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Identification of oral species of the genus *Veillonella* by polymerase chain reaction

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Introduction: Members of the genus *Veillonella* cannot be reliably distinguished by their biochemical characteristics and phenotypic features. Moreover, DNA–DNA hybridization and sequence analyses of the 16S ribosomal RNA gene including random fragment length polymorphism analysis, are complex and time-consuming procedures that are not well-suited to identifying oral species of *Veillonella*: *Veillonella atypica*, *Veillonella denticariosi*, *Veillonella dispar*, *Veillonella parvula*, and *Veillonella rogosae*.
Methods: In this study, five forward primers and a reverse primer were designed for polymerase chain reaction (PCR) according to the partial sequences of the *rpoB* genes of these oral *Veillonella* species.
Results: The forward primers were species-specific for these five *Veillonella* species, and could produce specific amplicons when used together with reverse primer and individual DNA templates of these species in PCR. These primer pairs were also found to discriminate between the respective species, and the *Veillonella* strains isolated from human oral cavities were successfully assigned to one of the five oral species of the genus *Veillonella* based on their specific products by PCR.
Conclusion: A simple two-step PCR procedure using the five sets of primer pairs developed in the present study is a rapid and reliable method for the identification of the recognized oral *Veillonella* species.

Key words: identification; oral species; polymerase chain reaction; *rpoB*; *Veillonella*

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The genus *Veillonella* consists of small, strictly anaerobic, gram-negative cocci that lack flagella, spores, and capsule. Taxonomically, the genus *Veillonella* belongs to the family Acidaminococcaceae of the phylum Firmicutes, and is widely distributed in the oral, genitourinary, respiratory, and intestinal flora of humans and animals.

The genus *Veillonella* is subdivided into 10 species: *Veillonella atypica*, *Veillonella caviae*, *Veillonella criceti*, *Veillonella denticariosi*, *Veillonella dispar*, *Veillonella montpellierensis*, *Veillonella parvula*, *Veillonella ratti*, *Veillonella rodentium*, and *Veillonella rogosae*. Of these, only *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, and *V. rogosae* have been isolated from human oral cavities (2, 3, 6, 11, 20, 22). The main habitats of the oral *Veillonella* species are the tongue, dental plaque, and buccal mucosa (1, 5, 20, 22). Also, *V. parvula* and other oral *Veillonella* species have been found in intraradicular infections (26, 28), including cases of abscess (12), in the apical root canal (4), and in the dentinal tubules at this region (21).

Veillonella are relatively easily identified to the genus level, but the discrimination of *Veillonella* strains at the species level remains difficult and inconclusive because there is a lack of conventional phenotypic and biochemical discriminating tests (6, 11, 14, 19, 24). Therefore, molecular methods based on 16S ribosomal RNA (rRNA) gene sequencing including polymerase chain reaction (PCR) random fragment length polymorphism analysis have been used to identify *Veillonella* strains at the species level (19, 24).

However, recent studies have shown that 16S rDNA-based approaches do not yield reliable data for the identification of *Veillonella* species, particularly oral species of *Veillonella* such as *V. dispar* and *V. parvula*, owing to the high level of sequence conservation in the 16S rRNA gene sequence among several *Veillonella*

species and to the relatively high level of intrachromosomal heterogeneity in some *Veillonella* isolates from human oral cavities (11, 19).

Recently, to overcome the limitations associated with using 16S rRNA gene sequences to identify *Veillonella* species, the sequence analysis of housekeeping genes, including *dnaK*, *gyrB*, and *rpoB*, has been suggested as an effective method for identification of oral *Veillonella* at the species level (3, 5). In fact, two new species, *V. denticariosi* and *V. rogosae*, have been proposed as new oral *Veillonella* based on the results of molecular methods using *dnaK* and *rpoB* gene sequencing in conjunction with sequence analysis of the 16S rRNA gene (2, 11). In addition, Beighton et al. (5) successfully used the *rpoB* gene sequence rather than the 16S rRNA sequence to discriminate between 253 *Veillonella* isolates from human tongues at the species level. However, analysis of the *rpoB* gene would be a fairly inconvenient and time-consuming method for the identification of many strains in many clinical samples.

In the present study, based on the results of a comparison of the *rpoB* gene sequences across the oral *Veillonella* spp. *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, and *V. rogosae*, a highly variable region (positions 2500–3100) in the *rpoB* gene was selected for the design of specific primers. Five forward primers were established from the sequences of this region as species-specific primers for the five species. In addition, one reverse primer was designed from the sequence of the conserved region of the *rpoB* gene. Finally, it was demonstrated that these primer sets could easily identify all of the oral *Veillonella* by their PCR products at species level.

Materials and methods

Bacterial strains and culture conditions

V. atypica ATCC 17744^T (where T indicates type strain), *V. dispar* ATCC 17748^T, and *V. parvula* ATCC 10790^T were obtained from the American Type Culture Collection (Rockville, MD). *Veillonella denticariosi* N38 and *V. rogosae* I6 were used as reference strains of the respective species of *Veillonella*, because these strains were identified by comparison studies of their *rpoB* gene sequences with the type strains, *V. denticariosi* CIP 109448^T and *V. rogosae* DSM 18960^T, respectively. Also, 67 laboratory strains were taken from our departmental collection. They were gram-negative anaerobic small cocci, and the strains had been identified as members of the genus *Veillonella* on the basis of their ability to grow on improved *Veillonella* agar (23) and their typical colonial appearance: colonies were 2–4 mm in diameter, regular, slightly domed in shape with an entire edge, opaque, and grayish white. These colonies had no hemolytic activity in blood agar.

All bacterial strains were grown in tryptic soy broth supplemented with 0.6% sodium lactate at 37°C for 3 days in an anaerobic glove box containing 80% N_2, 10% H_2, and 10% CO_2.

Preparation of bacterial chromosomal DNA

Chromosomal DNA was extracted from bacterial cells according to the method of Smith et al. (25). Briefly, chromosomal DNA was obtained after treatment with sodium dodecyl sulfate, proteinase K, phenol/chloroform/isoamyl alcohol extraction, and ethanol precipitation, followed by RNAase treatment, phenol/chloroform/isoamyl alcohol extraction, and ethanol precipitation.

PCR protocol

PCR was performed using 1 μl template, 2.5 μl of each primer (10 pmol/ml), 19 μl H_2O, and 25 μl master mix from a HotStarTaq Master mix kit (Qiagen, Valencia, CA). These mixtures were subjected to preheating at 94°C for 15 min followed by 20 cycles of 92°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. Then, the PCR products were applied to a 1.5% agarose gel. After electrophoresis, the gel was stained with ethidium bromide.

DNA sequence analysis

For DNA sequence analysis, PCR products were purified using a QIAquick spin PCR purification kit (Qiagen) following the manufacturer's protocol. Cycle sequencing was performed with a mixture composed of sample DNA, a forward or reverse primer (1 pmol/ml) and an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). After reaction, the mixtures were purified with a spin column and denatured using Template suppression reagent (Applied Biosystems) at 95°C for 2 min. DNA sequence was analysed with the ABI PRISM 310 DNA sequencer (Applied Biosystems). The level of sequence similarities was estimated by using the simple homology program DNASIS (Hitachi, Tokyo, Japan).

Design of primer pairs

After alignment of each DNA partial sequence of the *rpoB* gene, the species-specific primers for the oral *Veillonella* spp. were designed by the standard manual method.

Results and discussion

The accession numbers of the *rpoB* gene sequences of reference strains from the GenBank data base (http://www.ncbi.nlm.nih.gov/) were EF185159 for *V. atypica*, EF185162 for *V. denticariosi*, EF185161 for *V. dispar*, EF185181 for *V. parvula*, and EF211831 for *V. rogosae*. From the results of a similarity search of these *rpoB* gene sequences, a high degree of polymorphism was observed in the region from position 2500 to 3100 in all the oral *Veillonella* species. The similarities among the partial sequences of the *rpoB* gene regions of these species were found to be from 75.9 to 91.3% (Table 1).

To confirm the high polymorphism of the region, we analysed the DNA sequence from positions 2500 to 3100 of the *rpoB* gene of *Veillonella* strains isolated from human oral cavities and stored in the departmental collection. The partial sequencing studies indicated that the region of the *rpoB* gene of oral *Veillonella* strains showed universally high variability (data not shown). This suggested that this region of the *rpoB* gene contained suitable sequences for the design of specific primers to discriminate between oral *Veillonella* at the species level by PCR.

In the present study, according to the sequences of the variable regions in the *rpoB* gene of oral *Veillonella* species, five forward primers were designed as species-

Table 1. Level of *rpoB* partial sequence similarity among oral *Veillonella* species

	Percentage similarity with			
Species	*V. atypica*	*V. denticariosi*	*V. dispar*	*V. parvula*
V. denticariosi	75.9			
V. dispar	83.3	77.3		
V. parvula	78.4	79.4	84.4	
V. rogosae	78.2	81.4	83.8	91.3

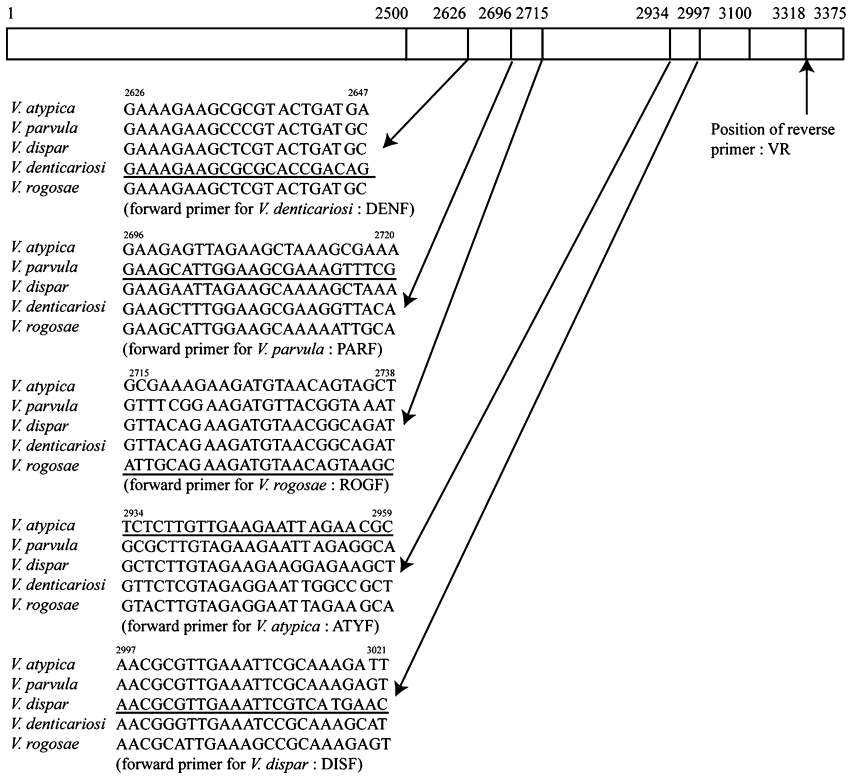

Fig. 1. Location and sequence of the species-specific primer in the *rpoB* gene of oral *Veillonella* spp. Underlines indicate the nucleotide sequences of each forward primer for *Veillonella* spp. Numbers indicate the nucleotide positions in the *rpoB* gene of the *Veillonella* spp.

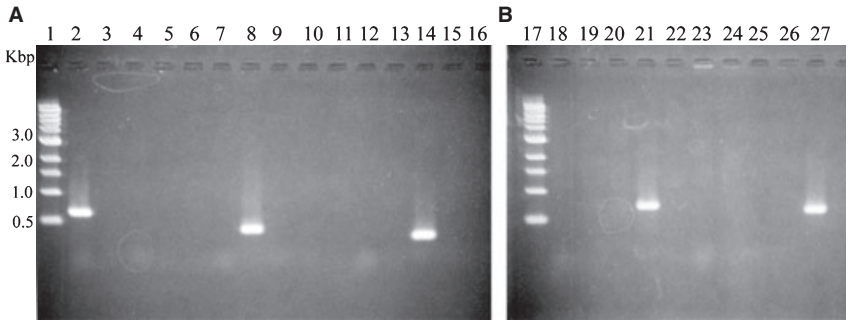

Fig. 2. The polymerase chian reaction products obtained by using species-specific primer pairs. (A, B) Molecular weight marker in lanes 1 and 17. Primer pairs: PARF-VR, lanes 2, 7, 12, 18, and 23; ATYF-VR, lanes 3, 8, 13, 19, and 24; DISF-VR, lanes 4, 9, 14, 20, and 25; DENT-VR, lanes 5, 10, 15, 21, and 26; ROGF-VR, lanes 6, 11, 16, 22, and 27. Template DNA from *Veillonella parvula*; lanes 2–6; *Veillonella atypica*, lanes 7–11; *Veillonella dispar*, lanes 12–16; *Veillonella denticariosi*, lanes 18–22; and *Veillonella rogosae*, lanes 23–27.

specific primers. These specific forward primers were designated as DENF for *V. denticariosi*, as PARF for *V. parvula*, as ROGF for *V. rogosae*, as ATYF for *V. atypica*, and as DISF for *V. dispar* (Fig. 1). In addition, one reverse primer was constructed using the sequence of the conserved region of the *rpoB* gene and was designated as VR (5′-GTGTAACAAGG-GAGTACGGACC-3′) for all of the oral *Veillonella* species (Fig. 1).

To examine the specificity of the five primer pairs (DENF-VR, PARF-VR, ROGF-VR, ATYF-VR, and DISF-VR), PCR was performed with DNA templates from the type or the reference strain of each oral *Veillonella* species. The electrophoretically detected amplicons of PCR are shown as species-specific products (Fig. 2). The molecular weights of the PCR products were also identical to the theoretical values, which were 623 base

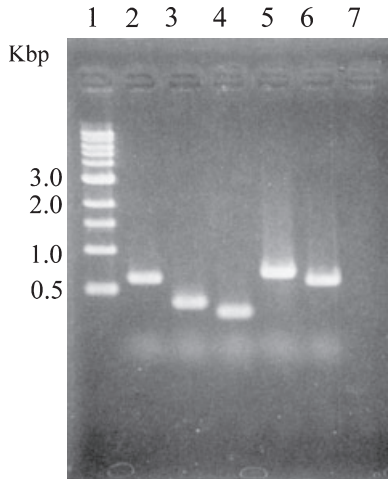

Fig. 3. The polymerase chain reaction products obtained by using the mixture of primers. Molecular weight marker is in lane 1. Primer was a mixture of PARF, ATYF, DISF, DENF, ROGF, and VR. Template DNA from *Veillonella parvula*, lane 2; *Veillonella atypica*, lane 3; *Veillonella dispar*, lane 4; *Veillonella denticariosi*, lane 5; and *Veillonella rogosae*, lane 6.

pairs (bp) for *V. parvula*, 384 bp for *V. atypica*, 321 bp for *V. dispar*, 604 bp for *V. rogosae*, and 693 bp for *V. denticariosi* (Fig. 2). These results demonstrated that these five primer pairs could effectively identify the recognized oral species of genus *Veillonella.*

As an additional step to realize faster and more convenient identification of the oral *Veillonella* species, we examined the simultaneous use of all five primers in a single PCR mixture. When DENF, PARF, ROGF, ATYF, DISF, and VR were mixed in one PCR tube with DNA template, only one PCR product was detected against each of the DNA templates from the oral *Veillonella* species individually (Fig. 3). The molecular weights of the PCR products were also identical to the theoretical values as indicated before. These results indicated that the oral *Veillonella* species could also be species specifically identified by PCR using a mixture of primers, DENF, PARF, ROGF, ATYF, DISF, and VR.

However, when all DNA templates from the five *Veillonella* species were used at once in the PCR procedure, it was quite difficult for the mixture of the five primers to clearly identify *V. denticariosi*, *V. parvula*, and *V. rogosae* because the PCR products from these three species were located close to each other on the agarose gel after electrophoresis. Therefore, we devised a two-step PCR procedure to identify all five species of oral *Veillonella*. The first step of PCR was performed with a mixture composed of four primers,

PARF, ATYF, DISF, and VR. The second step of PCR was carried out with a mixture of three primers, DENF, ROGF, and VR, for organisms that were not identified in the first step. *V. denticariosi* and *V. rogosae* could be identified by the second step of the PCR procedure.

The two-step PCR method was applied to 67 laboratory strains that had been identified as members of the genus *Veillonella* to confirm its efficacy. All 67 laboratory strains were identified as one of the five *Veillonella* species as follows: four strains *of V. atypica*, 55 strains of *V. parvula*, one strain of *V. dispar*, two strains of *V. denticariosi*, and five strains of *V. rogosae*. These results verified the usefulness and propriety of the two-step-method of PCR for identification of oral *Veillonella* strains at the species level.

Recently, three new species of the genus *Veillonella* were isolated (2, 6, 11). Those reports stated that comparison of the 16S rRNA gene sequence was not a method for identifying these new species, and so alternative gene sequences were compared to identify these *Veillonella* spp. In particular, it was reported that sequence analysis of the *rpoB* gene of *Veillonella* spp. was useful for the identification of these microorganisms (2). The *rpoB* gene sequences have also been used for the identification and phylogenic study of various bacterial species (13, 15, 16).

Members of the oral *Veillonella* are known as volatile sulfur-compound-producing bacteria and are a cause of oral malodor (5, 8–10, 17, 18, 27). However, the prevalence of the oral *Veillonella* spp. in the tongue biofilm, in the oral cavity, and in the region of endodontic infection has not been fully clarified because of the difficulty of identifying oral *Veillonella* at species level.

Recently, *Veillonella* isolates from human tongues were identified by sequencing their *rpoB* genes (5). However, the sequence analysis of the *rpoB* genes of all microorganisms in many samples would be a time-consuming procedure. On the other hand, the identification by detection of amplicons of PCR would be very easy and relatively fast.

To resolve the above-mentioned problems, we have established species-specific primers for PCR by using the partial sequences of *rpoB* genes to identify the five species of recognized oral *Veillonella*. In addition, we developed the two-step method of PCR as a convenient and time-saving means of identifying oral *Veillonella* species in many samples. This is the first report indicating that PCR products targeting the *rpoB* gene can identify oral *Veillonella* at species level.

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