Boffin Access
FOSTERING KNOWLEDGE

Open Access Journal | Volume: 3.3

Journal of Dentistry and Dental Medicine

ISSN 2517-7389

Multiplex PCR for Simultaneous Detection and Identification of Six *Slackia Species* Including *Slackia Exigua* Involved In Peri-Implantitis

Taira Kobayashi[1*]
Satoshi Uchibori[1]
Osamu Tsuzukibashi[2]
Chihiro Uezato[1]
Mana Fuchigami[2]
Yuji Takahashi[1]
Hiroyuki Tamaki[1]
Koji Umezawa[3]
Takaaki Tanaka[1]

[1]Department of Fixed Prosthodontics and Oral Implantology, Nihon University School of Dentistry at Matsudo, Chiba, Japan

[2]Department of Oral Health Science, Division of Laboratory Medicine for Dentistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan

[3]Department of Special Needs Dentistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan

Abstract

The genus Slackia comprises six species. Among this genus, *Slackia exigua* is isolated from human oral lesions, and other *Slackia species* were isolated from human feces, canine feces, and sheep rumen. Recently, it has reported that the sulcus around oral implants with peri-implantitis harbors high levels of *S. exigua*. A suitable method to each identify six *Slackia species* has not been yet developed because of the phenotypic and genetic similarities between these microorganisms. Moreover, it has been unclear whether *Slackia species* including *S. exigua* are a part of normal oral flora. The purpose of the present study was to design primers to identify six *Slackia species*, and to investigate their distributions in human oral cavities with multiplex PCR method. Polymerase chain reaction (PCR) primers were designed based on partial sequences of the 16S rDNA of six *Slackia species*. Their distributions in gingival crevice fluids (GCFs) were investigated with this multiplex PCR. The proportion of these microorganisms in gingival crevicular fluids (GCF) collected from 30 periodontally healthy subjects (PH) and 30 chronic periodontitis subjects (CP) was examined. These primers were able to distinguish each six *Slackia species* and did not display cross-reactivity with representative oral bacteria or other *Slackia species*. Moreover, we developed a multiplex PCR method with the ability to identify and differentiate six *Slackia species* (i.e., *S. exigua, S. equolifaciens, S. faecicanis, S. heliotrinireducens, S. isoflavoniconvertens, and S. piriformis*) using one PCR tube per sample. In GCFs from PH and CP subjects, S. exigua was detected at 33.3%, and 100%, respectively. On the other hand, *Slackia species* except *S. exigua* were not detected at all. The present results indicate that our multiplex PCR method with these primers is useful for identifying six *Slackia species*. The detection and identification of six Slackia species using this method only takes approximately 2 hours. Thus, the method described herein will allow the prevalence of six Slackia species in various sites of humans and their involvement in various infectious diseases to be fully clarified in future studies.

Keywords

Genus Slackia*; Slackia exigua*; Multiplex PCR; Oral cavity

Introduction

The genus Slackia is within the family Coriobacteriaceae in the phylum Actinobacteria. Currently, six *Slackia species* have been described: *Slackia exigua, S. heliotrinireducens, S. faecicanis, S. isoflavoniconvertens, S. equolifaciens* and *S. piriformis* (http://www.bacterio.net/slackia.html). The name "slackia" was named by honor Geoffrey Slack, a distinguished British microbiologist and dental researcher. Slackia belongs to domain: Bacteria, phylum: Actinobacteria, class: Actinobacteria, order: Coriobacteriales, family: Coriobacteriaceae. It consists of Gram-positive, non-spore-forming, non-motile, and strictly anaerobic rods. *S. exigua*, previous known as *Eubacteriumexiguum* was isolated from human oral lesions [1,2]. *S. heliotrinireducens*, previously known as *eptococcus heliotrinreducans* and *S. faecicanis* were isolated from the rumen of a sheep and canine faeces, respectively [3,4]. *S. isoflavoniconvertens* and *S. equolifaciens*, which produce equal, and *S. piriformis* were recently isolated from fumanfaeces[5-7]. Although the genus Slackia currently comprises six species, only *S. exigua* has been reported to cause infections in humans. *S. exigua* has

Article Information

DOI:	10.31021/jddm.20203154
Article Type:	Research
Journal Type:	Open Access
Volume:	3 **Issue:** 3
Manuscript ID:	JDDM-3-154
Publisher:	Boffin Access Limited
Received Date:	23 March 2020
Accepted Date:	04 April 2020
Published Date:	06 April 2020

***Corresponding author:**

Taira Kobayashi
Department of Fixed Prosthodontics and Oral Implantology
Nihon University School of Dentistry
Matsudo,Sakaechou-nishi, Matsudo City
Chiba, Japan
Fax: +81-47-360-9382
E-mail: kobayashi.taira@nihon-u.ac.jp

Citation: Kobayashi T, Uchibori S, Tsuzukibashi O, Uezato C, Fuchigami M, et al. Multiplex PCR for Simultaneous Detection and Identification of Six *Slackia Species* Including *Slackia exigua* Involved In Peri-Implantitis. J Dents Dent Med. 2020 Apr; 3(3): 154.

frequently been isolated from necrotic pulps and periradicular lesions [8-10]. These facts suggest that this species may play a pathogenic role in oral infectious diseases, including pulpal infections that spread to the periradicular tissues. In addition, this organism has been isolated from other oral sites, human wound infections and abscesses, a severe empyema with acute respiratory distress syndrome, polymicrobial feculent meningitis, and a bacteremia [11-19]. More interestingly, *S. exigua* has been significantly associated with chronic periodontitis and peri-implantitis[20,21]. Moreover, in our previous study, it was indicated the monitoring of *S. exigua* levels was useful as a clinical indicator for the diagnosis of peri-implantitis[22].

The accurate identification and enumeration of *Slackia species* are required in order to clarify the prevalence of six *Slackia species* in various sites of humans and their involvement in various infectious diseases. Although a sequence analysis of several target genes is the most reliable method, it is expensive, laborious, and time-consuming. Thus, a simple and more reliable assay for identifying *Slackia species* is desired. The purpose of the present study was to design primers for the identification of six *Slackia species* using multiplex PCR, and to investigate the proportion of these microorganisms in gingival crevicular fluids (GCF) collected from 30 periodontally healthy subjects (PH) and 30 chronic periodontitis subjects (CP) with this method.

Materials and Methods

Bacterial strains and culture conditions

The following bacterial strains were used in the present study: *S. exigua* JCM 11022, *S. heliotrinireducens* JCM 14554, *S. faecicanis* JCM 14555, *S. equolifaciens* JCM 16059, *S. pirifoemis* JCM 16070, *S. isoflavoniconvertens* JCM 16137, *S. exigua* clinical isolate Num-Sex 6965, *Streptococcus oralis* ATCC 10557, *S. salivarius* JCM 5707, *S. anginosus* ATCC 11391, *S. mutans* NCTC 10449, *Actinomycesnaeslundii* ATCC 12102, *A oris* ATCC 27044, *Rothiadentocariosa* JCM 3067, *R. mucilaginosa* JCM 10910, *Corynebacterium matruchotii* ATCC 14266, *C. durum* ATCC 33449, *Neisseria sicca* ATCC 29256, and *Aggregatibacteractinomycetemcomitans* ATCC 33384. *Slackia species* 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 (5μg/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, U.S.A) with 80% N2, 10% H2, and 10% CO2. S. exigua isolates NUM-Sex 6965 were obtained with non-selective medium, i.e., CDC, from the human oral cavity in our previous studies. Strains other than *Slackia species* were maintained by cultivating them on BactTM Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). These organisms were cultured at 37°C overnight in an atmosphere of 5% CO2 in a CO2 incubator (NAPCO® Model 5400; Precision Scientific, Chicago, IL, USA).

Design of species-specific primers for six *Slackia species*

The 16S rRNA sequences of *S. exigua* (accession no. AF101240), *S. heliotrinireducens* (accession no. AB559823), *S. faecicanis* (AJ608686), *S. equolifaciens* (EU377663), *S. pirifoemis* (AB490806), and *S. isoflavoniconvertens* (EU826403) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan), and a multiple sequence alignment analysis was performed with the CLUSTAL W program; i.e., the 16S rRNA sequences of six *Slackia species* were aligned and analyzed. Homology among the primers selected for six *Slackia species* and their respective 16S rRNA sequences was confirmed by a BLAST search.

Development of a multiplex PCR method using designed primers

The strains other than genus Slackia and *Slackia species* were cultured in BHIY broth and 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 for 24 h, respectively. 1 ml samples were then collected in microcentrifuge tubes and resuspended at a density of 1.0 McFarland standard (approximately 107 colony-forming units (CFU)/ml) 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 0.2 μM of each primer of each group, 10 μl of 2 × MightyAmp Buffer Ver.2 (Takara Bio Inc., Shiga, Japan), 0.4 μl of MightyAmp DNA Polymerase (Takara), and 5 μl of the template in a final volume of 20 μl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). 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 70 °C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being 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.

Clinical samples

Sixty patients attending Nihon University Hospital, School of Dentistry at Matsudo, participated in the present study. 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 contraceptive drugs; pregnant women. Gingival crevicular fluid (GCF) was collected using endodontic paper points, from periodontal sites of 30 periodontally healthy subjects (PH) and 30 chronic periodontitis subjects (CP; pocket depth > 4 mm) and placed in a sterile microcentrifuge tube containing 50 μl 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). A total of 5 μl of each sample was used as a PCR template. This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 17-014).

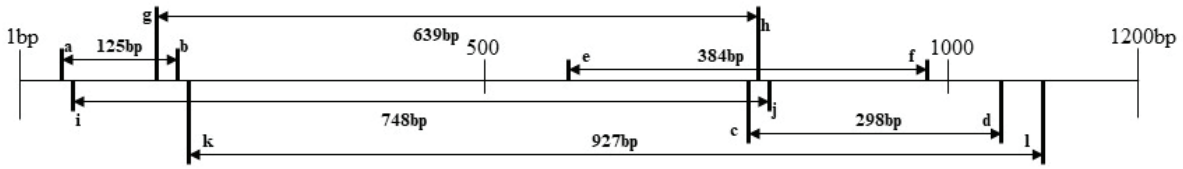
Results

Primer design

Twelve specific primers covering the upstream regions of the 16S rDNA sequences of six *Slackia species* were designed in the present study (Figure 1). The specific forward primers were designated as ShF for *S. heliotrinireducens*, SfF for *S. faecicanis*, SiF for *S. isoflavoniconvertens*, SpF for *S. pirifoemis*, SexF for *S. exigua*, and SeqF for *S. equolifaciens*, whereas the specific reverse primers were designated as ShR for *S. heliotrinireducens*, SfR for *S. faecicanis*, SiR for *S. isoflavoniconvertens*, SpR for *S. pirifoemis*, SexR for *S. exigua*, and SeqR for *S. equolifaciens*. The amplicon sizes of *S. heliotrinireducens*, *S. faecicanis*, *S. isoflavoniconvertens*, *S. pirifoemis*, *S. exigua*, and *S. equolifaciens* were 125 bp, 298 bp, 384 bp, 639 bp, 748 bp, and 927 bp, respectively.

Multiplex PCR

Detection limit: Our multiplex PCR method for the simultaneous detection and identification of six *Slackia species* successfully amplified DNA fragments of the expected size 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 x 103 and 5 x 105 CFU per PCR template (3.6 μl) for the *S. heliotrinireducens* -specific primer set with strain JCM 14554, the *S. faecicanis*-specific primer set with strain JCM 14554, the *S. isoflavoniconvertens*-specific primer set with strain JCM 16137, the *S. pirifoemis*-specific primer set with strain JCM 16070, the *S. exigua*-specific primer set with strain JCM 11022, and the *S. equolifaciens*-specific primer set with strain JCM 16059 (data not shown).



a: Forward primer for *Slackia heliotrinireducens*
ShF : ACCGCCCTC--GGGCGGACATAC 47-676bp

S. heliotrinireducens	ACCGCCCTC—GGGCGGACATAC
S. faecicanis	GGCGCCTTC—GGGCGCGAATAG
S. isoflavoniconvertens	GACGCCTTC—GGGCGTGGATAG
S. piriformis	GGCGCCTTC—GGGCGCGCATAG
S. exigua	GGCCCCCTTTAGGGGGCGCATAG
S. equolifaciens	GGCGCCTTC—GGGCGCGTATAG

b: Reverse primer for *Slackia heliotrinireducens*
ShR : CCGCAGCATGCGCTGGTGCC 173-154bp

S. heliotrinireducens	CCGCAGCATGCGCTGGTGCC
S. faecicanis	GCCCCCCATGCGGGGGGCCC
S. isoflavoniconvertens	GCATCCCATGCGGGGTGCCC
S. piriformis	CCCGGCCATGCGGCCCGGCG
S. exigua	GGCCCCCATGCGGGGGCCCC
S. equolifaciens	AGGGGGCATGCGTCCTCTCC

c: Forward primer for *Slackia faecicanis*
SfF : AGCCATTCCTCCGTGCCGC 785-803bp

S. heliotrinireducens	CCG-GACCCCCCGTGCCGC
S. faecicanis	AGCCATTCCTCCGTGCCGC
S. isoflavoniconvertens	GGAATCTCCTCCGTGCCGA
S. piriformis	AGATGTCCCTCCGTGCCGC
S. exigua	ACACGACCCTCCGTGCCGC
S. equolifaciens	CGACATGCCTCCGTGCCGA

d: Reverse primer for *Slackia faecicanis*
SfR : GGCATTACCCGATGGCAACATA 1082-1061bp

S. heliotrinireducens	AACTTGAT—GCTGGCAACATG
S. faecicanis	GGCATTACCCGATGGCAACATA
S. isoflavoniconvertens	AACTGAAT—GCTGGCAACACG
S. piriformis	AACTCAAT—GCTGGCAACACG
S. exigua	AACTGAAT—GCTGGCAACATG
S. equolifaciens	ATCGTTACATGCTGGCAACATA

e: Forward primer for *Slackia isoflavoniconvertens*
SiF : CCCCGAACTGGAAGCCTCGGA 590-610bp

S. heliotrinireducens	ACCCGAACTGGCCGGCTCGAG
S. faecicanis	GGCCGAACTGGGGGGATCGAG
S. isoflavoniconvertens	CCCCGAACTGGAAGCCTCGGA
S. piriformis	GGCCGAACTGGGGGGATCGAG
S. exigua	ACCCGAACTGGCGGGCTCGAG
S. equolifaciens	GGCCGAACTGGGGGGCTCGAG

f: Reverse primer for *Slackia isoflavoniconvertens*
SiR : CGGATCTCTCCGGCTTCACC 973-954bp

S. heliotrinireducens	GGCGTTTCCGCCGCTTCACC
S. faecicanis	GGCGTCTCCGCCGGCTCACG
S. isoflavoniconvertens	CGGATCTCTCCGGCTTCACC
S. piriformis	GCCGTCTCCGGCGGCTCACG
S. exigua	GGCGTCTCCGCCGCTTCACC
S. equolifaciens	GGCGTCTCCGCCGGCTCACG

g: Forward primer for *Slackia piriformis*
SpF : CGCCGGGCCGCATGGCCGGG 154-173bp

S. heliotrinireducens	GGCNGCAGCGCATGCTGCGG
S. faecicanis	GGGCCCCCCGCATGGGGGGC
S. isoflavoniconvertens	GGGCACCCCGCATGGGATGC
S. piriformis	CGCCGGGCCGCATGGCCGGG
S. exigua	GGGGCCCCCGCATGGGGGCC
S. equolifaciens	GGAGAGGACGCATGCCCCCT

h: Reverse primer for *Slackia piriformis*
SpR : GACATCTCCCCCCACACCTA 792-773bp

S. heliotrinireducens	GTC-CGGCCCCCCACACCTA
S. faecicanis	AATGGCT-CCCCCACACCTA
S. isoflavoniconvertens	AGATTCCTCCCCCACACCTA
S. piriformis	GACATCTCCCCCCACACCTA
S. exigua	GTCGTGTCCCCCCGCACCTA
S. equolifaciens	CATGTCG-CCCCCACACCTA

i: Forward primer for *Slackia exigua*
SexF : CCCCTTTAGGGGGCGCATAG 50-69bp

S. heliotrinireducens	GCCCTC—GGGCGGACATAC
S. faecicanis	GCCTTC—GGGCGCGAATAG
S. isoflavoniconvertens	GCCTTC—GGGCGTGGATAG
S. piriformis	GCCTTC—GGGCGCGCATAG
S. exigua	CCCCTTTAGGGGGCGCATAG
S. equolifaciens	GCCTTC—GGGCGCGTATAG

j: Reverse primer for *Slackia exigua*
SexR : AGGGTCGTGTCCCCCCGCA 797-779bp

S. heliotrinireducens	GGGGTC-CGGCCCCCCACA
S. faecicanis	AGGAATGGCT-CCCCCACA
S. isoflavoniconvertens	AGGAGATTCCTCCCCCACA
S. piriformis	AGGGACATCTCCCCCCACA
S. exigua	AGGGTCGTGTCCCCCCGCA
S. equolifaciens	AGGCATGTCG-CCCCCACA

k: Forward primer for *Slackia equolifaciens*
SeqF : AGAGGACGCATGCCCCCT 176-193bp

S. heliotrinireducens	CNGCAGCGCATGCTGCGG
S. faecicanis	GCCCCCCGCATGGGGGGC
S. isoflavoniconvertens	GCACCCCGCATGGGATGC
S. piriformis	CCGGGCCGCATGGCCGGG
S. exigua	GGCCCCCGCATGGGGGCC
S. equolifaciens	AGAGGACGCATGCCCCCT

l: Reverse primer for *Slackia equolifaciens*
SeqR : TCGTTACATGCTGGCAACATA 1102-1082bp

S. heliotrinireducens	ACTTGAT--GCTGGCAACATG
S. faecicanis	GCATTACCCGATGGCAACATA
S. isoflavoniconvertens	ACTGAAT--GCTGGCAACACG
S. piriformis	ACTCAAT--GCTGGCAACACG
S. exigua	ACTGAAT--GCTGGCAACATG
S. equolifaciens	TCGTTACATGCTGGCAACATA

Figure 1: Locations and sequences of species-specific primers for the 16S rDNA of six *Slackia species*. The nucleotide sequence of each primer has been underlined.

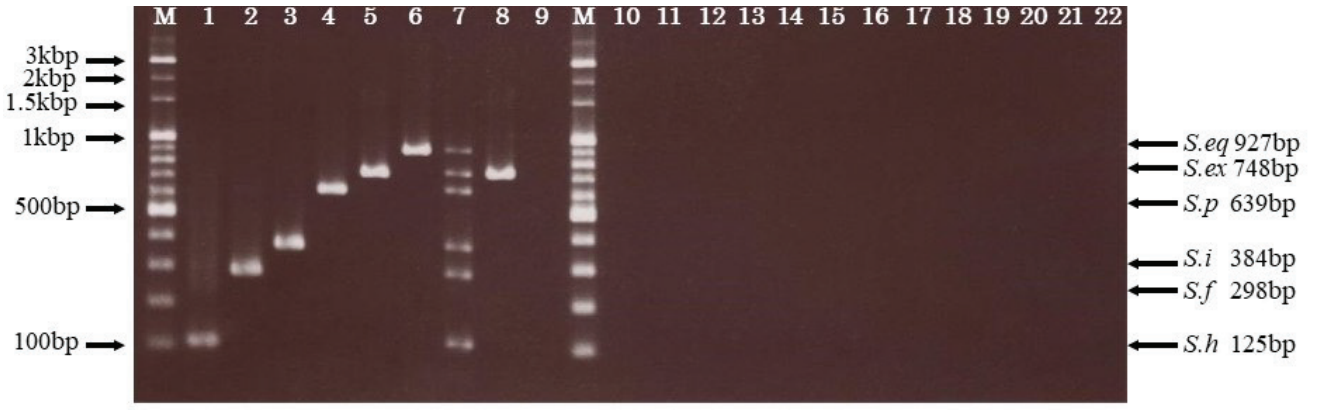


Figure 2: Multiplex PCR assay to detect six *Slackia* species

The primer mixture contained ShF, ShR, SfF, SfR, SiF, SiR, SpF, SpR, SexF, SexR, SeqF, and SeqR. Lanes: 1, *S. heliotrinireducens* JCM 14554; 2,*S. faecicanis* JCM 14555; 3, *S. isoflavoniconvertens* JCM 16137; 4, *S. pirifoemis* JCM 16070; 5,*S. exigua*JCM 11022; 6, *S. equolifaciens* JCM 16059; 7,mixture of six *Slackia species*; 8,*S. exigua* positive GCF sample; 9, *S. exigua* negative GCF sample; 10, *Streptococcus oralis* ATCC 10557; 11, *S. salivarius* JCM 5707; 12, *S. anginosus* ATCC 11391; 13, *S. mutans* NCTC 10449; 14, *Actinomyces naeslundii* ATCC 12102; 15,*A orisA*TCC 27044; 16, *Rothia dentocariosa* JCM 3067; 17, *R. mucilaginosa* JCM 10910; 18,*Corynebacterium matruchotii* ATCC 14266; 19, *C. durum* ATCC 33449; 20,*Neisseria sicca* ATCC 29256; 21,*Aggregatibacter actinomycetemcomitans* ATCC 33384; 23.

M, molecular size marker (100-bp DNA ladder).

Bacterial species	Periodontally healthy (PH)	Chronic periodontitis (CP)
	n=30	n=30
	(%, frequency)	(%, frequency)
S. heliotrinireducens	0 (0)	0 (0)
S. faecicanis	0 (0)	0 (0)
S. isoflavoniconvertens	0 (0)	0 (0)
S. pirifoemis	0 (0)	0 (0)
S. exigua	10 (33.3)	30 (100)
S. equolifaciens	0 (0)	0 (0)

Table 1: Frequency of six *Slackia species* in GCFs from 2 groups

Assay of representative oral bacteria: As representative oral bacteria, some genera Streptococcus, *Corynebacterium, Rothia*, Neisseria, and Aggregatibacter were subjected to PCR using the designed primer sets. However, no amplicons were produced from any of the representative oral bacteria.

Clinical examination: Table 1 shows the frequencies of six Slackia species in gingival crevicular fluids (GCF) collected from 30 periodontally healthy subjects (PH) and 30 chronic periodontitis subjects (CP) with this method. In GCFs from PH and CP subjects, *S. exigua* was detected at 33.3%, and 100%, respectively. On the other hand, *Slackia species* except *S. exigua* were not detected at all.

Discussion

Currently, six species of the genus Slackia have been described: *S. exigua*, *S. equolifaciens*, S. *faecicanis*, *S. heliotrinireducens*, *S. isoflavoniconvertens*, and *S. piriformis*. *S. exigua*, *S. heliotrinireducens*, and *S. faecicanis* was isolated from human oral lesions, the rumen of a sheep, and canine faeces, respectively [1-4]. *S. isoflavoniconvertens*, *S. equolifaciens*, and *S. piriformis* were recently isolated from fumanfaeces [5-7]. Cells of *S. exigua*, *S. equolifaciens*, *S. faecicanis*, and *S. isoflavoniconvertens* are short rods. Those of *S. heliotrinireducens* are cocci and coccobacilli. Those of *S. piriformis* form pear-shaped to irregularly shaped rods. *S. faecicanis* and *S. piriformis* are able to grow on medium containing 2 % oxgall, but the number of colonies decreased to about 50 % and 5 %, respectively, compared with the control medium without oxgall. In contrast, *S. exigua* and *S. heliotrinireducens* are not able to grow on the oxgall-containing medium. *S. exigua*, *S. equolifaciens*, *S. faecicanis*, *S. isoflavoniconvertens* and *S. piriformis* are asaccharolytic. *S. heliotrinireducens* produces acetate as the fermentation products from glucose [7]. Only *S. exigua* has ever been reported to cause infections in humans. This organism has been significantly associated with oral infectious diseases such as chronic periodontitis and peri-implantitis[20,21]. Moreover, in our previous study, it was indicated the monitoring of *S. exigua* levels was useful as a clinical indicator for the diagnosis of peri-implantitis[22]. In addition, this organism has been isolated from extra-oral infections occasionally [11-19].

A suitable method to each identify six *Slackia species* has not been yet developed because of the phenotypic and genetic similarities between these microorganisms. Moreover, *Slackia species* are easily missed under standard laboratory protocols due to the fastidious and slow grown and lack of activity in biochemical tests [14]. In addition, it has been unclear whether *Slackia species* including *S. exigua* are a part of normal oral flora. The accurate identification and enumeration of *Slackia species* have been required in order to clarify the prevalence of six *Slackia species* in various sites of humans and their involvement in various infectious diseases. When the biochemical characteristics and MALDI-TOF MS findings indicate a pathogen that is either very rare or unidentifiable, a molecular method such as 16S rRNA gene sequencing should be regarded as the gold standard for accurate identification. However, the technique is still relatively expensive, time-consuming and technically demanding, whereas the PCR method provides a rapid and relatively inexpensive assessment. Moreover, it may efficiently and accurately detect specific microorganisms in clinical samples, regardless of whether they are alive or dead. Also, it might be an innovative method for detection of microorganisms difficult to culture using traditional microbiological techniques. The 16S rDNA region of the bacterial genome provides an ideal target for species identification using PCR [23]. Moreover, multiplex-PCR is a rapid tool that allows for the simultaneous amplification of more than one sequence of target DNA in a single reaction, thereby saving time and reagents [24].

In the present study, we designed species-specific primers with the already mentioned means, for the identification of six *Slackia species* using a PCR method. These primers were able to distinguish each *Slackia species* and did not display cross-reactivity with representative oral bacteria. Moreover, we developed a multiplex PCR method with the ability to identify and differentiate six *Slackia species* (i.e., *S. exigua*, *S. equolifaciens*, *S. faecicanis*, *S. isoflavoniconvertens* and *S. piriformis*) using only two PCR tubes per sample.The proportion of these microorganisms in GCFs collected from 60 subjects was investigated with this multiplex PCR method. In GCFs from PH and CP subjects, *S. exigua* was detected at 33.3%, and 100%, respectively. On the other hand, *Slackia species* except *S. exigua* were not detected at all. These results indicated that *Slackia species* except *S. exigua* were not a part of normal oral flora and *S. exigua* might be associated with chronic periodontitis.Our multiplex PCR method is easy because the use of MightyAmp DNA Polymerase Ver.3 (Takara) means that DNA extraction may be avoided, and species identification using this method only takes approximately 2 hours. Thus, the method described herein will allow the prevalence of oral *Slackia species* and their involvement in oral infections to be fully clarified in future studies.

References

1. Poco SE, Nakazawa F, Ikeda T, Sato M, Sato T, et al. *Eubacteriumexiguum sp.*nov., isolated from human oral lesions. Int J SystBacteriol. 1996 Oct; 46(4):1120-1124.
2. Wade WG, Downes J, Dymock D, Hiom SJ, Weightman AJ, et al. The family Coriobacteriaceae: reclassification of Eubacteriumexiguum, (Poco et al. 1996) and *Peptostreptococcus heliotrinreducens* (Lanigan 1976) as *Slackiaexigua* gen. nov., comb. nov. and *Slackia heliotrinireducens* gen. nov., comb. nov., and *Eubacteriumlentum* (Pre´vot 1938) as Eggerthellalenta gen. nov., comb. nov.Int J SystBacteriol. 1999 Apr;49 Pt 2:595-600.
3. Lanigan GW. *Peptococcus heliotrinreducens*, sp. nov., a cytochrome producing anaerobe which metabolizes pyrrolizidine alkaloids. J Gen Microbiol. 1976 May; 94(1):1-10.
4. Lawson PA, Greetham HL, Gibson GR, Giffard C, Falsen E, et al. *Slackiafaecicanis sp*. nov., isolated from canine faeces. Int J Syst Evol Microbiol. 2005 May;55(Pt 3):1243-1246.
5. Matthies A, Blaut M, Braune A. Isolation of a human intestinal bacterium capable of daidzein and genistein conversion. Appl Environ Microbiol. 2009;75:1740-1744.
6. Jin JS, Kitahara M, Sakamoto M, Hattori M, Benno Y. *Slackiaequolifaciens sp*. nov., a human intestinal bacterium capable of producing equol. Int J Syst Evol Microbiol. 2010 Aug; 60(Pt 8): 1721-1724.
7. Nagai F, Watanabe Y, Morotomi M. *Slackia piriformis sp*. nov. and *Collinsellatanakaei sp*. nov., novel members of the family Coriobacteriaceae, isolated from human faeces. Int J Syst Evol Microbiol. 2010 Nov; 60(Pt 11): 2639-2646.
8. Hashimura T, Sato M, Hoshino E. Detection of Slackiaexigua, *Mogibacteriumtimidum* and *Eubacteriumsap henum* 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 E, Iwaku M. Bacteria invading periapical cementum. J 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(6):269-275.

11. Hill GB, Ayers OM, Kohan AP. Characteristics and sites of infection of *Eubacteriumnodatum*, *Eubacteriumtimidum*, *Eubacteriumbrachy*, and other asaccharolytic eubacteria. J Clin Microbiol. 1987 Aug;25(8):1540-1545.
12. Moore WEC, Holdemen LV, Cato EP, 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 unclassified 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 *Slackiaexigua* isolated from human wound infections, including abscesses of intestinal origin. J Clin Microbiol. 2010 Apr;48(4):1070-1075.
15. Roingeard C, Jaubert J, Guilleminault L. A large and unusual lung abscess with positive culture to *Slackiaexigua.* Int J Infect Dis. 2015 Nov;40:37-38.
16. Man MY, Shum HP, Wum A, Lee RA, Yan WW. A case of severe empyema with acute respiratory distress syndrome caused by *Slackiaexigua*re quiring veno-venous extracorporeal membrane oxygenation. Anaerobe. 2017 Dec;48:7-11.
17. Bükki J, Huttner HB, Lee DH, Jantsch J, Janka R, et al. Polymicrobial feculent meningitis with detection of *Slackiaexigua* in the cerebrospinal fluid of a patient with advanced rectal carcinoma. J Clin Oncol. 2010 Dec;29(35):e852-854.
18. Lee MY, Kim YJ, Gu HJ, Lee HJ. A case of bacteremia caused by *Dialisterpneumosintes* and *Slackiaexigua* in a patient with periapical abscess. Anaerobe. 2016 Apr;38:36-38.
19. Kawasuji H, Kaya H, Kawamura T, Ueno A, Miyajima Y, et al. Bacteremia caused by *Slackiaexigua*: A report of two cases and literature review. J Infect Chemother. 2020 Jan;26(1):119-123.
20. 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.
21. Shiba 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 Aug;6:30997.
22. Kobayashi T, Uchibori S, Tsuzukibashi O, Uezato C, Tamaki H, et al. Isolation and identification methods for *Slackiaexigua* and investigation of the relationship between this organism and peri-implantitis. J Dents Dent Med. 2018;1(3).
23. Panya S, Fliefel R, Probst F, Tröltzsch M, Ehrenfeld M, et al. Role of microbiological culture and polymerase chain reaction (PCR) of actinomyces in medication-related osteonecrosis of the jaw (MRONJ). J Craniomaxillofac Surg, 2017 Mar;45(3):357-363.
24. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques. 1997 Sep;23(3):504-511.