Study of Restriction Site Heterogeneity of *P. intermedia serotype c* and Its Relationship to Clinical Disease Entity

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I. Introduction

Enumeration of specific bacterial species has been considered as a potential tool for differential diagnosis among periodontal diseases. Cultural methods are probably not worth considering for this type of diagnosis for a variety of reasons but reliable, rapid means of identifying some species are becoming available. These include indirect immunofluorescence microscopy and nucleotide probes.

A DNA probe is a piece of DNA, with a detectable label, that binds to a target nucleic acid molecule under appropriate condition. Whole genomic probe analysis demonstrated 100% effectiveness in detecting *Aa* and *B. intermedius,* and 91% effectiveness in detecting *B. gingivalis* at culture positive levels[1]. In addition, probe assay frequently identified these pathogens in samples that were culture negative and are useful for identifying different species that exhibit similar or identical phenotypic characteristics since the idenitification procedure is not based on the expression of a measurable phenotype, but directly based on DNA-DNA hybridization[2]. Although whole genomic probe have been used for detection of various subgingival species by many investigators[3,4,5,6], and could be adapted for the direct identification of microorganisms in subgingival plaque sample[7,8], its cross-reactivity makes it far from a complete diagnostic tool[9]. This could be improved by cutting into the DNA of the target organism with a restriction endonuclease and cloning the random fragments. Randomly cloned DNA probes have been used in the identification of *Bacteroides thetaiotamicron*[10], to demonstrate restriction site heterogeneity among clinical isolates of *Salmonella* Species[11] and to support evidence that the structure of natural populations of encapsulated strains of *Haemophilus influenzae* is clonal[12]. In relation to oral pathogenic bacteria, it was shown that cloned chromosomal DNA fragments from *A actinomycetemcomitans* may be useful for studing the epidemiology of localized juvenile periodontitis[13].

Prevotella intermedia is Gram negative, strictly anaerobic, rod shaped bacteria which produce black or brown pigmented colonies on blood agar plates. It has been implicated in many forms of periodontal diseases including adult periodontitis, rapidly progressing periodontitis, refractory periodontitis, pubertal gingivitis, pregnancy gingivitis and acute necrotizing ulcerative gingivitis[14,15,16,17,18], and is frequently detected in subgingival plaque samples of periodontal patients. Among the three serogroups[19], serotype c is prevalent in severe forms of periodontitis and demonstrates highest in vitro fibroblast cytotoxicity[20]. The purpose of this study is to prepare randomly cloned DNA probe from

P. intermedia G8-9K-3(serotype c) strain to examine restriction endonuclease patterns and restriction fragment length polymorphism(RFLP) of various strains of *P. intermedia* serotype c from rapidly progresssing periodontitis(RPP) and refractory periodontitis(RP) patients and to determine if particular genetic heterogeneity is associated with clinical disease entity.

II. Materials and Methods

1. Bacterial strains and culture conditions

The bacterial strains used in this study and their sources are listed in Table 1. The *Prevotella* species were anaerobically grown in 10 ml of prereduced. BHI(Brain Heart Infusion) broth(DIFCO Laboratories, Detroit, Michigan, U. S. A.) supplimented with 5μℓ/ml of hemin and 0.5μℓ/ml of Vit. K for 2−3 days at 37℃. Subsequently these 10−ml cultures were inoculated into flasks containing 100 ml of broth and incubated for an additional 2−3 days. Clinical isolates of *Prevotella* species were obtained through Seoul National University Hospital from 5 patients with rapidly progressing periodontitis and another 5 patients with refractory periodontitis patients.

Subgingival plaques were removed with 3 sterile endodontic paper point #40(Johnson Fine Absorbent Points, Johnson & Johnson, East Windsor, N. J., U. S. A.) and immediately placed into test tube containing 2 ml of PRAS Ringer solution. Following voltex mixing for 2 minutes, sample were serially diluted with 10 ml of Ringer solution. A 50μℓ aliquot was removed from each sample and inoculated onto Blood Agar Plate containing 5% rabbit blood,

Table 1. Bacterial strains used in this study.

Strain	Source/Reference	Serotype
Prevotella intermedia		
25611	ATCC	a
9336	NCTC	b
G8-9K-3	SUNYab	c
R1−R5	RP* patients isolates	c
P1−P5	RPP** patients isolates	c
Porphyromonas gingivalis		
2561	ATCC	a
A7A1−28	SUNYab	c
HG 564	Netherlands	ND***
W 50	ND	b
381	Forsytu	a
9−14K−1	SUNYab	ND
Aa**** 75	SUNYab	a
Aa Y4	Forsytu	b
Aa 67	SUNYab	c

*RP : refractory periodontitis

**RPP : rapidly progressing periodontitis

***ND : not determined

****Aa : *Actinobacillus actinomycetemcomitance*

5μℓ/ml of hemin and 0.5μℓ/ml of Vit. K and incubated in anaerobic chamber(Coy anaerobic chamber, MI, USA) for 5−7 days. Clinical isolates identified as strains of *P. intermedia* serotype c based on the results of biochemical analyses and serotyping were used in this study, designated R1−R5 and P1−P5.

2. Preparation of bacterial genomic DNA[21]

Cell pellets obtained by centrifugal seperation for 10 minutes were resuspended gently in 9.5 ml TE buffer(Tris-EDTA buffer : 10 mM Tris-Hcl, 1 mM EDTA, pH 8.0), 0.5 ml of 10% SDS and 50μℓ of 20 mg/ml proteinase K, mixed thoroughly and incubated 1 hr at 37℃. Following incubation, 1.8 ml of 5 M NaCl and 1.5 ml of hexadecyltrimethyl ammonium bromide(CTAB)/NaCl solution were added and incubated 20 min at 65℃. Then, sequential extraction was performed with equal volume of phenol / chloroform / isoamylalcohol (25 : 24 : 1). Extracted DNA sol. was precipitated with 0.6 volume of isopropanol, cleaned with 70% ethanol and dried. The DNA preparations were stored in 200μℓ of TE buffer at 4℃. DNA concentrations were determined spectro- photometrically at A_{260}.

3. Purification and labeling of DNA probe

Insert DNA fragments were recovered from hybrid plasmid containing *P.intermedia* G8-9K-3 genome DNA EcoRI restriction fragment. Recombination plasmid DNA was restricted with EcoRI and examined on 1.4% agarose(1X TAE) slab gels. A portion of gels containing restriction fragment of genomic DNA was cut and purified by QIAEX agarose gel extraction technique(QIAGEN). Then purified DNA fragment was labeled with digoxigenin-dUTP by random primed DNA labeling technique.

The DNA was denaturated by heating in a boiling water bath(100℃) for 10 minutes and chilling quickly on ice/NaCl. In a microcentrifuge tube, 1ug of freshly denatured DNA, 2μℓ of hexanucleotide mixture, 2μℓ of dNTP labeling mixture, and 1μℓ of Klenow enzyme were mixed and total volume was adjusted to 19μℓ with sterile redistilled H_2O. Following 20 hours' incubation at 37℃, the reaction was stopped by adding 2μℓ of EDTA solution (0.2M, pH 8.0). The labeled DNA was precipitated with 2.5μℓ of LiCl and 75μℓ of prechilled (-20℃) ethanol and stored in 50μℓ of TE buffer at -20℃. The labeling efficiency was tested by dot blot assay.

4. Southern blotting and DNA/DNA hybridization[22]

For Southern blot anaylsis, 10μg of each DNA sample were restriction digested with 100 units of EcoRI and PstI for 2 hours at 37℃. The DNA fragments were seperated on 0.7% agarose gels in Tris-Acetate-EDTA (TAE) buffer. The gels were stained with ethidium bromide, photographed and the DNA fragments were transferred to nylon membranes(Boeringer Mannheim Biochemicals, Indianapolis, IN, USA) using 20X SSC(3M NaCl, 300 mM sodium citrate, pH 7.0) buffer. The transferred DNA was fixed to the membrane by baking in an oven at 80℃ for 2 hours and stored in plastic bag at -20℃. Prehybridization was undertaken by placing the membrane in a hybrdization bag containing 20 ml standard prehybridization solution(50% formamide, 5X SSC, 2% blocking agent, 0.1% N-lauronylsarcosine, 0.02% SDS) for 1 hour at 42℃. DIG-labeled probe was denatured by heating in a boiling water bath for 10 minutes and chilling immediately on ice. Following disposal of prehybridization solution , the hybridization solution containing the DIG-labeld probe was added and allowed to hybridize overnight at 42℃. Following hybridization, the blots were washed twice, 5 minutes per wash, in 2X wash solution (2XSSC containing 0.1% SDS) at room temperature for removing unbound probe. Then high stringency washing was undertaken in 0.1X wash solution twice, 15 minutes per wash, at 68℃.

5. Chemiluminescent Detection with Lumigen PPD

After hybridization and post-hybridization washes, the membrane was equilibrated in buffer 1 (100mM maleic acid, 150mM NaCl, pH 7.5) for 1 minute and blocked by gentle agitating it in 100ml of buffer 2 (1% blocking reagent in buffer 1) for 30−60 min. After removing buffer 2, the membrane was incubated for 30 minutes in the antibody soluton (anti-DIG-alkaline phosphatase 1 : 10,000 in buffer 2) and washed twice with buffer 1 (buffer 1 with 0.3% Tween 20). Then the membrane was placed between two sheets of acetate (plastic page protectors), added approximately 1.5 ml of diluted Lumigen PPD (1 : 100 in buffer 3), and incubated for 15 minutes. The detection of the chemiluminescent signal was done by exposing the membrane to standard X-ray film for 1 hour.

III. Result

1. Determination of the hybridization specificity of the cloned DNA fragments.

The size of each insert fragment, determined from EcoRI digests of *Prevotella intermedia* G8-9K-3 genomic DNA, was 2.4 Kb, 5.3 Kb, 2.1 Kb, 17.5 Kb, 1.4 Kb, 1.7 Kb, 1.3 Kb, 1.1 Kb and 1.2 Kb. One clone containing a 1.4 Kb insert fragment was selected, designated probe 8-8, and used as a randomly cloned DNA probe. This probe could be proved to be a piece of genomic DNA of *P. intermeia* G8-9K-3 by detecting formation of hybridization signal with 1.4 Kb fragment of homologous species (Fig. 2, Table 2). No hybridization was observed to DNA from heterologous species-varoius strains of *P. gingivalis and Aa.* Though the probe was species-specific (Fig. 3, Table 3), it was not serospecific since it hybridized with DNA of *P. intermedia* 25611(serotype a) and 9336(serotype b).

Table 2. Hybridization signal on Southern blot of EcoRI-digested genomic DNA of bacterial strains using cloned EcoRI fragments of *Prevotella intermedia* G8-9K-3 genomic DNA (8−8 probe).

Bacterial strain		Hybridization band (estimated m. w.)
P. intermedia	25611	1.4 Kb
	9336	1.4 Kb
	G8−9K−3	1.4 Kb
	R1−R5	1.4 Kb
	P1−P5	1.4 Kb

2. The restriction endonuclease pattern

The restriction pattern for laboratory strains and clinical isolates of *P. intermedia* are shown in Fig. 1. Each restriction pattern contained about 50 bands representing fragments between 1 and 25 Kb in size.

While there showed some difference of restric-

Table 3. Hybridization signal on Southern blot PstI-dgested genomic DNA of bacterial strains using cloned EcoRI fragments of *Prevotella intermedia* G8-9K-3 genomic DNA (8−8 probe).

Bacterial strains		Hybridization band (estimated m. w.)
P. intermedia	25611	7.8 Kb
	9336	16.0 Kb
	G8−9K−3	16.0 Kb
P. gingivalis	9−14K−1	−
	381	−
	W 50	−
	HG 564	−
	A7A1−28	−
	2561	−
Aa	Y4	−
	67	−
	75	−

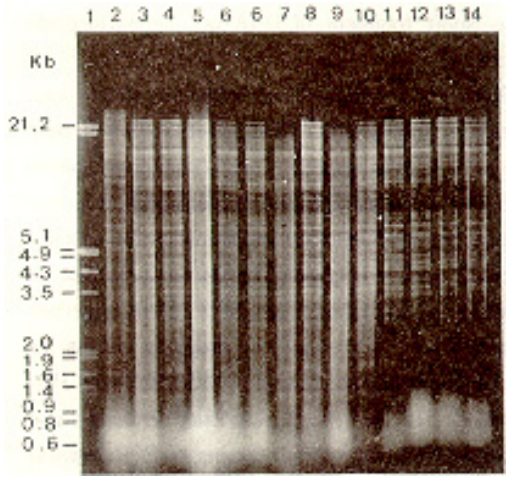

Fig.1 The restriction endonucelase pattern of *P. intermedia.* All clinical isolates are serotype c.

Lane 1, λ DNA m. w. marker : lane 2−4, *P. intermedia* 25611, 9336, G8-9K-3 : lane 5−9, *P. intermedia* R1−R5 : lane 10−14, *P. intermedia* P1−P5.

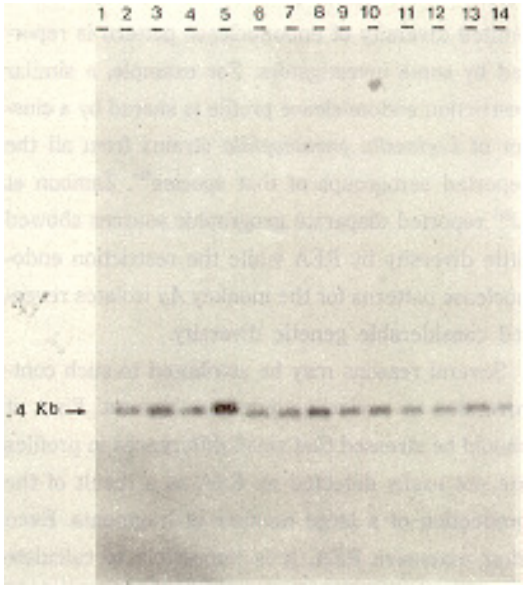

Fig. 2 Southern hybridization of the probe 8-8 to EcoRI digests of DNAs from strains of *P. intermedia.*

Lane 1, λ DNA m. w. marker : lane 2−4, *P. intermedia* 25611, 9336, G8-9K-3 : lane 5−9, *P. intermedia* R1−R5 : lane 10−14, *P. intermedia* P1−P5.

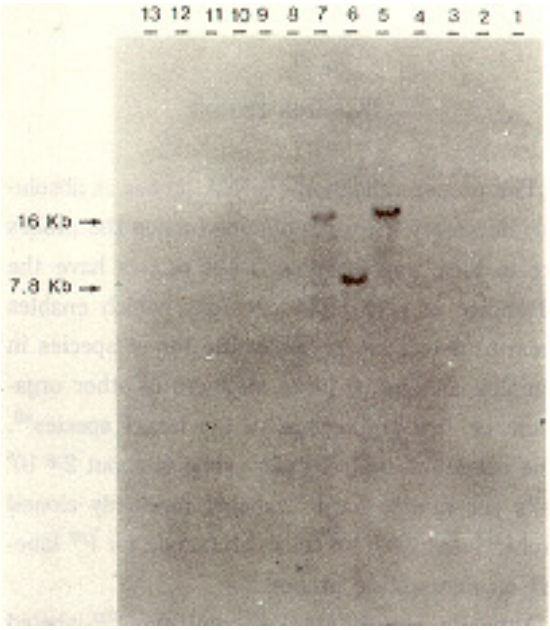

Fig. 3 Southern hybridization of the probe 8-8 to PstI digests of DNAs from various strains of *P. intermedia, P. gingivalis* and Aa.

Lane 1, λ DNA m. w. marker : lane 2−4, Aa 67, Y4, 75 : lane 5−7, *P. intermedia* 9336, 25611, G8−9 K−3 : lane 8−13, *P. gingivalis* 9−14K−1, 381, W50, HG564, A7A1-28, 2561.

tion pattern among laboratory strains, all clinical isolates from RP or RPP patients exhibited identical patterns. No difference was found between restriction patterns of RP and RPP patients by visual assessment.

3. Restriction site heterogeneity of patient isolates.

The probe 8-8 hybridized to the same size restriction fragment in 13 different strains of *P. intermedia* when the genomic DNA of each strain was digested with EcoRI (Fig. 2). Therefore, restriction site heterogeneity did not appear neither among laboratory strains nor among patient isolates in Fig. 2. However when genomic DNAs were digested with PstI, restriction site heterogeity could be found

among laboratory strains of *P. intermedia* (Fig. 3)

Ⅳ. Discussion

The proper validation of DNA probes is absolutely necessary if results obtained using the probes are to have any meaning. DNA probes have the advantage of very high specificity, which enables them to detect low levels of the target species in samples containting large numbers of other organism, i.e. low proportions of the target species[23]. The detection limit of DNA probe is about $2*10^5$ cells per sample for P^{32} labeled randomly cloned probe[24], and $2.9*10^4$ cells per sample for P^{32} labeled oligonucleotide probe[25,26].

Although earlier studies employed ^{32}P-labeled DNA for hybridization, this labeling method has some disadvantages. The radioactive materials are dangerous to manipulate, require long exposure time (7 days) and cannot be recorded permanantly. In this study we used nonradioactive labeling and chemiluminescence detection method which are rapid (15 minute's exposure) and have sensitivity equal to that of radioactive labels.

In the presence of low numbers of organisms, the cultural method is more sensitive than the DNA probe method. As a result, in some samples species may be detected by culture which cannot be detected by the DNA probes. One approach facilitating the application of DNA probe identification to small samples, in which probe sensitivity may be a problem, is the colony lift method[27]. This method makes use of the self amplification of species by their growth in culture, combined with the specificity of DNA probe method.

We used the probe 8-8 that was not specific for serotype c strains of *P. intermedia* since serospecificity of the probe was not essential requirement for the purpose of this study. In fact it is thought that to examine RFLP pattern, whole genomic probe or cloned probe from repetitive DNA are more valuable than cloned probe from non-repetitive, short fragment of DNA or oligonucleodtide probe.

Restriction endonuclease fragments of chromosomal DNA produce distinct patterns upon electrophoresis in agarose gels. Such "fingerprint" patterns have been used for strain identification in studies of the epidemeology and transmission of a number of species, including *Streptococcus mutans*, an etiologic agent of dental caries[28,29]. DNA fingerprints also have been used for grouping strains for taxonomic studies of strains of *Neisseria*[30] and *Pasteurella*[31]. In this study restriction endonuclease analysis(REA) of various strains of *P. intermedia* serotype c was observed. It is curious that DNA fragments from 10 patient isolates exhibited identical restriction patterns. Rudney et al[32] studied REA of oral viridans *Streptococci* and reported that information from REA was too strain-specific for species identification suggesting considerable genotypic diversity within panel species. On the other hand, limited diversity of endonuclease pattern is reported by some investigators. For example, a similar restriction endonuclease profile is shared by a cluster of *Legionella pneumophila* strains from all the reported serogroups of that species[33]. Zambon et al[34] reported disparate geographic sources showed little diversity by REA while the restriction endonuclease patterns for the monkey *Aa* isolates revealed considerable genetic diversity.

Several reasons may be attributed to such controversies in reviewing previous reports. First, it should be stressed that small differences in profiles are not easily detected by REA as a result of the production of a large number of fragments. Even after extensive REA, it is impossible to calculate how many restriction site changes have occured in strains that seem to have only minor differences in REA. It is known that by using conventional REA, toxigenic strains of *Corynebacterium diphtheriae* are indistinguishable from nontoxigenic strains[35]. As a consequence, small differences in REA are not

readily detected. Other techniques are needed to explore how "small" small differences in REA really are.

Second, *Streptococci* studied in Rudney's report are non-pathogenic in healthy hosts and studies of other bacteria support the notion that commensal strains are genotypically diverse[36]. However, pathogenic strains recovered during outbreaks of disease showed a much more limited range of electrophoretic types. The limited diversity of the human pathogenic isolates may be the result of selective pressures found in the human oral cavity or it may be the result of a biased sample. The pathogenic strains recovered from people with an infectious disease may provide a biased sample of the universe of bacteria. They can possess virulence factors which facilitate transmission in host populations[37,38]. Rapid transmission decreases time available for genetic divergence in different hosts, while pathogenicity increases the likelihood that isolates will be obtained for diagnosis and eidemiology. In those circumstances, culture collections may be likely to contain related strains with similar fingerprint patterns.

Similar results are reported in RFLP studies using labeled DNA probes. Mazurek et al[39] showed DNA figerprint patterns of clonal *M.tuberculosis* isolates are identical or, at most, minimally different and Moshitch et al[40] suggested that *S. typhi* comprise one clone around the world after testing genetic heterogeneity among *Salmonella typhi* isolates using rDNA and fliC DNA probes. In our study DNA hybridization patterns of 10 clinical isolates from RPP and RP patients did not show RFLP. RPP is associate with scantier amount of plaque and calculus. Page and colleagues[41] have described this condition as a distinct clinical entity with the following characteristics : seen most commonly in young adults in their twenties, but can occur up to the age of 35 : extreme inflammation : hemorrhage : proliferation of the marginal gingiva : exudation : and rapid bone loss. It may subside and become quiscent or progress to tooth loss. Most patients have serum antibodies for various species of *Bacteroies, Actinobacillus,* or both and show defects in either neutrophil or monocyte chemotaxis. Cases that do not response to therapy and/or recur soon after adequate treatment for unknown reasons have been refered to as refractory periodontitis. Deterioration in these cases occurs either by new involvement of additional teeth or increasing bone and attachment loss in previously treated areas. Pretreatment clinical findings and severity are not diagnosetic of RP. According to Page, RP is due to one or more of the following mechanisms : abnormal host response, resistant organisms or untreatable morphologic problem. Cases of RP may be similar to those identified by Hirschfeld and Wasserman[42] as "extreme down-hill cases", which composed 4.2% of the 600 patients they studied. Anyway, RPP and RP are classfied not on the basis of pathogenic bacteria, but on the rate of tissue destruction and some clinical features. In this respect, it is not suprising that RFLP was not found between DNA hybridization patterns of RPP and RP patients isolates.

Further studies are needed to determine which factors are involved in the selected colonization of periodontally diseased human oral cavity by this subset of *P. intermedia.*

V. Conclusions

This study was performed to examine restriction endonuclease pattern and RFLP of various strains of *P. intermedia* serotype c from rapidly progressing periodontitis and refractory periodontitis patients and to determine if particulaar genetic heterogeneity is associated with clinical disease entity using a randomly cloned DNA probe.

The conclusions are as follows :

1. 1.4 Kb fragment of genomic DNA from P. intermedia G8-9K-3 was prepared to randomly-cloned DNA probe.

2. The probe was species-specific but not strain-specific.
3. The REA patterns from 10 clinical isolates were identical.
4. Restriction site heterogeneity did not appear among patient isolates from RP and RPP.

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