Identification of Veillonella spp. on Tongue Plaque and Saliva Using Real-Time PCR

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Abstract

Veillonella spp., Gram-negative obligate anaerobic cocci bacteria, amounts to 3% in the oral cavity, relies on the fermentation of lactate as a carbon and energy source for growth. The bacteria are considered anti-cariogenic as they metabolize lactic acid into propionic acid which increases oral environment's pH and reduces demineralization rate of tooth structure. Identification of Veillonella spp. using traditional methods is difficult due to the lack of conventional phenotypic and biochemical tests. Thus, the biomolecular methods are suitable for the specific detection and identification of Veillonella spp. One of the biomolecular methods that can be used is real-time Polymerase Chain Reaction (PCR), which the results can be qualitative and quantitative. This study aimed to identify Veillonella spp. in tongue plaque's and saliva's samples using Real-time PCR. The DNA of Veillonella spp. derived from 36 samples, 18 samples of tongue plaque and 18 samples of saliva, were extracted using a freeze-thaw method and then quantified by real-time PCR using forward primer 5'-CCG TGA TGG GAT GGA AAC TGC-3' and reverse primer 5'-CCT TCG CCA CTG GTG TTC TTC-3'. Veillonella spp. in 18 samples of tongue plaque was 3,06 x 107 CFU/ml and in 18 saliva samples was 1,51 x 105 CFU/ml. It was concluded real-time PCR can detect Veillonella spp. from all tongue plaque's and saliva's samples.

Keywords: Veillonella spp., real-time PCR, tongue plaque, saliva

1. Introduction

Veillonella species are Gram-negative strictly anaerobic cocci that lack of flagella, spores, and capsule. This species is frequently isolated from the oral cavity and intestinal tract of humans and other animals.[1,2] V. atypica, V. denticariosi, V. dispar, V. parvula, V. rogosae, and V. tobetsuensis have been isolated from human oral cavities. The main habitats of these oral Veillonella spp. are tongue, saliva, dental plaque biofilm, and buccal mucosa.[2] They are often found in subgingival biofilm samples of patients with chronic periodontal diseases.[3] Veillonella spp. are unable fermenting carbohydrates and relying on the fermentation of lactate, pyruvate, malate, fumarate, and/or oxaloacetate as carbon and energy source for growth. 4 Veillonella spp. are considered as anti-cariogenic as they metabolize lactate into propionic acid and weaker acetic, where the weaker acids are less capable of demineralizing enamel. They commonly associated with bacteria that are capable of fermenting carbohydrates into lactic acids, such as Actinomyces, Streptococcus, and Lactobacillus.[4]

Identification of Veillonella spp. using conventional methods is difficult due to the lack of conventional phenotypic and biochemical tests. Thus, the biomolecular methods are suitable for the specific detection and identification of this bacteria. One of the biomolecular methods that can be used is real-time PCR, which the results can be qualitative and quantitative.

The method of PCR is noted as one of the most widely used technique in molecular biology due to its simplicity and time efficiency. Technically, PCR is a method to amplify a single or a few copies of specific sequences of DNA across several orders of magnitude, generating thousands to millions of copies of DNA sequence. Veillonella spp. identification using PCR, a molecular method based on 16S rDNA gene sequencing, including sequence-specific oligonucleotides, DNA polymerase, and thermal cycling, have been published.[2,5,6].

Unlike conventional PCR, quantifies the amount of DNA by the end of last PCR cycle, real-time PCR measures the amount of DNA at each cycle via fluorescent dyes that yield an increased fluorescent signal in direct proportion to the number of PCR product molecules (amplicons) generated. Real-time PCR offers advantages such as an ability to monitor the progress of the PCR reaction as it occurs in real time and to precisely measure the amount of the amplicon at each cycle. In Real-time PCR, the amplification and detection occurs in a single tube thus eliminating post-PCR manipulations.[6]

A key step in designing real-time PCR assay is selecting the chemistry to monitor the amplification of the target sequence. The variety of fluorescent chemistries can be categorized into 2 major types: DNA-binding dyes (SYBR Green I) and dye-labelled, sequence-specific oligonucleotide primers or probes.[7] The aim of this study is to identify Veillonella spp. on tongue plaque's and saliva's samples using real-time PCR.

2. Material and Method

2.1. **Ethical Approval**

All procedures were approved by Research Ethics Committee of Faculty of Dentistry, University of Indonesia (No: 58 / Ethical Clearance / FKGUI / VII / 2014).

2.2. **DNA strains**

In this study, Veillonella parvula ATCC 10790 was used as a standard bacteria.

2.3. **Subjects and samplings**

Early Childhood Caries (ECC) is a condition where there are one or more of primary tooth surface that are decayed (with or without cavitated lesions), absent (due to caries) or filled in a preschool-age (71 months or less) children.8 Thus, the subjects in this study are children aged 3-5 years old.

Eighteen subjects with the age of 3-5 years old were selected for this study. Informed consent obtained from parents of all subjects before performing the study. All of the subjects were students at Sanggar Talenta, Jakarta, Indonesia. Saliva samples were collected using 3 ml pipettes, while tongue plaque samples were collected by swabbing dorsal surface of tongue 10 times of each of subjects with cotton buds. The samples were immediately placed in 1,5 ml microtube, which consists of 1 ml of phosphate-buffered saline (PBS).

2.4. **Culture conditions**

Veillonella parvula ATCC 10790 were cultured overnight on Brain Heart Infusion (BHI) broth at 37 °C under anaerobic conditions.

2.5. DNA extraction

Freeze-thaw method following the standard procedure was performed to extract the DNA of all samples. The DNA concentration was determined using spectrophotometer based on the absorption at wavelength A260 and A280.

2.6. Specific primer pairs

Forward primer for Veillonella spp. was 5'-CCG TGA TGG GAT GGA AAC TGC-3' and the reverse primer was 5'-CCT TCG CCA CTG GTG TTC TTC-3'. These specific primer pairs are based on rpoB genes.9

2.7. Real-time PCR protocol

Real-time PCR (StepOne™ Real Time PCR System, Applied Biosystem, Thermo Fisher Inc., Massachusetts, USA) was performed using 5 µl Sybr Green, 1 µl forward primer 10µM, 1 µl reverse primer 10µM, 1 µl nuclease free water, and 2 µl templates. This real-time PCR consists of 3 steps, which repeated for 30-40 cycles in the experiment. The steps consist of denaturation by preheating the DNA usually to 98 °C for 5 min, annealing them at 54 °C for 30s, and followed by performing extension at 72 °C for 5 min.10,11

2.8. Qualification of Veillonella spp.

Veillonella spp. was quantified using absolute quantification method. This quantification was based on a standard curve which has been made earlier from extracted DNA of Veillonella parvula ATCC 10790 after serially diluted from 101 to 109.

3. Results

Blood glucose level before as well as after 2 and 4 weeks STZ injection increased simultaneously with the diabetic duration (Table 1). Blood glucose level was evaluated highest in the diabetic group after 4 weeks (215.63±35.63mg/dL) and the lowest in the negative control group (86.10±15.88mg/dL) before treatment (baseline). Hence, STZ injection promotes hyperglycemia condition. Diabetes Mellitus (DM) was evaluated histologically from pancreas samples. The result showed degeneration and decreasing of β-cell number in the Langerhans islets in the diabetic group and lead to diabetes type II (Fig. 1).

The melt curve of Veillonella spp. showed only 1 peak (Figure 1) and the standard curve of Veillonella spp. has R2 value equal to 0,99 (Figure 2). It suggested a great efficiency of earlier 101 to 109 serial dilution. Mean quantity of Veillonella spp. in 18 samples of tongue plaque was 3,06 x 107 CFU/ml and in 18 saliva's samples was 1,51 x 105 CFU/ml (Figure 3). Veillonella spp. quantities of samples were obtained from proportioning the CT of amplicons with the standard curve.

According to the results, Veillonella spp. was detected on all the subjects (Figure 1). It indicated that Veillonella spp. is accessible in both tongue plaque and saliva of 3-5 years old subjects.

4. Discussion

As an anti-cariogenic bacteria, Veillonella spp. utilizes lactate produced by Streptococcus mutans, the main pathogen for caries, then produces weaker acid such as propionic acid.[12] There are two options for Veillonella's detection, such as traditional and biomolecular methods. The traditional method of bacterial identification is based on phenotypic identification using Gram staining, culture and biochemical methods. However, these methods have two weaknesses. First, they can only be used for organisms that are in vitro cultivated. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species.[13] As we know, the identification of Veillonella spp. at the species level remains difficult by using traditional method due to a lack of conventional phenotypic and biochemical tests.2 Therefore, it can be simply done using biomolecular methods, and one of the methods is real-time PCR. The previous study stated that comparison of the 16S rDNA gene sequence was unsuitable for identifying Veillonella spp., so we used

rpoB gene sequence to identify these bacteria.2

Using DNA-based assay, one can simply detect bacterial strains directly from clinical samples or from the small amounts of cultured bacterial cells. This improving the sensitivity and decreases the time required for bacterial identification. PCR has been particularly useful in this regard, which relies on primer sequences designed to facilitate bacterial identification at any level of specificity.

Real-time PCR technique is different from conventional PCR due to its ability to determine not only the quality but also the quantity of detection target. Besides, real-time PCR offers other advantages such as high sensitivity and accuracy, time efficiency as it doesn't require electrophoresis gel, and less probability of contamination.[13] According to the result of this study, real-time PCR could detect Veillonella spp. on tongue plaque and saliva of 3-5 years old subjects.

Extraction of DNA is used to prepare the DNA samples as a template for further steps using real-time PCR. Many different methods are available for DNA extraction including chemical and physical techniques. Chemical disruption uses several enzymes, such as proteinase K and lysozyme, to dissolve and disrupt the cell membranes. Physical techniques are often the first method of choice for DNA extraction. Some examples from these techniques are freeze-thaw and DNA kit. The freeze-thaw method causes the cell to swell and shrink until the cell membrane breakage and release DNA for further experiment.[14] This method is way more price-reasonable and less complicated, yet the extraction results had less-purity compared to DNA kit. For this study, the freeze-thaw method was selected due to its effectiveness and cost-efficiency. As with it, real-time PCR was able to detect DNA despite its small amount and low purity level.

The first step of designing a real-time PCR experiment is deciding the best type of assay for the experiment. Next, a key step in designing a real-time PCR assay is selecting the chemical to monitor the amplification of the target sequence. This depends on the type of the assay, whether it would be a singleplex or multiplex assay. In this study, we used Sybr Green as the fluorescence chemical. The advantages of using Sybr Green are its simplicity (only requires two primers), its cost efficiency, and its ability to perform melt-curve analysis to examine the specificity of the amplified reaction.[7] In this study, the specific primer set for Veillonella was derived from rpoB gene sequence. Specific primers were used because Sybr Green assay needs a pair of PCR primers that amplify a specific region within the target sequence of interest.7

Real-time PCR method with Sybr Green allows the use of melt curve analysis to confirm specificity from the amplified product (amplicons) without any gel electrophoresis or sequencing. A DNA strand, which consists of approximately 200 bp, has 4200 different possible sequences. It makes any small changes in the sequence can lead to detectable differences. On the other hand, the melting temperature (Tm), when represents the temperatures when double-stranded DNA

dissociates into single-stranded DNA, varies only 40 °C range. Thus, the melt curve profiles will have a high degree of similarity between samples from one species, and a clear distinction between one species to another. The 15 Tm was seen as one point located on the peak of the curve. Hence, one-peak on Veillonella's melt curve from this study indicated a high specificity level of amplification.[6]

Earlier in this study, the serial dilution of Veillonella parvula ATCC 10790 was made as a reference to perform the standard curve. The x-axis showed the amount of DNA bacteria's quantity in log10, while the y-axis of the standard curve showed the number of amplicons on amplification cycles. The R2 value on the standard curve described dilution effectivity. The closer R2 value to 1, the better effectivity of the dilution was determined. In this study, the R2 value of Veillonella's standard curve was 0,99 (Figure 2).

The dorsal surface of tongue occupies a huge area of oral mucosa and harbor many microorganisms.16 There are more than 700 species of bacteria colonizes at the surface of mouth and tongue, which has the largest bacterial load of any oral tissues. There are more than 100 bacterias may be attached to a single epithelial cell on the dorsal tongue, whereas only about 25 bacterias attached to each cell of other oral cavity's area.[17,18] In this study, the result showed that the bacteria's quantity on tongue plaque was higher than in saliva (Figure 3) as suggested by references.

5. Conclusion

It has been shown that real-time PCR can detect Veillonella spp. from all tongue plaque's and saliva's samples accurately based on rpoB gene sequence. Veillonella spp. was 100% detected in all subjects. This study restricted only on genus level. Thus the further study was recommended to provide more information about Veillonella spp.

6. Acknowledgement

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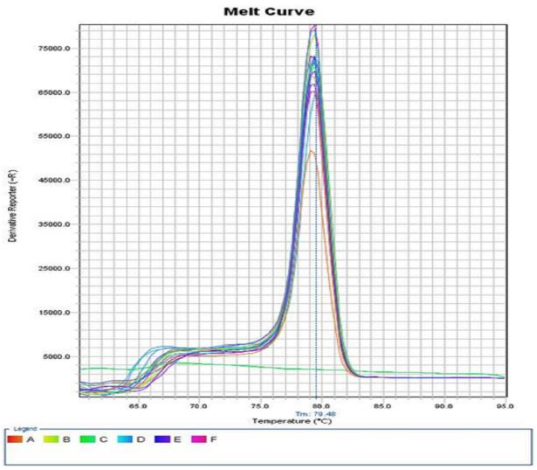
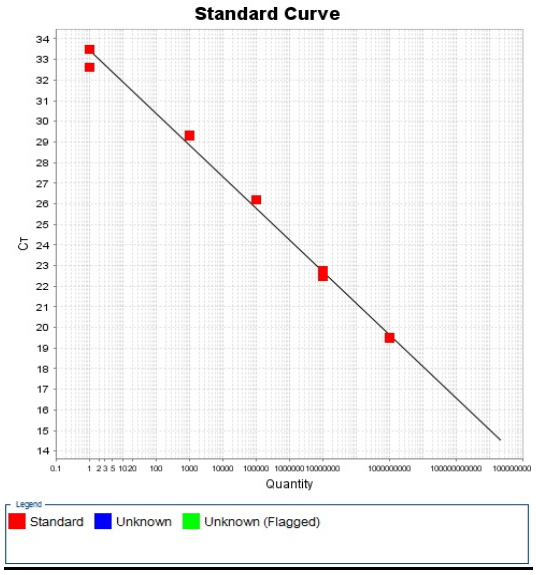
8. Figures

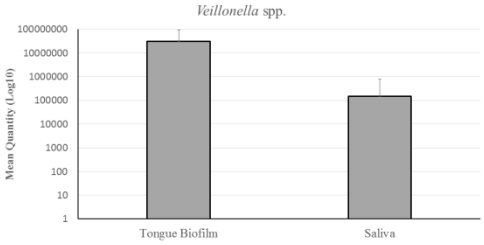
1. Figure 1. Melt curve of Veillonella spp.



2. Figure 2. Standard curve of Veillonella spp.



3. Figure 3. Mean quantity of Veillonella spp.



9. Tables

1. Table 1. Veillonella spp. Detection

Samples	n	%
Tongue plaque	18	100
Saiva	18	100