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Molecular Detection of Oral *Veillonella* Species in the Saliva of Children with Different Oral Hygiene Statuses

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A B S T R A C T

This study examined the distribution and frequency of oral *Veillonella* species in the saliva of 107 Thai children with different oral hygiene statuses (good, moderate, and poor). A total of 1609 *Veillonella* strains were isolated and confirmed by PCR with genus-specific primers. Oral *Veillonella* isolates were detected at 2-fold higher frequency in subjects with poor than with good or moderate oral hygiene. *Veillonella* species were identified by one-step PCR using species-specific primers based on *rpoB* of oral *Veillonella* species. *Veillonella rogosae* prevalence was significantly lower in the poor oral hygiene group than in the good oral hygiene groups. *Veillonella parvula*, *V. tobetsuensis*, and the unclassified *Veillonella* isolate were significantly more prevalent in the poor oral hygiene group. *Veillonella tobetsuensis* was not detected in the good oral hygiene group. Thus, the detection rate of oral *Veillonella* species such as *V. rogosae*, *V. parvula*, and *V. tobetsuensis* in the saliva indicates the oral hygiene status of children. This is the first report indicating an association between the distribution and frequency of oral *Veillonella* species in saliva and oral hygiene status of children. Other *Veillonella* species and novel species of the genus *Veillonella* may inhabit the oral cavity of children.

Introduction

Dental caries represent a significant problem affecting young children in both developed and developing countries, particularly in socio-economically disadvantaged areas (De Grauwe *et al.*, 2004). Several previous studies have indicated that diet, lifestyle, and socio-economic status affect the bacterial profile in the oral cavity (Belstrøm *et al.*, 2014). Oral hygiene habits could also influence the oral microbiota, both qualitatively and

quantitatively (Haffajee *et al.,* 2006; Tanwir *et al.,* 2009). Studies of dental caries have indicated a change in the fraction of *Veillonella* species in mixed-microbial colonies with *Streptococcus* species during the formation of early dental biofilms (Chalmer *et al.,* 2008). The metabolic interaction among these genera has been suggested as a pathogenic driver of dental caries; the carbon source for *Veillonella* species is lactic acid produced by *Streptococcus* species conducive to caries (Delwiche *et al.,* 1985; Hsu *et al.,* 1994; Hughes *et al.,* 1988; Leuckfeld *et al.,* 2010).

The genus *Veillonella* consists of small, non-fermentative, strictly anaerobic, gram-negative cocci lacking flagella, spores, and capsules (Igarashi *et al.,* 2009; Sutter, 1984). They are characterized by their ability to obtain energy from short-chain organic acids (Delwiche *et al.,* 1985). Members of this genus have been isolated mainly from the oral cavity and intestinal tract of humans and other animals (Delwiche *et al.,* 1985; Sutter, 1984). Thirteen species have been established in the genus *Veillonella*. Of these, only *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis* have been isolated from human oral cavities as oral *Veillonella* species (Mashima *et al.,* 2016). The main habitats of oral *Veillonella* species are tongue biofilms, dental biofilms, buccal mucosa, and saliva (Hughes *et al.,* 1988; Mashima *et al.,* 2016; Arif *et al.,* 2008; Liljemark and Gibbons, 1971). Oral *Veillonella* species, particularly *V. parvula*, have been detected in severe early childhood caries (Kanasi *et al.,* 2010) and intraradicular infections (Sundqvist, 1992), including abscesses (Khamaleelakul *et al.,* 2002), apical root canals (Baumgartner and Falkler Jr., 1991), and dental tubules (Peters *et al.,* 2001). In addition, oral *Veillonella* species have been detected in saliva (Takeshita *et al.,* 2009) and subgingival biofilm specimens (Heller *et al.,* 2012; Mashima *et al.,* 2015; Silva-Boghossian *et al.,* 2013) from patients with chronic periodontitis. However, there are no reliable reports of the pathogenic roles of *Veillonella* species in different oral hygiene statuses.

Periasamy and Kolenbrander (2010) reported that *Veillonella* species play a central role as early colonizers to establish multispecies oral biofilm communities comprised of initial, middle, and late colonizers. Oral biofilms are known to cause many human oral infectious diseases such as periodontitis and dental caries. Mashima *et al.,* (2015) reported an association between *V. parvula* and chronic periodontitis. In addition, Delwiche *et al.,* (1985) reported that *Veillonella* species produce large amounts of lipopolysaccharides. They also showed that in *V. parvula*, lipopolysaccharide-stimulated cytokine induction and p38 MAPK activation were Toll-like receptor 4-dependent (Matera *et al.,* 2009). These properties of *Veillonella* make it difficult to treat associated periodontitis.

Oral *Veillonella* species are known to form biofilms, often with *Streptococcus* species. These genera prefer human hosts with poor oral health (Olson *et al.,* 2011). *Veillonella* species comprise as much as 10% of the bacterial community that initially colonizes the enamel. These species are found throughout the entire oral cavity, particularly on the tongue dorsum and in the saliva (Aas *et al.,* 2005; Diaz *et al.,* 2006; Mager *et al.,* 2003).

Gross *et al.,* (2012) reported that among children without caries, the presence of *Veillonella* or other acid-producing species, including *Streptococcus mutans*, predicted the future development caries, suggesting that *Veillonella* levels are sensitive clinical bio-indicators and early warning signs of acid production. Therefore, when determining

methods for treating or preventing oral infectious diseases in children, it is important to understand the distribution and frequency of *Veillonella* species in oral biofilms.

As an easily collectable and non-invasive biological material, the saliva is suitable for medical investigation; several health and disease-associated factors are reflected in the saliva (Lee and Wong, 2009). In addition, the salivary microbiome has been shown to be highly diverse and dependent on lifestyle and diet (Nasidze *et al.*, 2009, 2011), including oral hygiene (Pereira *et al.*, 2012). Thus, saliva may influence the bacterial profile of oral diseases. However, the *Veillonella* species composition in the saliva in the context of childhood oral health has not been investigated.

The aim of this study was to determine the distribution and frequency of oral *Veillonella* species in the saliva of children in the context of oral hygiene status. We also compared our results with those of previous reports on the identification of oral *Veillonella* species at different intra-oral sites.

Materials and Methods

Statement of human rights

The Ethics Committee of Mahidol University, Bangkok, Thailand approved our study protocol under process number MU-DT/PY-IRB 2015/DT028. Saliva samples were collected at Mahidol University Dental Hospital. The participants and their parents were made aware of the objectives and procedures of the study, and written informed consent was obtained from all individual participants in the study.

Subjects

One hundred and seven school-going children (51 males and 56 females; aged 7–15 years) participated in the present study. Children with a history of immunosuppression or systemic diseases (such as diabetes and human immunodeficiency virus), children with conditions that require antibiotics for monitoring or treatment (such as heart conditions or joint replacements), children with mucosal lesions, children who had been under chemotherapy or radiation therapy, children under medication that reduces saliva flow, and children under antimicrobial treatment within the last 3 months were excluded from the study.

Clinical oral examination

The subjects were evaluated based on the Simplified Oral Hygiene Index (OHI-S) according to the criteria of Greene and Vermillion (1964). Based on this evaluation, subjects were divided into three groups. The first group (good oral hygiene) included 27 children (9 males and 18 females) with OHI-S scores of 0–1.2. The second group (moderate oral hygiene) included 35 children (17 males and 18 females) with OHI-S scores of 1.3–3.0. The third group (poor oral hygiene) included 45 children (25 males and 20 females) with OHI-S scores of 3.1–6.0.

Sample collection

Approximately 1.5-mLstimulated saliva specimens were collected after paraffin chewing for ~1 min at the Mahidol University Faculty of Dentistry Dental Hospital. Subjects were asked to refrain from eating or cleaning their teeth for at least 2 h prior to collection. The samples were collected in a sterile tube and transported in an anaerobic box (HIRASAWA WORKS, Inc., Osaka, Japan) with 80% N_2, 10% CO_2, and 10% H_2 (<1 h from the time of collection). The samples (1 mL each)were homogenized for 1 min with a Bio Masher®II (Nippi Incorporated Protein Engineering Office, Tokyo, Japan) for dispersion and then

serially diluted by 10-fold, from 10^{-3} to 10^{-8}, with sterile saline.

Culture conditions

Aliquots (100 µL) of the 10-fold diluted samples were inoculated in Bacto™ Brain Heart Infusion (BHI, Difco Laboratories, Detroit, MI, USA) supplemented with 5% (v/v) defibrinated sheep blood (BHI agar), hemin (10 µg/mL, Wako, Osaka, Japan), menadione (5 µg/mL, Wako), 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°C; *Veillonella* agar was incubated for 5 days, while BHI agar was incubated for 7 days.

The total number of cultivable bacteria in the samples was determined by counting the total number of colonies on BHI agar, and the number of *Veillonella* species was determined by counting the total number of typical *Veillonella* colonies on the *Veillonella* agar.

Bacterial cells of typical *Veillonella* colonies were confirmed by observation with a light microscope after gram staining.

DNA extraction

Genomic DNA was extracted from individual bacterial cells using an Insta Gene Matrix Kit (Bio-Rad Laboratories, Hercules, CA, USA). The DNA concentration was determined based on fluorescence using a Qubit® 3.0 Fluorometer (Invitrogen life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. 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 controls.

Protocol and primers for PCR

Before identifying oral *Veillonella* at the species level, we used a PCR primer pair to identify *Veillonella* at the species level, Veill-rpoBF and Veill-rpoBR, based on the protocols described by Arif *et al.,* (2008) and Beighton *et al.,* (2008). For species-level identification, we used a one-step PCR method with the species-specific primer sets ATYR, DENR, DISR, PARR, ROGR, TOBR, and VF (Mashima *et al.,* 2016).

The PCR products were subjected to electrophoresis in a 2.0% agarose gel. After electrophoresis, the gels were stained with SYBR® Safe DNA gel stain (Invitrogen life Technologies).

Statistical analysis

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

Results and Discussion

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 1.7 (±0.47) × 10^8 with a median of 5.4 × 10^7 in the good oral hygiene group (Table 1), 7.4 (±4.03) × 10^8 with a median of 1.2 × 10^8 in the moderate oral hygiene group (Table 2), and 2.1 (±1.30) × 10^9 with a median of 6.6 × 10^7in the poor oral hygiene group (Table 3).

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

single cells, although some short chains were visible. The detection limit was <0.1% of the total colony count. Of the 107 subjects in this study, oral *Veillonella* species were detected in 101 subjects from all oral hygiene groups (Tables 1–3). The number of *Veillonella* species in subjects with poor oral hygiene status was higher than that in those with good or moderate oral hygiene status (Tables 1–3).

The average number of CFU/mL (±SE) of *Veillonella* species per subject was 1.0 (±0.70) × 10^6 with a median of 1.0 × 10^4 in the good oral hygiene group (Table 1), 2.1 (±1.00) × 10^6 with a median of 1.6 × 10^5 in the moderate oral hygiene group (Table 2), and 4.3 (±1.74) × 10^6 with a median of 2.0 × 10^4 in the poor oral hygiene group (Table 3).

From the good (27 subjects), moderate (35 subjects), and poor (45 subjects) oral hygiene groups, 384, 517, and 708 isolates, respectively, were identified as *Veillonella* species by PCR with a genus-specific primer set (1609 isolates). Using the one-step PCR method with species-specific primer sets, 1442 of 1609 isolates were identified as *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, or *V. tobetsuensis* (Tables 1–3). Of the 354 isolates from the good oral hygiene group, 34, 6, 9, 24, and 281 isolates were identified as *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, and *V. rogosae*, respectively. *Veillonella tobetsuensis* was not detected in the good oral hygiene group. In addition, 54, 1, 18, 36, 360, and 10 isolates of the 479 isolates from the moderate oral hygiene group and40, 1, 16, 120, 415, and 17 isolates of the 609 isolates from the poor oral hygiene group were identified as *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis*, respectively.

Figure 1 shows the ratio between the total number of each *Veillonella* species and total number of *Veillonella* isolates in the good, moderate, and poor oral hygiene groups. *Veillonella rogosae* was the predominant species detected in all groups. In addition, the number of *V. rogosae* decreased as oral hygiene quality decreased; its detection rates were 73.2%, 69.6%, and 58.6% in the good, moderate, and poor oral hygiene groups, respectively. There was statistically significant difference in the detection rates of *V. rogosae* between the good and poor oral hygiene groups (Fig. 1).

In contrast, the detection rates of *V. parvula* increased as oral hygiene quality decreased; its detection rates were 6.3%, 7.0%, and 16.9% in the good, moderate, and poor oral hygiene groups, respectively. The differences between the detection rates of *V. parvula* between the good and poor oral hygiene groups, and moderate and poor oral hygiene groups were significant (Fig. 1). *Veillonella tobetsuensis* was detected in only five subjects in the moderate oral hygiene group (Table 2) and in eight subjects in the poor oral hygiene group (Table 3). *Veillonella denticariosi* was also isolated in small numbers: six isolates from three subjects in the good oral hygiene group, one isolate from one subject in the moderate oral hygiene group, and one isolate from one subject in the poor oral hygiene group (Table 1–3).

Among the 1609 *Veillonella* strains isolated in this study, 30 isolates from 13 subjects in the good oral hygiene group, 38 isolates from 15 subjects in the moderate oral hygiene group, and 99 isolates from 29 subjects in the poor oral hygiene group were not assigned to any oral *Veillonella* species (total 167 isolates), as they did not show any PCR products with the species-specific primer sets (Table 1–3). The number of these unclassified *Veillonella* isolates and number of subjects with these unclassified *Veillonella* isolates in the poor oral hygiene group were higher than those in the good and moderate oral hygiene

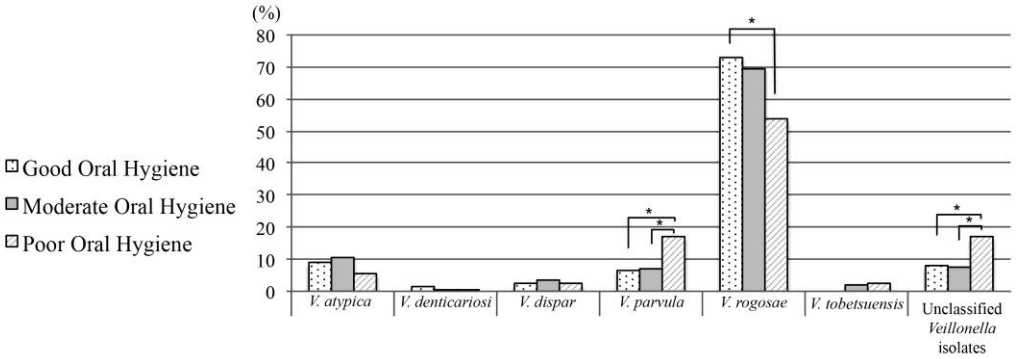
groups (Table 1–3). There were also significant differences in the detection rates of these unclassified *Veillonella* isolates between the good and poor oral hygiene groups and between the moderate and poor oral hygiene groups (Fig. 1).

As shown in tables 1–3, the CFU count of all cultivable bacteria in BHI agar, including *Veillonella* species, in saliva was associated with oral hygiene status. Additionally, *Veillonella* species were 2-fold more likely to be detected on *Veillonella* agar in a subject with poor oral hygiene than in a subject with good or moderate oral hygiene.

In a previous study, Mashima *et al.*, (2016) investigated the distribution and frequency of oral *Veillonella* at the species level in tongue biofilms of 89 children. The study reported that 101 strains of *Veillonella* species were detected in only 10 of the 89 subjects. In the present study, *Veillonella* isolates in the saliva samples were detected in nearly all subjects from the three groups: the total number of isolates was 1609 from 101 subjects. Thus, the proportion of detectable *Veillonella* species in salivary isolates was higher than that in the tongue biofilm isolates.

The bacterial profile of saliva is known to include bacteria from different oral surfaces (Belstrøm *et al.*, 2014). However, Liljemark and Gibbons (1971) detected *Veillonella* species both in the saliva and on the tongue surface (54 healthy young adults in the USA; aged 19 years; oral hygiene statuses were not reported). It has been suggested that most salivary bacteria were washed off the tongue surface (Gibbons *et al.*, 1964). However, Liljemark and Gibbons (1971) also reported that the proportion of *Veillonella* species on the tongue surface was higher than that detected in the saliva. They suggested that *Veillonella* species adhered to oral epithelial surfaces. Similarly, Mager *et al.*, (2003) also reported that *V. parvula* was more abundant in the saliva and on the tongue surface, particularly the tongue dorsum, than at other intra-oral sites (225 healthy subjects in the USA; aged >18 years; oral hygiene statuses were not reported). However, *V. parvula* was the only oral *Veillonella* species that was investigated in these limited studies.

Fig.1 Percentages of the six oral *Veillonella* species (including unclassified *Veillonella* isolates belonging to the genus *Veillonella*)



Total isolated number of each *Veillonella* species isolated was expressed as a percentage of the total number of *Veillonella* species isolated from all samples (n = 27) in the good oral hygiene group, samples (n = 35) in the moderate oral hygiene group, and samples (n = 45) in the poor oral hygiene group. Significant difference in detection rates of *V. parvula*, *V. rogosae*, and unclassified *Veillonella* isolates based on oral hygiene status * $P < 0.05$

Table.1 Ratio of the number of isolates of each *Veillonella* species to the total number of *Veillonella* isolates from the good oral hygiene group

Good oral hygiene (OHIs 0 - 1.2) group												
Subject	**Age**	**Sex**	**Total Number**		**Isolated *Veillonella* spp.**							
			All Bacteria CFU/mL ($\times 10^8$)	***Veillonella spp.* CFU/mL ($\times 10^6$)**	**Total number (100%)**	***V. atypica* number (%)**	***V. denticariosi* number (%)**	***V. dispar* number (%)**	***V. parvula* number (%)**	***V. rogosae* number (%)**	***V. tobetsuensis* number (%)**	**Unclassified *Veillonella* Number (%)**
G1	10	M	0.06	0	-	-	-	-	-	-	-	-
G2	10	F	0.009	0	-	-	-	-	-	-	-	-
G3	10	F	3.0	2.3	23	3 (13.0)	0	2 (8.7)	4 (17.4)	12 (52.2)	0	2 (8.7)
G4	9	F	5.2	1.5	15	3 (20.0)	0	0	0	10 (66.7)	0	2 (13.3)
G5	12	M	2.3	1.8	18	0	0	0	0	18 (100.0)	0	0
G6	15	F	0.4	0.13	13	0	0	0	0	11 (84.6)	0	2 (15.4)
G7	13	M	0.5	0.11	11	0	2 (18.2)	0	0	9 (81.8)	0	0
G8	11	F	0.2	0.11	11	5 (45.5)	0	0	0	4 (36.4)	0	2 (18.2)
G9	11	F	0.9	0.01	10	0	0	0	0	10 (100.0)	0	0
G10	12	F	0.5	0.19	19	0	0	0	1 (5.3)	17 (89.5)	0	1 (5.3)
G11	10	F	2.5	0.0019	19	0	0	0	0	19 (100.0)	0	0
G12	11	F	1.1	19.0	19	0	0	1 (5.3)	0	16 (84.2)	0	2 (10.5)
G13	9	F	0.7	0.0014	14	2 (14.3)	0	2 (14.3)	0	7 (50.0)	0	3 (21.4)
G14	12	M	0.3	1.3	13	5 (38.5)	0	0	1 (7.7)	4 (30.8)	0	3 (23.1)
G15	8	F	3.03	0.13	13	2 (15.4)	2 (15.4)	0	1 (7.7)	5 (38.5)	0	3 (23.1)
G16	13	M	0.2	0.0018	18	0	0	0	0	18 (100.0)	0	0
G17	12	M	0.1	0.000002	2	0	0	0	0	2 (100.0)	0	0
G18	11	M	0.2	0.0019	19	2 (10.5)	0	0	4 (21.1)	13 (68.4)	0	0
G19	13	F	0.3	0.0021	21	8 (38.1)	0	0	0	9 (42.9)	0	4 (19.0)
G20	9	F	1.2	0.002	20	4 (20.0)	2 (10.0)	0	4 (20.0)	10 (50.0)	0	0
G21	7	F	10.8	0.16	16	0	0	0	0	16 (100.0)	0	0
G22	11	M	3.6	0.0016	16	0	0	0	4 (25.0)	11 (68.8)	0	1 (6.3)
G23	10	F	0.07	0.000012	12	0	0	2 (16.7)	0	8 (66.7)	0	2 (16.7)
G24	10	F	0.03	0.009	9	0	0	0	1 (11.1)	8 (88.9)	0	0
G25	10	F	5.7	0.16	16	0	0	0	3 (18.8)	13 (81.3)	0	0
G26	11	M	2.7	0.0017	17	0	0	0	0	17 (100.0)	0	0
G27	10	F	0.5	0.2	20	0	0	2 (10.0)	1 (5.0)	14 (70.0)	0	3 (15.0)

Total colony counts of anaerobic bacteria on BHI agar, total colony counts of *Veillonella* species on *Veillonella* agar, and number of isolates from each subject (n = 27) in the good oral hygiene group, identified using species-specific primer sets. CFU: colony-forming unit; detection limit <0.1% of the total count. Individual species are denoted as a percentage of the number of isolates from each subject, identified using species-specific primer sets.

Table.2 Ratio of the number of isolates of each *Veillonella* species to the total number of *Veillonella* isolates from the moderate oral hygiene group

Moderate oral hygiene (OHIs > 1.2 - 3.0) group												
Subject	**Age**	**Sex**	**Total Number**		**Isolated *Veillonella* spp.**							
			All Bacteria CFU/mL ($\times 10^8$)	***Veillonella spp.* CFU/mL ($\times 10^6$)**	**Total number (100%)**	***V. atypica* number (%)**	***V. denticariosi* number (%)**	***V. dispar* number (%)**	***V. parvula* number (%)**	***V. rogosae* number (%)**	***V. tobetsuensis*number (%)**	**Unclassified *Veillonella* Number (%)**
M1	9	F	0.1	0	-	-	-	-	-	-	-	-
M2	11	M	2.3	0	-	-	-	-	-	-	-	-
M3	10	M	0.6	0.1	10	0	0	0	0	10 (100.0)	0	0
M4	11	F	4.9	2.0	20	5 (25.0)	0	8 (40.0)	0	5 (25.0)	0	2 (10.0)
M5	13	M	0.09	0.13	13	0	0	0	0	13 (100.0)	0	0
M6	13	F	3.7	0.13	13	0	0	2 (15.4)	0	8 (61.5)	0	3 (23.1)
M7	12	M	0.7	0.13	13	0	0	0	3 (23.1)	10 (76.9)	0	0
M8	11	F	0.4	0.1	10	2 (20.0)	0	0	0	8 (80.0)	0	0
M9	10	F	0.7	0.2	20	5 (25.0)	0	0	0	12 (60.0)	0	3 (15.0)
M10	11	F	0.02	0.18	18	0	0	1 (5.6)	0	14 (77.8)	0	3 (16.7)
M11	11	M	1.2	0.2	20	1 (5.0)	0	0	0	16 (80.0)	1 (5.0)	2 (10.0)
M12	10	M	0.3	0.006	6	0	0	0	2 (33.3)	4 (66.7)	0	0
M13	9	F	2.0	0.17	17	7 (41.2)	0	0	4 (23.5)	6 (35.3)	0	0
M14	11	F	0.2	20.0	20	0	0	0	0	20 (100.0)	0	0
M15	10	M	1.1	26.0	26	5 (19.2)	0	5 (19.2)	2 (7.7)	7 (26.9)	1 (3.8)	6 (23.1)
M16	11	M	2.3	0.14	14	0	0	0	0	14 (100.0)	0	0
M17	10	M	3.2	0.018	18	2 (11.1)	0	0	1 (5.6)	12 (66.7)	0	3 (16.7)
M18	10	M	1.6	0.01	10	0	0	0	0	9 (90.0)	1 (10.0)	0
M19	10	F	3.7	2.0	20	0	0	0	0	17 (85.0)	0	3 (15.0)
M20	11	M	2.6	0.14	14	0	0	0	1 (7.1)	13 (92.9)	0	0
M21	11	F	9.6	0.16	16	1 (6.3)	0	0	2 (12.5)	13 (81.3)	0	0
M22	11	F	0.10	0.16	16	0	0	0	0	14 (87.5)	0	2 (12.5)
M23	10	M	2.2	1.7	17	5 (29.4)	0	0	0	12 (70.6)	0	0
M24	12	M	139.0	0.14	14	1 (7.1)	0	0	5 (35.7)	7 (50.0)	0	1 (7.1)
M25	12	F	27.6	1.9	19	5 (26.3)	0	0	0	14 (73.7)	0	0
M26	12	F	0.9	0.21	21	5 (23.8)	0	0	3 (14.3)	11 (52.4)	0	2 (9.5)
M27	14	F	28.6	1.4	14	0	0	0	0	13 (92.9)	0	1 (7.1)
M28	11	M	0.2	0.012	12	1 (8.3)	1 (8.3)	2 (16.7)	2 (16.7)	6 (50.0)	0	0
M29	15	M	0.5	0.13	13	0	0	0	8 (61.5)	4 (30.8)	0	1 (7.7)
M30	12	M	1.0	0.2	20	5 (25.0)	0	0	2 (10.0)	11 (55.0)	0	2 (10.0)
M31	13	M	2.2	0.2	20	2 (10.0)	0	0	0	18 (90.0)	0	0
M32	11	F	0.8	0.19	19	0	0	0	0	14 (73.7)	5 (26.3)	0
M33	11	F	11.4	16.0	16	1 (6.3)	0	0	0	11 (68.8)	0	4 (25.0)
M34	11	F	2.1	0.08	8	1 (12.5)	0	0	0	7 (87.5)	0	0
M35	11	F	0.4	0.01	10	0	0	0	1 (10.0)	7 (70.0)	2 (20.0)	0

Total colony counts of anaerobic bacteria on BHI agar, total colony counts of *Veillonella* species on *Veillonella* agar, and number of isolates from each subject (n = 35) in the moderate oral hygiene group, identified using species-specific primer sets. CFU: colony-forming unit; detection limit <0.1% of the total count. Individual species are denoted as a percentage of the number of isolates from each subject, identified using species-specific primer sets.

Table.3 Ratio of the number of isolates of each *Veillonella* species to the total number of *Veillonella* isolates from the poor oral hygiene group

Poor oral hygiene (OHIs 3.1 - 6) group												
Subject	**Age**	**Sex**	**Total Number**		**Isolated *Veillonella* spp.**							
			All Bacteria CFU/mL ($\times 10^6$)	***Veillonella spp.* CFU/mL ($\times 10^6$)**	**Total number (100%)**	***V. atypica* number (%)**	***V. denticariosi* number (%)**	***V. dispar* number (%)**	***V. parvula* number (%)**	***V. rogosae* number (%)**	***V. tobetsuensis* number (%)**	**Unclassified*Veillonella* number (%)**
P1	11	F	0.03	0	-	-	-	-	-	-	-	-
P2	11	M	0.1	0	-	-	-	-	-	-	-	-
P3	9	M	2.9	17.0	17	4 (23.5)	0	0	0	9 (52.9)	1 (5.9)	3 (17.6)
P4	11	M	0.02	0.002	20	0	0	0	16 (80.0)	0	0	4 (20.0)
P5	13	M	0.4	0.17	17	3 (17.6)	0	3 (17.6)	0	9 (52.9)	0	2 (11.8)
P6	13	F	2.4	0.7	7	0	0	4 (57.1)	2 (28.6)	0	0	1 (14.3)
P7	11	F	0.4	0.17	17	0	0	2 (11.8)	0	10 (58.8)	0	5 (29.4)
P8	12	F	3.4	0.013	13	1 (7.7)	0	0	1 (7.7)	10 (76.9)	0	1 (7.7)
P9	11	F	1.5	1.7	17	3 (17.6)	1 (5.9)	0	4 (23.5)	3 (17.6)	2 (11.8)	4 (23.5)
P10	12	M	0.5	0.004	4	0	0	0	2 (50.0)	0	0	2 (50.0)
P11	11	F	0.1	0.011	11	0	0	0	3 (27.3)	8 (72.7)	0	0
P12	10	F	0.06	0.0015	15	0	0	0	0	15 (100.0)	0	0
P13	10	F	0.2	0.0018	18	6 (33.3)	0	0	1 (5.6)	9 (50.0)	0	2 (11.1)
P14	11	F	0.4	0.022	22	2 (9.1)	0	0	4 (18.2)	12 (54.5)	0	4 (18.2)
P15	12	M	3.0	0.16	16	0	0	2 (12.5)	4 (25.0)	1 (6.3)	5 (31.3)	4 (25.0)
P16	10	M	12.0	16.0	16	1 (6.3)	0	0	0	11 (68.8)	0	4 (25.0)
P17	11	F	0.1	0.002	20	0	0	0	0	20 (100.0)	0	0
P18	9	M	8.7	0.19	19	0	0	0	9 (47.4)	5 (26.3)	0	5 (26.3)
P19	11	M	0.2	0.16	16	0	0	0	0	16 (100.0)	0	0
P20	11	F	0.3	0.0013	13	0	0	0	4 (30.8)	5 (38.5)	0	4 (30.8)
P21	9	M	52.4	70.0	7	0	0	1 (14.3)	2 (28.6)	3 (42.9)	1 (14.3)	0
P22	11	M	1.5	16.0	16	4 (25.0)	0	0	5 (31.3)	3 (18.8)	0	4 (25.0)
P23	11	F	0.8	0.013	13	2 (15.4)	0	0	4 (30.8)	5 (38.5)	0	2 (15.4)
P24	11	F	0.3	0.15	15	0	0	0	1 (6.7)	12 (80.0)	0	2 (13.3)
P25	11	F	0.01	0.0017	17	0	0	0	0	17 (100.0)	0	0
P26	10	F	10.0	0.0016	16	1 (6.3)	0	0	5 (31.3)	8 (50.0)	2 (12.5)	0
P27	11	M	0.3	0.0018	18	0	0	0	4 (22.2)	6 (33.3)	4 (22.2)	4 (22.2)
P28	10	F	0.6	0.018	18	0	0	0	0	14 (77.8)	0	4 (22.2)
P29	13	M	0.9	0.021	21	0	0	0	0	12 (57.1)	0	9 (42.9)
P30	12	M	0.7	0.02	20	0	0	0	0	18 (90.0)	0	2 (10.0)
P31	14	M	1.4	0.002	20	0	0	0	0	20 (100.0)	0	0
P32	9	M	0.1	0.0017	17	2 (11.8)	0	0	5 (29.4)	10 (58.8)	0	0
P33	13	M	8.0	20.0	20	0	0	0	10 (50.0)	10 (50.0)	0	0
P34	12	F	0.3	0.2	20	0	0	0	3 (15.0)	14 (70.0)	1 (5.0)	2 (10.0)
P35	13	M	8.0	0.0016	16	0	0	0	9 (56.3)	7 (43.8)	0	0
P36	10	M	18.0	1.3	13	0	0	0	8 (61.5)	5 (38.5)	0	0
P37	7	M	2.3	0.008	8	0	0	0	3 (37.5)	5 (62.5)	0	0
P38	12	M	11.9	0.02	20	8 (40.0)	0	3 (15.0)	0	6 (30.0)	0	3 (15.0)
P39	11	M	0.5	0.2	20	0	0	0	0	20 (100.0)	0	0
P40	11	M	0.4	0.027	27	0	0	0	0	22 (81.5)	1 (3.7)	4 (14.8)
P41	9	F	13.0	18.0	18	3 (16.7)	0	0	3 (16.7)	10 (55.6)	0	2 (11.1)
P42	11	F	254.0	14.0	14	0	0	0	2 (14.3)	11 (78.6)	0	1 (7.1)
P43	11	M	1.3	0.02	20	0	0	0	0	18 (90.0)	0	2 (10.0)
P44	11	F	0.2	0.002	20	0	0	0	0	12 (60.0)	0	8 (40.0)
P45	11	M	514.0	16.0	16	0	0	1 (6.3)	6 (37.5)	4 (25.0)	0	5 (31.3)

Total colony counts of anaerobic bacteria on BHI agar, total colony counts of *Veillonella* species on *Veillonella* agar, and the number of isolates from each subject (n = 45) in the poor oral hygiene group, identified using species-specific primer sets. CFU: colony-forming unit; detection limit <0.1% of the total count. Individual species are denoted as a percentage of the number of isolates from each subject, identified using species-specific primer sets.

These differences observed between the studies were likely related to differences in geographical location, age, diet, lifestyle, socio-economic status, and oral hygiene status, all of which may affect 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.

This study showed that *V. rogosae* was the predominant species in saliva samples from all oral hygiene groups. A previous study also demonstrated that *V. rogosae* was the predominant *Veillonella* species isolated from tongue biofilms of children (Mashima *et al.*, 2016). Beighton *et al.*, (2008) investigated the predominant cultivable *Veillonella* species in tongue biofilms of healthy adults in the UK (11 subjects; gender and age not reported), and found *V. rogosae* as one of the predominant species. Based on the results of previous studies and the present results, *V. rogosae* is the predominant species of oral *Veillonella* in the saliva and tongue biome.

In this study, *V. denticariosi* was isolated in small numbers from the saliva samples of all oral hygiene groups. Similarly, Mashima *et al.*, (2011) detected *V. denticariosi* from the tongue biofilm of only one young Japanese adult.

They did not detect *V. denticariosi* in any of tongue biofilm specimens of Thai children (Mashima *et al.*, 2016). These results are consistent with the results of Beighton *et al.*, (2008), who also detected no *V. denticariosi* in any subjects in their study. Therefore, *V. denticariosi* may be the least common oral *Veillonella* species in the saliva and tongue biome. These observations revealed that the oral *Veillonella* species composition in the saliva closely resembles that of the tongue biofilm.

The present study investigated the distribution and frequency of oral *Veillonella* species in saliva samples. Interestingly, we found that the detection rates of *V. rogosae* in the saliva significantly decreased with oral hygiene quality (Fig. 1). Similarly, Arif *et al.*, (2008) detected *V. rogosae* only in carious-free lesions of dental plaques. These data indicate that an oral cavity with a good hygiene status is a suitable environment for *V. rogosae*.

In contrast, the detection rates of *V. parvula* in the saliva significantly increased as oral hygiene quality decreased (Fig. 1). This data is consistent with the results of other studies, in which *V. parvula* was frequently detected in active occlusal carious-lesions (Arif *et al.*, 2008) and periodontal pockets (Mashima *et al.*, 2015). Similarly, Hughes *et al.*, (1988) reported that *V. parvula* was present in subgingival biofilm samples. These data suggest that a suitable environment for *V. parvula* is an oral cavity with poor hygiene status.

Veillonella tobetsuensis was not detected in subjects with good oral hygiene (Table 1). However, *V. tobetsuensis* was detected in 5 of 35 subjects (14.3%) in the moderate oral hygiene group and in 8 of 45 subjects (17.8%) in the poor oral hygiene group (Tables 2 and 3). The ratio between the number of subjects with *V. tobetsuensis* and total number of subjects in each group increased slightly with decreasing oral hygiene quality. These results suggest that the presence of *V. tobetsuensis* in the saliva is an index of deteriorating oral hygiene.

Of the 1609 isolates, 167 (10.4%) could not be assigned to any of the six known oral *Veillonella* species through one-step PCR using species-specific primers, although they were confirmed by PCR using a *Veillonella* genus-specific primer set as members of the genus *Veillonella*. Mashima *et al.*, (2015) also reported that 43 (9.7%) of the 442 *Veillonella* isolates from periodontal pockets and gingival sulcus could not be identified as any of the six known oral *Veillonella* species. Although only six species were isolated from human oral cavities as oral *Veillonella* in previous studies, these results suggest that other *Veillonella* species can inhabit human oral cavities. These results also suggest the presence of novel *Veillonella* species in these unclassified

Veillonella isolates. To evaluate these possibilities, a phylogenetic study with housekeeping genes, such as 16S rDNA, *dnaK*, *rpoB*, and *gyrB*, is currently underway.

This is the first report to investigate the distribution and frequency of oral *Veillonella* species in the saliva of children in the context of oral hygiene. Our results indicate that changes in the number of some oral *Veillonella* species in the saliva of children, such as increased *V. parvula*, *V. tobetsuensis*, and unclassified *Veillonella* isolates and decreased *V. rogosae*, can serve as indices for deteriorating oral hygiene.

These results may provide a useful indicator of oral hygiene status, and thus help prevent further deterioration. However, further studies involving country- and age-specific cohorts, including differences in intra-oral cavity isolation sites, are needed to better understand the distribution and frequency of oral *Veillonella* species in the context of oral hygiene. The distribution and frequency of oral *Veillonella* species in the saliva of subjects from different countries will be investigated in future studies. The present study also revealed the potential numbers of novel *Veillonella* species in the oral cavity of children.

Conflicts of interest

The authors declare no conflicts of interest.

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