Original Article

Comparison of cluster analysis of *Porphyromonas gingivalis* by arbitrarily primed-polymerase chain reaction between healthy and chronic periodontitis subjects

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Abstract

Introduction: Periodontitis is a chronic destructive inflammatory disease of the oral cavity. The main causative agent is presence of biofilm formed due to different micro-organisms. Among different micro- organisms "red complex" bacteria is known to be the main causative agent in progression of periodontitis. *Porphyromonas gingivalis* out of the red the complex organism plays a major role in progression of periodontitis. *P. gingivalisis* present in both in healthy and diseased individuals. The difference in the strains will determine the virulence factor of the organism and also progression of disease. Only few studies have been done showing variation in strains present between healthy and diseased.

Aims: To check the difference in heterogeneity of *P. gingivalis* in chronic periodontitis and healthy individuals through Arbitrarily Primed-PCR (AP-PCR).

Materials and Methods: A total of 400 subjects (200 each of chronic periodontitisandhealthy individuals) were included. Sub-gingival plaque was collected in the Reduced transport fluid (RTF) medium and processed at the institutional central research laboratory. Presence of *P. gingivalis* was, confirmed by culture andphenotypical analysis. Further confirmed cases were processed for PCR after DNA extraction using 16S rRNA. Positive cases of *P. gingivalis* were subjected for AP-PCR for clonal analysis using the specific 272 primer.

Results: In 152(76%) and 98(49%) were confirmed for *P. gingivalis* in chronic periodontitis and healthy individual respectively by PCR. AP-PCR analysis showed 6 clusters with similarity index in CP and 3 clusters with similarity index in Healthy individuals.

Conclusion: The present study showed difference in clusters between chronic periodontitis and healthy individual'sthussuggestive variantin genetic heterogeneity of *P. gingivalis* strain between healthy and chronic periodontitis. AP- PCR appears to be a promising tool for clonal analysis of *P. gingivalis*.

Keywords: Arbitrarily primed-polymerase chain reaction, chronic periodontitis, cluster, healthy, *Porphyromonas gingivalis*, subgingival plaque

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INTRODUCTION

Porphyromonas gingivalis is a black-pigmented anaerobic Gram-negative microorganism known to be a putative periodontopathogen.[1] It is present in the subgingival plaque of healthy and diseased individuals, although with a lower prevalence of *P. gingivalis* in the healthy oral cavity.[2] The strains and virulence of this *P. gingivalis* vary between healthy and diseased periodontium. Hence, it is suggested that there are specific clonal types that are responsible for the progression of the disease. There is the presence of clonal heterogeneity of subpopulation with both high and low levels of pathogenicity which was suggested to be present in isolates from healthy and diseased populations. Strains that are present in healthy sites represent relatively avirulent strains, whereas certain specific virulent strains of *P. gingivalis* may cause chronic periodontitis (CP).[3]

Based on these findings, several studies in the literature have demonstrated the variation of *P. gingivalis* strains concerning their association in disease and also virulence.[4-7] Efforts are also being made to study the discrimination of bacterial strains among *P. gingivalis* and identify different genetic clusters associated with oral health and disease. Many molecular methods have been applied to ascertain the genetic variations in *P. gingivalis* which include restriction endonuclease analysis (REA) of whole chromosomal DNA, ribotyping; repetitive extragenic palindromic polymerase chain reaction (PCR). Each one of them has its own advantages and disadvantages.[8]

Recently, a method with a simple procedure and excellent resolution has been developed that is arbitrarily primed–PCR (AP-PCR), which is one of the most popular techniques applied for molecular typing for oral microbes including *P. gingivalis*.[9] In the literature search, we found a lack of studies comparing the difference in the strains of *P. gingivalis* in the healthy oral cavity and with CP. Hence, this study was ventured to find out the difference in genetic clusters in *P. gingivalis* between healthy and CP using AP-PCR.

MATERIALS AND METHODS

The present study was performed at the Central Research Laboratory of our Institution. Two hundred patients each with CP and apparently healthy individuals between the age group of 18–60 years belonging to both sexes were enrolled for the study. Approval of the Institutional Ethical Committee was obtained before the initiation of the study. The study duration was between June 2015 and 2016. The inclusion criteria for patients with CP were the presence of more than 20 natural teeth *in situ*, clinical attachment loss ≥3 mm in at least 4 or more teeth and bleeding on probing. The criteria for defining healthy participants were probing depth ≤3 mm, no clinical signs of inflammation, no clinical attachment loss and no tooth mobility. Participants who had received periodontal therapy, antibiotics/antimicrobials within 3 months before sampling, pregnant and lactating women, smokers and subjects using smokeless tobacco, presence of diabetes, or other systemic diseases were excluded from the study. Participants who met the inclusion and exclusion criteria were considered for the study. After obtaining willing informed consent from the participants, subgingival plaque was collected.

Microbiological sampling

After stripping off the supragingival plaque, the subgingival plaque sample was collected with sterile Gracey curettes from at least four teeth in healthy subjects and four deepest pocket sites or most diseased sites in the CP patients. The samples were transferred to reduced transport fluid (RTF) and brought to the laboratory at the earliest. Upon receipt in the laboratory, the samples were divided into two aliquots: one was utilized for culture and the second portion was stored in cryopreservative agent at −80°C as a backup.

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Anaerobic culture was performed to detect the presence of *P. gingivalis* in plaque samples. The samples were vortexed for 30 s and diluted thioglycollate broth in 1:50 dilution. The samples were plated on to blood agar (with hemin and Vitamin k) and kanamycin blood agar. After inoculation, plates were kept in an anaerobic jar with the gas pack at 37°C for 72 h. After 72 h, the culture plates were examined for the presence of black/brown colonies with or without hemolysis. Further, the colonies were analyzed for Gram-negative nature of bacilli by gram staining. Upon confirmation, they were further subjected to a series of phenotypic tests to confirm *P. gingivalis*. The biochemical test performed showed oxidase negative, catalase negative, indole positive and reduced nitrate and did not ferment glucose, sucrose, cellobiose, lactose, xylose, salicin, arabinose and mannose which were considered to be *P. gingivalis*.[10,11] After identification, the isolated colonies

were transferred to 15% glycerol broth and stored at −80°C for further processing.

The procedure of DNA extraction

The stored colonies were later subjected to DNA extraction by the modified proteinase K method. The sample was vortexed thoroughly and centrifuged at 5000 RPM for 5 min. Supernatant RTF buffer was removed, and then, the pellet was suspended in fresh Tris-EDTA (TE) buffer.

Washing with TE buffer was repeated three times, and supernatant TE buffer was discarded following centrifugation at 5000 RPM for 5 min. Bacterial cells were lysed by using lysis buffer I (10 mM Tris buffer, 1 mM EDTA) and lysis buffer II (50 mM Tris HCL, 50 mM KCL, $MgCl_2$ 2.5 mM, Tween 20 0.45%, nonident *P* 0.45%) following lysis, proteinase K (10 mg/ml) was added to degrade the protein contaminants. The sample was incubated at 60°C in a water bath for 2 h. Then tubes were transferred to a boiling water bath for 10 min for the deactivation of enzyme. The sample were centrifuged at 5000 RPM for 5 min and then supernatant containing DNA was collected in a fresh microcentrifuge tube and DNA was purified using sodium acetate and alcohol. These were stored at −20°C unit further use for PCR.

PCR was carried out to identify the species *P. gingivalis* using species-specific primer 16S rRNA gene with primer sequence forward 3' AGG CAG CTT GCC ATA CTG CG 5' and reverse 3' ACT GTT AGC AAC TAC CGA TGT 5'.[12] The samples which were positive for *P. gingivalis* through PCR were further subjected to AP-PCR to analyze the strain of *P. gingivalis* [Figure 1].

Procedure for arbitrarily primed-polymerase chain reaction

AP-PCR was carried out in a Veriti thermal cycler (Applied biosystem California, USA) to specifically detect *P. gingivalis* diversity. AP randomized sequence 272 (5'-A GCGGGCCAA-3')[13,14] with a concentration of 20 pmole/μl was used in the reaction. Total reaction volume of 20 μl was prepared in a 0.2 ml PCR tube. Amplification Taq PCR master mix (Amplicon Denmark) was used which contains ×10 PCR buffer with 1.5 mM $MgCl_2$, 10 mM of dNTP mix, AmpliTaq polymerase 2.5 units/reaction and an inert red dye. The composition of the reaction mixture was as per the manufacturer's instruction (ampliquon). 2 μl of DNA template (Approx-100 μg/ml) was added to the reaction mixture. The final volume to 20 μl was made up of molecular grade water. The tubes were placed in a thermal cycler and DNA was amplified by using standard PCR conditions. Initial denaturation was carried out at 94°C for 5 min, followed by 35 cycles of denaturation was carried out at 94°C for 1 min, annealing at 32°C for 2 min proceeded with extension at 72°C for 2 min and a final extension at 72°C for 5 min. Following AP-PCR, the amplified product with 272 bp was detected by running amplified samples on 1.5% agarose. The electrophoresis was run at 70V for 2 h. The gel was stained with ethidium bromide (0.5ug/ml) and then was visualized and documented on ultraviolet transilluminator (Gel documentation system, Major Science, USA). The bands of the samples were compared with 100 bp DNA ladder (Genetix Asia Pvt Ltd, New Delhi, India) to detect the differences in the molecular sizes of each sample. The analysis was done using Total lab software (Newcastle upon Tyne, UK) purpose using Phoretix 1D pro-gel software (Total lab, UK).

RESULTS

Out of total 400 samples, 227 (56.75%) were female and 173 (43.25%) were male. Out of 200 samples each in healthy and CP, 179 (89%) cases were positive for *P. gingivalis* in CP and 109 (54.5%) cases were positive in healthy plaque samples through culture.

Positivity for *P. gingivalis* was more in females when compared to males. Out of 179 culture-positive cases of CP, 152 samples showed positivity for *P. gingivalis*, and for 109 healthy out of 98 showed positivity for PCR. The cases which were positive for PCR were subjected to AP-PCR which showed a difference in *P. gingivalis* strains between healthy and CP. Analysis of the dendrogram pattern generated using Phoretix 1D pro–gel software (Total lab, UK) revealed a difference in the number of clusters and sub-clusters between healthy and CP. The healthy samples showed a similarity index in three clusters while CP showed six clusters with similarity index [Figures 2 and 3].

On calculating similarity coefficients by comparing the bands pattern among healthy individuals and CP. We found

Table 1: Percentage of similarity index between healthy and chronic periodontitis

Groups	Similarity coefficient (%)
CP versus H	0-66.66
CP versus CP	0-33.33
H versus H	0-66.66

CP: Chronic periodontitis, H: Healthy individuals

Table 2: Similarity coefficient band pattern among healthy and chronic periodontitis

Groups	Number of bands	Mean
Healthy	0-14	7.5
CP	1-12	6.5

CP: Chronic periodontitis

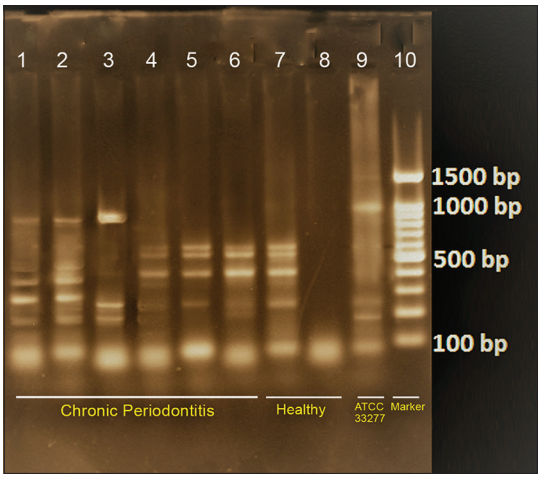

Figure 1: Photograph showing arbitrarily primed-polymerase chain reaction with different banding patterns determining the heterogeneity of *Porphyromonas gingivalis* in healthy and chronic periodontitis (except band eight all other samples are positive for *Porphyromonas gingivalis*)

highest diversity with healthy versus healthy and CP versus healthy [Table 1].

The mean number of bands and intensity was slightly higher in healthy compared to CP [Table 2].

DISCUSSION

CP is a chronic microbial disease and the true nature of the disease largely remains speculative. However, identification of few putative periodontopathogens has been major milestone in the research of periodontal microbiology and "red complex" organism comprising *Tannerella forsythia, P. gingivalis* and *Treponema denticola* have been implicated in the disease progression of CP. Among these three organisms, *P. gingivalis* has shown to be associated strongly with disease process.[15]

Various studies in the literature have demonstrated the presence of *P. gingivalis* both in healthy and diseased sites. These findings led researchers to find out the difference in *P. gingivalis* isolated from healthy and diseased sites. Various laboratory techniques were used to identify the heterogeneity in the clone of *P. gingivalis* such as serotyping multilocus enzyme electrophoresis, DNA fingerprinting, ribotyping, whole-genomic restriction fragment length polymorphism, heteroduplex PCR and AP-PCR.[15]

Even though many techniques have been used to identify the difference in strains and virulence factor of *P. gingivalis* between healthy and diseased sites, there are limited data available to substantiate the findings. Hence, the study was conducted on larger sample size to determine the difference in the heterogeneity in clusters of *P. gingivalis* between healthy and CP participants.

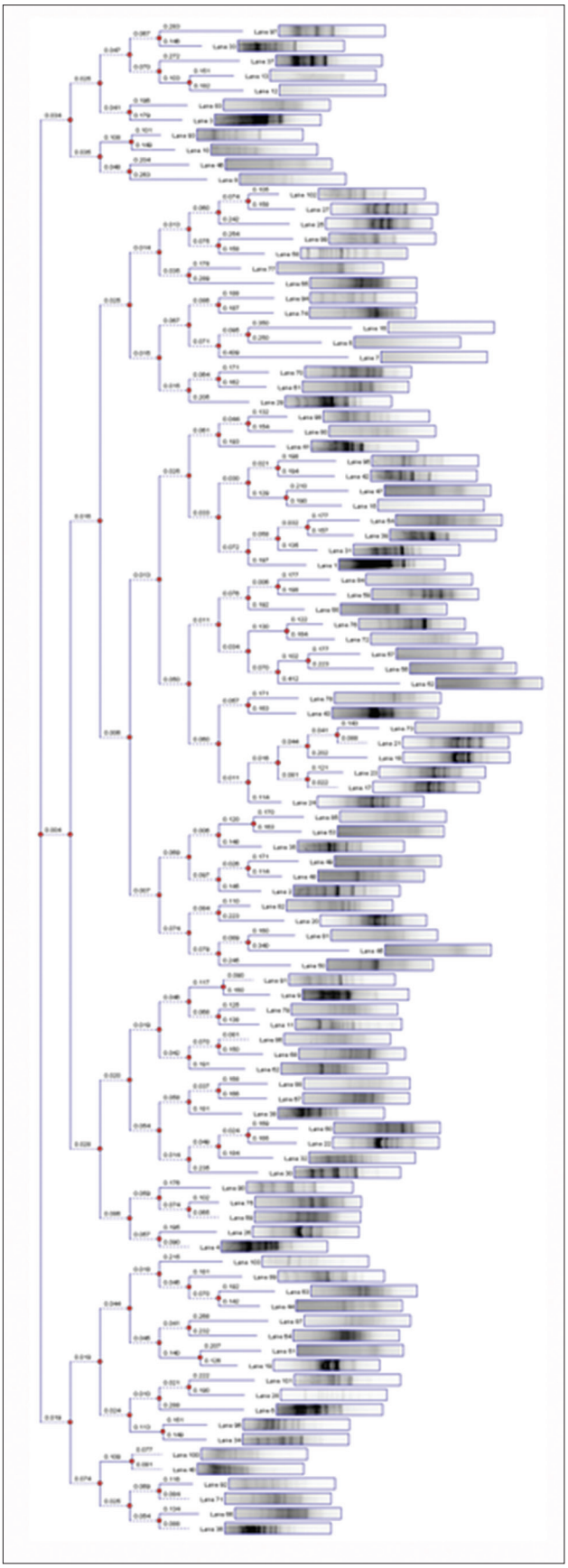

Figure 2: Dendrogramic image showing healthy samples with three clusters

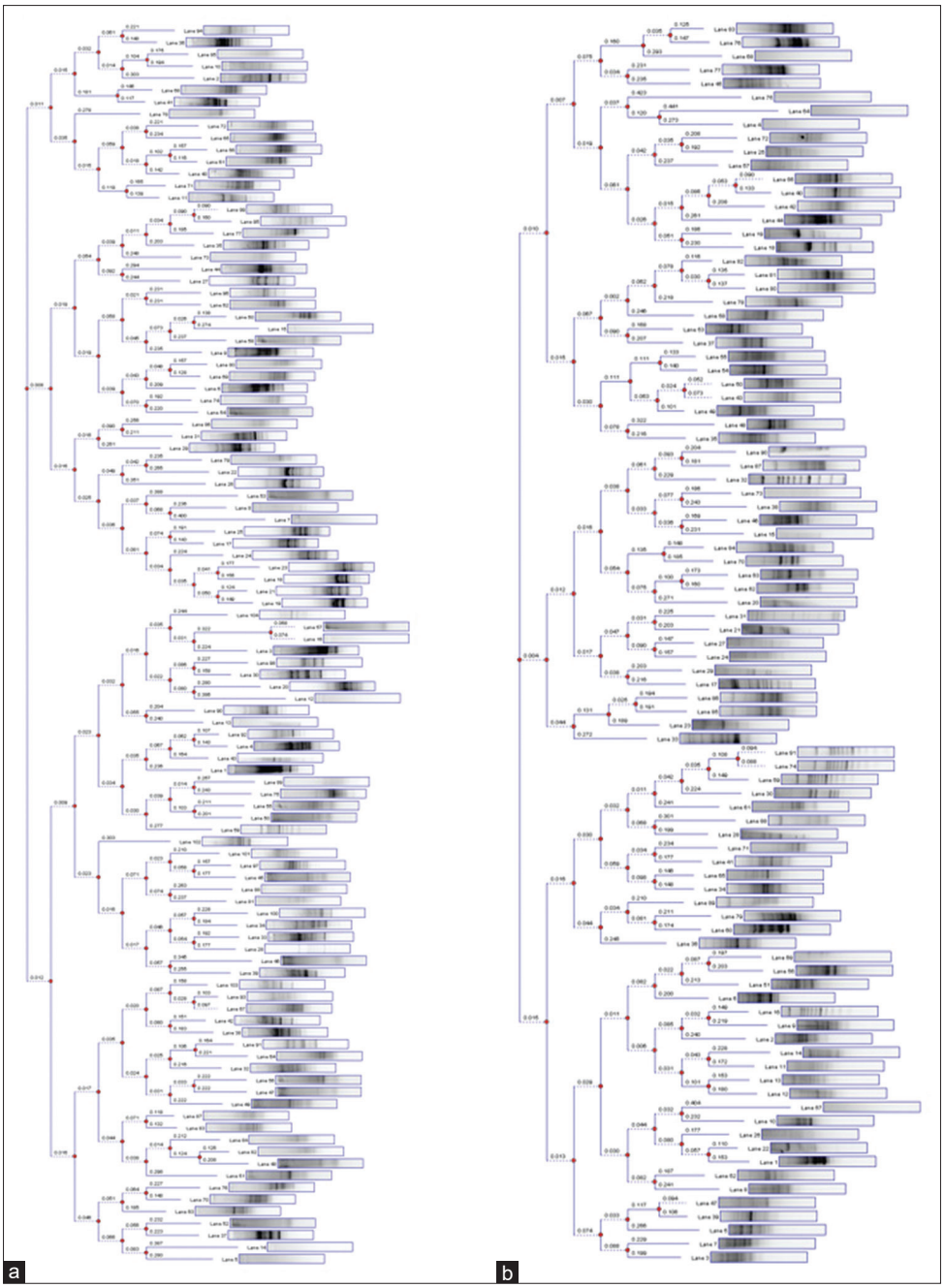

Figure 3: (a and b) Dendrogramic image showing chronic periodontitis samples with six clusters

The present study was conducted on a total 400 participants were sub gingival plaque sample was collected (200 each of healthy and 200 of CP) and AP-PCR was performed. In CP, 152 (76%) participants showed positivity for *P. gingivalis* in CP and in healthy 98 (49%) participants showed positivity for *P. gingivalis*. Our finding is similar to the observation made by Missailidis *et al.* who found 89.4% of patients with CP showed positivity for *P. gingivalis*[16] low positivity was observed by Shibli *et al.* with only 12.1% positivity[17] and Fernandes *et al.* in edentulous patients who suffered with CP observed 26.6% positivity for *P. gingivalis*.[18] The prevalence of *P. gingivalis* has shown variation with the geographical locations. In Japan, it is 78%–95%[4] and in Korea 100%.[19] Whereas in the USA, it is between 50% and 87%.[20] In Brazil, it is between 78%.[21] In this study, we reported a prevalence of 76% in CP, and in healthy, it is 49%. This suggests that there might be certain variation in clonal heterogeneity of strain between each population which led us to identify the clonal difference between healthy and CP.

AP-PCR method is used for genetic analysis of prokaryotic and eukaryotic cells. The main advantage of AP-PCR is

its ability to furnish highly specific DNA profiles with no prerequisite for knowing the DNA sequences. This method utilizes single 10–20 base pair random sequences primer that will anneal to the nonspecified target DNA sites that allows for mismatched base pairing. The DNA regions between two annealed primers are subsequently amplified by PCR and used as polymorphic markers. Hence, AP-PCR is proven to be fast, reproducible and highly sensitive method to study genetic polymorphism for gene mapping and to study population genetics.[22] Thus, in the present study, we used AP-PCR to analyze genetic variation in *P. gingivalis* among healthy and CP individuals. In the present study, the primer sequence used for the first time to check the heterogeneity in *P. gingivalis,* it has given very good results.

Through AP-PCR, the healthy samples showed three clusters with similarity index of 0%–66.6%, whereas in CP, it was six clusters with similarity index of 0%–33.3%. On comparing the similarity coefficient percentage, we found diversity between healthy and CP.

In a study done by Gonçalves *et al*. examine the genetic diversity of *P. gingivalis* or *Prevotella nigerscens* using AP-PCR recovered from infected root canal and subgingival plaque showed *P. gingivalis* isolated from each of three patients was unique for each patient, suggesting that the population structure of the species consists of multiple clonal lines confirming to the taxon a high level of genetic diversity.[23]

In a study by Griffen *et al*. to determine the strain variability of *P. gingivalis* in periodontitis and healthy human subject by heteroduplex method. They found association of individual heteroduplex types of *P. gingivalis* with periodontitis showed the presence of one type (hW 83) was highly statistically significant. Two other that is h49417 and hHG1691 were also significantly associated with disease. Their observation showed variation among virulent strains of *P. gingivalis* in periodontitis compared to healthy. Thus, this study suggests that these observations may offer an insight into the mechanism of pathogenesis of disease. However, the limitation of the study was small sample size.[7]

Clonal analysis of *P. gingivalis* by AP-PCR was done by Chen and Slots on 72 patients with periodontitis and also standard ATCC strain. They demonstrated genetic heterogeneity of *P. gingivalis* by AP-PCR. They concluded that AP-PCR offers a sensitive and simple method to delineate genetic polymorphism among *P. gingivalis* strains. Similarly, we also found genetic variation of *P. gingivalis* between healthy and CP, also there were variation in numbers of band between healthy and CP. Chen and Slots analyzed 10–20 base pair oligonucleotide primers and found two primers produced clear and distinct DNA bands. These two primers which were selected for the analysis, and on this basis, 73 strains were classified into 23 genotyping for one primer and for other was 45 genotypes.[22]

In a study on mouse model to identify the strains which are periodontopathic. The study observed the certain strains of *P. gingivalis* were highly invasive and can produce abscesses. Whereas in another study on rat model, they found two different *P. gingivalis* strains. One strain causing horizontal bone loss and other strain showed vertical bone loss.[24] Thus suggesting difference in strain show different clinical outcome.

Another study found 33 isolates of *P. gingivalis* from periodontitis patients or infected root canals analyzed through REA. The majority of in dependent *P. gingivalis* isolates had a unique DNA finger print indicating extensive genetic heterogeneity within this species.[25]

The literature review of various studies done is based on different molecular cloning method, and model, either by using animal model or human dental samples. They have found genetic variation in the strains of *P. gingivalis*. In our study also, we found heterogeneity in the strains of *P. gingivalis* between healthy and CP suggesting that the high level of diversity existing in *P. gingivalis* and suggests that all clonal types of *P. gingivalis* would be equally effective in colonizing the human host and that they share a common virulence potential. Moreover, the virulence factor varies between healthy and CP.

CONCLUSION

The present study we noticed the difference in the cluster of *P. gingivalis* formed between healthy and CP, suggesting genetic heterogeneity existing in *P. gingivalis* between healthy and CP. However, further studies are needed for comparing the clinical parameters of CP with clonal heterogeneity of *P. gingivalis* among these individuals to ascertain the virulent factors present in CP when compared to healthy individuals.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000 1999;20:168-238.
2. Igboin CO, Griffen AL, Leys EJ. *Porphyromonas gingivalis* strain diversity. J Clin Microbiol 2009;47:3073-81.
3. Kubinowa M, Inaba K, Amano A. Genotyping to distinguish microbial pathogenicity in periodontitis. Periodontol 2000 2010;54:136-59.
4. Amano A, Kubinowa M, Nakagawa C, Akiyama S, Morisaki I, Hamada S. Prevalence of specific genotypes of *Porphyromonas gingivalis* fim A and periodontal health status. J Dent Res 2000;79:1664-8.
5. Amano A, Nakagawa I, Kalaoka M, Morisaki I, Kamada S. Distribution of *Porphyromonas gingivalis* strains with fim A genotypes in periodontitis patients. J Clin Microbiol 1999;37:1426-30.
6. Grenier D, Mayrand D. Selected characteristic of pathogenic and non- pathogenic strain of Bacteroides gingivalis. J Clin Microbiol 1987;25:738-40.
7. Griffen AL, Lyons SR, Beeker MR, Moeschberger ML, Leys EJ. Porphyromonas strain variability, and periodontitis. J Clin Microbiol 1999;37:4028-33.
8. Teanpaisan R, Douglas CW. Molecular fingerprinting of *Porphyromonas gingivalis* by PCR of repitative extra gene pallidromic (REP) sequence and comparison with other finger printing method. J Med Microbiol 1999;48:741-9.
9. Menard C, Brousseau, Mouton C. Application of polymerase chain reaction with arbitrary primer (AP-PCR) to strain identification of Porphyromonas (Bacteriodes) Gingivalis. FEMS Microbiol Lett 1992;95:163-8.
10. Olsen I, Yilmaz O. Modulation of inflammsome activity by *Porphyromonas gingivalis* in Periodontitis and associated systemic diseases. Oral Microbiol 2016;8:1-11.
11. Chapple IL. Periodontal diagnosis and treatment—where does the future lie? Periodontol 2000 2009;51:9-24.
12. Chaudhary V, Bhat KG, Rao S, Ingalagi P, Kugaji M. Comparison of culture and PCR for detection of *Porphyromonas gingivalis* in patients with chronic periodontitis and healthy subjects. J Pharm Biomed Sci 2013;34:1670-5.
13. Nanvazadeh F, Khosravi AD, Zolfaghari MR, Parhizgari N. Genotyping of *Pseudomonas aeruginosa* strains isolated from burn patients by RAPD-PCR. Burns 2013;39:1409-13.
14. Clarridge JE 3rd. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 2004;17:840-62.
15. Kulkarni MR, Bhat KG, Thomas BS, Bhat GS, Kulkarni RD. Identification of multiple strains of *Porphyromonas gingivalis* using heteroduplex polymerase chain reaction in varying severity of chronic periodontitis. Indian J Med Microbiol 2018;36:81-6.
16. Missailidis CG, Umeda JE, Ota-Tsuzuki C, Anzai D, Mayer MP. Distribution of fimA genotypes of *Porphyromonas gingivalis* in subjects with various periodontal conditions. Oral Microbiol Immunol 2004;19:224-9.
17. Shibli JA, Melo L, Ferrari DS, Figueiredo LC, Faveri M, Feres M. Composition of supra- and subgingival biofilm of subjects with healthy and diseased implants. Clin Oral Implants Res 2008;19:975-82.
18. Fernandes CB, Aquino DR, Franco GC, Cortelli SC, Costa FO, Cortelli JR. Do elderly edentulous patients with a history of periodontitis harbor periodontal disease? Anaerobe 2007;13:244-8.
19. Choi BK, Park SH, Yoo YJ, Choi SH, Chai SK, Cho KS, *et al*. Detection of mayor putative periodontopathogens in Korean advanced adult periodontitis patients using a nucleic acid based approach. J Periodontol 2000;71:1387-94.
20. McClellan DL, Griffen AL, Leys EJ. Age and prevalence of *Porphyromonas gingivalis* in children. J Clin Microbiol 1996;34:2017-9.
21. Avila-Campos MJ, Velasquez-Melendez G. Prevalence of putative periodontopathogens from periodontal patients and healthy subjects in Sao Paulo, SP, Brazil. Rev Inst Med Trop Sao Paulo 2002;44:1-5.
22. Chen C, Slots J. Clonal analysis of *Porphyromonas gingivalis* by the arbitrarily primed polymerase chain reaction. Oral Microbiol Immunol 1994;9:99-103.
23. Gonçalves RB, Robitaille M, Mouton C. Identical clonal types of *Porphyromonas gingivalis* or *Prevotella nigrescens* recovered from infected root canals and subgingival plaque. Oral Microbiol Immunol 1999;14:197-200.
24. Ozmeric N, Preus HR, Olsen I. Genetic diversity of *Porphyromonas gingivalis* and its possible importance to pathogenicity. Acta Odontol Scand 2000;58:183-7.
25. Loos BG, Mayrand D, Genco RJ, Dickinson DP. Genetic heterogeneity of Porphyromonas (Bacteriodes) gingivalis by genomic DNA fingerprinting. J Dent Res 1990;69:1488-93.