The Sensitivity of Selective *Veillonella* Medium as Confirmed by Gram Staining and *Veill-rpoB* Polymerase Chain Reaction

Ariadna Adisattya Djais[*1], Citra Fragantia Theodorea[1], Harun Asyiq Gunawan[1], Elza Ibrahim Auerkari[1]

1.Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia.

Abstract

The number of *Veillonella* bacteria in the oral cavity is suggested to be a biological indicator and early warning sign of oral acid production, and therefore may help to predict the development of imminent dental caries. A simple technique is recommended in the present study, which aimed to assess the sensitivity of the approach. The potential use of Rogosa agar as a *Veillonella*-selective growth medium was tested by incubating the microbes in an anaerobic atmosphere for several days. Afterwards, the colony and cellular morphology of 173 Rogosa agar plates were observed under light microscope after Gramstaining. The presence of microbes was confirmed using the polymerase chain reaction (PCR) technique, which detects genuses with *Veill-rpoB* primer. Gram-stain observation revealed Gram-negative cocci, which underwent DNA extraction and *Veillonella* genus identification viaPCR. As a *Veillonella*-selective medium, Rogosa agar has 80.3% selectivity, as determined by Gram-negative cocci (100% of which were members of the *Veillonella* genus) and confirmed by PCR. Rogosa agar's sensitivity to *Veillonella* was 80.3%.

Experimental article (J Int Dent Med Res 2017; 10(Special Issue): pp. 764-768)

Keywords: *Veillonella*-selective medium, *Veill-rpoB* PCR, *Veillonella* spp.

Received date: 18 August 2017 **Accept date:** 20 September 2017

Introduction

Veillonella bacteria have the potential to balance *Streptococcus mutans*, the most important microbe in the etiology of dental caries[1]. Lactic acid can reduce pH below 5.5,the critical pH forenamel demineralization. Lactic acid produced by *S.mutans* can be used by *Veillonella* for metabolism and adjustment of further production of propionic acid[2,3].

Previous studies suggest that the level of *Veillonella* in an oral cavity may be a sensitive biological indicator and an early warning sign of acid production. Therefore, the presence of *Veillonella* could be useful to predict future development of dental caries[4,5]. Recent standard procedures to cultivate these bacteria have used both brain heart infusion (BHI) agar as a rich medium and Rogosa agar as a *Veillonella*-selective medium. When incubated anaerobically, the bacterial colonies growing on rich medium such as BHI agar shouldbe representative of alloral anaerobic bacteria. Bacterial colonies growing on selective Rogosa agar may describe *Veillonella* spp. of oral anaerobic bacteria.

The composition of Rogosa agar is able to distinguish *Veillonella* from other bacteria[3]. The components of the medium, such as tryptone, yeast extract, sodium thioglycolate, basic fuchsin, and vancomycin, are expected to hinder the development of other bacteria.

It should be noted that it is difficult to achieve absolute sensitivity (100%) for a selective medium. Rogosa agar was used for simple detection of *Veillonella* at the genus level to predict bacteria growth. It was therefore essential to confirm the sensitivity of this selective medium. In this study, to confirm the sensitivity of Rogosa agar medium, we observed its micro bialcellular and bio-molecular characteristics. A bacteria colony of 173 *Veillonella* in selective medium agar was subjected to morphological observation of the colony's characteristics under a light microscope after Gramstaining. The *Veillonella*

Corresponding author:
Ariadna Adisattya Djais
Department of Oral Biology
Faculty of Dentistry, Universitas Indonesia
E-mail: ariedjais26@gmail.com

genus consists of small, strictly anaerobic Gram-negative cocci in the *Veillonellaceae* family of the bacterial phylum *Firmicutes*[6].

Rogosa agar can distinguish *Veillonella* from other bacteria[3]. The typical *Veillonella* colony on these selective medium were 2–4 mm in diameter, regular and slightly domed in shape with an entireedge, opaque, and grayish white[7]. Gramstaining and observation under a light microscope revealed that the colonyis composed of small, Gram-negative coccal cells, most of which grew as single cells but had some visible short chains[7]. Bio-molecular confirmation was performed with polymerase chain reaction (PCR) and visualized by electrophoresis.

This is the first report to confirm the sensitivity of Rogosa agar by combining selective *Veillonella* medium, colony morphology, Gram-stain observation, and PCR using primer (*Veill-rpoB*F, *Veill-rpoB*R) to identify bacteria in the *Veillonella* genus.

Methods

Preparation of media

BHI agar, a rich medium, is composed of Bacto™ Brain Heart Infusion (Difco Laboratories, BD) supplemented with 5% (vol) defibrinated sheep blood, hemin (10 µg/mL), and menandione (5µg/mL). Rogosa agar, a selective *Veillonella* medium, is composed of Bacto tryptone (5 g/L, Difco), Bacto yeast extract (5 g/L, Difco), sodium thioglycolate (0.75 g/L, Sigma), and Bacto basic fuchsin (0.002 g/L, Difco). The pH values of the media were adjusted to 7.5 prior to auto claving, and vancomycin (7.5µg/mL, Sigma) was added after autoclaving[7].

Before use, this selective *Veillonella* medium was incubated for 24 h at 37ºCunder anaerobic conditions (10% H_2,85% N_2, 5% CO_2) in an anaerobic box (Hirasawa Works Inc.).

Bacterial cultivation

Twelve (12) Eppendorf tubes containing frozen human saliva were thawed and serially diluted tenfold with sterile phosphate buffer saline (PBS)from 10^{-3} to 10^{-8}, and 100 µL was placed on BHI agar and then on Rogosa agar. The saliva sample was spread evenly using small sterile glass marbles and the surface plate and was shaken for one minute. The laboratory procedure was carried out under anaerobic conditions in an anaerobic box. The bacterial colonies growing on selective medium (*Veillonella*-selective agar) were counted, and the mean number of bacterial colonies obtained from plates derived from each Eppendorf tube was documented (Table 1).

After arranging several plates from the same source, 20 colonies were randomly selected. Each colony was re-cultured and spread on new Rogosa agar by incubation at 37ºC under anaerobic conditions(10% H_2, 85% N_2, 5% CO_2) in an anaerobic box (Hirasawa Works Inc.) for 5 days. This was the first step towards obtaining pure bacterial colonies. From the growing colonies, a new set was selected, re-cultured, and spread on a new Rogosa agar, and incubated at 37ºC in an anaerobic atmosphere. This was the second step towards obtaining pure bacterial colonies, and it was performed to ensure that the sample would represent a single colony. The color, size, and morphology of the colonies were visually inspected, and then one colony was taken and confirmed with light microscopy after Gram staining to include Gram-negative cocci.

DNA Extraction

Genomic DNA was extracted from individual bacterial cells isolated on *Veillonella*-selective agar medium using the InstaGene Matrix Kit (Bio-Rad). DNA concentration was based on fluorescence using a Qubit® 3.0 fluorometer (Invitrogen life technologies) in accordance with the manufacturer's instructions.

Identification of oral *Veillonella*

For identification of oral *Veillonella* at the genus level, one primer pair of *Veill-rpoB*F (5'-GTA ACA AAGGTGTCGTTTCTCG-3') and *Veill-rpoB*R (5'-GCACCR TCA AAT ACA GGT GTA GC-3') was used.[7-10]PCR was performed at the genus level in accordance with protocols described elsewhere[7-10] using 1 µl template, 2.5 µl of each primer (10 pmol/ml), 19 ul H_2O and 25 µl ampli Taq Gold ® 360Master Mix (Applied Biosystems). These mixtures were subjected to preheating at 94ºC for 15 min, followed by 20 cycles of 92ºC for 1 min and extension at 72ºC for 1 min, with a final extension at 72ºC for 5 min.

Then, the PCR products were applied to 1.5% agarose gel. After electrophoresis, the gel was stained with SYBR® Safe DNA Gel Stain

(Invitrogen™). The DNA band was visualized by the Cooled CCD Camera System Model AE-6981 Light Capture System (ATTO Corporation). The outcome was printed out to document the results.

Table 1. Colony Forming Unit (CFU/mL) and Gram stain confirmation.

No	Total number		Colony Growth (selective media)	Gram Stain		*Veillonella* genus by PCR
	Oral anaerob bacteria CFU/mL	*Veillonella* spp CFU/mL		Coccus Gram-negative	Others	
1	1.04E+07	9.90E+05	20 / 20	20	0	20
2	4.00E+06	1.86E+06	15 / 20	12	3	12
3	5.30E+08	2.24E+07	12 / 20	8	4	8
4	7.40E+08	4.56E+06	16 / 20	15	1	15
5	7.92E+09	3.80E+05	13 / 20	11	2	11
6	4.10E+09	8.28E+07	09 / 20	8	1	8
7	2.48E+09	2.58E+08	06 / 20	5	1	5
8	2.08E+09	5.85E+07	20 / 20	10	10	10
9	4.40E+06	1.50E+06	16 / 20	12	4	12
10	8.00E+05	2.20E+05	15 / 20	15	0	15
11	2.50E+06	4.00E+04	13 / 20	13	0	13
12	1.20E+06	6.70E+05	18 / 20	10	8	10
	1.49E+09	3.60E+07	173 / 240	139	34	139

Results

The growth of oral anaerobic bacterial colonies on rich media (BHI agar) in terms of Colony Forming Unit per milliliter (CFU/mL) ranged from 8.00E+05 to 7.92E+09 (mean: 1.49E+09). The amount (CFU/mL) of growth on selective *Veillonella* agar medium ranged from 4.00E+04 to2.58E+08 (mean: 3.60E+07). The percentage of *Veillonella* spp. (CFU/mL) compared to all oral anaerobic bacteria was 2.41% (Table 1).

During the first step towards obtaining pure bacterial colonies, the adaptive factors of bacterial growth resulted in 173–240 cultivated plates with single-colony growth (Table 1).

Of the 173 Rogosa agar plates with bacterial colony growth, 139 (80.3%) plates showed Gram-negative cocci under light microscope after Gramstaining, and 34 plates (19.6%) included Gram-positive cocci and Gram-negative short rod bacteria. All of the bacterial colonies (139) consisting of Gram-negative cocci were later identified to be members of the *Veillonella* genus using PCR methods and *Veill-rpoB* primer.

Discussion

In this study, the main source of the sample was human saliva. It is easily collected, a non-invasive biological material, and suitable for medical investigation, reflecting several health and disease-associated factors. Saliva is being thoroughly investigated in general and in the field of dental medicine in order to search for health and disease biomarkers.[11-14]

Consisting of 99% water, saliva has a complex composition that includes urea, ammonia, uric acid, glucose, cholesterol, fatty acid, triglycerides, neutral lipid, glycolipid, amino acid, steroid hormones, mucin, amylase, lectin, glycoprotein, lysozyme, peroxidase, and lactoferrin.[15] Fluid saliva bathes both cleaned surfaces and attached cells with a variety of species suspended in saliva. A highly selective mechanism of co-aggregation (inter bacterial adherence) between species is involved in the development of multispecies communities.[16] Many of these inter- and intra species co-aggregation mechanisms can be reversed by adding simple sugars such as lactose. The initial

community comprised of two streptococcal species and a *Veillonella* sp. Co-aggregated with each other, and some of the co-aggregations were lactose-reversible.[17]

Veillonellae have been shown to coaggregate with many oral bacteria, including *S.salivarius, S.mutans*, *Eubacterium saburreum*, and *Actinomyces viscosus*. With regard to the potential interdependence of oral bacteria and their co-aggregation partners, *Veillonellae* is a particularly interesting group of organisms to consider. First, for metabolic energy, they utilize pyruvate and lactate. Second, *Veillonellae* appear to have a limited ability to adhere to host tissue. *Veillonellae* may derive particular benefit from adherence to and interaction with other bacterial species.[18]

Table 1 shows the results of a sample of saliva poured onto 12 Rogosa agar plates, with the entire plates growing bacteria. The condition of growing bacteria is caused by coaggregation of *Veillonella* with other bacteria and a supply of lactic acid to fertilize the growth of Gram-negative coccus bacteria. *Veillonellae* use lactic acid for growth in saliva, and they communicate metabolically with initial, early, middle, and late colonizers to establish multispecies communities on enamel.[18]

In the first step to achieving pure bacterial colonies, bacterial colonies grew on 173 of the 240 Rogosa agar plates in this study. The growth of the bacteria is not due to absolute single colony re-breed cannot afford to adapt to Rogosa agar media. Rogosa agar media or selective *Veillonella* medium agar consisted of Bacto tryptone (5g/L, Difco), Bacto yeast extract (5g/L, Difco), sodium thioglycolate (0.75 g/L, Sigma), Bacto basic fuchsin (0.002 g/L, Difco), 60% sodium lactate (21mL/L, Sigma), and Bacto agar (15 g/L Difco). The pH values of the media were 7.5,and vancomycin (7.5 µg/mL, Sigma) was added to each.[17] The inoculated plates were incubated at 37ºC in an anaerobic box containing 80% N_2, 10% CO_2, and 10% H_2 for 5days.[8] This media was able to grow bacteria on all Rogosa agar plates (173/173) in the second step towards achieving pure colonies. In the second step, the bacteria were likely to adapt to the Rogosa agar media in anaerobic conditions.

In our study, the total number of oral anaerobic bacteria in saliva was detected by cultivation on rich medium agar (BHI agar)[9], and the mean number was 1.49E+09. Anaerobic Gram-negative cocci in saliva were detected by *Veillonella*-selective medium[9], with a mean of 3.60E+07.The percentage of these bacteria in comparison to all oral anaerobic bacteria was not more than 5%. This data corresponds to a previous report showing that the majority of oral anaerobic bacteria were Gram-positive cocci[19] and that oral anaerobic Gram-negative cocci (*Veillonellae*) constitutes as much as 5% of the initial plaque biomass.[18]

Isolates are presumptively identified as *Veillonella* spp. based on their ability to grow on the selective medium.In terms of biochemical identification, *Veillonella* can be further characterized as it does not involve fermented glucose or any other carbohydrate. Indole is not produced, gelatin is not liquefied, nitrate is reduced, and H_2S is produced. Propionic acid and acetic acids, CO_2 and H_2, are produced from lactate during growth, and pyruvic, oxaloacetic, malic, fumaric, and succinic acids are metabolized by resting cells, but citric, isocitric, and malonic acid are not.[15] Identifying members of the *Veillonella*genus viabiochemical reaction is time-consuming and the procedures are multifarious because they must take place in an anaerobic box with limited space.

In our study, isolates are presumptively identified as *Veillonella* spp. if they featured the typical opaque or grayish-white colonies with a diameter of 2–4 mm, a regular and slightly domed shape with an entire edge, and Gram-negative spherical cells of 0.3–0.5 µm under a microscope.[20]

Absolute identification of the *Veillonella* genus with the PCR technique is possible using the *rpoB* primer.[9,21] The small sub unit ribosomal RNA gene is the gold standard for estimating microbial phylogenetic diversity. The use of the *rpoB* gene offers various potential advantages over standard 16S rRNA gene-based approaches. The *rpoB* is a protein-encoding gene, and the data generated from this marker is more readily interpreted in an evolutionary framework.[22]

In our study, all colonies that grew in Rogosa medium, were incubated in anaerobic conditions, featured the typical colonial appearance of *Veillonella*, and were identified as Gram-negative cocci by Gramstaining, were identified as members of the *Veillonella* genus. Detection of the genus of *Veillonella* bacteria from clinical samples was efficient and effective,

leaving the biochemical and bio-molecular stage test. Accurate selective *Veillonella* medium, anaerobic incubation in anaerobic boxes, and observations of the typical colony morphology and cell morphology after Gramstaining can identify members of the *Veillonella* genus.

Conclusions

In this study, the sensitivity of Rogosa agar as a *Veillonella*-selective medium was shown to be 80.3%.Bacterial cells of typical *Veillonella* colonies were confirmed by light microscopy after Gramstaining, and100% of the Gram-negative cocci were identified as members of the genus *Veillonella*. This was further confirmed by PCR using the primers *Veill-rpoB*F and *Veill-rpoB*R.

Acknowledgements

The authors wish to convey our gratitude to Professor Futoshi Nakazawa from the Health Sciences Department of Hokkaido University Japan for support and laboratory materials.

The publication of this manuscript is supported by Universitas Indonesia.

References

1. Sim CP, Dashper SG, Reynolds EC. Oral Microbial Biofilm Models and Their Application to the Testing of Anticariogenic Agents. J Dent 2016;50:1-11.
2. Qiong Zhou, Xiurong Qin, Man Qin & Lihong Ge. Genotypic Diversity of Streptococcus mutans and Streptococcus sobrinus in 3-4 Year-Old Children with Severe Caries or without Caries. Int J Paediatr Dent 2011;21(6):422-31.
3. Liu J, Wu C, Huang IH, Merritt J, Qi F. Differential Response of Streptococcus Mutans towards Friend and Foe in Mixed-Species Cultures. Microbiology 2011;157(Pt 9):2433-44.
4. Gross EL, Beal CJ, Kutsch SR, Firestone ND, Leys EJ, Griffen AL. Beyond Streptococcus mutans Dental Caries Onset Linked to Multiple Species by 16S rRNA Community Analysis. PLoS One 2012;7(10):e47722.
5. Byun R, Carlier JP, Nicholas A, Jacques, Marchandin H, Hunter N. Veillonella denticariosa sp. nov., Isolated from Human Carious Dentine. Int J Syst Evol Microbiol 2007;57:2844-8.
6. Tammi Vesth, Asli Ozen, Sandra C Andersen, et al. Veillonella, Firmicutes: Microbes Disguised as Gram Negatives. Stand Genomic Sci2013;9(2):431-48.
7. Arif N, Do T, Byun R et al. Veillonella rogosae sp. nov., an Anaerobic Gram-Negative Coccus Isolated from Dental Plaque. Int J Syst Evol Microbiol 2008;58(Pt 3):581-4.
8. Beighton D, Clark D, Hanakura B,Gilbert S, Do T. The Predominant Cultivable Veillonella spp. of the Tongue of Healthy Adults Identified Using rpoB Sequencing. Oral Microbiol Immunol 2008;23(4):344-7.
9. Mashima I, Kamaguchi A, Nakazawa F. The Distribution and Frequency of Oral Veillonella spp. in the Tongue Biofilm of Healthy Young Adults. Curr Microbiol 2011;63(5):403-7.
10. Igarashi E, Kamaguchi A, Fujita M,Miyakawa H, Nakazawa F. Identification of Oral Species of the Genus Veillonella by Polymerase Chain Reaction. Oral Microbiol Immunol 2009;24(4):310-3.
11. Lee YH, Wong DT. Saliva: An Emerging Biofluid for Early Detection of Diseases. Am J Dent 2009;22(4):241-8.
12. Giannobile WV, McDevitt JT, Niedbala RS, Malamud D. Translation and Clinical Application of Salivary Diagnostics. Adv Dent Res 2011;23(4):375-80.
13. Rathnayake N, Akerman S, Klinge B, et al. Salivary Biomarkers of Oral Health: A Cross-Sectional Study.J Clin Periodontol 2013;40(2):140-7.
14. Rathnayake N, Akerman S, Klinge B, et al. Salivary Biomarkers for Detection of Systemic Diseases. PLoS One 2013;8(4):e61356.
15. Zhang CZ, Cheng XQ, Li JY, et al. Saliva in the Diagnosis Diseases. Int. J Oral Sci 2016;8(3):133-7.
16. Kolenbrander PE, Palmer RJ Jr, Periasamy S, Jakubovics NS. Oral Multispecies Biofilm Development and the Key Role of Cell-Cell Distance. Nat Rev Microbiol 2010;8(7):471-80.
17. Kolenbrander PE. Multispecies Communities: Interspecies Interactions Influence Growth on Saliva as Sole Nutritional Source. Int J Oral Sci 2011;3(2):49-54.
18. Hughes CV, Kolenbrander PE, Andersen RN, Moore LV. Coaggregation Properties of Human Oral Veillonella spp: Relationship to ColonizationSite and Oral Ecology. Appl Environ Microbiol 1988;54(8):1957-63.
19. Murphy EC, Frick IM. Gram-Positive Anaerobic Cocci-Comensals and Opportunistic Pathogens. FEMS Microbiol Rev 2013;37(4):520-3.
20. Jumas-Bilak E, Carlier JP, Pierre HJ. et al. Veillonella montpellierensis sp. nov., a Novel Anaerobic Gram-Negative Coccus Isolated from Human Clinical Samples.Int J Syst Evol Microbiol 2004;54:1311-6.
21. Mashima I, Theodorea CF, Thaweboon B, Thaweboon S, Nakazawa F. Identification of Veillonella Species in the Tongue Biofilm by Using a Novel One-Step Polymerase Chain Reaction Method. PLoS One2016;11(6):e0157516.
22. Vos M, Quince C, Pijl AS, de Hollander M, Kowalchuck GA. A Comparison of rpoB and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity. PLoS One 2012;7(2):e30600.