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Original Article

Identification of Multiple Strains of *Porphyromonas gingivalis* using Heteroduplex Polymerase Chain Reaction in Varying Severity of Chronic Periodontitis

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Abstract

Aim: Research has demonstrated that there are multiple strains of *Porphyromonas gingivalis* with varying potency to cause periodontal disease. The current study aims at using heteroduplex polymerase chain reaction (PCR) to detect the strain diversity of *P. gingivalis* in periodontitis lesions of varying severity in a sample of the Indian population. **Materials and Methods:** Subgingival plaque samples were collected from 60 individuals with varying severity of chronic periodontitis and 30 individuals with a clinically healthy periodontium. The samples were subjected to PCR analysis to identify *P. gingivalis,* followed by heteroduplex analysis to identify the strain diversity in a given sample. Bacterial culture was carried out as a comparative standard. **Results:** Of the 56 samples that were positive for *P. gingivalis* by PCR, 54 samples yielded eight different heteroduplex patterns. Analysis of these patterns indicated that two strains of *P. gingivalis* were present in 41 individuals (45.6%) and three strains were present in 13 individuals (14.4%). Detection of *P. gingivalis* by PCR was significantly more in the periodontitis group as compared to the healthy group. **Conclusions:** Species-specific PCR and heteroduplex analysis provide a simple and accurate method to analyse the strain diversity of *P. gingivalis. P. gingivalis* was detected in both healthy periodontal sites as well as sites with periodontitis. The presence of two or three *P. gingivalis* strains was seen in 60% of the samples.

Keywords: Chronic periodontitis, heteroduplex analysis, molecular biology, polymerase chain reaction, *Porphyromonas gingivalis*

Introduction

The true nature of the process of periodontal disease causation remains largely speculative, but there are a few aspects of the disease process for which strong evidence is available. A major milestone in the research of periodontal microbiology was the identification of the 'Red complex' that comprises of *Tanerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola*. All these bacterial species have shown a strong association with periodontitis with *P. gingivalis* showing the strongest association.[1]

The association of *P. gingivalis* to periodontal disease is substantiated by numerous studies,[2,3] but at the same time, a few studies have reported the presence of *P. gingivalis* at periodontally healthy sites.[4] The isolation of *P. gingivalis* from healthy periodontal sites led researchers to suspect that there may be differences in the virulence of various strains of this organism.[5] Various techniques have been used for strain typing of *P. gingivalis*. These include serotyping,[6] multilocus enzyme electrophoresis,[7] DNA fingerprinting,[8] ribotyping,[9] whole genomic restriction fragment length polymorphism,[10] arbitrarily primed polymerase chain reaction (PCR)[11,12] and heteroduplex PCR,[13,14] among others.

Heteroduplex analysis has been used extensively to identify allelic variation among mammalian genes. It provides a rapid and reliable method for determining and cataloguing minor differences between two closely related DNA sequences. The

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geographical distribution of various strains and heteroduplex types of *P. gingivalis* needs to be investigated and catalogued to create an epidemiological database for *P. gingivalis*. In the present study, an attempt has been made to study the number of heteroduplex types of *P. gingivalis* prevalent in a sample of the Indian population.

Materials and Methods

This study was approved by the Institutional Ethics Committee of the Manipal University, Karnataka, India. All the individuals were recruited from among the patients attending the Manipal College of Dental Sciences and Hospital, Manipal University.

The study population consisted of 90 individuals recruited based on certain predetermined inclusion and exclusion criteria and categorised into three groups.

Group 1 or healthy group included 30 subjects with a clinically healthy periodontium. Group 2 included 30 subjects with mild or moderate periodontitis and Group 3 included 30 subjects with severe periodontitis. Group 1 was the control group whereas Groups 2 and 3 formed the 'Periodontitis' group.

Inclusion criteria for the periodontitis group were a diagnosis of mild, moderate or severe periodontitis,[15] presence of bleeding on gentle probing and the presence of at least one periodontal pocket in every quadrant. Patients were included in the periodontitis group only if they fulfilled the 1999 criteria suggested by AAP.[15,16] Individuals with a clinical attachment loss of 1–4 mm were classified as having mild-to-moderate periodontitis (Group 2) and those with clinical attachment loss of ≥5 mm were classified as having severe periodontitis (Group 3)

The following groups were excluded from the study

1. Patients who had received any antimicrobial therapy within the previous 3 months
2. Patients who had received oral prophylaxis or periodontal surgery at the site to be sampled within the last 6 months
3. Current or past smokers
4. Pregnant or lactating females
5. History of diabetes mellitus or any systemic disease affecting periodontal disease severity.

Criteria for periodontally healthy sites (control group) were as follows

1. Probing depth <3 mm
2. No clinical attachment loss
3. Absence of bleeding on probing.

The microbiology and molecular biology data included:

1. Culture results for *P. gingivalis*
2. Colony forming units (CFUs) of *P. gingivalis*
3. Detection of *P. gingivalis* using a species-specific PCR
4. Detection of *P. gingivalis* strain variability by heteroduplex analysis.

Plaque sampling

Supragingival plaque was removed using hand scalers and curettes. The deepest pocket in every quadrant was chosen for plaque sampling (a total of 4 sites per subject were chosen). Sites to be used for subgingival plaque sampling were isolated using sterile cotton rolls. A sterile Gracey curette was then inserted in the pocket until resistance was felt and a plaque sample was taken. This sample was transferred into a sterile vial containing reduced transport fluid. Samples from all the 4 sites were pooled together into one tube. Plaque samples that were contaminated with blood or saliva were discarded. The samples were transported to the laboratory within 24 h of collection.

Laboratory procedures

Culture

Well-mixed plaque samples were plated on blood agar and Kanamycin blood agar (both supplemented with haemin and menadione) and incubated anaerobically in an anaerobic jar with gas pack for 3 days. At the end of incubation, the colonies were inspected, and the identity of *P. gingivalis* was confirmed by black pigmentation, gram staining, inability to ferment glucose, production of indole and absence of fluorescence under ultraviolet light, and the total number of CFUs was determined.

A second aliquot of the plaque sample was subjected to DNA extraction using a column based DNA isolation kit (Chromous Biotech). Manufacturer's instructions were strictly adhered to during the entire procedure. The concentration of isolated DNA was measured using Bio photometer (Eppendorf) and stored at −20°C till use.

A two-step, nested PCR was performed for the amplification of *P. gingivalis* specific gene.[13] In the first step, universal prokaryotic primers (785, 422) were used to amplify the spacer region between 16s and 23s rRNA. Then, the products of the first PCR were used to amplify *P. gingivalis* specific gene by employing universal primers (241) and *P. gingivalis*-specific primers (pg8). Details of primer sequences are shown in Table 1.

All PCRs were performed in a total volume of 100 µl containing 50 mM KCl, 10 mM TrisHCl (pH 8.8), 3 mM $MgCl_2$, 0.1% Triton X-100 and 200 µM of each dNTP. For the first PCR step, 0.036 µg of primers 785 and 0.36 µg of primers 422 were used. For the second amplification step 0.03 µg of each primer 241 and pg8 were used.

The cycling conditions for the 1st PCR were initial denaturation of 94°C for 5 min followed by 25 cycles of 94°C for 1 min,

Table 1: Sequences of oligonucleotide primers

Primer	Sequence
PG13	CATCGGTAGTTGCTAACAGTTTTC
785	GGATTAGATACCCTGGTAGTC
422	GGAGTATTTAGCCTT
Pg8R	TGTATATGACTGATGGTGAAAACC
241	TTCGCTCGCCGCTACT

42°C for 3 min and 72°C for 1 min with a final extension of 72°C for 2 min. For the 2nd PCR, cycling conditions included initial denaturation of 94°C for 3 min followed by 25 cycles of 94°C for 1 min, 52°C for 2 min and 72°C for 3 min with a final extension of 72°C for 2 min.

DNA isolated from a reference strain of *P. gingivalis* (ATCC 33277) was used as positive control throughout the study.

The amplified PCR products by the above-mentioned method were used for heteroduplex analysis. Equal quantities of amplified product from each clinical sample and control strain were mixed in a 0.2 ml PCR tube to make a final quantity of 12 µl. For formation of heteroduplexes, the mixture was incubated at 95°C for 5 min to melt the double-stranded DNA followed by cooling to 25°C at the rate of 1°C per minute for reannealing. The tubes were then immediately placed on ice and subjected to polyacrylamide gel electrophoresis for visualisation of heteroduplexes.

Samples were mixed with gel loading buffer and loaded on to 10% polyacrylamide gel and electrophoresed at 120V for 3.5 h. With each run, a molecular weight marker of 100–1000 bp was included. After the run was complete, the gel was stained with ethidium bromide (1 µg/ml) for 15 min and visualised under UV gel transilluminator (Major Science) for inspection and presence/absence of duplexes at various locations were noted.

The recorded data were subjected to statistical analysis. The analysis was performed using the software SPSS 16.0 for Windows®. The mean and standard deviation values for the recorded clinical parameters were calculated for inter-group comparison. Independent sample *t*-test and 'ANOVA' with '*post-hoc*' Tukey's test was used for comparison of clinical parameters and colony counts. 'Chi-square' rest was used for comparing the distribution of *P. gingivalis* among the study groups. $P < 0.001$ was considered to be statistically significant. All the statistical analyses were done by an experienced statistician.

Results

Samples from 90 subjects were included in this study. Of the 90 samples, 30 were collected from periodontally healthy controls (Group 1), 30 from subjects with mild-to-moderate periodontitis (Group 2) and 30 from subjects with severe periodontitis (Group 3). The mean difference in clinical attachment level between Group 1 and Group 2 was 2.80 mm ($P < 0.001$), between Group 2 and Group 3 was 3.07 mm ($P < 0.001$) and between Group 1 and Group 3 was 5.87 mm ($P < 0.001$).

Culture

From the total sample set of 90, 39 (35.6%) samples yielded *P. gingivalis*, 42 (47.8%) samples did not grow *P. gingivalis* and contamination was observed in 9 samples. Culture positivity rose to 39.0% after exclusion of contaminated samples.

In Group 1, detection of *P. gingivalis* by culture was positive for 9 (32.1%) samples (after excluding contaminated samples) and was negative for 19 (67.9%) samples. In Group 2, detection of *P. gingivalis* by culture was positive for 15 (53.6%) samples and negative for 13 (46.4%) samples. In Group 3, detection of *P. gingivalis* by culture was positive for 15 (60%) samples and negative for 10 (40%) samples. A Chi-square test revealed that the differences in culture positivity between Groups 1 and 2, Groups 2 and 3 and Groups 1 and 3 were not statistically significant ($P = 0.075$) [Table 2].

Detection of *Porphyromonas gingivalis* by a species-specific polymerase chain reaction

Of the 90 samples, 56 (62.2%) samples tested positive for *P. gingivalis* when a species-specific PCR was used. In Group 1, PCR was positive for *P. gingivalis* in 10 (33.3%) samples and negative in 20 samples. In Group 2, PCR was positive for *P. gingivalis* in 22 (73.3%) samples and negative in 8 samples. In Group 3, PCR was positive for *P. gingivalis* in 24 (80.0%) samples and negative in 6 samples. A Chi-square test revealed that the difference of PCR positivity between Group 1 and Group 2 and between Group 1 and Group 3 was statistically significant with a $P < 0.001$ [Table 3].

Comparison between culture and polymerase chain reaction for the detection of *Porphyromonas gingivalis*

Of the 90 samples, 39 tested positive for *P. gingivalis* by culture as against 56 positive tests by the PCR method. From the 9 samples that showed contamination on culture, 4 samples tested positive for *P. gingivalis* on PCR. From the 42 culture-negative samples, 13 were PCR positive for *P. gingivalis*. The difference between the two techniques for the detection of *P. gingivalis* was seen to be statistically significant ($P < 0.001$) [Table 4].

Detection of *Porphyromonas gingivalis* strain variability by heteroduplex analysis

Out of the 56 samples that were positive for *P. gingivalis* by PCR, 54 samples yielded heteroduplex patterns [Figure 1].

Table 2: Detection of *Porphyromonas gingivalis* in the plaque samples as observed by culture

Culture	Group code			Total samples ($n=90$)*	*P*
	1, *n* (%)	2, *n* (%)	3, *n* (%)		
Negative	19 (67.9)	13 (46.4)	10 (40.0)	42	0.1
Positive	9 (32.1)	15 (53.6)	15 (60.0)	39	

*Contamination was seen in nine samples

Table 3: Detection of *Porphyromonas gingivalis* by a species-specific polymerase chain reaction

PCR	Group code			*P*
	1, *n* (%)	2, *n* (%)	3, *n* (%)	
Negative	20 (66.7)	8 (26.7)	6 (20.0)	<0.001
Positive	10 (33.3)	22 (73.3)	24 (80.0)	

PCR: Polymerase chain reaction

These heteroduplex patterns could be classified into eight distinct patterns [Figure 2]. Heteroduplex patterns 1, 2, 3 and 6 indicated the presence of two strains in the sample and patterns 4, 5, 7 and 8 indicated the presence of three strains in the sample.

Of the 54 samples that formed heteroduplex patterns, 21 samples (38.9%) showed pattern 3, 16 samples (29.6%) showed pattern 1, 9 samples (16.7%) showed pattern 4, 2 samples (3.7%) showed pattern 2, 2 samples (3.7%) showed pattern 5, 2 samples (3.7%) showed pattern 6, 1 sample (1.9%) showed pattern 7 and 1 sample (1.9%) showed pattern 8.

The above observations indicated that two strains of *P. gingivalis* were present in 41 individuals (45.6%) and three strains were present in 13 individuals (14.4%). The differences in heteroduplex patterns among the three groups were not statistically significant [Table 5].

Discussion

P. gingivalis has been called an opportunistic pathogen and is known to be a key pathogen in the process of periodontal disease causation and progression.[17] Multiple strains of *P. gingivalis* have been identified. Some strains are known to be avirulent (ATCC 33277) and some strains show a strong association with periodontal disease (W83 and W50). These strains actively express a multitude of virulence factors.[5,14] Igboin *et al*.[14] have also demonstrated a geographic variation in the distribution of clonal types of *P. gingivalis*. It will be interesting to look for the existence of multiple pathotypes of *P. gingivalis* akin to those seen in *Escherichia coli* where commensals and pathotypes can be clearly demarcated.[18]

Clonal variations are seen to exist at macroscopic levels, like geographic variations as well as at microscopic levels, like site-specificity in the oral cavity. This has stimulated researchers and epidemiologists to carry out a detailed profiling of this pathogen. Identification of specific patterns of distribution and association will contribute immensely to the development of treatment modalities that will target-specific strains or genes encoding specific virulence factors. PCR-based assays are highly sensitive and specific tools for identification of known genetic sequences in various samples such as dental plaque, gingival crevicular fluid and tissue samples.[19] Sequence polymorphisms and length variations found in the 16S–23S rRNA intragenic spacer region are increasingly being used as tools for the differentiation of bacterial species and subspecies.[20,21] Heteroduplex analyses has been shown to be a rapid and accurate method to identify genetic variants of *P. gingivalis*.[13]

A sample population with a wide spectrum periodontal health status was used for the present study. In every subject, one site with the deepest probing depth in each quadrant was used for plaque sampling.[22] All the samples were pooled into one vial in accordance with the study by Leys *et al*.[13] Plaque samples were collected using sterile Gracey curettes.[23,24]

The method of directly extracting DNA from the plaque sample and then using a two-step nested PCR to detect *P. gingivalis*

Table 4: Comparison of culture and polymerase chain reaction for the detection of *Porphyromonas gingivalis*

Culture	PCR		*P*
	Negative, *n* (%)	Positive, *n* (%)	
Negative	27 (100.0)	13 (25.0)	<0.001
Positive	0	39 (75.0)	

PCR: Polymerase chain reaction

Table 5: Detection of *Porphyromonas gingivalis* strain variability by heteroduplex polymerase chain reaction analysis

Heteroduplex analysis	Group code			*P*
	1, *n* (%)	2, *n* (%)	3, *n* (%)	
Two strains	1 (12.5)	9 (40.9)	8 (33.3)	0.345
Three strains	7 (87.5)	13 (59.1)	16 (66.7)	

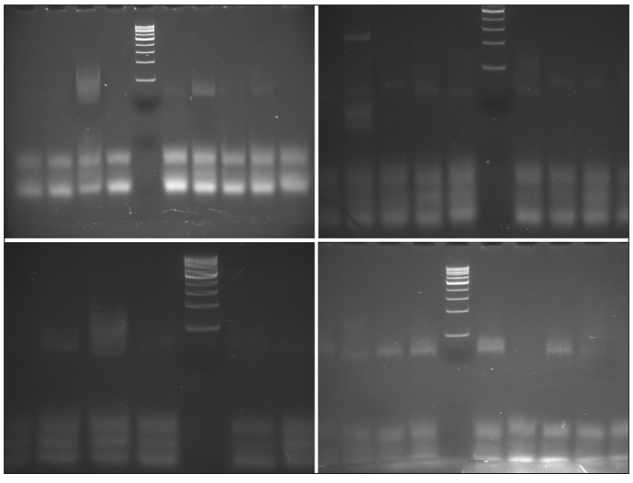

Figure 1: Sample photographs of the heteroduplex band patterns

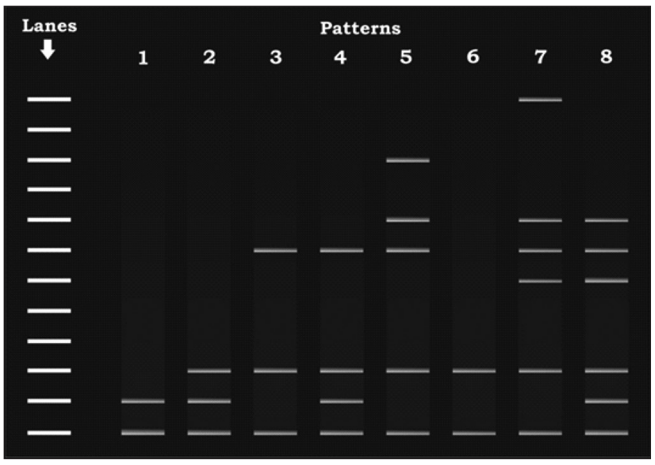

Figure 2: Graphical representation of the eight different heteroduplex band patterns observed in the present study

is similar to that used by Leys *et al*.[13] The samples that tested positive for *P. gingivalis* were then used for heteroduplex analysis.

Detection of *Porphyromonas gingivalis* by culture

Percentage detection of *P. gingivalis* in Group 1 was 32.1 that in Group 2 was 53.6 and that in Group 3 was 60. The figures for Group 1 are consistent with the findings by Lau *et al*.[25] who observed that the prevalence of *P. gingivalis* in healthy controls and subjects with gingivitis ranged from 18 to 40%. The difference in the detection of *P. gingivalis* between healthy and diseased groups did not reach statistical significance in the present study ($P = 0.075$). The percentage isolation of 50–60 for the group with periodontitis was less than the figure of 84.4% reported by Lau *et al*.[25] The figure observed in the present study was closer to the findings by Loesche *et al*.[26] and Tuite-Mcdonnell *et al*.[27]

Detection of *Porphyromonas gingivalis* by polymerase chain reaction

Percentage detection of *P. gingivalis* in the samples as seen by PCR was 62.2 (56 out of 90 samples tested positive). The percentage detection in Group 1 was 33.3, that in Group 2 was 73.3 and that in Group 3 was 80 [Table 3]. The difference between Group 1 and Group 2 and between Group 1 and Group 3 was statistically significant with a $P < 0.001$. This higher prevalence of *P. gingivalis* in subjects with periodontitis was expected and is in agreement with other studies.[25,28]

Comparison between culture and polymerase chain reaction

Percentage detection for *P. gingivalis* as seen by PCR was 62.2 and as seen by culture was 43.3 [Tables 2 and 3]. This discrepancy is because PCR is a more sensitive test for detection of bacteria in a given sample.[25] It is possible that the 18 samples that tested positive for *P. gingivalis* by PCR but negative by culture may harbour *P. gingivalis* in quantities below the detection limit of culture or may contain slow growing or exceptionally fastidious strains of this bacterium.

Heteroduplex analysis for detection of strain variability among *Porphyromonas gingivalis* isolates

When the PCR products are denatured at high temperature and permitted to re-anneal by lowering the temperature, DNA duplexes are formed. Only homoduplexes are formed when the sample contains a single strain of the bacterium. However, when the sample contains two or more strains heteroduplexes are formed in addition to homoduplexes. On electrophoresis, the number of bands formed is proportionate to the number of strains present in the sample. Of the 56 samples that tested positive for *P. gingivalis* by PCR, 54 samples demonstrated the formation of distinctive band patterns on heteroduplex analysis. These patterns were of eight different types. A graphical representation of these eight patterns is shown in Figure 1. The visualisation of 1 or 2 bands in addition to the homoduplex band indicated the presence of two strains in the given sample. The presence of 3–6 additional bands indicated the presence of three strains. Two or three strains of *P. gingivalis* were seen in 60% of the samples in the present study.

The identification of *P. gingivalis* by heteroduplex analysis was performed as per the studies by Leys *et al*.[13,29] and Igboin *et al*.[14] Leys *et al*.[13] have also observed that it was uncommon to find more than two or three strains of *P. gingivalis* in a single sample where they have identified 22 heteroduplex types of *P. gingivalis*. They also noted a geographic variation in the distribution of these 22 heteroduplex types of *P. gingivalis*. Of the 22 types, 6 types were seen to be widely distributed in the USA, Europe and East Asia and 14 rare types were found in isolated areas. Distribution of heteroduplex types of *P. gingivalis* has not been documented in the Indian population and is an important step in the epidemiologic research of this important periodontal pathogen.

It is known that avirulent strains of *P. gingivalis* are commonly carried by people in their oral cavities. A new strain gaining entry into the oral cavity may produce disease. Whether there is synergism among the strains to produce pathology or the pathogenic strains work independently is the intriguing question.

Conclusions

PCR with species-specific primers and heteroduplex PCR provide a simple and accurate method to analyse the strain diversity of *P. gingivalis* without the need to culture the organism. This makes it a useful tool for large-scale epidemiological studies.

This study is the first one to the best of our knowledge to investigate the strain diversity of *P. gingivalis* in an Indian population. The presence of two or three strains of *P. gingivalis* was seen in 60% of the samples in the present study. DNA of known *P. gingivalis* strains can be used to create heteroduplex patterns, and the presence of these strains in a given sample can be investigated further. Similar studies using a larger sample size are essential to elaborate the various heteroduplex patterns that can be found in the Indian population.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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