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Original

Development of a novel clinical prototype kit that detects health-indicator bacteria for periodontitis risk assessment

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Abstract
Purpose: Recently, high-throughput 16S rRNA pyrosequencing studies have reported that *Rothia dentocariosa* was the dominant bacteria found in health-associated subgingival plaque communities. The monitoring of *R. dentocariosa* levels as an indicator that reflects healthy periodontal tissue conditions may be useful for the prevention of periodontitis. The purpose of this study was to develop a useful clinical examination kit for estimating *R. dentocariosa* levels in gingival crevicular fluid（GCF）samples.
Methods: The kit employed a modified selective medium for the isolation of *R. dentocariosa*（RDSM）. To confirm the clinical effectiveness of the kit, GCF samples were collected from periodontally healthy subjects and patients with periodontitis, and the levels of *R. dentocariosa* in the GCF samples were quantified using the kit and compared to those quantified using the RDSM.
Results: The growth of oral bacteria other than *R. dentocariosa* was markedly inhibited with this kit. The kit could distinctly distinguish between periodontally healthy subjects and patients with periodontitis. Moreover, it was able to accurately estimate the *R. dentocariosa* levels in GCF samples; the levels were significantly higher in periodontally healthy subjects than in patients with periodontitis.
Conclusions: This developed kit appears to be useful for estimating the condition of periodontal tissue.

Key words：*Rothia dentocariosa*, healthy periodontal tissue, periodontitis, gingival crevicular fluid

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1. Introduction

Periodontal disease, which includes gingivitis and periodontitis, is an inflammatory disease that affects the soft and hard tissues surrounding the teeth. In general, past findings have indicated that bacterial infection might be the primary cause of periodontal disease[1–3].

The diagnosis of this disease is generally based on clinical findings and radiographic images. Although these examinations are useful for estimating past and present periodontal tissue conditions, they are not suitable for accurately predicting future periodontal tissue destruction[4]. Furthermore, they are not suitable for large-scale epidemiological studies since they are expensive and labor-intensive. In contrast, the sampling of gingival crevicular fluid (GCF) is comparatively safe, easy, and noninvasive[5]. GCF samples can be collected with paper strips or points in gingival crevices. Several biochemical parameters of GCF have been proposed as predictors of periodontal disease activity[6, 7]. Recently, GCF samples have also been clinically applied for bacteriological examinations to qualitatively or quantitatively detect periodontopathic bacteria, such as red complex bacteria, including *Porphyromonas gingivalis*[8, 9]. However, it seems that the results of these examinations do not precisely reflect the condition of periodontal tissue, because each individual type of periodontopathic bacteria might not always be consistently detected from unhealthy sites. Therefore, we considered that it was difficult to use periodontopathic bacteria as an indicator of unhealthy periodontal tissue conditions, and instead, we explored the levels of good bacteria as an indicator of healthy periodontal tissue conditions. Recent high-throughput 16S rRNA pyrosequencing studies have reported that *Rothia dentocariosa* dominated health-associated subgingival plaque communities[10–12]. These results suggested that *R. dentocariosa* may be useful as an indicator of healthy periodontal tissue. Furthermore, we have already reported on a novel selective medium for the detection of *R. dentocariosa*, i.e., *R. dentocariosa* selective medium (RDSM)[13]. As such, *R. dentocariosa*, which is part of the normal oral flora, was investigated as an indicator of health in this study.

Periodontitis is caused mainly by bacterial infection. It presents as symptoms that include soft tissue inflammation and support bone resorption, and asymptomatically progresses over time. In severe periodontitis, the tooth may fall out due to support bone resorption, which can remarkably reduce the quality of life of the patient. To prevent the onset of periodontitis, it is necessary to establish a useful bacteriological examination system.

The purpose of this study was to develop a useful clinical examination kit for estimating *R. dentocariosa* levels in GCF samples, and to investigate whether the monitoring of these levels is useful for periodontitis risk assessment. We performed according to STARD (standard for reporting an epidemiologic diagnosis accuracy study) guidelines[14].

2. Materials and methods

2. 1. Bacterial strains and culture conditions

All bacterial strains used in this study are listed in Table 1. These strains were maintained on 1.5% agar with Bact™ Brain Heart Infusion (BHI; Becton, Dickinson and Company, Sparks, USA). They were cultured at 37℃ for 24 h in a CO_2 incubator (NAPCO® Model 5400; Precision Scientific, Chicago, USA) with an atmosphere of 5% CO_2. The *R. dentocariosa* isolates (NUM-Rd 6012, NUM-Rd 6014, and NUM-Rd 6018) were obtained from human oral cavities in our previous study[15].

2. 2. Composition of the modified RDSM

The RDSM that was developed in our previous study comprises 25 g/l of Heart Infusion (HI; Becton, Dickinson and Company), 0.5 mg/l of lincomycin (Sigma Chemical Co., St. Louis, USA), 10 mg/l of colistin (Wako Pure Chemicals Co., Tokyo, Japan), 400 g/l of sucrose (Wako Pure Chemicals Co.), and 15 g/l of agar[13]. In the present study, a modified RDSM was developed by adding 17 mg/l of bromocresol purple (Wako Pure Chemicals Co.), which is a pH indicator, into the RDSM.

2. 3. Recovery of R. dentocariosa *and other representative oral bacteria on the modified RDSM*

The bacterial strains listed in Table 1 were used. *Rothia* species, including *R. dentocariosa*, were pre-incubated in BHI broth at 37℃ for 24 h under aerobic conditions. Bacteria other than *Rothia* species were pre-incubated in BHI broth at 37℃ for 24 h under an atmosphere of 5% CO_2 in a CO_2 incubator. Ten-fold dilutions of cultures were made using 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2), and aliquots of 0.1 ml were spread onto the test media. The plates on which *Rothia* species were inoculated were cultured at 37℃ for 72 h under aerobic conditions, while the plates on which the other bacteria were inoculated were cultured at 37℃ for 72 h under an atmosphere of 5% CO_2 in a CO_2 incubator. After cultivation, the number of colony-forming units (CFU)/ml was counted. Recovery of *R. dentocariosa* and other representative oral bacteria on the modified RDSM was calculated by comparing the CFU/ml on the modified RDSM with that on HI agar.

2. 4. Development of a clinical examination kit for estimating R. dentocariosa *levels*

The liquid selective medium (test solution) used in the clinical examination kit for estimating *R. dentocariosa* levels comprised modified RDSM without agar. The kits were prepared by pouring 0.1 ml of the test solution into sterile 0.2-ml polymerase chain reaction (PCR) tubes (Funakoshi Co., Tokyo, Japan). *Rothia* species were pre-incubated in BHI broth at 37℃ for 24 h under an aerobic condition. Ten-fold dilutions of cultures were made using 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2). Then, 10-μl aliquots were inoculated into each of the PCR tubes of the kit, and 100-μl aliquots were inoculated onto each HI agar plate. Color changes in the test solution of the kit were observed over time during cultivation. Furthermore, the CFU/ml values of pre-incubated cultures were calculated by counting the number of colonies on HI agar plates after cultivation at 37℃ for 72 h under an aerobic condition.

Table 1 Recovery of *R. dentocariosa* and other bacteria on HI agar and the modified RDSM

		HI agar (CFU/ml, $\times 10^8$)	Modified RDSM (CFU/ml, $\times 10^8$)	Recovery, %
R. dentocariosa				
	JCM3067	1.0 ± 0.1[a]	0.9 ± 0.1	85.4
	NUM-Rd6012	2.0 ± 1.2	2.0 ± 1.2	101.7
	NUM-Rd6014	2.1 ± 0.7	1.9 ± 0.7	90.3
R. mucilaginosa				
	JCM10910	1.2	0.000001	<0.0[b]
R. aeria				
	JCM11412	0.8	0.0003	<0.0
Streptococcus salivalius				
	HHT	3.2	0	0
Actinomyces naeslundii				
	ATCC12104	1.4	0	0
Neisseria sicca				
	ATCC29256	2.4	0	0
Corynebacterium matruchotii				
	ATCC14266	0.8	0	0

[a]Average ± SD; [b]less than 1×10^2 CFU/ml

2. 5. Clinical samples

A total of 60 patients who visited Nihon University Hospital, School of Dentistry at Matsudo, Japan, participated in the present study. This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC15-025). Informed consent was obtained from all subjects.

The subjects were divided into two groups: a periodontally healthy group (PH group) and a periodontitis group (PD group; Table 2). Patients were allocated to the PH group when the percentage of sites of bleeding on probing (% BOP) was <20% and the periodontal pocket depth (PPD) was <4 mm, and no attachment loss or resorption of the alveolar bone were detected in X-ray findings. Patients were allocated to the PD group when the % BOP was ≥ 20% and the PPD was ≥ 4 mm, and attachment loss and resorption of the alveolar bone were detected in X-ray findings.

The exclusion criteria were as follows: infants; pregnant women; patients with orthodontic treatment; patients with systematic diseases; patients who received periodontal therapy within the last 6 months; patients who used immunosuppressive agents or antibiotics within the last 6 months; or patients with the long-term use of contraceptive drugs.

2. 6. Estimation of R. dentocariosa *levels in GCF*

Figure 1 shows a flowchart of the procedure for the test kit. GCF was collected in the PH group and the PD group from periodontal sites using two endodontic paper points. The two paper points were inserted into the same site of each individual subject for 20 s each. Then, one of the two paper points was placed in a sterile microcentrifuge tube containing 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2), and dispersed by sonication for 30 s in an ice bath (50 W, 20 kHz, Astrason® System model XL2020; MISONIX Co., Farmingdale, USA). Aliquots (0.1 ml) of the dilutions of these samples were plated in triplicate on BHI agar plates for total cultivable bacteria and on modified-RDSM plates. The BHI agar plates and modified-RDSM plates were cultured at 37℃ for 72 h under an atmosphere of 5% CO_2 in a CO_2 incubator or under aerobic conditions, respectively. After cultivation, the CFU/ml was calculated. The other paper point was inserted into the test solution in a test tube of the clinical examination kit and cultured at 37℃ under an aerobic condition. The color of the test solution of the kit was observed after cultivation.

2. 7. Identification of the R. dentocariosa *species isolated from clinical samples*

Twenty-four of the approximately 50 colonies that grew on the modified RDSM per subject were randomly isolated and subcultured, then the bacterial species were confirmed by PCR analysis. The PCR method for identifying *R. dentocariosa* was performed as previously described[14]. In addition, the cultures of the clinical examination kit were used as PCR templates. The PCR method was performed as previously described[14].

2. 8. Statistical analysis

The CFUs of *R. dentocariosa* on the modified RDSM were compared between the PH and PD groups using the Mann-Whitney U test. Values of $P < 0.05$ were considered to be significant.

Table 2 Subjects and clinical parameters

Subjects	Sex (male : female)	Age (years)	Probing depth (mm)
Periodontally healthy (n = 30)	14 : 16	30.2 (range : 25–43)	2.4 (range : 2–3)
Periodontitis (n = 30)	18 : 12	66.2 (range : 46–78)	5.9 (range : 5–9)

3. Results

3. 1. Recovery of R. dentocariosa *and inhibition of other representative oral bacteria on the modified RDSM*

Table 1 shows the recovery rates of the *R. dentocariosa* reference strain JCM3067 and isolates on the modified RDSM relative to those on the HI agar. The recovery rates of the *R. dentocariosa* reference strain and isolates ranged from 85.4% to 108.2% (average 96.4%) on the modified RDSM relative to those on the HI agar. The results shown in Table 1 also indicated that the growth of other representative oral bacteria, including *Rothia mucilaginosa* and *Rothia aeria*, was markedly inhibited on the modified RDSM relative to the HI agar.

3. 2. Clinical examination kit for estimating R. dentocariosa *levels*

Figure 2 shows the results of the clinical examination kit after the inoculation of *R. dentocariosa* strains and cultivation for 24 h. In this kit, the color of the medium changes from purple to yellow when the level of *R. dentocariosa* surpasses 10^4 CFU/ml after cultivation for 24 ± 3 h under an aerobic condition. In contrast, at levels less than 10^4 CFU/ml, the color remains purple. As such, the development of a yellow color after cultivation for 24 ± 3 h under an aerobic condition was considered to indicate a positive result for *R. dentocariosa* strains in this study.

3. 3. Comparison of the R. dentocariosa *levels between the PH and PD groups using the modified RDSM and the developed kit*

The comparison of *R. dentocariosa* levels between the PH (n = 30) and PD (n = 30) groups using the modified RDSM and the developed kit is shown in Table 3 and Figure 3. With the modified RDSM, the mean levels of *R. dentocariosa* in the PH and PD groups were 1.6×10^4 (16,000) CFU/ml (standard deviation (SD), ± 3.65×10^3) and 2.0×102 (200) CFU/ml (SD, ± 1.89×10^2), respectively. The mean level was significantly higher in the PH group than in the PD group ($P < 0.05$). The modified RDSM inhibited the growth of bacteria other than *R. dento-*

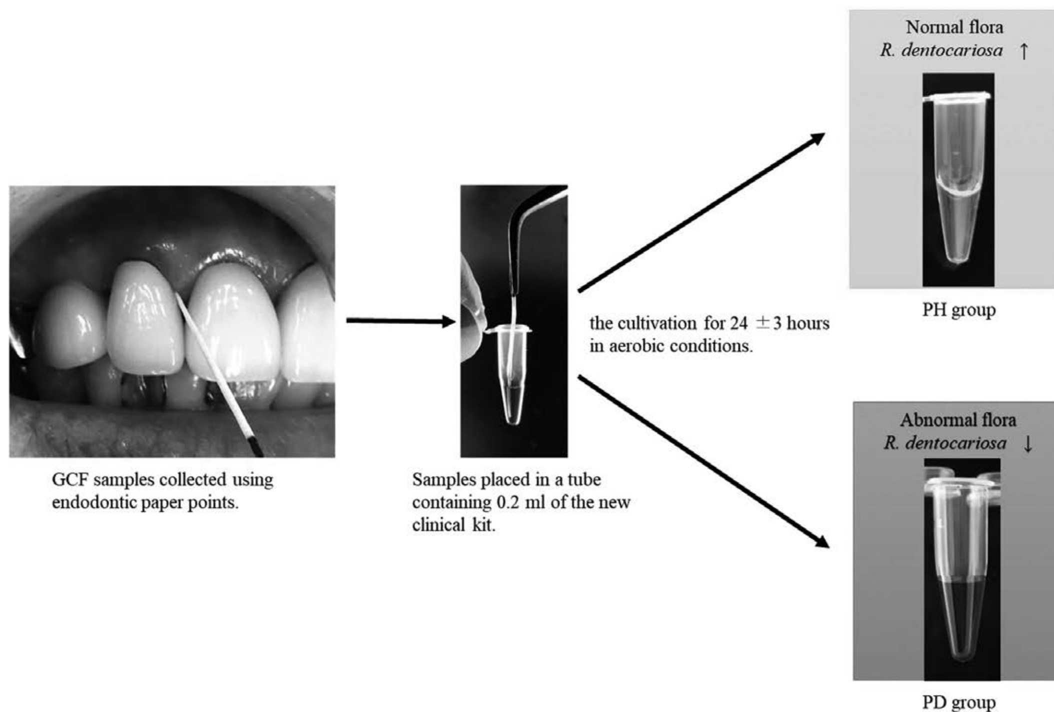

Figure 1 Flowchart of the novel clinical kit

GCF was collected in the PH group and the PD group from periodontal sites. The paper point was inserted into the test solution in a test tube of the clinical examination kit and cultured at 37℃ under an aerobic condition. The color of the test solution of the kit was observed after cultivation.

cariosa in the GCF samples. In the primary isolation, the *R. dentocariosa* colonies on the modified RDSM commonly had a yellow, smooth, and circular appearance. With the developed kit, the numbers of positive samples were 30 and 0 in the PH and PD groups, respectively.

4. Discussion

R. dentocariosa was first isolated from dentin caries in humans by Onishi[16]. The organism is a highly pleomorphic facultative anaerobic bacteria that shows both coccoid and branched filamentous elements. It produces an extracellular levan from sucrose[16]. Several *Rothia* species have been isolated from the oral cavity of humans, including *R. dentocariosa*, *R. mucilaginosa*, and *R. aeria*[18, 19]. *R. dentocariosa* has been isolated in several laboratories from a variety of human sources, most commonly from the oral cavity, carious teeth, and periodontal disease, but also from blood, respiratory secretions, abscesses, wounds, and the eye[16, 20–23]. *R. dentocariosa* has been reported to be a cause of endocarditis and lung cancer, and may also cause opportunistic infections[24–26].

In this study, a modified RDSM was developed by adding bromocresol purple into RDSM. The modified RDSM could distinguish *R. dentocariosa* from other bacteria by detecting the production of acid from sucrose; the acid production by *R. dentocariosa* is detected as a color change from purple to yellow. All tested *R. dentocariosa* strains grew well on the modified RDSM, while the growth of other bacteria was

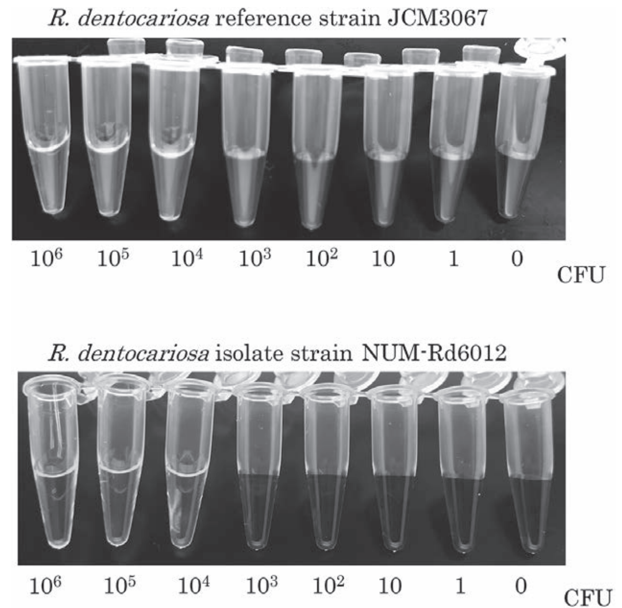

Figure 2 Estimation of the *R. dentocariosa* level using the kit
The clinical examination kit after the inoculation of *R. dentocariosa* strains and cultivation for 24 h. In this kit, the color of the medium changes from purple to yellow when the level of *R. dentocariosa* surpasses 10^4 CFU/ml after cultivation for 24 ± 3 h under an aerobic condition.

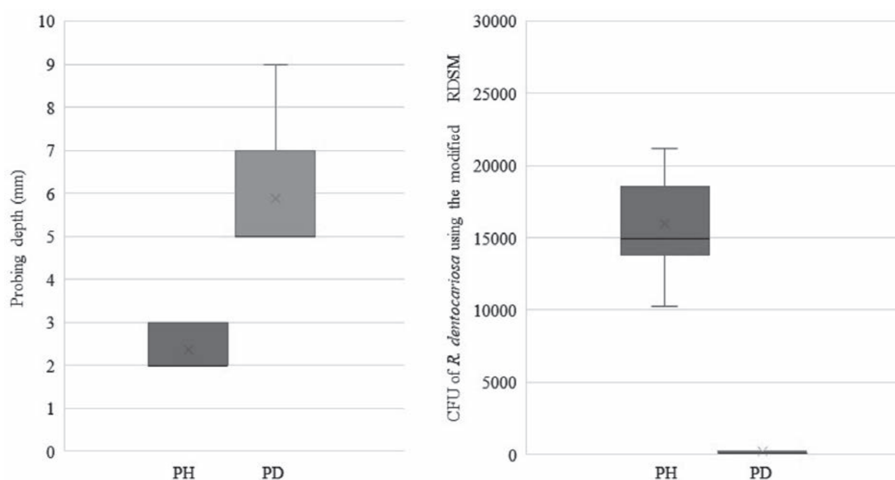

Figure 3 Box-and-whisker plots showing distributions of probing depth (mm) and CFU of *R. dentocariosa* using the modified RDSM in PH (Periodontally healthy) and PD (Periodontitis) groups
The boxes represent the 25th to 75th percentiles, and horizontal lines within the box represent median values. The whiskers represent the lowest and highest value. X in the plot represent mean values.

Table 3 Comparison of the *R. dentocariosa* levels between the PH and PD groups using the modified RDSM and the developed kit

	Periodontally healthy (n = 30)	Periodontitis (n = 30)
CFU of *R. dentocariosa* using the modified RDSM	1.6×10^{4}* (SD： $\pm 3.65 \times 10^{3}$)	2.0×10^{2}* (SD： $\pm 1.89 \times 10^{2}$)
Number of positive samples using the kit	30	0

*Mann-Whitney U test; $P < 0.05$

markedly inhibited (Table 1). This indicated that the modified RDSM may be extremely useful for the detection of *R. dentocariosa*.

In methods for nucleic acid amplification, loop-mediated isothermal amplification (LAMP) and real-time PCR are employed to develop a rapid and simple detection system for periodontal pathogen[27, 28]. However, even a dead pathogen may become positive by these methods. Thus, inspection positive may not necessarily mean that there is an infection power. In contrast, bacteriological examinations are clinically used to qualitatively or quantitatively detect periodontopathic bacteria, such as red complex bacteria, which are the targets of the current treatments for periodontal disease. However, it seems that the results of these examinations do not precisely reflect the condition of periodontal tissue, because each individual type of periodontopathic bacteria might not always be consistently detected from unhealthy sites. We have been exploring the levels of good bacteria as an indicator that reflects healthy peri-implant tissue conditions. Recently, several studies have reported that *R. dentocariosa* was detected more frequently in periodontally healthy subjects than in patients with chronic periodontitis[10–12]. As such, this organism, which is a part of the normal oral flora, was investigated as an indicator of health in the present study. We found that the mean level of *R. dentocariosa* in GCF samples was significantly higher in the PH group than in the PD group ($P < 0.05$). This result indicated that the monitoring of the levels of this bacterium may be useful for periodontitis risk assessment.

In this study, the clinical examination kit developed for the evaluation of the *R. dentocariosa* level showed high diagnostic performance since it could distinguish healthy periodontal sites from unhealthy ones. This inexpensive kit can be easily used clinically to estimate the *R. dentocariosa* level within a period of 24 h. Moreover, GCF sampling is safe and noninvasive, and can be performed quickly.

In conclusion, the clinical examination kit developed in this study could accurately estimate the *R. dentocariosa* level in GCF samples. This kit may be useful for predicting the future onset of periodontitis.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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