The Detection of *Veillonella* spp. in the Oral Mucosa of Indonesian High-Risk Early Childhood Caries by Absolute Quantitative Real-Time PCR

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Abstract

The presence of oral *Veillonela* spp., *V. atypica, V. denticariosi, V. dispar, V. parvula* and *V. rogosae*, in oral mucosa biofilms and saliva have been associated with dental cary development in young children. However, identifying oral *Veillonella* spp. is difficult since there is no useful differential phenotypic test. To reliably detect *Veillonella*- related early childhood caries (ECC), we used a real-time PCR assay based on SYBR Green I, followed by a melting curve analysis. The q-PCR identified the oral *Veillonella* spp. directly from the clinical specimen. The oral mucosa biofilm collected from ECC was dominated by *V. dispar*, while the minor species was *V. rogosae.* This is the first report indicating the profile of oral *Veillonella* spp. in the oral mucosa biofilm of high-risk EEC in Indonesian subjects.

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Introduction

Early childhood caries (ECC) is a serious public health problem in both developing and industrialized countries. The literature shows that the highest prevalence of ECC is found in Africa and Southeast Asia. EEC is reported to cause detrimental effects on the dental health and quality of life of children.[1,2,3]

The literature shows that *Veillonella* levels in the oral cavity may serve as a sensitive biological indicator and an early warning sign of acid production by oral bacteria.[4,5] The genus *Veillonella* consists of small, strictly anaerobic, Gram-negative cocci that lack flagella, spores, and capsules. This genus is subdivided into 12 species: *V. atypica*, *V. caviae, V. criceti, V.denticariosi, V. dispar, V. magna, V. montpellierensis, V. parvula, V. ratti, V. rodentium, V. rogosae*, and *V. tobetsuensis.*[5-10].

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Veillonela spp. (*V. atypica, V. denticariosi, V. dispar, V. parvula*, and *V. rogosae*) have been reported as isolates on oral mucosa surfaces,[6,10,11] and *V. parvula* is the most common isolate detected in biofilms on oral mucosa. These species could participate in co-aggregations with many oral bacteria. *Veillonella* may derive particular benefit from both adherence to and interaction with other oral bacterial species. The inter bacterial adherence plays a functional role in the bacterial colonization of different habitats within the oral cavity.[10] However, the identification of *Veillonella* spp. is difficult, since no useful differential phenotypic methods are available. Therefore, molecular methods, including PCR and PCR-RFLP, have been used to identify *Veillonella* strains at the species level.[11] This method has many advantages over conventional PCR, including increased speed due to a reduced cycle number, the lack of post-PCR gel electrophoresis detection of products, and higher sensitivity of the fluorescent dyes used for the detection of the amplicon. The application of real-time PCR (q-PCR) also permits convenient detection and quantitation of *Veillonella* spp. directly from clinical

specimens, contributing to improved epidemiological investigations and control of diseases due to oral *Veillonella* spp.

Therefore, discerning the presence of *Veillonella* can be used as a method to predict the development of future caries.[4] The present study describes the use of an absolute quantitative-real time PCR (q-PCR) assay that rapidly discriminates *Veillonella* spp. from genera that are commonly found in the oral mucosa of ECC. Moreover, the specificity of the q-PCR assay was assessed with a melting curve analysis. This is the first report indicating the profile of oral *Veillonella* spp. from the oral mucosa biofilms collected from Indonesian children's association with high-risk ECC.

Methods

Subject population

This study was performed after receiving approval provided by the Ethics Committee Faculty of Dentistry Universitas Indonesia, Jakarta, Indonesia, under process number 101/Ethical Clearance/FKGUI/X/2014.

Sixteen (16) children under five years old from a rural area in south Kalimantan, Indonesia, were selected as subjects in this study after informed consent was obtained from each subject's mother's. The ECC indicators were determined according guidelines provided by the American Academy of Pediatric Dentistry (AAPD).

Sample collection

Oral mucosa biofilm samples were taken using a sterile cotton bud, and the saliva was blocked using a cotton roll by applying it firmly to the dorsal surface of the tongue and buccal mucosa. Afterward, the cotton bud was inoculated to a micro centrifuge tube containing 1 ml of phosphate buffer saline (PBS, pH 7.2), and the samples were stored at (−) 20°C before the extraction of genomic DNA in the laboratory.

DNA extraction

After thawing, the samples were centrifuged at 10 000 rpm for 5 min, and the supernatant was removed. The DNA was extracted from the sample by immersing biofilm-covered pegs in 40 µL of sterile ultrapure water plus 160 µL of 0.05 M sodium hydroxide. After incubation at 60°C for 60 min, 18.4 µL of 1M Tris-HCl pH 7.0 was added to neutralize the pH. The extracted DNA was further used as a template for the q-PCR amplification.

q-PCR

The primers and q-PCR assay were applied as previously reported.[11,12] The first PCR utilized a pair of universal 16S rRNA gene primers: 27f (5' - AGAGTTTGATCC TGGCTCAG - 3') and 1,492r (5 '- ACGGCTACCTTG TTACGACTT - 3'), generating a 1466 bp fragment.[13] For the second PCR targets on the *Veillonella* genus level, we used two primer sets: *Veill-rpoB*F (5'- GTAACAAAGGTGTCGTTTCTC G - 3') and *Veill-rpoB*R (5'-GCACCRTCAAATAAT ACAGGTGTAGC-3').[11,12] For the third PCR target of oral *Veillonella* at the species level, we used five species-specific primer sets: DENF (5'-GAAAGAAGCGCGCGCACCGACAGT -3') and VR (5' - GTGTAAACAAGGGAGTACGGACC -3') for *V. denticariosi*, PARF (5'-GAA GCATTGGAAGCGAAAGTTTCG-3') and VR (5'-GTGTAAACAAGGGAGTACGGACC-3') for *V. parvula*, ROGF (5'ATTGCAGAAGATGTA ACATGAAGC-3') and VR (5'-GTGTAA ACAAGGGAGTACGGACC-3') for *V. rogosae,* ATYF (5'-TCTCTTTGGGAAGAATTAGAA CGC-3') and VR (5'-GTGTAAACAAGGGAGTACGGACC-3') for *V. atypica*, and DISF (5'-AACGCGTTGAAATTCGTCATGAAC-3') and VR (5'-GTGTAAACAAGGGAGTACGGA CC-3') for *V. dispar.*[11,12]

The bacterial genomic DNA used for the standard curves was extracted from overnight cultures of *Veillonella* sp. strain PK 1910, with a DNA extraction InstaGene Matrix kit (Bio-Rad) according to the manufacturer's instructions, and the genomic DNA was stored at (-) 20°C [14] until used.

A q-PCR analysis using SYBR green dye was performed to quantify the *Veillonella* spp. in the samples. The q-PCR mixture (25

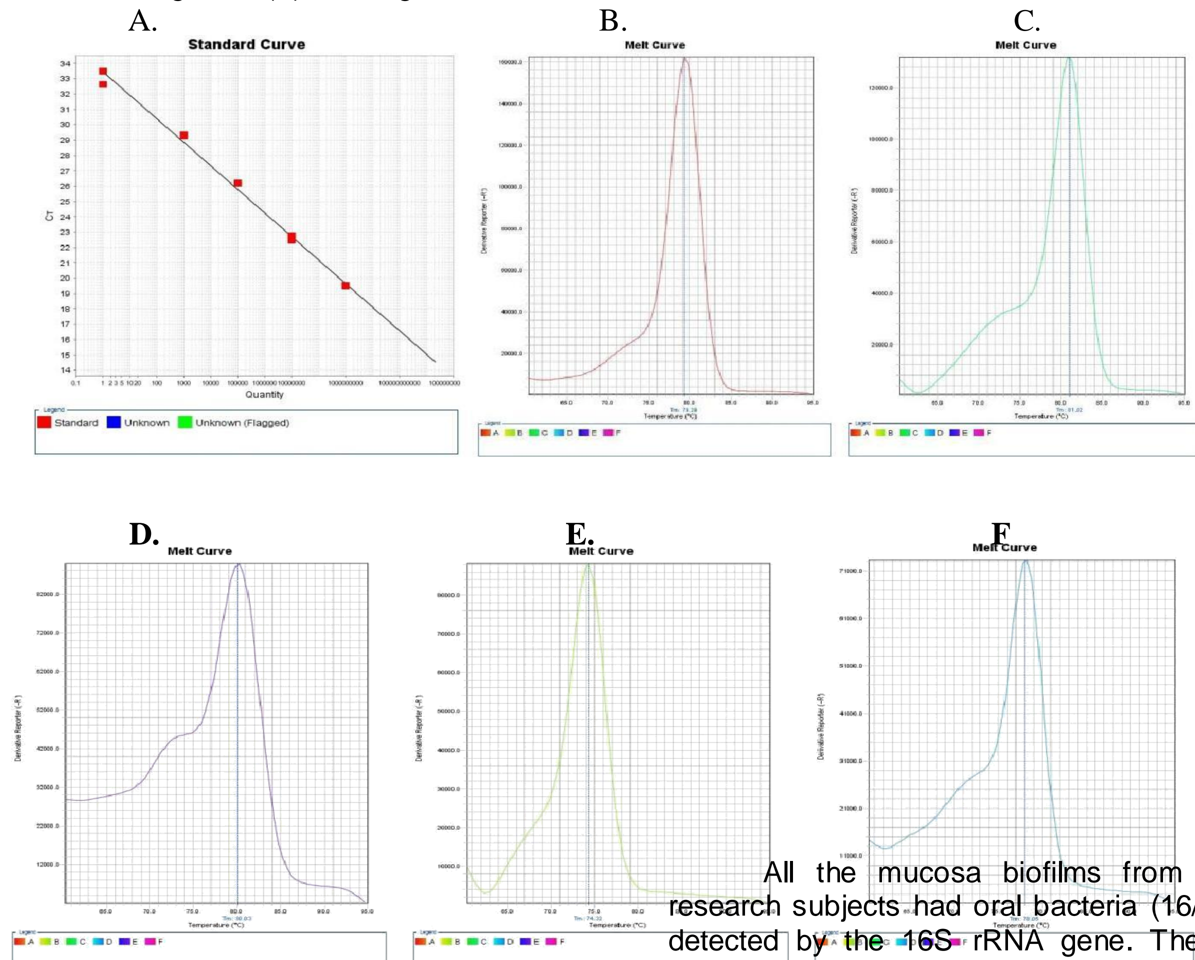
μL) was made up of 3 μL template, 3.5 μL of diethyl pyrocarbonate-treated ultrapure water, 12.5 μL of Power SYBR green PCR master mixture (Applied Biosystems), and 3 μL each of the forward and reverse primers (375 nM).[14] A q-PCR analysis was conducted in an MX3005P thermocycler (Stratagene) with the following thermal cycle recommended for the power SYBR green PCR mixture: 95°C for 10 min and then 40 cycles of 30 s at 95°C and 1 min at 56°C. Dissociation curves were generated by incubating the reaction products at 95°C for 1 min and at 56°C for 30 s and by incrementally increasing the temperature to 95°C. Fluorescence data were collected at the end of the 56°C primer annealing step for 40 amplification cycles and throughout the dissociation curve analysis.

Results

The analysis of the melting curves with both primer sets revealed a single sharp peak. This was further used to determine the species of *Veillonella* genus in each sample. The amplified DNA concentrations (ng/mL) were calculated based on the standard curve obtained by using tenfold serial dilutions of bacterial DNA relative to Ct.

To assess the differences between the *Veillonella* species in each sample, we used a melting curve analysis on the different amplicons to discriminate the species from the genus. This allowed us to determine each *Veillonella* species under investigation. As shown in Fig. 1, the melting curve shown by genus is represented by one peak (as is each *Veillonella* species), ranging from 65°C to 95°C.

Figure 1. (A) standard curve of tenfold dilution of *V. parvula* ATCC 10790T. (B). Melting curve of *V. atypica*. (C). Melting curve of *V. parvula*. (D). Melting curve of *V. dispar*. (E). Melting curve of *V. rogosae*. (F). Melting curve of *V. denticariosi*



All the mucosa biofilms from the research subjects had oral bacteria (16/16) detected by the 16S rRNA gene. The q-PCR analysis identified the genus of

Veillonella in 87.5% of the samples (14/16). In this study, the predominant oral *Veillonella* species was *V. dispar* (11/14), and the number of this species (mean) was 45.39 X 10^6. We also found *V. atypica* in 6 of 14 subjects (31.97 X 10^2), *V. denticariosi* in 4 of 14 subjects (12.58 X 10), *V. parvula* in 2 of 14 subjects (34.27 X 10^8), and *V. rogosae* in 1 of 14 subjects (1.21).

Discussion

In this study, according to the AAPD indicator, all of the research subjects were high-risk ECC individuals. The participants live in the rural area of South Kalimantan, Indonesia, with minimal economic status levels. The classic etiology of ECC involves bacterial, dietary, and host determinants with an interplay of multiple sociological and environmental factors.

Streptococcus mutans and *Streptococcus sobrinus*, the most common identified causative agentsof ECC, are acid-producing pathogens that cause damage by dissolving tooth structures in the presence of fermentable carbohydrates, such as sucrose, fructose, and glucose.[15-19]

The growth of the *Streptococcus* species leads to the formation of lactate, which is a favored substrate of the *Veillonella* species. This in turn accelerates the glycolytic rate in *Streptococcus* species by removing the inhibition of the end product (lactate). The lactate that is formed by *Streptooccus* species is converted to less potent acids, such as acetic acid, by *Veillonella* species. These less potent acids have been assumed to reduce susceptibility to the caries in the host.[10] The oral species of *Veillonella* have been reported to play a central role as early colonizers in forming the multispecies community involved in biofilm formation and in facilitating species succession in the development of oral biofilms *in vivo.*[20] The *Veillonella* genus consists of small, strictly anaerobic, gram-negative cocci that lack flagella, spores, or a cap sul.[5,12]

The species-level identification of *Veillonella* is very difficult due to the lack of conventional phenotypic and biochemical tests.[21] In this study, oral *Veillonella* species on oral mucosa biofilms were identified by a melting curve analysis of amplicons. The current study showed that the melting curve produced with each paired primer revealed that all species in *Veillonella* spp. could easily be distinguished from the oral mucosa of ECC.

Furthermore, an examination of the *Veillonella* differentiation melting curve, with the genus melting curve as the baseline, revealed that the melting curve shown by each species' amplicon was different, suggesting that all the examined *Veillonella* spp. species identified via the melting curve were indeed different. This result confirmed that the species-specific primers used in this study detected a sufficient region to distinguish the target *Veillonella.*

An uncultivated method was used because the species-level identification of *Veillonella* is very difficult due to the lack of conventional phenotypic and biochemical tests.[5,7,11] The phenotypic identifications of *Veillonella* spp. are based on the fact that *Veillonella* grow on a selective agar.[8] These procedures are time consuming and laborious, and a lot of materials are needed. The molecular microbial technique with the direct amplification and sequencing of 16S rRNA genes from the environment[22] has revolutionized microbial ecology and changed the way of studying prokaryotes in the environment. In our study, nested PCR procedures were used to identify oral *Veillonella.*

The first PCR utilized a pair of universal 16S rRNA gene primers—27f and 1,492r—to discriminate between bacteria DNA and eukaryotic DNA. The 16S rRNA genes are used as the standard for the classification of microbes because the gene is present in most microbes and shows the proper changes. In this study, the 16S rRNA gene was detected in all the mucosa biofilm (100%, 16/16) samples. Molecular methods based on 16S rRNA gene sequencing, including PCR-RFLP analysis, have been used to identify strains from the *Veillonella* genus at the species level.[11] However, recent studies have shown that the identification of members of *Veillonella*

genus by using only 16S rRNA gene sequencing is not reliable.[11] The 16S rRNAgene is not without potential drawbacks, and the use of alternative markers has been proposed, including the beta sub unit of DNA polymerase, *rpoB.*[11,23,24]

To define species of the genus *Veillonella,* partial sequence analyses of the housekeeping genes, including *rpoB, dnaK*, and *gyrB*, have been used.[21] In our study, for the second PCR targets for the *Veillonella* genus level, we used two primer sets—*Veill-rpoB*F and *Veill-rpoB*R—using a method designed by Igarashi et al. (2009).[11] Fourteen of the sixteen samples (87.5%) were discerned to be from the *Veillonella* genus. For the third PCR targets of this study for oral *Veillonella* at the species level, five individual forward primers and a shared reverse primer from a highly variable region (position 2500–3100) of the *rpoB* gene were used.

This implies that *rpoB* can be a more efficient marker than the 16S rRNA gene when a subset of the total diversity is targeted.[25] The use of the *rpoB* marker has disadvantages as well. First, it is not conserved enough to be used as a universal marker, and only a subset of the microbial community can be targeted. Second, assigning a taxonomy to the sequences is problematic because no appropriate database and classifiers are available. Third, experiments using complex but defined communities are necessary to rigorously test for primer bias.[26]

The five oral *Veillonella* spp. (*V. atypica, V. denticariosi, V. dispar, V. parvula*, and *V. rogosae*) can be readily identified via q-PCR. A real-time PCR (q-PCR) based on SYBR green dye was reported for the specific detection of species in *Veillonella* spp.[14] SYBR green is a dye that binds the minor groove of double-stranded DNA. When SYBR green dye binds to double-stranded DNA, the intensity of the fluorescent emissions increases.

As more double-stranded amplicons are produced, SYBR green dye signals increase. SYBR green dye binds to any double-stranded DNA molecule, while a 5' nuclease assay is specific to a predetermined target. According to the results in Table 1, in each of subjects 1, 2, 3, 4, 5, 6, 7, 8, 10, 15 and 16, *V.dispar* and two or three other species were isolated. In 11 of 16 subjects, *V. dispar* was the most frequently isolated species, at a percentage of more than 68%. Moreover, in subjects 2, 3, 8, 10, and 15, *V. dispar* and *V. atypica* were isolated.

This data indicates that *V. dispar* likely coexists with *V. atypica.* Previously, Mashima et al.[27] reported that *V. dispar* was likely to coexist with *V. atypica,* and these bacteria were predominant in tongue biofilms. Beigton et al.[11]alsoreported that the predominant species in tongue biofilms were *V. atypica*, *V. dispar*, and *V.rogosae.* The difference in our study compared to Mashima et al. and Beigton et al. is that they used cultivable *Veillonella* spp. In our study, a q-PCR procedure was used to identify oral *Veillonella,* without cultivation on *Veillonella* selective agar.

Based on the melting curve analysis, our data showed that *V. denticariosi* was identified in 6 out of 16 subjects (37.5%). Beighton et al. and Mashima et al.[11] reported that *V. denticariosi* was not isolated on tongue biofilm from any subjects in their study. Several factors likely influenced these differences, such as geographic issues and the methods used for identification. As reported by Mashima et al.,[11] *V. rogosae* was isolated in 7 of 11 subjects (62.5%). In our study, *V.rogosae* was identified in only 1 out of 16 subjects (6.25%).

Although no clear explanation isapparent at present, it may be that this discrepancy occurred because of the use of different methods. Mashima et al. detected the species by PCR from a bacterial colony, which it retrieved from a medium selective *Veillonella* agar. Dewhirst et al. reported that *V. parvula,* a Gram-negative strictly anaerobic coccus, is one of the most predominant bacterial species of the oral microbiome.[28]. In this study, *V. parvula* was identified only in one subject. Beighton et al.[11] reported similarly results in tongue

biofilms, suggesting that *V. parvula* is a minor species in oral mucosa biofilms.

Conclusions

In conclusion, based on the results of the present research, *V. dispar* is identified as the predominant species, whereas *V. dispar* is likely to coexist with *V. atypica.* The application of q-PCR also permits the convenient identification of *Veillonella* spp. directly from clinical specimens. This is the first report indicating that the profile of oral *Veillonella* spp. in oral mucosa biofilms of individuals with high-risk ECC, as determined using q-PCR.

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Declaration of interest

The authors report no conflict of interest.

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