative strains of the 49 unknown strains were compared to the 14 established *Veillonella* species (Mashima et al. 2018). The constructed phylogenetic tree revealed that these 9 strains formed distinct taxa within the established *Veillonella* species. Although two strains, S13-5 and S19-4 were found to be closely related to *V. infantium* based on the *rpoB* gene, and one strain was found to be closely related to *V. infantium*, the remaining strains were distinct from them and from the established *Veillonella* species based on *dnaK* gene. Thus, the phylogenetic analysis with partial *rpoB* and *dnaK* sequences suggested that these unknown *Veillonella* isolates were taxonomically heterogeneous and were likely novel *Veillonella* species (Fig. 3 and 4). However, biochemistry analysis will be needed to definitively establish a novel species in the genus *Veillonella* (Sato et al., 1997 ; Marchandin et al., 2003 ; Byun et al., 2007 ; Arif et al., 2008 ; Mashima et al., 2018).

Conclusion

This report is the first to demonstrate that the distribution and frequency of the oral *Veillonella* species in saliva associated with oral hygiene status in Hokkaido children, as determined by a one-step PCR method using species-specific primer sets. The results of this study indicate that changes in number of some oral *Veillonella* species in the saliva might

serve as one of the index for deteriorating oral hygiene status. These results may provide a useful indicator of oral hygiene status, which can prevent further deterioration. However, as described above, further studies of various country− and age−specific cohorts are needed, including differences in intra−oral cavity isolation sites, to explore the distribution and frequency of oral *Veillonella* species in the context of oral hygiene status. In addition, the distribution and frequency of oral *Veillonella* species isolated from saliva in other countries will be investigated in the near future.

In this study, phylogenetic analysis indicated novel oral *Veillonella* species in the oral cavity of children. This study showed a new perspective on oral biofilm communities in early stages of development, which be investigated by discovering the novel *Veillonella* species. In addition, these results may lead to a better understanding of the ecological succession and intricate relationships of bacteria associated with oral biofilm formation that cause many human oral infectious diseases.

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Conflict of Interest

The authors declare no conflicts of interest.

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