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Health Indicator Bacteria That Is Useful for Risk Assessment of Peri-Implantitis

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Abstract

We established a novel identification method for oral *Rothia* species using one-step multiplex PCR analysis to investigate whether the monitoring of oral *Rothia* species levels is useful for peri-implantitis risk assessment, and to examine the oxygen concentration that these organisms need for growth *in vitro*. The mean number and proportion of *Rothia aeria* in peri-implant sulcus fluid (PISF) samples was significantly higher in the healthy implant group than in the peri-implantitis group ($P < 0.05$). Moreover, *R. aeria* under aerobic conditions vigorously grew compared with those under anaerobic conditions, and this organism grew only at the upper layer where high oxygen concentrations existed in a semi-liquid nutrient medium. Therefore, the monitoring of *R. aeria* levels may be suitable as an indicator of healthy peri-implant tissue conditions for the prevention of peri-implantitis.

Keywords

Peri-Implantitis, Multiplex PCR, *Rothia aeria*, Oral Cavity

1. Introduction

The successful use of jawbone-anchored (osseointegrated) titanium dental implants for the rehabilitation of edentulous and partially dentate patients has been well documented [1]-[7]. The results of implant treatment have mostly been satisfactory with survival rates of 85% to 99%. However, infections such as implant mucositis and peri-implantitis occur around dental implants [8] [9] [10] [11] [12]. The prevalence of peri-implant infections, defined as bone loss ≥ 3.1 mm compared with one-year radiographic data after placement of the supra-structure, and bleeding on probing (BOP) approaches 20% of cases within a 15-year follow-up

period [13]. Patients with a history of periodontitis also appear to be more susceptible to developing peri-implant infections [14]. Smoking is another risk factor that has been associated with peri-implant infections [15] [16] [17] [18].

Shortly after the installation of titanium implants, an implant sub-mucosal microbiota is established [19]. In fact, the initial colonization of peri-implant pockets with bacteria associated with periodontitis has been demonstrated to occur within two weeks [20]. This early colonization pattern may contribute to the development of peri-implant lesions. Leonhardt *et al.* [21] reported that peri-implantitis lesions contain not only periodontopathic bacteria but also staphylococci, enteric species, and yeasts, indicating that a complex microbiota is associated with the infections of tissues surrounding implants. Such observations are consistent with the hypothesis that an extensive unknown microbiota may be associated with periodontitis [22].

Currently, bacteriological examinations of implant treatments target periodontopathic bacteria such as red complex bacteria, including *Porphyromonas gingivalis*, and detect them qualitatively or quantitatively. However, those examinations do not reflect the peri-implant tissue conditions precisely, because periodontopathic bacteria may be detected from healthy peri-implant sites [23]. Consequently, we concluded that it is suboptimal to use periodontopathic bacteria as an unhealthy indicator, and have instead explored bacteria that indicate healthy peri-implant tissue conditions. We chose oral *Rothia* species, which are part of normal oral flora, to be examined as potential health indicators. These species include *Rothia mucilaginosa*, *Rothia dentocariosa*, and *Rothia aeria* [24].

In addition to periodontitis, peri-implantitis is primarily caused by bacterial infection and presents symptoms such as soft tissue inflammation and bone resorption, but often progresses asymptomatically. However, peri-implantitis rapidly progresses compared with periodontitis, and therapeutics for periodontitis have limited effectiveness against peri-implantitis [25] [26] [27]. The detachment of the implant body in severe peri-implantitis cases occurs by resorption of the supporting bone, thereby reducing the quality of life of patients. In order to prevent the onset of peri-implantitis, it is necessary to establish a useful bacteriological examination system.

In the present study, we established a high-precision novel identification method for oral *Rothia* species using one-step multiplex PCR analysis to investigate whether oral *Rothia* species levels are useful for peri-implantitis risk assessment. We also examined the oxygen concentrations that this organism needs for growth *in vitro*. Furthermore, the relationship between red complex bacteria most involved in periodontal disease and peri-implantitis was also investigated using PCR analysis.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

The following bacterial strains were used in the present study: *R. mucilaginosa*

JCM 10910, *R. dentocariosa* JCM 306, *R. aeria* JCM 11412, *R. aeria* Num-Ra7006, *Rothia terrae* JCM 15158, *Rothia amarae* JCM 11375, *Rothia nasimurium* JCM 10909, *Rothia endophytica* JCM 18541, *Streptococcus mitis* ATCC 49456, *Streptococcus oralis* ATCC 10557, *Streptococcus sanguinis* ATCC 10556, *Streptococcus salivarius* JCM 5707, *Streptococcus anginosus* ATCC 33397, *Streptococcus mutans* NCTC 10449, *Actinomyces naeslundii* ATCC 12104, *Actinomyces oris* ATCC 27044, *Actinomyces odontolyticus* ATCC 17929, *Actinomyces israelii* ATCC 12102, *Neisseria sicca* ATCC 29256, *Corynebacterium matruchotii* ATCC 14266, *Corynebacterium durum* ATCC 33449, and *P. gingivalis* ATCC 33277. Bacterial strains other than *P. gingivalis* were maintained by cultivation on Bact™ Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). All bacterial strains were cultured at 37˚C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator (MCO-18AIC; Sanyo Electric Co., Tokyo, Japan). *P. gingivalis* was cultured at 37˚C for 48 h under anaerobic conditions with a gas pack system (AnaeroPack®; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

2.2. Design of Species-Specific Primers for Oral *Rothia* Species

The design of species-specific primers for oral *Rothia* species was performed as described previously [24]. Briefly, the 16S rDNA sequences of *R. dentocariosa* (accession no. M59055), *R. mucilaginosa* (accession no. X87758), and *R. aeria* (accession no. AB071952) were obtained from the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/services.html, Mishima, Japan), and a multiplex sequence alignment analysis was performed using the CLUSTALW program; *i.e.*, the 16S rDNA sequences of seven *Rothia* species were aligned and analyzed. Homologies among the primers selected for *R. dentocariosa*, *R. mucilaginosa*, and *R. aeria* were confirmed by a BLAST search.

2.3. Development of a Novel One-Step Multiplex PCR Method Using Designed Primers

Bacterial cells were cultured in BHI supplemented with 0.5% yeast extract for 24 h, and 1 ml samples were then collected in microcentrifuge tubes and resuspended at a density of 1.0 McFarland standard [approximately 10^7 colony-forming units (CFU)/ml] in 1 ml of sterile distilled water. A total of 5.6 µl of the suspension was then used as the PCR template. The detection limit of PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 0.2 µM of each primer, 10 µl of 2 × MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 5.6 µl of the template in a final volume of 20 µl. PCRs were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Forster City, CA, USA). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 25 cycles consisting of 98˚C for 10 s and 68˚C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis and then

visualized by electrophoresis in 1 × Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker. All experiments were performed in triplicate.

2.4. Clinical Samples

Sixty patients attending Nihon University Hospital, School of Dentistry at Matsudo, participated in the present study. They were divided into two subject groups: healthy implant (HI) and peri-implantitis (PI) groups. Thirty HI and thirty PI subjects were selected by inclusion criteria for peri-implantitis as follows: patients who underwent dental implantation treatments between 2015 and 2019; patients with at least one dental implant for more than half a year; according to the Guidelines of Periodontology, PI was defined as bleeding of probing (BOP) and/or probing pocket depth (PPD) ≥ 4 mm, accompanied by bone tissue loss under the first thread of the implant (*i.e.*, bone absorption ≥ 2 mm). HI was defined as PPD ≤ 3 mm, and the absence of BOP, pus discharge, and bone absorption. Exclusion criteria were as follows: patients with systematic diseases; patients receiving periodontal therapy within six months; taking immunosuppressive agents or antibiotics; the long-term use of contraceptive drugs; pregnant women.

Peri-implant sulcus fluid (PISF) samples were collected using endodontic paper points from all subjects and placed in a sterile microcentrifuge tube containing 1 ml of Tris-HCl buffer (0.05 M, pH 7.2). Samples were dispersed by sonication for 30 s in an ice bath (50 W, 20 kHz, Astrason® System model XL 2020, NY., USA). Portions (100 μl) of appropriate dilutions of these samples were plated, in triplicate, on Anaerobic Blood Agar (CDC), that consists of a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with vitamin K_1, hemin, L-cysteine, yeast extract, and sheep blood, and on selective medium plates that were developed for the isolation of oral *Rothia* species in our previous report [24]. Selective medium plates for oral *Rothia* species were cultured at 37˚C for three days in an atmosphere of 5% CO_2 in a CO_2 incubator. CDC plates for total cultivable bacteria were cultured at 37˚C for five days under anaerobic conditions with a gas pack system. After the cultivation, the number of CFU was calculated. Also, the detection frequencies of red complex bacteria, *i.e.*, *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia* in the remainder of each PISF sample were determined using PCR as previously described [28]. This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 19-033).

2.5. Examination of Oxygen Concentrations that *R. aeria* and Anaerobic Bacteria Needed for Growth *in Vitro*

2.5.1. Comparison of the Growth of *R. aeria* between Two Culture Conditions

BHI agar plates on which *R. aeria* were inoculated were cultured at 37˚C for three days under aerobic conditions and under anaerobic conditions with a gas

pack system. After cultivation, the number of CFUs was calculated, and the CFUs of *R. aeria* under the two culture conditions were compared.

2.5.2. Comparison of Growth in a Semi-Liquid Nutrient Medium between *R. aeria* and Anaerobic Bacteria

A semi-liquid nutrient medium that consisted of BHI and 0.8% agar was prepared in a glass test tube. Bacterial suspensions of *R. aeria* JCM 11412 and *P. gingivalis* ATCC 33277 that were preincubated were inoculated into each medium with an inoculating needle and were cultured at 37˚C for two days under aerobic conditions. After cultivation, the growth of *R. aeria* and *P. gingivalis* in semi-liquid nutrient medium was macroscopically compared.

2.6.Statistical Analysis

The numbers of oral *Rothia* species and total bacteria in the PISF samples from the HI and PI groups were compared using the Mann-Whitney *U* test. Values of $P < 0.05$ were considered significant. The detection frequencies of red complex bacteria in both groups were compared using Fisher's exact test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Multiplex PCR

3.1.1. Primer Design

Six specific primers covering the upstream regions of the 16S rDNA sequences of three oral *Rothia* species were designed in the present study (**Figure 1**). The specific forward primers were designated as RMFF for *R. mucilaginosa*, RDFF for *R. dentocariosa*, and RAFF for *R. aeria*, whereas the specific reverse primers were designated as RMFR for *R. mucilaginosa*, RDFR for *R. dentocariosa*, and RAFR for *R. aeria*. The amplicon sizes of *R. mucilaginosa*, *R. dentocariosa*, and *R. aeria* were 356 bp, 541 bp, and 924 bp, respectively.

3.1.2. Detection Limit

Our one-step multiplex PCR method for identifying three oral *Rothia* species, *R. mucilaginosa*, *R. dentocariosa*, and *R. aeria*, successfully amplified DNA fragments of the expected sizes for each species (**Figure 2**). The detection limit was assessed in the presence of titrated bacterial cells, and the sensitivity of the PCR assay was between 5 × 1 and 5 × 10 CFU per PCR template (5.0 µl) for the *R. mucilaginosa*-specific primer set with strain JCM 10910, the *R. dentocariosa*-specific primer set with strain JCM 306, and the *R. aeria*-specific primer set with strain JCM 11412 (data not shown).

3.1.3. Assay of Representative *Rothia* Species and Representative Oral Bacteria

The one-step multiplex PCR method used to identify *R. mucilaginosa*, *R. dentocariosa*, and *R. aeria* produced positive bands from each respective strain (**Figure 1**) and did not produce any amplicons from other *Rothia* species or with *Streptococci*,

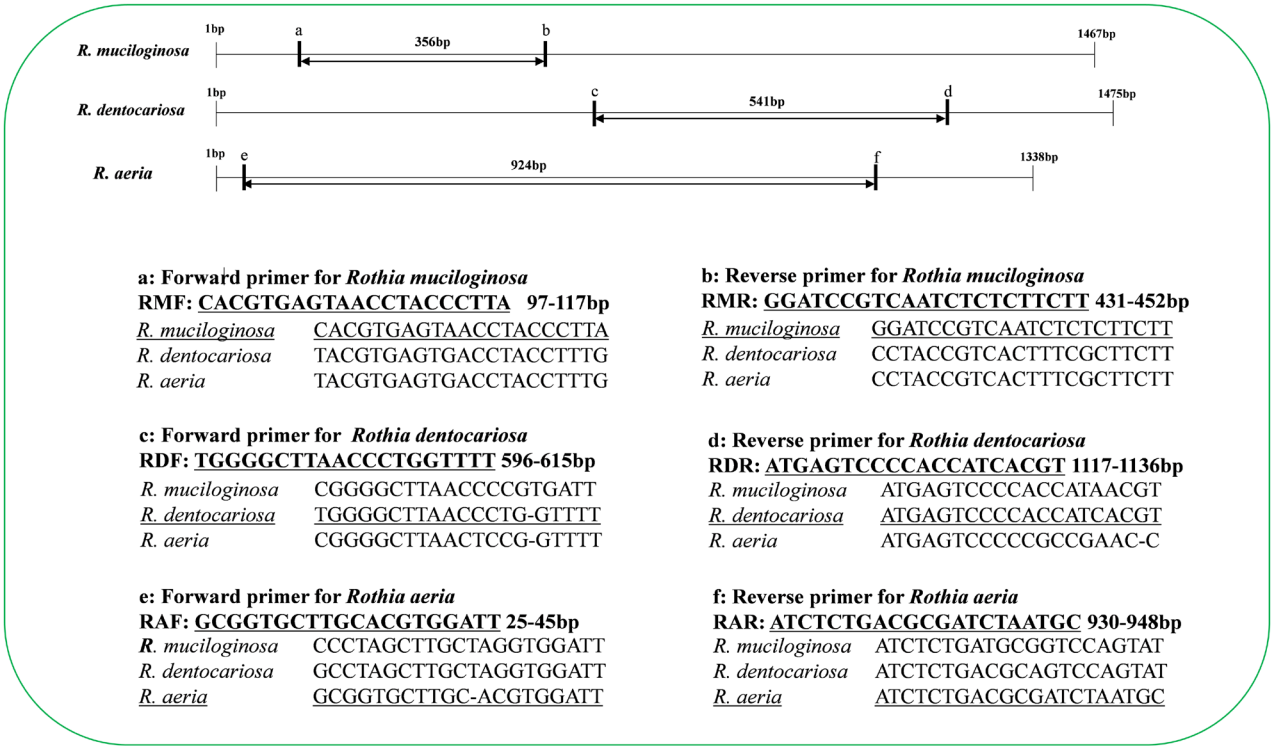
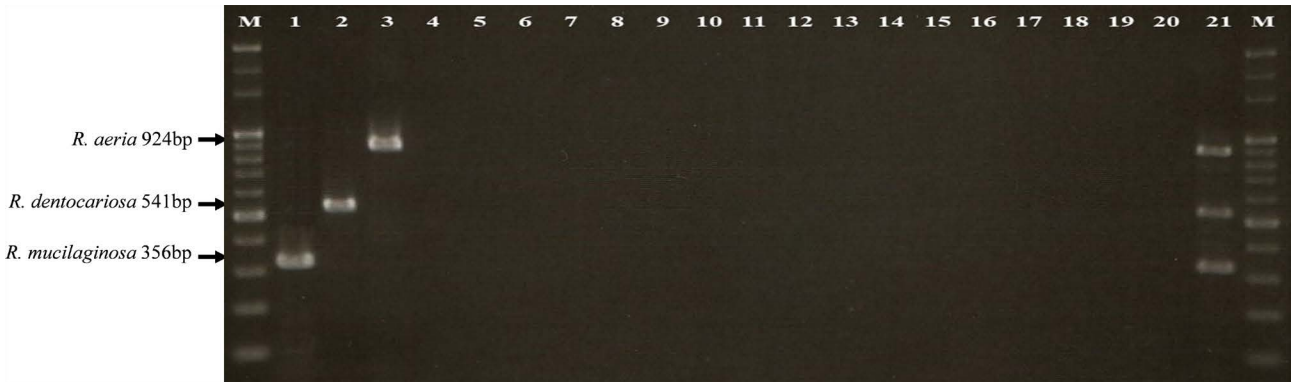


Figure 1. Locations and sequences of species-specific primers for 16S rDNA of oral *Rothia* species.



Lanes: 1: *R. mucilaginosa* JCM 10910; 2: *R. dentocariosa* JCM 3067; 3: *R. aeria* JCM 11412; 4: *R. terrae* JCM 15158; 5: *R. amarae* JCM 11375; 6: *R. nasimurium* JCM 10909; 7: *R. endophytica* JCM 18541; 8: *S. mitis* ATCC 49456; 9: *S. oralis* ATCC 10557; 10: *S. sanguinis* ATCC 10556; 11: *S. salivarius* JCM 5707; 12: *S. anginosus* ATCC 33397; 13: *S. mutans* NCTC 10449; 14: *A. naeslundii* ATCC 12104; 15: *A. oris* ATCC 27044; 16: *A. odontolyticus* ATCC 17929; 17: *A. israelii* ATCC 12102; 18: *C. matruchotii* ATCC 14266; 19: *C. durum* ATCC 33449; 20: *N. sicca* ATCC 29256; 21: Mixture of *R. mucilaginosa* JCM 10910, *R. dentocariosa* JCM 3067, and *R. aeria* JCM 11412; M, molecular size marker (100-bp DNA ladder).

Figure 2. Multiplex PCR assay for identifying oral *Rothia* species.

Actinomyces, *Neisseria*, or *Corynebacterium* species used as representative oral bacteria with the designed primer sets. Moreover, three bands equivalent to *R. mucilaginosa*, *R. dentocariosa*, and *R. aeria* were produced from a mixed sample of the three oral *Rothia* species.

3.2. Clinical Examination

The clinical parameters of HI and PI groups are shown in (Table 1). The average

ages and PPDs of HI and PI groups were 51 (range: 35 - 63) and 57 (range: 45 - 66), and 2.35 mm and 7.90 mm, respectively. A comparison of the mean numbers of total bacteria and oral *Rothia* species between the two groups is shown in (Table 2). The mean number of total bacteria in the HI group was 1.24×10^6 CFU. The mean numbers of *R. dentocariosa*, *R. mucilaginosa*, and *R. aeria* in the HI group were 1.05×10^3 CFU, 4.97×10^2 CFU, and 6.66×10^3 CFU, respectively. The mean number of total bacteria in the PI group was 7.16×10^6 CFU. The mean numbers of *R. dentocariosa*, *R. mucilaginosa*, and *R. aeria* in the PI group were 4.23×10^3 CFU, 6.40×10^2 CFU, and 6.25×10^2 CFU, respectively. The mean number of *R. aeria* in PISF samples was significantly higher in the HI group than in the PI group ($P < 0.05$).

A comparison of the proportions of oral *Rothia* species between the two groups is shown in (Figure 3). *R. aeria* in the PISF samples of the HI and PI groups was detected at 0.998% and 0.008%, respectively, of total bacteria. *R. mucilaginosa* in the HI and PI groups was detected at 0.030% and 0.010%, respectively, of total bacteria. *R. dentocariosa* in the HI and PI groups was detected at 0.176% and 0.079%, respectively, of total bacteria. The proportion of *R. aeria* in PISF samples was significantly higher in the HI group than in the PI group ($P < 0.05$).

The detection frequencies of red complex bacteria in PISF samples obtained from the two groups are shown in (Table 3). While the detection frequencies of all red complex bacteria, *P. gingivalis*, *T. forsythia*, and *T. denticola*, in PISF samples were significantly higher in the PI group than in the HI group ($P < 0.01$), these organisms were also detected from some of the HI group.

3.3. Comparison of the Growth of *R. aeria* between Two Culture Conditions

A comparison of the growth of *R. aeria* between two culture conditions is shown

Table 1. Clinical parameters of the two groups.

Group	Subject		Clinical findings			
	No. of subjects (male:female)	Average age (range)	BOP	Pus discharge	Bone loss	Average PPD (range)
Healthy implants	30 (17:13)	51 (35 - 63)	-	-	-	2.35 mm (2 - 3 mm)
Peri-implantitis	30 (14:16)	57 (45 - 66)	+	+	+	7.90 mm (5 - 11 mm)

Table 2. Comparison of the numbers of total bacteria and oral *Rothia* species between the two groups.

	No. of total bacteria	No. of *R. dentocariosa*	No. of *R. mucilaginosa*	No. of *R. aeria*
	(CFU)			
Healthy implants (n = 30)	1.24×10^6	1.05×10^3	4.97×10^2	$6.66 \times 10^{3*}$
Peri-implantitis (n = 30)	7.16×10^6	4.23×10^3	6.40×10^2	$6.25 \times 10^{2*}$

*Mann-Whitney *U* test; $p < 0.05$.

in (Figure 4). *R. aeria* under aerobic conditions vigorously grew compared with under anaerobic conditions.

3.4. Comparison of Growth in Semi-Liquid Nutrient Medium between *R. aeria* and Anaerobic Bacteria

A comparison of growth in semi-liquid nutrient medium between *R. aeria* and anaerobic bacteria (*P. gingivalis*) is shown in (Figure 5). *R. aeria* grew only at

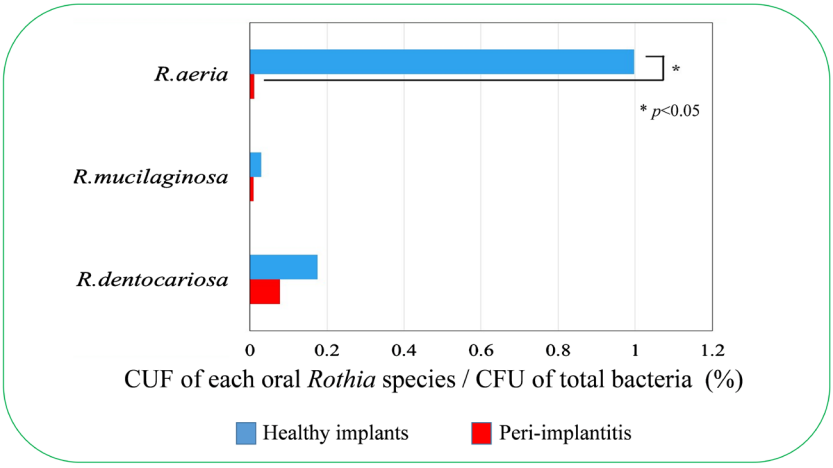


Figure 3. Comparison of the proportions of oral *Rothia* species between the two groups.

Table 3. Detection frequencies of red complex bacteria in PISF samples of the two groups.

Species	Healthy implants (n = 30)	Peri-implantitis (n = 30)	Fisher's exact test
	No. of positive samples (%; frequency)		
P. gingivalis	6 (20)	24 (80)	$p < 0.01$
T. forsythia	8 (27)	23 (77)	$p < 0.01$
T. denticola	5 (17)	18 (60)	$p < 0.01$

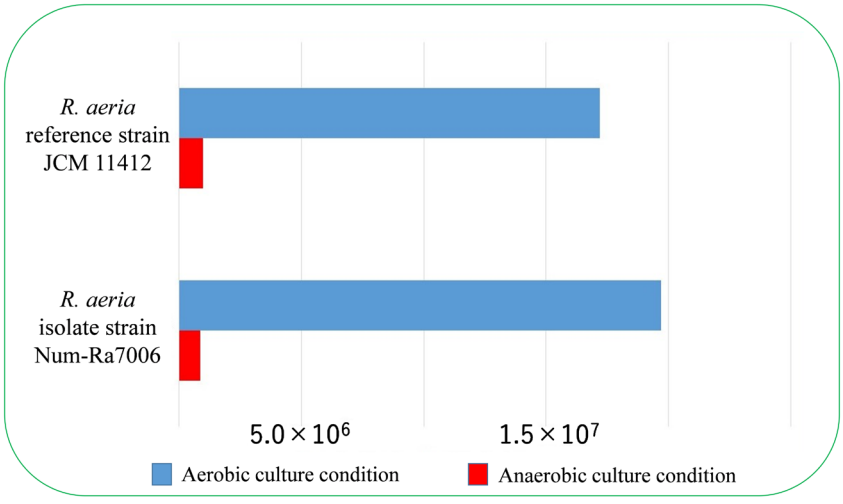


Figure 4. Comparison of growth of *R. aeria* between two culture conditions.

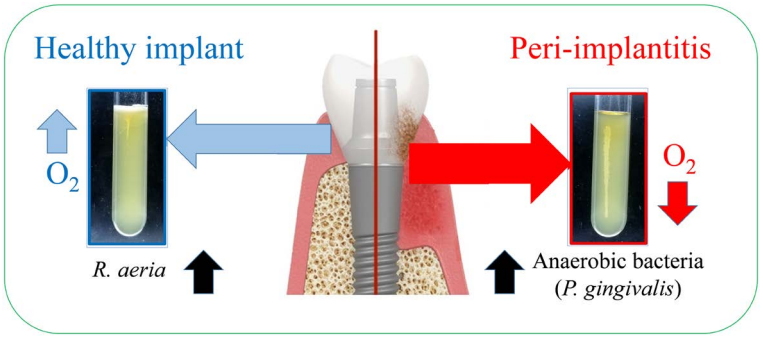


Figure 5. Comparison of growth in semi-liquid nutrient medium between *R. aeria* and anaerobic bacteria.

the upper layer where high oxygen concentrations existed. In contrast, *P. gingivalis* did not grow in the upper layer, but instead grew in the middle-under layer where little or no oxygen existed.

4. Discussion

The genus *Rothia* is Gram-positive and includes seven species, *R. aeria*, *Rothia amarae*, *R. dentocariosa*, *Rothia endophytica*, *R. mucilaginosa*, *Rothia nasimurium*, and *Rothia terrae* [24]. Among the *Rothia*, *R. aeria*, *R. dentocariosa*, and *R. mucilaginosa* inhabit the human oral cavity and pharynx. All three have been identified as opportunistic pathogens that can cause septicemia, endocarditis, and other serious infections [29] [30] [31]. *R. aeria*, which was originally classified as *R. dentocariosa* genomovar II, was first isolated from air and condensation water samples in the Russian space station Mir [32]. There was only one report in which *R. aeria* was detected in the mouths of healthy individuals [33] until our previous study demonstrated that *R. aeria* is part of the normal flora in the oral cavity [24].

Upon clinical microbiological examination, *Rothia* species can be mistaken for bacteria such as *Dermabacter hominis*, *Actinomyces viscosus*, *Propionibacterium avidum*, *Corynebacterium matruchotii*, and *Nocardia* spp., because many laboratories are unfamiliar with these organisms, which may be difficult to culture due to having the same gram-positive rods and to their varying aero-tolerance [34] [35] [36]. Moreover, some studies have previously reported that it is difficult to identify isolates, and that routine biochemical tests might misidentify *R. aeria* as *R. dentocariosa* [37] [38]. In addition, *R. aeria* can be mistaken for *Nocardia* spp. due to morphological similarities, and discrimination between *R. aeria* and *Nocardia* spp. needs further analyses, such as 16S rRNA sequencing [39]. Sequence analysis of several target genes is the most reliable method. However, it is expensive, laborious, and time-consuming. Thus, a simple and more reliable assay for identifying oral *Rothia* species is required.

In the present study, species-specific primers to identify oral *Rothia* species were designed using a one-step multiplex PCR method. These primers were able to distinguish *R. mucilaginosa*, *R. dentocariosa*, and *R. aeria* and did not react with representative oral bacteria or other *Rothia* species. Moreover, the novel

one-step multiplex PCR analysis could directly use bacterial cells using MightyAmp DNA Polymerase Ver. 3 (Takara) and be completed in approximately 1.5 h. Our previous study also used a multiplex PCR method for the identification of oral *Rothia* species [24]. The previous method took approximately 2 h; therefore, the new method was shorter by 30 min.

In the present study, oral *Rothia* species were investigated as an indicator of healthy peri-implant tissue conditions. Currently, bacteriological examinations of implant treatments target periodontopathic bacteria such as red complex bacteria, which are detected qualitatively or quantitatively. However, those examinations do not precisely reflect the peri-implant tissue conditions, because periodontopathic bacteria may be detected at healthy peri-implant sites [23]. We have been searching for bacteria that are suitable as an indicator for healthy peri-implant tissue conditions. Recently, several studies have reported that oral *Rothia* species are detected more frequently in periodontally healthy subjects compared with chronic periodontitis patients [40] [41]. We therefore chose oral *Rothia* species, which are part of the normal oral flora, as possible indicator species in the present study. As a result, the mean number and proportions of *R. aeria* in PISF samples were significantly higher in the HI group than in the PI group ($P < 0.05$); however, those of *R. dentocariosa* and *R. mucilaginosa* did not demonstrate significant differences between the groups. Moreover, the detection frequencies of all red complex bacteria were significantly higher in the PI group than in the HI group ($P < 0.01$); however, these organisms were also detected in some samples of the HI group. Renvert *et al.* also reported that the prevalence of red complex bacteria, considered as key pathogens in periodontitis, is low and does not seem to differ by implant status [23]. In addition, *R. aeria* under aerobic conditions vigorously grew compared with anaerobic conditions and grew only at the upper layer where high oxygen concentrations existed in a semi-liquid nutrient medium. These results indicated that a shallow healthy peri-implant sulcus, where high oxygen concentration exists, is preferable for *R. aeria* growth.

We developed a one-step multiplex PCR method for the identification of oral *Rothia* species. The method described herein will be useful for determining the distribution and role of these organisms in various locations in humans. Moreover, the monitoring of *R. aeria* levels may be suitable as an indicator reflecting healthy peri-implant tissue conditions to aid in the prevention of peri-implantitis.

Authors' Contributions

Suzuki H, Tsuzukibashi O, Fukatsu A, corrected the data. Suzuki H wrote the manuscript. The concept of this manuscript was devised by Suzuki H. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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