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Comparison of Detection Rate of *Prevotella intermedia* and *Prevotella nigrescens* in Subgingival Plaque from Korean Periodontitis Patients

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색인 : Detection rate, periodontitis, *Prevotella intermedia*, *Prevotella nigrescens*

1. Introduction

Periodontitis refers to a group of diseases that cause inflammation and a loss of the supporting structures of the teeth[1]. The major cause of periodontitis is dental plaque, and the putative pathogens known to be involved in destructive periodontal diseases include *Actinobacillus actinomycetemcomitans*, *Tannerella forsythensis* (*Bacteroides forsythus*), *Campylobactor rectus*, *Eikellena corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and spirochetes[2].

The human oral cavity harbors a wide variety of moderately saccharolytic species from the bacterial genus *Prevotella*, some of which have clinical significance. The two most prevalent species of the group are *Prevotella intermedia* and *Prevotella nigrescens*. Formerly considered a single species, they were first separated 10 years ago[3,4] after multiple DNA homology studies[5,6], serological experiments[7,8], isoenzyme screenings[9], and whole-protein analyses[10] provided evidence for at least two distinct subgroups. Several studies have

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reported significant differences in the prevalence and/or abundance of the two species in samples collected from various oral sites. It is believed that *P. intermedia* is associated with the periodontal disease sites while *P. nigrescens* has been isolated from healthy sites within the mouth as well as endodontic infection sites[9] and oral abscesses[10]. However, some studies have reported contradictory results regarding the occurrence of *Prevotella* in the oral cavity[11,12]. There is little data regarding the prevalence of both *P. intermedia* and *P. nigrescens* in the subgingival plaque from healthy tissue, gingivitis, or periodontitis lesions in Korean patients. The aim of this study was to examine the detection rate of both *P. intermedia* and *P. nigrescens* in subgingival plaque samples from Korean periodontitis patients using a polymerase chain reaction with species-specific primers designed from the 16S-rDNA nucleotide sequence.

2. Materials and Methods

2.1. Subjects and Subgingival Plaque Sample

All patients who had undergone a previous periodontal treatment and who had not received antibiotics over the previous 6 months before treatment were selected. The following parameters were measured at 6 sites on each tooth: the plaque index (PI)[13], the gingival index (GI), bleeding on probing (BOP), the probing depth (PD), and the attachment level (AL). The sites were divided, into 3 groups according to the PD: 1) a shallow PD (sPD): $\leq$3 mm, 2) a moderate PD (mPD); 4 - 5 mm, 3) a deep PD (dPD): $\geq$6 mm.

After removing the supragingival plaque with a sterile curette, subgingival plaque samples were taken from 4 healthy sites, 103 gingivitis sites, and 26 periodontitis sites from 34 patients, who visited the Department of Preventive Dentistry, Dental Hospital, Chosun University, by inserting 3 sterile paper points in the periodontal pockets for approximately 30 seconds. The paper points were placed immediately in 0.5 ml of 1X PBS. All the samples were immediately frozen at -20℃.

2.2. DNA extraction from the plaque samples

The bacterial DNAs from the plaque samples were extracted using the direct DNA extraction method described by Lee *et al.*[14]. A 50 μl aliquot of a plaque sample was mixed with 50 μl of a 2X lysis buffer (2 mM EDTA-1% Triton X-100), and the mixture was then boiled for 10 min. After centrifugation to remove the cell debris for 20 s at 1,3000 $\times g$ at room temperature, the supernatant was collected and used as a template for amplification using a polymerase chain reaction (PCR).

The reference DNAs from the *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC were extracted using a modification of the method reported by Lippke *et al.*[15]. Briefly, the cell pellets were resuspended by vortexing in 5 volumes of a cell lysis buffer [5M guanidine isothiocyanate, 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 2% S-lauryl sarcosinate, 140 mM 2-mercaptoethanol]. The bacterial genomic DNAs were purified using the phenol/chloroform extraction method instead of the cesium chloride method[16]. The DNA concentrations were determined by UV-

spectrophotometry (Ultrospec 2000, Pharmacia Biotech., Cambridge, UK) at wavelengths of 260 and 280 nm. The results served as the positive control for the primers used herein.

2.3. PCR amplification

Either the *P. intermedia*- or *P. nigrescens*-specific PCR primers was selected from the publications previously described[17] and obtained from BIONEER Corp (Daejeon, Korea). The PCR was performed using an *AccuPower*™ PCR PreMix (BIONEER Corp.), which contained 5 nM of each deoxynucleoside triphosphate, 0.8 μM KCl, 0.2 μM Tris-HCl (pH 9.0), 0.03 μM $MgCl_2$, and 1 unit of *Taq*DNA polymerase. The bacterial genomic DNA and 20 pM of each primer were added to a PCR PreMix tube. The PCR was carried out in a final volume of 20 μl. The PCR was run for 32 cycles on a Peltier thermal cycler (Model PTC-200 DNA engineTM, MJ Research Inc., Watertown, MA, U.S.A) under the following conditions: denaturation at 94℃ for 1 min, primer annealing at 60℃ for 30 sec, and extension at 72℃ for 1 min. The final cycle included an additional extension time of 10 min at 72℃. A 2 μl aliquot of the reaction mixture was analyzed by 1.5% agarose gel electrophoresis in a Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, [pH8.0]) at 100V for 30 min. The amplification products were stained with ethidium bromide and visualized by UV transillumination.

2.4. Data analysis

The detection frequencies are presented as the mean percentage of the sites testing positive for *P. intermedia* and *P. nigrescens*. The percentages of the shallow, moderate, and deep sites testing positive were compared for the two bacteria using a chi-square test ($P < 0.05$).

3. Results

The detection frequency of *P. intermedia* was higher than that of *P. nigrescens* in all groups (Table 1). The detection rate of *P. intermedia* was higher in the mPD sites than in the dPD sites. However, the level of *P. nigrescens* in the mPD sites was similar to that of *P. nigrescens* in the dPD sites. This study could not compare the detection frequencies of the two bacteria in the sPD and mPD or dPD sites because the sample size of the sPD sites was too small.

There was a higher level of *P. intermedia* in the BOP sites in all groups than *P. nigrescens* (Table 2).

4. Discussion

The results obtained in this study showed that *P. intermedia* was more associated with BOP than *P. nigrescens*. It was reported that the hemolytic activity of the *P. intermedia* isolates was significantly higher than those of the *P. nigrescens* isolates[18]. The high hemolytic activity of *P. intermedia* may be involved in the pathogenicity of *P. intermedia* in the progression of periodontal disease. *P. intermedia* and *P. nigrescens* require iron for growth. Hemoglobin or hemin resulting from the hemolytic degradation of red blood cells might be a major source of exogeneous iron for

Table 1. Number of sites positive for *P. intermedia* and *P. nigresence* of subgingival plques according to pocket depth(%)

PD[1]	Number of Positive Sites (%)								
	≤3			4-5			≥6		
BOP[2]	(-)	(+)	subtotal	(-)	(+)	subtotal	(-)	(+)	subtotal
	n = 4	n = 15	n = 19	n = 15	n = 103	n = 118	n = 4	n = 26	n = 30
Pi[3]	0 (0.0)	5 (33.3)	5 (26.3)*	7 (46.7)	66 (64.1)	73 (61.9)*	3 (75.0)	10 (38.5)	13 (43.3)*
Pn[4]	1 (25.0)	0 (0.0)	1 (5.3)	3 (20.0)	22 (21.4)	25 (21.2)	1 (25.0)	6 (23.1)	7 (23.3)

[1]Pocket depth (mm); [2]Bleeding on probing; [3]*Prevotella intermedia*; [4]*Prevotella* nigrescence
*Significantly different ($P < 0.05$)

Table 2. Number of sites positive for *P. intermedia* and *P. nigresence* of subgingival plques according to bleeding on probing(%)

BOP[1]	Number of Positive Sites (%)		
	(-)	(+)	Total
	n = 23	n = 144	n = 167
Pi[2]	10 (43.5)	81 (56.3)*	91 (54.5)
Pn[3]	5 (21.7)	28 (19.4)*	33 (19.8)

[1]Bleeding on probing; [2]*Prevotella intermedia*; [3]*Prevotella nigrescence*
*Significantly different ($P < 0.05$)

the growth of *P. intermedia* [19]. These results suggest that *P. intermedia* may play a significant role in the pathogenesis of periodontal disease via its hemolytic activity.

The data showed that there was a higher level of *P. intermedia* in the mPD and dPD sites than *P. nigrescens*. This indicates that *P. intermedia* is more associated with the progression of periodontal disease than *P. nigrescnes*. This study is a preliminary investigation into the difference in the detection rate of *P. intermedia* and *P. nigrescens* in periodontitis lesions in Korean patients. Several investigations have reported significant differences in the prevalence and/or abundance of the two species in samples collected from healthy and diseased sites. Therefore, more studies will be needed to confirm these results.

Many bacterial identification methods have been developed to explore the relationship between infectious diseases and their associated causative agents. Among them, some of nucleic acid-based methods, for example, DNA-DNA hybridization, DNA fingerprinting, DNA probes, 16S or 23S rRNA gene sequencing, and PCR[20]. PCR methods utilizing the 16S rRNA as a target have many advantages[1,21]. Recently, a real-time PCR method was introduced and has been used in epidemiological studies on periodontitis[22,23]. This method has sufficient sensitivity, and specificity and is a powerful tool for microbiological examinations of periodontal disease. However, it is expensive. Therefore, this study used a conventional PCR method.

5. Conclusion

This study examined the detection rate of both *P. intermedia* and *P. nigrescens* in subgingival plaque samples from Korean periodontitis patients using a conventional PCR method. *P. intermedia* is more associated with the BOP sites than *P. nigrescens*. In addition, there was a higher level of *P. intermedia* in the moderate (4-5 mm) pocket depth and the deep (≥6 mm) pocket depth sites than *P. nigrescens*. Overall, there was a higher level of *P. intermedia* in the BOP positive sites in Korean periodontal disease patients than *P. nigrescens*. *P. intermedia* may play a more important role in the pathogenesis of periodontal disease than *P. nigrescens*.

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국문초록

한국인의 치주질환 환자의 치은연하치면세균막에서 *Prevotella intermedia*와 *Prevotella nigrescens*의 검출율 비교

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본 연구의 목적은 한국인의 치주질환 환자의 치은연하치면세균막에서 *Prevotella intermedia*와 *Prevotella nigrescens*의 검출율을 비교하기 위하여 시행하였다. 34명의 환자로부터 총 133개의 치은연하치면세균막을 멸균된 paper point를 이용하여 채취하였으며, 이때 건강한 치주조직이 4개, 치은염을 보이는 치주부위가 103개, 치주염을 보이는 부위가 26개였다. 16S 라이보솜 RNA의 염기서열을 바탕으로 제작된 종-특이 프라이머을 이용한 중합효소연쇄반응법으로 *P. intermedia*와 *P. nigrescens*를 치면세균막으로부터 존재유무를 확인하였다. 실험 결과 *P. intermedia*는 *P. nigrescens*보다 탐침시 출혈을 보이는 환자에서 검출율이 더 큰 것으로 조사되었다. 또한 *P. intermedia*는 치주낭 깊이가 4 mm이상의 부위에서 *P. nigrescens*보다 상대적으로 높게 검출되었다. 이러한 결과는 *P. intermedia*가 *P. nigrescens*보다 치주질환의 진행에 더 연관성이 높다는 것을 간접적으로 시사한다.