Isolation and Identification Methods for Slackia Exigua and Investigation of the Relationship between this Organism and Peri-Implantitis

Taira Kobayashi 1*
Satoshi Uchibori 1
Osamu Tsuzukibashi 2
Chihiro Uezato 1
Hinyuki Tamaki 1
Koji Umezawa 1
Takaaki Tanaka 1
Mitsuhiro Ohta 4

1 Department of Fixed Prosthodontics and Oral Implantology, Nihon University, School of Dentistry at Matsudo, Chiba 271-8587, Japan
2 Department of Oral Health Science, Division of Laboratory Medicine for Dentistry, School of Dentistry at Matsudo, Nihon University, Chiba 271-8587, Japan
3 Department of Oral Diagnostics, Nihon University, School of Dentistry at Matsudo, Chiba 271-8587, Japan
4 Department of Oral Implantology, Department of Fixed Prosthodontics and Oral Implantology, Nihon University, School of Dentistry at Matsudo, Chiba 271-8587, Japan

Abstract
In the genus Slackia, Slackia exigua (formerly Eubacterium exiguum) is isolated from the human oral cavity. A relationship was recently reported between S. exigua and periodontal disease, including chronic periodontitis and peri-implantitis. A suitable selective medium for the isolation of S. exigua is needed in order to assess the veritable prevalence of this organism in various lesions of the oral cavity. The purpose of the present study was to develop selective media for the isolation of S. exigua and investigate whether the monitoring of S. exigua levels is useful as a clinical indicator for the diagnosis of peri-implantitis. In order to examine the bacterium population in the oral cavity, a novel selective medium (SEXSM) was herein developed for the isolation of S. exigua. SEXSM consists of tryptic soy agar, yeast extract, hemin, Vitamin K1, L-cysteine, sheep blood, 2-phenylethanol, trimethoprim, nalidixic acid, and polymyxin B. The average growth recovery of S. exigua on SEXSM was 98.3% that on CDC blood agar. The growth of other representative oral bacteria, i.e., the genera Streptococcus, Actinomyces, Neisseria, Corynebacterium, Veillonella, Fusobacterium, and Rothia, was strongly inhibited on the selective medium. PCR primers were designed based on partial sequences of the 16S rDNA genes of S. exigua. These primers reacted with S. exigua, but not with other representative oral bacteria. These results indicate that these primers are useful for identifying S. exigua. The proportion of S. exigua in Gingival Crevicular Fluids (GCF) collected from periodontally Healthy with Implants (HI) and Peri-Implantitis (PI) groups was examined. Colonies on SEXSM were subcultured for confirmation by a PCR analysis using the primers designed in the present study. The growth recoveries of S. exigua strains on SEXSM were very satisfactory. S. exigua in GCFs of the HI and PI groups were detected at 0.002%, and 1.7% to the total bacteria number, respectively. The selective medium, designated SEXSM, and a PCR method using the primers designed in the present study were useful for the isolation and identification of S. exigua. Moreover, the monitoring of S. exigua levels was useful as a clinical indicator for the diagnosis of peri-implantitis.

Keywords
Slackia exigua; Selective medium; Oral cavity; Peri-implantitis

Introduction
The genus Slackia comprises 6 species (http://www.bacterio.net/slackia.html). The name “slackia” was adopted in honor of Geoffrey Slack, a distinguished British microbiologist and dental researcher. Wade, et al reported a 16S rRNA gene sequence similarity of 94.5% between Eubacterium exiguum from human oral lesions and Peptostreptococcus heliotrinreducens from the sheep rumen as well as a 60 to 64 mol% G+C content for the DNA base composition of the two species [1]. Based on these sequence similarity data, the new genus Slackia was created for these two bacterial species, and E. exiguum and P. heliotrinreducens were reclassified as Slackia exigua and S. heliotrinreducens, respectively. Phenotypic and phylogenetic analyses have since revealed four more species in the genus Slackia: S. caecianis, S. isoflavoniconvertens, S. equilicienis, and S. piriformis. S. exigua was isolated from human oral lesions; S. heliotrinreducens was isolated from the sheep rumen; S. caecianis was isolated from dog feces; and S. isoflavoniconvertens, S. equilicienis, and S. piriformis were isolated from human feces [2-7].

S. exigua has frequently been isolated from necrotic pulp and periradicular lesions [8-10]. These findings suggest that this species plays a pathogenic role in oral infectious diseases as an important pathogen in the oral cavity. However, the veritable prevalence of S. exigua in oral infections remains untested. Moreover, the role of S. exigua in peri-implantitis has not been fully explored. Selective media for the isolation of S. exigua would be useful for the investigation of the relationship between this organism and peri-implantitis.
diseases, including pulpal infections that spread to pericardial tissues. Furthermore, this organism has been isolated from other oral sites, human wound infections and abscesses, severe empyema with acute respiratory distress syndrome, polymicrobial febrile meningitis, and bacteremia [11–18]. A relationship has been reported between S. exigua and chronic periodontitis and peri-implantitis [19,20]. Thus, a suitable selective medium is needed in order to assess the veritable prevalence of S. exigua, which is capable of causing periodontal disease, including chronic periodontitis and peri-implantitis.

A sequence analysis of several target genes is the most reliable method for identifying bacterial species, but is expensive, laborious, and time-consuming. Thus, a simple and more reliable assay, such as a Polymerase Chain Reaction (PCR) amplification method, for identifying S. exigua is needed. A 16S rDNA PCR amplification method is sensitive and useful [21].

The aims of the present study were to develop isolation and identification methods for S. exigua using selective medium and a PCR method, respectively, and to investigate whether the monitoring of S. exigua levels is useful as a clinical indicator for the diagnosis of peri-implantitis.

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

All bacterial strains used in the present study are listed in Table 1. The anaerobic bacteria (i.e., S. exigua, Vellonella parvula, and Fusobacterium nucleatum) used in the present study were maintained by cultivating them on Anaerobic Blood Agar (CDC), which has a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) base supplemented with vitamin K1 (10 μg/ml), hemin (5 μg/ml), L-cysteine (800 μg/ml), 0.5% yeast extract, and 5% sheep blood. These organisms were cultured at 37°C for 48 h in an anaerobic chamber (Forma Scientific Anaerobic System Model 1024, Forma Scientific, Marietta, OH, USA) with 80% N2, 10% H2, and 10% CO2. S. exigua isolates (NUM-Sex 6965, NUM-Sex 6967, NUM-Sex 6969, NUM-Sex 6972, and NUM-Sex 6977) were obtained with non-selective medium, i.e., CDC, from the human oral cavity in our previous studies.

Strains other than anaerobic bacteria were maintained by cultivating them on BactTM Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) base supplemented with vitamin K1 (10 μg/ml), hemin (5 μg/ml), L-cysteine (800 μg/ml), 0.5% yeast extract, and 5% sheep blood. These organisms were cultured at 37°C overnight in an atmosphere of 5% CO2 in a CO2 incubator (NAPCO® Model S400; Precision Scientific, Chicago, IL, USA).

**Development of the New Selective Medium**

**Evaluation of the base medium**: BHI supplemented with 1% yeast extract (BHI-Y), BHI-Y supplemented with 5% sheep blood (BHIY blood), and CDC were examined as the base medium in the selective medium. Ten-fold dilutions of cultures were made in 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 bacteria, except anaerobic bacteria, were inoculated were cultured at 37°C for 72 h in an atmosphere of 5% CO2 in a CO2 incubator and the plates on which anaerobic bacteria were inoculated were cultured at 37°C for 6 days under anaerobic conditions. After cultivation, the number of colony-forming units (CFU/ml) was counted.

**Susceptibility tests**: Preliminary studies of antibiotic selection were also performed using disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA). The microbroth dilution method was used for susceptibility testing [22].

**Recovery of S. exigua and other Representative Oral Bacteria**

The recoveries of the S. exigua reference strain, S. exigua isolates, and other representative oral bacteria were calculated as CFU/ml on selective medium and compared with those on CDC for total cultivable bacteria. All bacterial strains used in the present study are listed in Table 1.

All bacterial strains, except anaerobic bacteria, were pre-incubated in BHI broth at 37°C overnight in an atmosphere of 5% CO2, in a CO2 incubator. Anaerobic bacteria were pre-incubated in Tryptic soy broth (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with vitamin K1 (10 μg/ml), hemin (5 μg/ml), and 0.5% yeast extract at 37°C overnight under anaerobic conditions. Ten-fold dilutions of cultures were made in 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 bacteria, except anaerobic bacteria, were inoculated were cultured at 37°C for 72 h in an atmosphere of 5% CO2 in a CO2 incubator, and the plates on which anaerobic bacteria were inoculated were cultured at 37°C for 5 days under anaerobic conditions. After cultivation, the number of CFU/ml was counted.

**Clinical Samples**

One hundred and twenty patients attending Nihon University Hospital, School of Dentistry at Matsudo, participated in the present study. They were divided into two subject groups: periodontally healthy with implants (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 2013 and 2016; patients with at least 1 dental implant for more than half a year; according to the Guidelines of Periodontology, peri-implantitis was defined as Bleeding of Probing (BOP) and/or periodontal pocket depth (PDP) ≥ 4 mm, accompanied by bone tissue loss under the first thread of the implant (i.e., bone absorption ≥ 2 mm). Exclusion criteria were as follows: patients with systematic diseases; patients receiving periodontal therapy within 6 months; taking immunosuppressive agents or antibiotics; the long-term use of contraceptives; drugs; pregnant women.

Gingival Crevicular Fluid (GCF) was collected using endodontic paper points from all subjects and placed in a sterile microcentrifuge tube containing 0.9 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 CDC and selective medium plates. CDC plates for total cultivable bacteria and selective medium plates were cultured at 37°C for 5 days under anaerobic conditions. After cultivation, the number of CFU/ml was calculated. This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 17-014).

**Identification of S. exigua Species Isolated from Clinical Samples**

Twenty-four of the approximately 50 colonies that grew on the selective medium plate per subject were randomly isolated, subcultured, and their identity was then confirmed by a PCR analysis.

**Design of Species-Specific Primers for S. exigua**

The design of species-specific primers for S. exigua was performed as follows. Briefly, the 16S rRNA sequences of S. exigua (accession no. AF101240), S. heliotriniireducens (accession no. CP001684), S. faecicanis (accession no. AY608680), S. isoflavoniconvertens (accession no. EU377663), and S. piperiformis (accession no. AB490806) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan), and multiple sequence alignment analyses were performed using the CLUSTAL W program; i.e., the 16S rRNA sequences of six Slackia species were aligned and alignment analyses were performed using a BLAST search.

**Development of a PCR Method for Identifying S. exigua using Designed Primers**

A PCR method for identifying S. exigua using the designed primers was developed as follows. Bacterial cells were cultured in BHI broth overnight, and 1 ml of the sample was then collected in a microcentrifuge tube and resuspended at a density of 1.0 McFarland standard (approximately 107 CFU in 1 ml of sterile distilled water). A total of 3.6 μl of the suspension was then used as a 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 2 μM of each primer, 10 μl of 2 × MightyAmp Buffer Ver.2 (Takara Bio Inc., Shiga, Japan), 0.4 μl of MightyAmp DNA Polymerase (Takara), and 3.6 μl of the template in a final volume of 20 μl. PCR was performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Carlsbad, CA). PCR conditions included an initial denaturation step at 98°C for 2 min, followed by 30 cycles consisting of 98°C for 10 s and 68°C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1 × Trisborate-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.

### Statistical Analysis

The proportions of S. exigua to the total bacteria number in GCFs from the HI and PI groups were compared by the Kruskall-Wallis test (Mann-Whitney U test). Values of \( P < 0.05 \) were considered to be significant.

### Results

#### Development of the Selective Medium

**Selection of the base medium:** The selection of a base medium for the growth of S. exigua was performed. S. exigua grew well on CDC, but did not grow well and showed extremely small colonies on BHI-Y and BHI-Y blood (data not shown). CDC was ultimately selected as the base medium.

**Susceptibility to antibiotics:** S. exigua was more resistant to 2-phenylethanol than oral Gram-negative rods, such as *Fusobacterium* species. The minimal inhibitory concentration (MIC) of 2-phenylethanol for S. exigua was 10 μl/ml. Oral Gram-negative bacteria were sensitive to 1.5 μl/ml of 2-phenylethanol. S. exigua was more resistant to nalidixic acid than oral Gram-negative cocci, such as *Neisseria* and *Veillonella* species. The minimal inhibitory concentration (MIC) of nalidixic acid for S. exigua was 10 μl/ml. Oral Gram-negative bacteria were sensitive to 1.5 μl/ml of nalidixic acid.

### Table 1: Recovery of S. exigua and other bacteria on CDC blood and SEXSM

| Strain                  | CDC blood CFU/ml \(\times 10^8\) | SEXSM CFU/ml \(\times 10^8\) | Recovery % |
|-------------------------|---------------------------------|------------------------------|------------|
| *Slackia exigua*        |                                 |                              |            |
| JCM 11022               | 1.5 ± 0.2*                      | 1.5 ± 0.3                    | 99.3       |
| NUM-Sex 6965            | 2.2 ± 0.3                       | 2.1 ± 0.2                    | 98.9       |
| NUM-Sex 6967            | 1.0 ± 0.3                       | 1.0 ± 0.3                    | 99.1       |
| NUM-Sex 6969            | 1.1 ± 0.2                       | 1.1 ± 0.3                    | 98.0       |
| NUM-Sex 6972            | 0.7 ± 0.3                       | 0.7 ± 0.2                    | 96.6       |
| NUM-Sex 6977            | 0.9 ± 0.2                       | 0.9 ± 0.3                    | 98.1       |
| *Streptococcus oralis*  |                                 |                              |            |
| ATCC 10557              | 1.5                             | 0                            | 0          |
| *Streptococcus salivarius* |                               |                              |            |
| JCM 5707                | 2.8                             | 0                            | 0          |
| *Streptococcus anginosus* |                               |                              |            |
| ATCC 33397              | 3.1                             | 0                            | 0          |
| *Streptococcus mutans*  |                                 |                              |            |
| ATCC 25175              | 5.5                             | 0                            | 0          |
| *Streptococcus sobrinus* |                                 |                              |            |
| ATCC 33478              | 7.4                             | 0                            | 0          |
| *Actinomyces naeslundii* |                                 |                              |            |
| ATCC 12104              | 1.2                             | 0                            | 0          |
| *Actinomyces oris*      |                                 |                              |            |
| ATCC 27044              | 2.5                             | 0                            | 0          |
| *Actinomyces odontolyticus* |                           |                              |            |
| ATCC 17929              | 2.2                             | 0                            | 0          |
| *Corynebacterium matruchotii* |                          |                              |            |
| ATCC 14266              | 1.9                             | 0                            | 0          |
| *Corynebacterium durum* |                                 |                              |            |
| ATCC 33449              | 0.9                             | 0                            | 0          |
| *Rothia dentocariosa*   |                                 |                              |            |
| JCM 3067                | 0.8                             | 0                            | 0          |
| *Rothia mucilaginosa*   |                                 |                              |            |
| JCM 10910               | 0.7                             | 0                            | 0          |
| *Rothia aeria*          |                                 |                              |            |
| JCM 11412               | 1.1                             | 0                            | 0          |
| *Veillonella parvula*   |                                 |                              |            |
| ATCC 10790              | 1.1                             | 0                            | 0          |
| *Fusobacterium nucleatum* |                               |                              |            |
| ATCC 22586              | 2.1                             | 0                            | 0          |
| *N. sicca*              |                                 |                              |            |
| ATCC 29256              | 0.7                             | 0                            | 0          |

\( ^* \) Ave ± SD.
concentration (MIC) of nalidixic acid for \textit{S. exigua} was 200 μg/ml. Oral Gram-negative bacteria were sensitive to 30 μg/ml of nalidixic acid. \textit{S. exigua} was more resistant to trimethoprim than oral streptococci species, oral Corynebacterium species, and oral Actinomyces species (\textit{Actinomyces naeslundii}, \textit{A. oris}, and \textit{A. odontolyticus}) other than \textit{A. israelii} and \textit{A. meyeri}. The MIC of trimethoprim for \textit{S. exigua} was 5000 μg/ml. Oral streptococcus species, oral \textit{Corynebacterium} species, and oral Actinomyces species other than \textit{A. israelii} and \textit{A. meyeri} were sensitive to 125 μg/ml of trimethoprim. \textit{S. exigua} were more resistant to polymyxin B than \textit{A. israelii} and \textit{A. meyeri}. The MIC of polymyxin B for \textit{S. exigua} was 100 μg/ml. \textit{A. israelii} and \textit{A. meyeri} were sensitive to 5 μg/ml of polymyxin B.

Composition of the new selective medium: The new selective medium, designated \textit{S. exigua} selective medium (SEXSM), was composed of the following (per liter): 40 g of tryptic soy agar, 5 g of yeast extract, 5 mg of hemin, 10 mg of Vitamin K1, 800 mg of L-cysteine, 50 ml of sheep blood, 1.5 ml of 2-phenylethanol, 1000 mg of trimethoprim, 30 mg of nalidixic acid, and 5 mg of polymyxin B. Sheep blood, 2-phenylethanol, and antibiotics, i.e., trimethoprim, nalidixic acid, and polymyxin B, were added after the base medium had been sterilized and cooled to 50°C.

PCR Method for Identifying \textit{S. exigua}

**Primer design:** The specific primer set covering the upstream region of the 16S rDNA sequence of \textit{S. exigua} was designed in the present study (Table 2). The amplicon size of \textit{S. exigua} was 739 bp.

**Detection limit:** A PCR method was used to identify the \textit{S. exigua} -amplified DNA fragment of the expected size for this organism (Figure 1). The detection limit was assessed in the presence of titrated bacterial cells, and the detection sensitivity of the PCR assay was 50-100 CFU per PCR template (5.6 μl) for the \textit{S. exigua} -specific primer set with strain JCM 11022 (data not shown).

**Assay of \textit{S. exigua} and Representative Oral Acteria:** The PCR method used to identify \textit{S. exigua} produced positive bands from the \textit{S. exigua} reference strain JCM 11022 and \textit{S. exigua} clinical isolate NUM-Sex 6965, and did not produce any amplicons from other Slackia species (Figure 1).

Some \textit{Streptococci}, \textit{Actinomyces}, \textit{Neisseria}, \textit{Corynebacterium}, \textit{Rothia}, \textit{Veillonella}, and \textit{Fusobacterium} species were subjected as representative oral bacteria to PCR using the designed primer set. No amplicons were produced from any of the representative oral bacteria (Figure 1).

Recovery of \textit{S. exigua} and Inhibition of Other Representative Oral Bacteria on Selective Medium

Table 1 shows the recovery of the \textit{S. exigua} reference strain JCM 11022 and isolates on SEXSM relative to CDC. The growth recoveries of the \textit{S. exigua} reference strain and isolates ranged between 96.6% and 99.3% (average 98.3%) on SEXSM relative to that on CDC.

Table 1 also shows the inhibition of other representative oral bacteria on SEXSM relative to CDC agar. The growth of other representative oral bacteria was markedly inhibited on selective medium. Furthermore, the growth of \textit{S. faecalis} JCM 14555, \textit{S. equilfaciens} JCM 16059, \textit{S. piriformis} JCM 16070, and \textit{S. isoflavoniconvertens} JCM 16137 was inhibited on SEXSM (data not shown).

Clinical Examination

The detection frequencies of \textit{S. exigua} in GCFs from the 4 groups are shown in Table 3. In GCFs from the HI (n=30) and PI (n=30) groups, \textit{S. exigua} was detected at 33.3%, and 100%, respectively. The mean numbers of total cultivable bacteria and \textit{S. exigua} in the HI group were 1.0×104 CFU/ml (range: 2.2 × 103 - 3.6 × 104) and 1.8 × 101 CFU/ml (range: 0 - 1.8 × 10), respectively; and those in the PI group were 2.0 × 105 CFU/ml (range: 2.0 × 104 - 1.2 × 106) and 3.4 × 103 CFU/ml (range: 3.4 × 102 - 2.0 × 104), respectively. Comparisons of \textit{S. exigua} proportions to the total bacteria number in GCFs between the 2 groups are shown in Figure 2. \textit{S. exigua} in GCFs from the HI and PI groups accounted for 0.002% and 1.7% of all bacteria, respectively. The proportion of \textit{S. exigua} to the total bacteria number in GCF was significantly higher in the PI group than in the HI group (P<0.001).

In the first isolation, \textit{S. exigua} colonies on SEXSM commonly had a smooth and circular appearance. The colony color and average colony size of \textit{S. exigua} on SEXSM was light purple and 1.0 mm in diameter, respectively (Figure 3).

| Species     | Primer | Sequence         | Product size (bp) | Position | Accession number |
|-------------|--------|------------------|-------------------|---------|-----------------|
| \textit{S. exigua} | SEXF   | TGCTGTGCTGAGTGTTGT | 740               | 55-73   |                 |
|             | SEXR   | AAAGGACAGGCTGCTTC |                   | 794-775 | AF101240        |

Table 2: Locations and sequences of species-specific primers for the 16S rDNA of \textit{S. exigua}
**Discussion**

*S. exigua* is a non-sporing, non-motile, strictly anaerobic, asaccharolytic, and Gram-positive rod. Amino acids are important metabolic substrates for the growth of this organism, particularly arginine and lysine, which are produced by the enzymatic degradation of peptides by trypsin-like proteinases. *S. exigua* does not produce butyrate from ornithine, which may be one of the reasons for its poor growth [23].

Asaccharolytic and Gram-positive rods, including *S. exigua*, use peptides for their growth, and may be associated with proteolytic activities in sites of inflammation that are diseased. *S. exigua* was isolated from various oral sites, such as infected dental pulps, infected periapical tissues, periodontal pockets with chronic periodontitis, and acute dento-alveolar abscesses [8-10,12,24,25]. A relationship has been reported between *S. exigua* and peri-implantitis [20]. Dental implant-based reconstruction, which has been adapted to replace conventional fixed or removable partial dentures, has led to the emergence of peri-implantitis as a serious problem in 28–56% of recipients and a major cause of implant loss [26]. As such, effective prevention and management of peri-implantitis are essential for
improving the quality of life and health of patients. Thus, a suitable selective medium and reliable identification method are needed in order to assess the veritable prevalence of \textit{S. exigua}, which is capable of causing peri-implantitis.

In the present study, we designed species-specific primers to identify \textit{S. exigua} using a PCR method. These primers were able to distinguish \textit{S. exigua}, and did not react with representative oral bacteria or other Slackia species. Moreover, the PCR method in this study directly uses bacterial cells with Mighty Amp DNA Polymerase Ver2 (Takara) and is completed within approximately 2 hours.

A useful selective medium for isolating \textit{S. exigua} may contribute to the correct and rapid diagnosis of infectious diseases caused by this organism. However, a selective medium that is useful for the isolation of \textit{S. exigua} did not exist. In the present study, the \textit{S. exigua} strains used were more resistant to 2-phenylethanol, trimethoprim, nalidixic acid, and polymyxin B than other oral bacteria.

The growth of oral bacteria detected in the oral cavity was inhibited by the addition of 1.5 ml/L 2-phenylethanol, 1000 mg/L trimethoprim, 30 mg/L nalidixic acid, and 5 mg/L polymyxin B to CDC agar. All of the \textit{S. exigua} reference strains and isolates tested grew well on the new selective medium, designated as SEXSM, while the growth of other bacteria was markedly inhibited (Table 1). Moreover, SEXSM easily distinguished \textit{S. exigua} by its characteristic colony morphology. However, other than \textit{S. exigua}, \textit{S. faecicicus}, \textit{S. equolifaciens}, \textit{S. piriformis} and \textit{S. isoflavoniconvertens} did not grow on SEXSM (data not shown).

The distribution of \textit{S. exigua} in the oral cavity of humans has not yet been reported in detail. In the present study, \textit{S. exigua} in GCFs from the HI and PI groups was detected at 33.3% and 100%, respectively. Furthermore, the proportion of \textit{S. exigua} to the total bacteria number in GCF was significantly higher in the PI group than in the HI group (P < 0.001). These results indicate that the monitoring of \textit{S. exigua} levels is useful as a clinical indicator for the diagnosis of peri-implantitis. We developed a selective medium, designated SEXSM, to isolate \textit{S. exigua} in the oral cavity of humans. Since SEXSM is highly selective for \textit{S. exigua}, it will be useful for assessing the distribution and role of this organism at various locations in humans.

Selective medium (SEXSM) and a PCR method as isolation and identification methods for \textit{S. exigua} may contribute to the diagnosis of infectious diseases, including peri-implantitis, which are caused by this organism.

**Author’s Contributions**

TK, HG, TT, HT and KU corrected the data. TK, SU, OT MO, and CU drafted and wrote the manuscript. The concept of this manuscript was devised by TK All authors read and approved the final manuscript.

**References**

1. Wade WG, Downes J, Dymock D, Hiom SJ, Weightman AJ, et al. The family Coriobacteriaceae: reclassification of Eubacterium exiguum, (Poco et al. 1996) and Peptostreptococcus heliotrinireducens (Lanigan 1976) as Slackia exigua gen. nov., comb. nov. and Slackia heliotrinireducens gen. nov., comb. nov., and Eubacterium lentum (Pre`vot 1938) as Eggerthella lenta gen. nov., comb. nov. Int J Syst Bacteriol. 1999 Apr;49 Pt 2:595-600.
2. Lawson PA, Greetham HL, Gibson GR, Gifford C, Falsen E, et al. Slackia faeciaciscus sp. nov., isolated from canine faeces. Int J Syst Evol Microbiol. 2004 Sep;54(Pt 5):1581-1584.
3. Matthies A, Blaut M, Braune A. Isolation of a human intestinal bacterium capable of daidzein and genistein conversion. Appl Environ Microbiol. 2009 Mar;75(6):1740-1744.
4. Jin JS, Kitahara M, Sakamoto M, Hattori M, Benno Y. Slackia equolifaciens sp. nov., a human intestinal bacterium capable of producing equol. Int J Syst Evol Microbiol. 2010 Aug;60(Pt 8):1721-1724.
5. Nagai F, Watanabe Y, Morotomi M. Slackia piriformis sp. nov. and Collinsella tanakaei sp. nov., novel members of the family Coriobacteriaceae, isolated from human faeces. Int J Syst Evol Microbiol. 2010 Nov;60(Pt 11):2639-2646.
6. Poo SE, Nakazawa F, Ikeda T, Sato M, Sato T, et al. Eubacterium exiguum sp. nov., isolated from human oral lesions. Int J Syst Bacteriol. 1996 Oct;46(4):1120-1124.
7. Lanigan GW. Peptococcus heliotrinireducens, sp. nov., a cytochrome producing anaerobe which metabolizes pyrrolizidine alkaloids. J Gen Microbiol. 1976 May;94(1):1-10.
8. Hashimura T, Sato M, Hoshino E. Detection of Slackia exigua, Mogibacterium timidum and Eubacterium saphenum from pulpal and periradicular samples using the polymerase chain reaction (PCR) method. Int Endod J. 2001 Sep;34(6):463-470.
9. Kiryu T, Hoshino I, Iwaku M, 1994. Bacteria invading periapical cementum. Int Endod. 1994 Apr;20(4):169-172.
10. Sato T, Hoshino E, Uematsu H, Noda T. Predominant obligate anaerobes in necrotic pulps of human deciduous teeth. Microb. Ecol. Health Dis. 1993;6:269-275.
11. Hill GB, Ayers OM, Kohan AP. Characteristics and sites of infection of Eubacterium nodatum, Eubacterium timidum, Eubacterium brachy, and other asaccharolytic subbacteria. J Clin Microbiol. 1987 Aug;25(8):1540-1545.
12. Moore WEC, Holdemen JV, Cato ER, Smibert RM, Burmeister JA, et al. Bacteriology of moderate (chronic) periodontitis in mature adult humans. Infect Immun. 1983 Nov;42(2):510-515.
13. Wade WG, Lewis MA, Cheeseman SL, Absi EG, Bishop PA. An undiagnosed Eubacterium taxon in acute dento- alveolar abscess. J. Med. Microbiol. 1994 Feb;40(2):115-117.
14. Kim KS, Rowlinson MC, Bennion R, Liu C, Talan D, et al. Characterization of \textit{Slackia exigua} isolated from human wound infections, including abscesses of intestinal origin. J Clin Microbiol. 2010 Apr;48(4):1070-1075.
15. Roingeard C, Jaubert J, Guillemelnault L. A large and unusual lung abscess with positive culture to \textit{Slackia exigua}. Int J Infect Dis. 2015;40:37-38.
16. Man MY, Sham HP, Wum A, Lee RA, Yan WW. A case of severe empyema with acute respiratory distress syndrome caused by \textit{Slackia exigua} requiring veno-venous extracorporeal membrane oxygenation. Anaerobie. 2017 Jun;48:7-11.
17. Bükkj J, Huttnner HB, Lee DH, Jantsch J, Janka R, et al. Polymicrobial furuncul meningitis with detection of \textit{Slackia exigua} in the cerebrospinal fluid of a patient with advanced rectal carcinoma. J Clin Oncol. 2011 Dec;29(35):e852-854.
18. Lee MY, Kim YJ, Gu HJ, Lee HJ. A case of bacteremia caused by Dialister pneumosintes and \textit{Slackia exigua} in a patient with periapical abscess. Anaerobe. 2016 Apr;38:36-38.
19. Booth V, Downes J, Van den Berg J, Wade WG. Gram-positive anaerobic bacilli in human periodontal disease. J Periodontal Res. 2004 Aug;39(4):213-220.
20. Shibata T, Watanabe T, Kachi H, Koyanagi T, Maruyama N, et al. Distinct interacting core taxa in co-occurrence networks enable discrimination of polymicrobial oral diseases with similar symptoms. Sci Rep. 2016;6:30997.
21. Choi BK, Paster BJ, Dewhirst FE, Göbel UB. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect Immun. 1994 May;62(5):1889-1895.
22. Hirasawa, M., Takada, K. Susceptibility of Streptococcus mutans and Streptococcus sobrinus to cell wall inhibitors and development of a novel selective medium for \textit{S. sobrinus}. Caries Res. 2002 May-Jun;36(3):155-160.
23. Hiranmayi KV, Sirisha K, Rao MV, Sudhakar P. Novel pathogen in periodontal microbiology. J Pharm Bioallied Sci. 2017 Jul-Sep;9(3):155-163.
24. Uematsu H, Hoshino E, Predominant obligate anaerobes in human periodontal pockets. J Periodontal Res. 1992 Jan;27(1):15-19.
25. Wade WG, Moran J, Morgan JR, Newcombe R, Addy M. The effects of antimicrobial acrylic strips on the subgingival microflora in chronic periodontitis. J Clin Periodontol. 1992 Feb;19:127-134.

26. Zitzmann NU, Berglundh T. Definition and prevalence of peri-implant diseases. J Clin Periodontol. 2008 Sep;35(8 Suppl):286-291.