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Investigation of Bacteria Species Most Involved in Peri-Implantitis

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Abstract

Purpose: Currently, bacteriological examinations of implant treatments target periodontopathic bacteria such as red complex bacteria, including *Porphyromonas gingivalis*, and detect them qualitatively or quantitatively. However, it seems that those examinations do not reflect the peri-implant tissue conditions precisely, because periodontopathic bacteria are also frequently detected from healthy peri-implant sites. The purpose of the present study was to investigate bacteria species most involved in peri-implantitis using a PCR method. **Methods:** Polymerase chain reaction (PCR) primers in this study were designed based on partial sequences of 16S rDNA of bacteria species involved in peri-implantitis that were described in numerous previous studies. Peri-implant sulcus fluid (PISF) samples were collected from thirty periodontally healthy patients with implants (HI) and thirty patients with peri-implantitis (PI). Each detection frequency of bacteria species in PISFs of both groups was investigated using a PCR method, and was compared using Fisher's exact test. **Results:** In PI group, detection frequencies of *Corynebacterium durum*, *Fretibacterium fastidiosum* and *Slackia exigua* were significantly higher than those of HI group ($p < 0.01$). On the other hand, *P. gingivalis* and *Tannerella forsythia* belonging to red complex were frequently detected in the PISF samples of HI group ($p > 0.05$). **Conclusion:** It was suggested that monitoring *C. durum* and *F. fastidiosum* levels in PISF samples was useful as a clinical indicator for the evaluation of peri-implant tissue conditions.

Keywords

Peri-Implantitis, PCR Method, Bacteria Flora in Peri-Implant Sulcus, Red Complex

1. Introduction

At the present moment, dental implants have shown high survival rates of up to 99% for the last ten years [1] [2]. Even if many severe criteria for success are applied, the concept of dental implantology still appears promising [3] [4], despite the fact that certain limitations of the relevant techniques become evident. Besides minor prosthetic complications (such as crown loosening or ceramic chipping, which can mostly be resolved easily, and without big effort), peri-implantitis is the most common cause of biologic failure [4] [5]. The prognosis of peri-implantitis therapy, however, is far away from satisfactory today [6] [7]. The key feature of peri-implantitis is the progressive loss of marginal peri-implant bone as a symptom of chronic inflammation of the peri-implant tissues [6]. While particular co-factors, such as diabetes mellitus [8] [9], tobacco smoking [10] [11], and insufficient oral hygiene [12] [13], were found to accelerate the progress of bone destruction, the primary etiologic reason for the inflammation of peri-implant tissues is the oral biofilm [14].

Shortly after the installation of titanium implants, an implant sub-mucosal microbiota is established [15]. In fact, the initial colonization of peri-implant pockets with bacteria associated with periodontitis has been demonstrated to occur within two weeks [16]. This early colonization pattern may contribute to the development of peri-implant lesions. Leonhardt *et al.* [17] 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 [18].

Currently, bacteriological examinations of implant treatments target periodontopathic bacteria such as red complex bacteria, including *Porphyromonas gingivalis*, and detect them qualitatively or quantitatively. However, it seems that those examinations do not reflect the peri-implant tissue conditions precisely, because periodontopathic bacteria might be detected from healthy peri-implant sites [19]. Moreover, there is still some controversy among researchers about whether the composition of biofilm in peri-implantitis is really different from that in periodontitis-affected sites, or even from the microflora around healthy dental implants.

The purpose of the present study was to design species-specific primers in order to detect bacteria species involved in peri-implantitis that were described in numerous previous studies, and investigate bacteria species most involved in peri-implantitis using a PCR method.

2. Materials and Methods

2.1. Bacteria Species, Bacterial Strains and Culture Conditions

Bacteria species involved in peri-implantitis investigated using PCR method in this study are listed in **Table 1**. Bacterial strains were obtained from American Type Culture Collection (ATCC; USA), Culture Collection University of Gothenburg (CCUG; Sweden) and Japan Collection of Microorganisms (JCM; Japan). The following bacterial strains were used in the present study: *Eubacterium sulci* ATCC 35585, *Eubacterium saphenum* ATCC 49989, *Eubacterium limosum* JCM 6421, *Eubacterium nodatum* JCM 14550, *Eubacterium branchy* ATCC 33089, *Eubacterium yurii* ATCC 43714, *Eubacterium infirmum* ATCC 700433, *Eubacterium minutum* ATCC 700079, *Atopobium minutum* ATCC 33267, *Atopobium deltae* CCUG 65171, *Atopobium rimae* ATCC 49626, *Atopobium fossor* ATCC 43386, *Atopobium parvulum* ATCC 33793, *Atopobium vaginae* ATCC BAA-55, *Gemella morbillorum* JCM 12968, *Streptococcus mutans* JCM 5705, *Filifactor alocis* ATCC 35896, *Fretibacterum fastidiosum* JCM 16858, *Fusobacterium nucleatum* JCM 8532 and *Slackia exigua* JCM 11022. Anaerobic bacteria strains, *i.e.* genera *Eubacterium*, *Atopobium*, *Gemella*, *Filifactor*, *Fretibacterium*, *Fusobacterium* and *Slackia exigua*, were maintained by cultivating them on Fastidious Anaerobe Agar (FA; Neogen Co., Lansing, MI, USA) with sterile defibrinated sheep blood. These organisms were cultured at 37˚C for 48 h under anaerobic conditions with a gas pack system (AnaeroPack; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). *S. mutans* strains were maintained by cultivating them on Bact™ Brain Heart Infusion (BHI; Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). The organism was cultured at 37˚C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator (MCO-18AIC; Sanyo Electric Co., Tokyo, Japan).

2.2. Design of Species-Specific Primers for Bacteria Species Involved in Peri-Implantitis

Design of species-specific primers for some bacteria species involved in peri-implantitis, *i.e. E. sulci*, *E. saphenum*, *E. limosum*, *E. nodatum*, *E. branchy*, *E. yurii*, *E. infirmum*, *E. minutum*, *A. minutum*, *A. deltae*, *A. rimae*, *A. fossor*, *A. parvulum*, *A. vaginae*, *G. morbillorum*, *S. mutans*, *F. alocis*, *F. fastidiosum*, *F. nucleatum* and *S. exigua* was performed as described previously [20]. Briefly, the 16S rRNA gene sequences of *E. sulci* (accession no. AJ006963), *E. saphenum* (U65987), *E. limosum* (M59120), *E. nodatum* (Z36274), *E. branchy* (Z36272), *E. yurii* (GU269551), *E. infirmum* (Z36273), *E. minutum* (AB020885), *A. minutum* (M59059), *A. deltae* (KF537630), *A. rimae* (AB540986), *A. fossor* (AB015945), *A. parvulum* (AB558168), *A. vaginae* (Y17195), *G. morbillorum* (LC096237), *S. mutans* (AJ243965), *F. alocis* (AJ006962), *F. fastidiosum* (GQ149247), *F. nucleatum* (M58683) and *S. exigua* (AF101240) were obtained from the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/services/index.html, Mishima, Japan), and a multiple sequence alignment analysis was performed with the

CLUSTAL W program.

2.3. Development of a PCR Method for Detecting Bacteria Species Involved in Peri-Implantitis

A PCR method for detecting bacteria species involved in peri-implantitis, using the designed primers in this study and the previous reported primers [21]-[26] that were listed in Table 1, was developed as follows. Bacterial cells which were cultured on FA or BHI agar were suspended at a density of 1.0 McFarland standard (approximately 10^7 CFU in 1 ml of sterile distilled water). A total of 5.6 µl of the suspension was then used as a PCR template. The detection limit for PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The 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 × 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.

Table 1. Species-specific primers for bacteria species involved in peri-implantitis and detection frequencies of those organisms.

No.	Sequence			Size (bp)	Reference	Detection frequency Number of positive samples (%)	
						Healthy implant (n = 30)	Peri-implantitis (n = 30)
1	*Porphyromonas uenonis*	F	CGTCTACGTGTAGACGTTT	189	[21]	0 (0)	3 (10)
		R	CTAGAGAGTTTCAAAGGCAAGA				
2	*Porphyromonas endodontalis*	F	TGATTACAGATGGGCATG	257		6 (20)	16 (53.3)
		R	TCTCAGCTACACGTAGCTGC				
3	*Porphyromonas gingivalis*	F	ACAGAGGGGGATAACCCGTT	338		6 (20)	12 (40)
		R	ATGCAATACTCGTATCGCC				
4	*Porphyromonas asaccharolyticus*	F	TACTCCTTAGATCCCATGAG	466		0 (0)	4 (13.3)
		R	CTAGAGAGTTTCAAAGGCAAGA				
5	*Porphyromonas bennonis*	F	CTTAAGTACGCCTGTACATG	831		3 (10)	2 (6.7)
		R	GGTTTCCCAAGAGGCTCAC				
6	*Prevotella melaninogenica*	F	TTTGAAGTAAAGATTTATC	274		3 (10)	6 (20)
		R	AATAGGGACACGTCCCTAAC				

Continued

7	*Prevotella loescheii*	F	GGGGCGCTTGAGTGCGCTGA	394		3 (10)	0 (0)
		R	GCGGCGGCCCCGAAGGGCC				
8	*Prevotella intermedia*	F	CATATGGCATCTGACGTGGAC	659		3 (10)	6 (20)
		R	TAACGCCAGGCGCTAACAG				
9	*Prevotella nigrescens*	F	GTTTCATTGACGGCATCCGATA	394		3 (10)	8 (26.7)
		R	AAGCCCACGTCTCTGTGGG				
10	*Prevotella Denticola*	F	TTCGAAGCAAAGATCCGTC	1062		9 (30)	8 (26.7)
		R	GCTCGCGCCGCACCGGCAC				
11	*Tannerella forsythia*	F	GCGTATGTAACCTGCCCGCA	641		6 (20)	18 (60)
		R	TGCTTCAGTGTCAGTTATACCT				
12	*Treponema denticola*	F	TAATACCGAATGTGCTCATTTACAT	316		6 (20)	14 (46.7)
		R	TCAAAGAAGCATTCCCTCTTCTTCTTA		[22]		
13	*Aggregatibactor actinomycetemcomitans*	F	AAACCCATCTCTGAGTTCTTCTTC	557		0 (0)	0 (0)
		R	ATGCCAACTTGACGTTAAAT				
14	*Campylobactor rectus*	F	TTTCGGAGCGTAAACTCCTTTTC	598		9 (30)	18 (60)
		R	TTTCTGCAAGCAGACACTCTT				
15	*Helicobacter pylori*	F	ATAGTCAGTCAGGTGTGA	869	[23]	3 (10)	2 (6.7)
		R	CAATTTAGCATCCTGACTT				
16	*Solobacterium moorei*	F	TCGGAAGGCATCTTCTGGTT	452	[24]	3 (10)	6 (20)
		R	AAGTGGCTGGATTGGGTTGA				
17	*Rothia mucilaginosa*	F	GCCTAGCTTGCTAGGTGGA	400	[25]	21 (70)	6 (20)
		R	GCAGGTACCGTCAATCTCTC				
18	*Corynebacterium matruchotii*	F	TGGTGACGGTACCTTTGTTA	569		3 (10)	6 (20)
		R	ACCGGTCCCCACACCTAA		[26]		
19	*Corynebacterium durum*	F	ACATACGACCATGGCGTAGG	284		16 (53.3)	29 (96.7)
		R	AGGTGGGGCTTCGTCCCGG				
20	*Eubacterium sulci*	F	ATAAAGGAATGAAGCTTCG	122		0 (0)	0 (0)
		R	GTTATGGGGTATTAATCAC				
21	*Eubacterium saphenum*	F	CGTACCCTTAATCGGGTAT	275		0 (0)	4 (13.3)
		R	AAGATTTGCTCCCCCTTGCG				
22	*Eubacterium limosum*	F	TTGATGGATCTTCGGGTGAC	361		0 (0)	2 (6.7)
		R	TCCGAAAACCTTCTTCAC		In this study		
23	*Eubacterium nodatum*	F	TTAAGTAAGCGTAGGGTTT	433		0 (0)	8 (26.7)
		R	CTCAGTTTTAACCGAGCTT				
24	*Eubacterium branchy*	F	TTTTGAAAAGATTCTTCGGA	509		0 (0)	2 (6.7)
		R	AAGGCCACCTACGTACCC				
25	*Eubacterium yurii*	F	TCAACCTGTGACACACGGA	691		0 (0)	6 (20)
		R	TTCCTCCCGACACCTAGTGT				

Continued

26	*Eubacterium infirmum*	F	GATGCAAGAGATACACATGT	790	0 (0)	6 (20)
		R	GTTCCTGGTAAGGTTCTT			
27	*Eubacterium minutum*	F	TAAAAGGACACTTCGGTAG	936	0 (0)	2 (6.7)
		R	AACGGCATTACCCGATACT			
28	*Atopobium minutum*	F	TCTTTTAGATGTGTATAAAG	139	0 (0)	0 (0)
		R	TAGACGCTTTGTCTTGTGTG			
29	*Atopobium deltae*	F	TGTATTGATCGCATGGTAT	277	0 (0)	0 (0)
		R	ATAAGGCCTCGTCCCTGCTG			
30	*Atopobium rimae*	F	AGAATAGCTCTTCCGTGCC	432	6 (20)	2 (6.7)
		R	CGCCCTTGCGGGTTGGCAGCT			
31	*Atopobium fossor*	F	AGACCGCGTTCCGATACCG	500	0 (0)	0 (0)
		R	TGGCTGCCAGCTTAACCT			
32	*Atopobium parvulum*	F	GAGACTTCCGCATGGAAGACT	642	0 (0)	2 (6.7)
		R	GAAATACTCCCCCACACCT			
33	*Atopobium vaginae*	F	ATATTTCTCGCATGGCGAAT	817	0 (0)	0 (0)
		R	AGTGTTTCCACTGCTTCAC			
34	*Gemella morbillorum*	F	TATTTCTCGCATGAGAGATA	300	0 (0)	0 (0)
		R	TACATGTATAGTTACTACAT			
35	*Streptococcus mutans*	F	GAGCTTACCAAGGCGACGATA	332	18 (60)	12 (40)
		R	TCCTGACCGCCTGCGCTCC			
36	*Filifactor alocis*	F	AAGAAATGACAGTACCC	546	3 (10)	12 (40)
		R	GTCCTCGATTAAAAGGCTGTCATT			
37	*Fretibacterium fastidiosum*	F	TGGTAACACGGAATGGCATAC	738	5 (16.7)	20 (66.7)
		R	ACCAACATCTCTGCTCGC			
38	*Fusobacterium nucleatum*	F	AATAGGGCATCCTATAAT	1063	22 (73.3)	24 (80)
		R	TTCACAGCTTTGCAACTC			
39	*Slackia exigua*	F	TTTAGGGGGCGCATAGAGT	1175	4 (13.3)	18 (60)
		R	AAGGGATTCGCTCGCCCTCGCGGGTC			

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: periodontally healthy patient with implants (HI) and patients with 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 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). The detection frequencies of bacteria species involved in peri-implantitis in each PISF sample were determined using a PCR method. The present study was conducted in accordance with the principles of the Declaration of Helsinki, and was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 18-033).

2.5. Statistical Analysis

The detection frequencies of bacteria species involved in peri-implantitis in both groups were compared using Fisher's exact test. Values of $p < 0.05$ were considered significant. Moreover, bacteria species involved in peri-implantitis were divided into three hierarchies (Hierarchy I: $p < 0.01$; Hierarchy II: $p < 0.05$; Hierarchy III: $p > 0.05$).

3. Results

3.1. PCR Method for Detecting Bacteria Species Involved in Peri-Implantitis

3.1.1. Primer Design

Ten specific primers covering the upstream regions of the 16S rDNA of *E. sulci*, *E. saphenum*, *E. limosum*, *E. nodatum*, *E. branchy*, *E. yurii*, *E. infirmum*, *E. minutum*, *A. minutum*, *A. deltae*, *A. rimae*, *A. fossor*, *A. parvulum*, *A. vaginae*, *G. morbillorum*, *S. mutans*, *F. alocis*, *F. fastidiosum*, *F. nucleatum* and *S. exigua* were designed in the present study (**Table 1**). The amplicon sizes of *E. sulci*, *E. saphenum*, *E. limosum*, *E. nodatum*, *E. branchy*, *E. yurii*, *E. infirmum*, *E. minutum*, *A. minutum*, *A. deltae*, *A. rimae*, *A. fossor*, *A. parvulum*, *A. vaginae*, *G. morbillorum*, *S. mutans*, *F. alocis*, *F. fastidiosum*, *F. nucleatum* and *S. exigua* were 122 bp, 275 bp, 361 bp, 433 bp, 509 bp, 691 bp, 790 bp, 936 bp, 139 bp, 277 bp, 432 bp, 500 bp, 642 bp, 817 bp, 300 bp, 332 bp, 546 bp, 738 bp, 1063 bp and 1175 bp, respectively.

3.1.2. Assay of Bacteria Species Involved in Peri-Implantitis

The PCR method used to detect bacteria species involved in peri-implantitis produced positive bands from each reference strains (**Figure 1** and **Figure 2**).

Some *Streptococcus*, *Actinomyces*, *Neisseria*, *Corynebacterium* and *Rothia* species were used as representative oral bacteria in PCR using the designed primer set. No amplicons were produced from any of the representative oral bacteria (data not shown). 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 each species-specific primer set with each strain (data not shown).

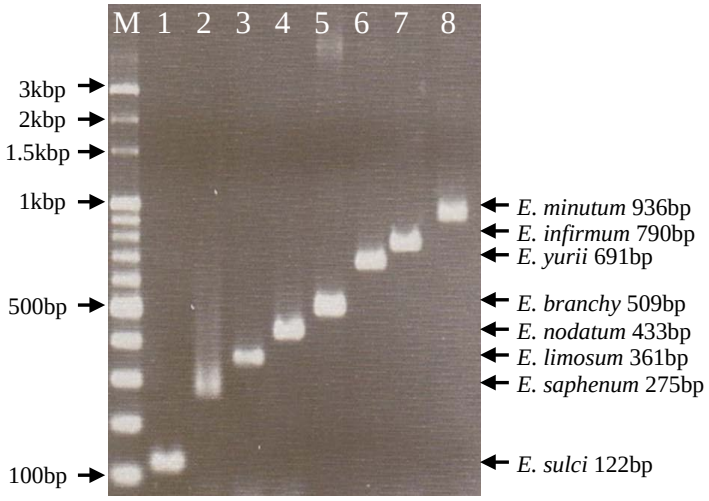

Figure 1. Specificity of PCR assays. Lanes: 1: *Eubacterium sulci* ATCC 35585; 2: *Eubacterium saphenum* ATCC 49989; 3: *Eubacterium limosum* JCM 6421; 4: *Eubacterium nodatum* JCM 14550; 5: *Eubacterium branchy* ATCC 33089; 6: *Eubacterium yurii* ATCC 43714; 7: *Eubacterium infirmum* ATCC 700433; 8: *Eubacterium minutum* ATCC 700079. M: molecular size marker (100-bp DNA ladder).

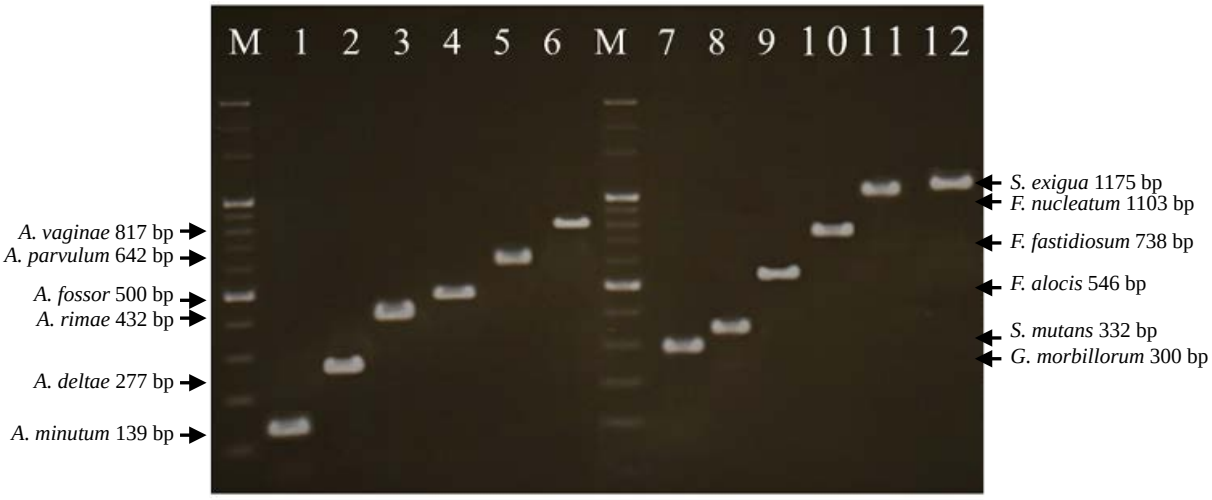

Figure 2. Specificity of PCR assays. Lanes: 1: *Atopobium minutum* ATCC 33267; 2*: Atopobium deltae* CCUG 65171; 3: *Atopobium rimae* ATCC 49626; 4: *Atopobium fossor* ATCC 43386; 5*: Atopobium parvulum* ATCC 33793; 6: *Atopobium vaginae* ATCC BAA-55; 7: *Gemella morbillorum* JCM 12968; 8: *Streptococcus mutans* JCM 5705; 9: *Filifactor alocis* ATCC 35896; 10: *Fretibacterum fastidiosum* JCM 16858; 11: *Fusobacterium nucleatum* JCM 8532; 12: *Slackia exigua* JCM 11022. M: molecular size marker (100-bp DNA ladder).

3.2. Clinical Examination

The clinical parameters of HI and PI groups are shown in Table 2. The average ages and PPDs of HI and PI groups were 56 (range: 37 - 68) and 58 (range: 44 - 71), and 2.32 mm and 7.78 mm, respectively. The detection frequencies of bacteria species involved in peri-implantitis in PISF samples obtained from the two groups are shown in Table 1. Also, Table 3 shows the hierarchy of bacteria species involved in peri-implantitis. In the PI group, detection frequencies of *Corynebacterium durum* ($p = 0.0001$), *F. fastidiosum* ($p = 0.0002$) and *Slackia exigua* ($p = 0.0004$) were significantly higher than those of the HI group, and were grouped into Hierarchy I ($p < 0.01$). Following those organisms, *Tannerella forsythia* ($p = 0.003$), *E. nodatum* ($p = 0.005$), *Porphyromonas endodontitis* ($p = 0.015$), *F. alocis* ($p = 0.015$), *E. yurii* ($p = 0.024$), *E. infirmum* ($p = 0.024$) and *Campylobacter rectus* ($p = 0.037$) were grouped into Hierarchy II ($p < 0.05$). On the other hand, *Porphyromonas gingivalis* ($p = 0.158$) and *Treponema denticola* ($p = 0.054$) belonging to red complex were frequently detected from some of the HI group, and were grouped into Hierarchy III ($p > 0.05$).

4. Discussion

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 [27] [28] [29]. 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, species-specific primers to detect some bacteria species involved in peri-implantitis were designed using a multiplex PCR method. These primers were able to distinguish each bacteria species and did not react with representative oral bacteria. Moreover, the PCR analysis in this study could directly use bacterial cells using MightyAmp DNA Polymerase Ver. 3 (Takara) and be completed in approximately 1.5 h.

Table 2. 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 (13:17)	56 (37 - 68)	-	-	-	2.32 mm (2 - 3 mm)
Peri-implantitis	30 (16:14)	58 (44 - 71)	+	+	+	7.78 mm (5 - 11 mm)

Table 3. Hierarchy of bacteria species involved in peri-implantitis.

Ranking	Bacteria species	Fisher's exact test (p-value)	Hierarchy
1	*Fretibacterium fastidiosum*	0.0001	Hierarchy I ($p < 0.01$)
2	*Corynebacterium durum*	0.0002	
3	*Slackia exigua*	0.0004	
4	*Tannerella forsythia*	0.003	Hierarchy II ($p < 0.05$)
5	*Eubacterium nodatum*	0.005	
6	*Porphyromonas endodontalis*	0.015	
7	*Filifactor alocis*	0.015	
8	*Eubacterium yurii*	0.024	
9	*Eubacterium infirmum*	0.024	
10	*Campylobactor rectus*	0.037	
11	*Treponema denticola*	0.054	Hierarchy III ($p > 0.05$)
12	*Porphyromonas asaccharolyticus*	0.112	
13	*Eubacterium saphenum*	0.112	
14	*Porphyromonas gingivalis*	0.158	
15	*Prevotella nigrescens*	0.181	
16	*Porphyromonas uenonis*	0.273	
17	*Prevotella melaninogenica*	0.472	
18	*Prevotella intermedia*	0.472	
19	*Solobacterium moorei*	0.472	
20	*Corynebacterium matruchotii*	0.472	

In the present study, bacteria species involved in peri-implantitis were investigated as an indicator of unhealthy peri-implant tissue conditions such as implant mucositis and peri-implantitis. Currently, bacteriological examinations of implant treatments target periodontopathic bacteria such as red complex bacteria, which are detected qualitatively or quantitatively. However, it seems that those examinations do not precisely reflect the peri-implant tissue conditions, because periodontopathic bacteria may be detected at healthy peri-implant sites [19]. We have been searching for bacteria that are suitable as an indicator for unhealthy peri-implant tissue conditions such as implant mucositis and peri-implantitis. Recently, peri-implantitis-specific bacteria species have been reported by several studies using DNA hybridisation and 16S rDNA sequencing [30] [31]. We, therefore, chose several bacteria species involved in peri-implantitis as possible indicator species in the present study. As a result, In the PI group, the detection frequencies of *C. durum* ($p = 0.0001$), *F. fastidiosum* ($p = 0.0002$) and *S. exigua* ($p = 0.0004$) were significantly higher than those of the HI group ($p < 0.01$); however, *P. gingivalis* ($p = 0.158$) and *T. denticola* ($p = 0.054$) belonging

to red complex were frequently detected from some of the HI group ($p > 0.05$). 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 [19].

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 *C. durum*, *F. fastidiosum* and *S. exigua* levels may be suitable as an indicator reflecting unhealthy peri-implant tissue conditions to aid in the prevention of peri-implantitis.

5. Conclusion

It was suggested that monitoring *C. durum*, *F. fastidiosum* and *S. exigua* levels in PISF samples was useful as a clinical indicator for the evaluation of peri-implant tissue condition.

Authors' Contributions

H. Murakami, O. Tsuzukibashi, A. Fukatsu, Y. Takahashi, K. Idei, K. Usuda, M. Fuchigami, C. Komine, S. Uchibori, K. Umezawa, S. Hayashi and T. Asano corrected the data. H. Murakami, O. Tsuzukibashi, A. Fukatsu, M. Wakami, T. Kobayashi and M. Fukumoto drafted and wrote the manuscript. The concept of this manuscript was devised by H. Murakami. 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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