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Original Article

COLONIZATION BY *PORPHYROMONAS GINGIVALIS* AND *PREVOTELLA INTERMEDIA* FROM TEETH TO OSSEOINTEGRATED IMPLANT REGIONS

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Abstract

Colonization by periodontopathic bacteria is a risk factor for peri-implantitis. The purpose of this study was to investigate the colonization by black-pigmented anaerobic bacteria that occurs between the time before fixture installation and 6 months after inserting superstructures in implant treatment in partial edentulous cases. Dental plaque was serially collected from around the natural teeth and implants in 12 patients in whom a dental implant was indicated, and *Porphyromonas gingivalis* and *Prevotella intermedia* were detected using polymerase chain reaction (PCR). One month after connecting the abutment, the detection rate of *P. gingivalis* per site from around the implants was 63.7% and that of *P. intermedia* was 50.8%. Six months after superstructure setting, the detection rate per site of *P. gingivalis* from around the implants was 56.8% and that of *P. intermedia* was 41.1%. When chromosomal DNA segmentation patterns in the isolated *P. gingivalis* and *P. intermedia* were compared using pulsed field gel electrophoresis (PFGE), the patterns in the natural teeth were in accordance with those in the implants in 3 of 4 cases (75.0%) in *P. gingivalis* and all cases in *P. intermedia.* This finding suggested that bacterial colonization around implants occurred early after the implant region was exposed to the intraoral cavity and that the bacteria were transmitted from the area around the natural teeth.

Key words: *Porphyromonas gingivalis*—*Prevotella intermedia*—Transmission—Colonization—Peri-implantitis

INTRODUCTION

Osseointegrated implantation has been used as a means of prosthetic treatment, and many cases with good results have been reported[21,34]. However, investigating the 10-year survival rate of implants in 127 Kennedy class I–III cases, Lekholm *et al.*[16] reported that approximately

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Table 1 Profile of dental implant patients

Patient No.	Gender	Age (y)	Location of implant site
1	F	33	36 37 45 46 47
2	F	18	11 21 22 23 24
3	M	51	46 47
4	F	65	46 47
5	M	57	36 37
6	M	39	24 25 26 27 44 45 46 47
7	M	52	11 12 13 14 15 16 17 21 22 23
8	M	53	24 25 26
9	F	42	24 25 26
10	M	35	12
11	M	46	14 16 18 34 36 37 46
12	M	39	36

10% of cases failed. Disintegration of implants occurred, and the implants were removed in most of the failed cases. Occlusal overload and peri-implantitis caused by specific bacterial flora infection were the major causes of implant disintegration[29]. The incidence of peri-implantitis in inserted implants has been reported to be 2–10%[6,19]. DNA probe detection of bacterial flora around the natural teeth and implants performed by Hultin *et al.*[12] showed that the detection rate of *Porphyromonas gingivalis* was 9.7% around the natural teeth, and 10.6% around the implant regions. Furthermore, periodontopathic bacteria were detected in all areas in which supporting tissue surrounding implants was lost. Gouvoussis *et al.*[11] and Sumida *et al.*[27] reported that the *P. gingivalis* frequently detected around implants were transmitted from local periodontal areas; however, the time of bacterial colonization has not been clarified.

The purposes of this study were to detect *P. gingivalis* and *Prevotella intermedia* from around the natural teeth and implants between before surgery and 6 months after setting the superstructure in dental implant treatment, to clarify the period of colonization by *P. gingivalis* and *P. intermedia* from periodontal pockets to the implant regions, to investigate whether *P. gingivalis* and *P. intermedia* are transmissible from the natural teeth to the implant regions, and to analyze the restriction enzyme segmentation patterns of the isolated bacterial chromosomal DNA.

MATERIALS AND METHODS

1. Subjected patients

The data of sites of implant from the subjected patients in this study are summarized in Table 1. The subjects were 12 patients (8 males and 4 females) aged 18–65 years (the mean age: 44.2 years) who underwent dental implant treatments. Microbial samples were obtained from 58 natural teeth and 118 implants (sum total of all sampling periods) and examined. We obtained the subjects' informed consent to this study.

We examined the periodontal status of the natural teeth. The data from all the patients enrolled in this study are summarized in Table 2. Four of the patients were smokers.

We applied the Branemark system implant for all patients; it is made from 100% titanium. The superstructure is made from metal backed porcelain. In cases in which we used a temporary superstructure between the abutment connection and the superstructure setting, it was made from resin.

One month before the Branemark system implant installation surgery, the subgingival plaque around the natural teeth adjacent to the area intended for implantation was

Table 2 Clinical periodontal status and smoking situation of patients subjected in this study

Patient	Gender	Average pocket depth (mm)	Average plaque control record (%)	Bleeding on probing/ Examined teeth	Smoking
1	F	2.4	34	0/20	+
2	F	2.9	65	0/22	+
3	M	3.8	40	0/23	+
4	F	1.8	10	0/23	−
5	M	3.8	77	0/24	−
6	M	4.2	68	0/19	−
7	M	4.0	69	0/15	+
8	M	2.8	25	0/24	−
9	F	2.0	15	0/24	−
10	M	2.4	21	0/26	−
11	M	3.1	18	0/19	−
12	M	2.4	19	0/24	−

Table 3 Primers used for detecting *Porphyromonas gingivalis* and *Prevotella intermedia*

Species	Sequence	Product size
P. gingivalis	5′-ATA ATG GAG AAC AGC AA-3′ 5′-TCT TGC CAA CCA GTT CCA TTG C-3	131 (bp)
P. intermedia	5′-TTT GTT GGG GAG TAA AGC GGG-3′ 5′-TCA ACA TCT CTG TAT CCT GCC T-3′	575 (bp)

collected to detect *P. gingivalis* and *P. intermedia* (the first sampling). Dental plaque sampling from the implant region was performed one month after abutment connection (the second sampling), one month after superstructure setting (the third sampling), and six months after superstructure setting (the fourth sampling). Amoxicillin (750 mg per day) was administered on the days of fixture installation surgery and abutment connection surgery, and for 4–7 days postoperatively. We instructed all patients to perform tooth brushing carefully at least three times a day, and they did. For the cleaning of implant regions, we recommended using an intertooth brush to all patients. In addition, we instructed all patients to use a 0.2% benzethonium chloride solution for rinsing after tooth brushing.

2. Sampling of subgingival plaque

Briefly, the subgingival plaque was collected from both 4–6 natural teeth and all implants. The obtained plaque sample in each case was transferred to 100 μl of reduced transport fluid (RTF)[28], and the sample was dispersed[13]. The samples were serially diluted, and part of each suspension was cultured. The microorganisms in the RTF without dilution were sedimented by centrifugation at 15,000 $\times g$ at 4°C for 10 minutes.

3. Detection of *P. gingivalis* and *P. intermedia* with polymerase chain reaction (PCR) using specific primers designed from 16s RNA sequence

The obtained samples were examined for the presence of *P. gingivalis* and *P. intermedia* by PCR as described by Ashimoto *et al.*[4]. Primers used in this study are summarized in Table 3. Sedimented microorganisms were suspended in 100 μl of boiling buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% triton X-100), and boiled at 100°C for 10 minutes.

After the removal of cell debris by centrifugation at 15,000 × *g* at 4°C for 15 minutes, the bacterial DNA was extracted with phenol and precipitated by ethanol. The specific primer pairs used in the PCR are summarized in Table 3. Briefly, 5 μl of sample was added to 45 μl of reaction mixture, which was comprised of PCR buffer (Takara Co., Shiga, Japan) containing 2 mM dNTP, 50 μM of the specific primer pairs listed in Table 2, and 0.25 U Taq DNA polymerase (Takara Co., Shiga, Japan). The amplification for 16s RNA locus of *P. gingivalis* were performed using a thermal cycler (Gene Amp PCR system 9700, PE Biosystems, Foster City, CA) under the following conditions: 36 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and an extension step at 72°C for 1 minute. The PCR assays for *P. intermedia* were performed using a thermal cycler as follows: 36 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and an extension step at 72°C for 1 minute. The PCR products were electrophoresed on 2% agarose gel and visualized under UV light following staining with ethidium bromide. Bands showing an intensity stronger than the control for each species demonstrating at least 5×10^2 cells were judged positive.

4. Analysis by pulsed field gel electrophoresis (PFGE)

Part of each plaque sample was diluted with RTF solution in a gradient of 1:10 steps down to $1:10^{-5}$, and 100 μl of each dilution was inoculated onto Tryptic soy agar (BBL, Cockeysville, MD) containing 5 μg/ml hemin, 0.5 μg/ml menadione, and 10% horse defibrinated blood (blood agar plate). The plates were cultured in an anaerobic chamber containing 10% CO_2, 10% H_2, and 80% N_2 at 37°C for 5 to 7 days. Black-pigmented colonies were re-inoculated on blood agar plates for isolation. All isolated strains of black pigmented colony were identified by sequences of the 16s rRNA locus. From these colonies, *P. gingivalis* and *P. intermedia* were subjected to pulsed field gel electrophoresis (PFGE) analysis. Single colonies of *P. gingivalis* and *P. intermedia* taken from each patient were inoculated into tryptic soy broth (BBL) containing 5 μg/ml of hemin and 0.5 μg/ml of menadione and cultured for two days. The bacterial cells were then harvested by centrifugation at 15,000 × *g* at 4°C for 15 minutes. The cells obtained were treated using the method described by Nakayama[23]. Briefly, after embedding in agarose gel blocks, cells were lysed with a solution containing 2 mg/ml 1.0% SDS, 500 μl of proteinase K and 0.5 M EDTA. After washing the block, genomic DNA was digested with *Not* I. Using a 1% agarose gel, electrophoresis with a CHEF-DR III apparatus (Biorad, Hercules, CA) was performed on the resulting material under the following conditions: potential difference, 6 volts/cm; electrode angle, 120°; initial switch time, 5.3 seconds; final switch time, 49.9 seconds; and total duration, 20 hours. After the completion of electrophoresis, the gel was placed in 0.1 μg/ml ethidium bromide for 60 minutes, and, after being stained, was photographed under ultraviolet light.

5. Statistical analysis

The relationships between colonization by the two bacterial species from natural teeth and from implant regions were analyzed by Mann-Whitney U test.

RESULTS

PCR detections of *P. gingivalis* and *P. intermedia* from teeth of 12 patients before implant treatment are summarized in Table 4. We detected *P. gingivalis* and *P. intermedia* in samples from eleven patients. The detection rates of *P. gingivalis* from periodontal regions were 85.4%. The *P. intermedia* detection rates from periodontal regions were 60.0%.

The detection rates of *P. gingivalis* from implant sites by PCR at one month after abutment connection, one month after superstructure setting, and six months after superstructure setting were 63.7, 58.5, and 56.8%, respectively as shown in Table 5.

The detected percentage rates of

Table 4 PCR detection rates of *Porphyromonas gingivalis* and *Prevotella intermedia* in samples from natural teeth one month before fixture installation

Patient No.	Age	Gender	1st sample of natural teeth number examined	*P. gingivalis*		*P. intermedia*	
				Positive site	%	Positive site	%
1	33	F	6	6	100	6	100
2	18	F	5	5	100	5	100
3	51	M	6	6	100	3	50
4	65	F	5	5	100	0	0
5	57	M	4	4	100	4	100
6	39	M	5	5	100	0	0
7	52	M	5	5	100	1	20
8	53	M	5	5	100	5	100
9	42	F	4	1	25	2	50
10	35	M	4	0	0	0	0
11	46	M	4	4	100	4	100
12	39	M	5	5	100	5	100
Total			58				
Average	44.2				85.4		60.0

Table 5 PCR detected percent of *Porphyromonas gingivalis* in samples from peri-implant regions by PCR one month after abutment connection, and one month after superstructure setting and sixth month after superstructure setting

Patient No.	Number of implant examined sites	Detected percent of examined sites		
		1M after A	1M after set	6M after set
1	5	40	80	60
2	5	100	100	100
3	2	100	0	0
4	2	0	0	0
5	2	100	100	100
6	8	100	100	100
7	7	14.3	14.3	14.3
8	3	100	100	100
9	3	66.7	50	50
10	1	0	0	100
11	7	42.9	57.1	57.1
12	1	100	100	0
	Average	63.7	58.5	56.8

P. intermedia from implant sites by PCR at one month after abutment connection, one month after superstructure setting, and six months after superstructure setting were 50.8, 52.7, and 41.1% respectively as shown in Table 6.

We isolated *P. gingivalis* or *P. intermedia* from both periodontal regions of teeth and implant sites from 4 (patient No. 1, 5, 6 and 9) and 4 (patient No. 2, 3, 7 and 11) in 12 patients respectively. We confirmed that there were no identical PFGE patterns of isolated *P. gingivalis* and *P. intermedia* strains from different patients examined in this study[2,10]. The PFGE patterns of 9 strains of *P. gingivalis* isolated from patient No. 6 are shown in Fig. 1. The PFGE patterns of all tested strains are identical. From the analysis of PFGE patterns,

Table 6 PCR detected percent of *Prevotella intermedia* in samples from peri-implant regions by PCR one month after abutment connection, and one month after superstructure setting and sixth month after superstructure setting

Patient No.	Number of implant examined sites	Detected percent of examined sites		
		1M after A	1M after set	6M after set
1	5	0	40	0
2	5	100	100	100
3	2	100	100	100
4	2	0	0	0
5	2	0	100	100
6	8	100	0	0
7	7	14.3	14.3	14.3
8	3	100	100	100
9	3	66.7	50	50
10	1	0	0	0
11	7	28.6	28.6	28.6
12	1	100	100	0
	Average	50.8	52.7	41.1

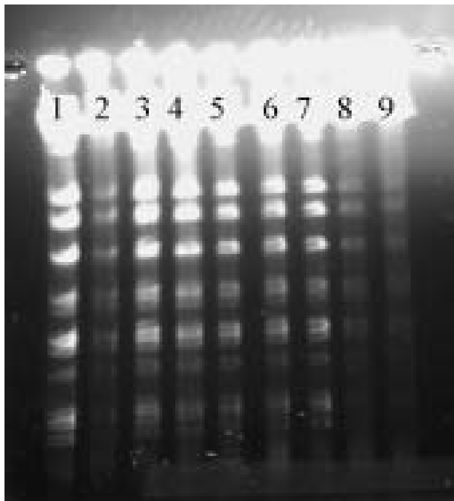

Fig. 1 PFGE patterns of *P. gingivalis* strains isolated from Patient 6

Lines 1 and 2; natural teeth
(one month before fixture installation):
Lines 3, 4 and 5; implants
(one month after abutment connection):
Lines 6 and 7; implants
(one month after superstructure setting):
Lines 8 and 9; implants
(six month after superstructure setting):

we found that 3 out of 4 patients from whom we isolated *P. gingivalis* strains from teeth and implant regions colonized the identical clone of the microorganism as well as Sumida *et al.* reported[27].

There were no different PFGE patterns of *P. intermedia* isolated from patients. Identical PFGE patterns of *P. intermedia* isolated from both teeth and implant regions in each patient were found. These results indicate that clonal *P. intermedia* strains colonize in the 4 respective patients.

DISCUSSION

The PCR detection rate of *P. gingivalis* one month after abutment connection was 63.7%, a high value, which was similar to that 6 months after superstructure setting. Thus the colonization by *P. gingivalis* occurred at the comparatively early stage by the time of superstructure setting. The PCR detection rates of *P. intermedia* in samples obtained from implant sites were similar to those of *P. gingivalis*. These results suggest that the high susceptibility of patients to peri-implantitis due to the combination of immunological reaction in the soft tissue around implants caused by the penetration of the implant abutment through the mucous membrane[17] and bacterial flora in the implant region which become similar to that around remaining teeth[15], is greatly related to peri-

implantitis and implant prognosis.

The detection rates of *P. gingivalis* and *P. intermedia* were about 50–60% at one month after setting abutment. *P. gingivalis* and *P. intermedia* have been reported to be risk factors for periodontitis[33]. Therefore, the high detection rates indicate that the risk of failure is higher in patients colonized by *P. gingivalis* and *P. intermedia*, and the importance of thorough maintenance such as professional dental plaque control has been suggested. The relationship between dental plaque control conditions in the implant region and marginal bone loss has been reported[20,30–32], suggesting the importance of plaque control in the implant region. In recent years, implant treatment has been performed in patients with periodontal disease. However, the survivability rate of implants in previous studies has been significantly lower than that in patients without periodontal disease[18,26]. Karoussis *et al.*[15] reported that, although a strict maintenance program such as cumulative interceptive supportive therapy was performed for patients with previous chronic periodontitis, the survivability rate of implants was low. A recent study showed that *P. gingivalis* and *P. intermedia* were promptly transmitted from teeth to the implant region[27]. It can be speculated that this transmission caused peri-implantitis and lowered the survivability rate of implants. Pontoriero *et al.*[25] reported that both the natural teeth and implants showed a higher gingival index and plaque index with increases in dental plaque and that the bacteria detected around the natural teeth were almost the same as those around the implants.

In this study, the analysis using PFGE showed that electrophoretic patterns of *P. gingivalis* and *P. intermedia* around the natural teeth were in accordance with those in the implant region in 7 of 8 cases (87.5%), indicating that bacterial colonization in the implant region originated from those around the natural teeth. Amano *et al.* reported that there were separate and multiple binding sites for proline-rich protein 1 (PRP1) statherin in the *P. gingivalis* fimbrillin and that the combination of all of binding sites were indispensable in establishing stable bacterial adherence to saliva-coated surfaces in oral cavity[1]. This report suggested that *P. gingivalis* is able to spread from colonized teeth to non-infected teeth or implants by its ability to adhere to saliva protein.

Periodontopathic bacteria in nature occur as biofilms, which are firm clusters of bacteria adhering in periodontal regions. The glycocalyx of many bacterial species, including those of periodontopathic bacteria, play significant roles, not only in forming biofilms, but also in escaping from host defense mechanisms such as phagocytosis and killing by leukocytes[6]. Kolenbrander demonstrated that dental plaque is a unique ecosystem and these multiple bacterial species form a community[14]. Recent studies have demonstrated that periodontopathic bacteria produced extracellular signals[5,8,9]. Moreover, although the immunological response related to phagocytosis is able to eliminate bacteria on the surface of biofilm, phagocytic cells cannot phagocytose or kill the bacterial cells within the biofilm[14]. Although disinfectants and antibiotics are effective against floating bacteria, these agents cannot penetrate as deeply as the central bacterial cells in biofilm[14]. In addition, because bacteria in the central region are in the stationary phase, bacteriostatic antibiotics inhibiting metabolism are not effective. In order to reduce the numbers of biofilm microorganisms, mechanical cleansing using anti-microbial agents is an essential method. Recent studies have clearly demonstrated that active periodontal therapy for implant patients consisting of motivation, instruction in oral hygiene practices, scaling and root planning, and periodontal surgery scheduled at three and six months, resulted in a reduction of the plaque index, gingival index and sulcus bleeding index, and probing pocket depths and recession in mm, optimal oral hygiene was reinstituted[7,11,22,26]. In addition, the effect of the rotating brush with anti-microbial agents on experimentally reduced peri-implantitis lesions was reported in dogs[24]. Recently, Asano *et al.* showed that the PFGE patterns of *P. gingivalis* strains from

matched husbands and wives were identical at a high rate[3]. They indicated the possible transmission of *P. gingivalis* in spouses. It is possible that planktonic cell periodontopathogens such as *P. gingivalis* are transmissible from biofilm to other sites of teeth and implants. These findings indicate that periodontal therapy for eliminating biofilm periodontal regions is an essential treatment before dental implant therapy.

The addition of our results to these findings suggests that treatment for elimination of periodontopathogens before implant installation is important and that sufficient subsequent continuous maintenance for reduction of periodontopathogens is necessary for dental implant patients.

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