

Polymerase Chain Reaction: A New Era in Detection of Periodontopathogens

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ABSTRACT

Aim: Various techniques like cultural methods, Immunofluorescence and ELISA have largely been used for studies of microbial ecology. But nowadays PCR is used as a diagnostic tool that can detect even small numbers of periodontal pathogens with a high degree of accuracy. In this study, the quantity of periodontal pathogens (*P.gingivalis*, *T.denticola*, *T.forsythia* and *A.actinomycetemcomitans*) in saliva, preoperatively and postoperatively after scaling and root planing was compared by using multiplex PCR test.

Materials and Method: Unstimulated saliva of 30 chronic periodontitis patients was taken preoperatively by spitting method in eppendorf vial. After treatment i.e scaling and root planing again saliva of the same patient was taken and sent to the laboratory of Maratha Mandal Dental College, Department of Microbiology, Belgaum, Karnataka for PCR analysis.

Results: Quantity of three bacteria (*T.d*, *A.a*, *P.g*) were reduced significantly after scaling and root planing as compared to *T.f* which did not show statistically significant reduction.

Conclusion: Non-surgical therapy like scaling and root planing plays a very important part in treating the patients of chronic periodontitis.

Keywords: Hydrogen peroxide, Oxygen radicals, Tooth bleaching.

INTRODUCTION

Periodontitis is defined as an inflammatory disease of the supporting tissues of the teeth caused by specific microorganisms or complexes of microorganisms resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both¹. The composition of the subgingival microbial flora and the level of pathogenic species differ from



subject to subject as well as from site to site². The microbial populations involved in periodontal diseases are known to be highly complex and Haffajee & Kamma considered four bacteria i.e *Aggregatibacter actinomycetemcomitans* (*A.a*), *Tannerella forsythia* (*T.f*), *Porphyromonas gingivalis* (*P.g*) and *Treponema denticola* (*T.d*) to be putative periodontopathogenic microorganisms³. Various chemotherapeutic agents like chlorhexidine, dentifrices, antibiotics have been used for plaque removal. But plaque being a biofilm, chemotherapeutic agents alone without the mechanical therapy cannot destroy it.

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Fig 1: DNA Extraction Kit.



Fig 2: PCR Thermocycler (Inside).

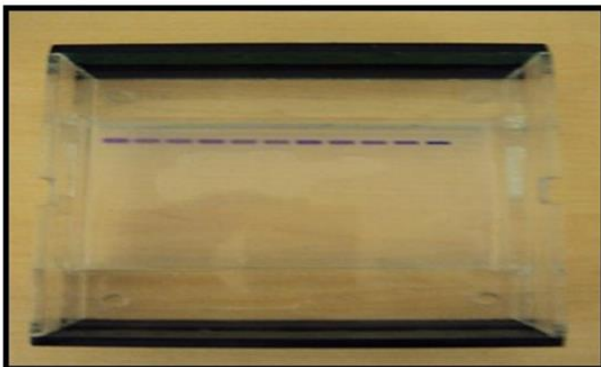


Fig 3: Agarose Gel Tray Loading.

It has always been a difficult task to detect the specific microorganisms associated with periodontitis. Various techniques like blood agar, Immunofluorescence and Enzyme linked immunosorbent assay (ELISA) have largely been used for studies on microbial ecology^{4,5}. But, it is difficult to use these techniques for evaluating a varied number of species in very large number of



Fig 4: Photographic Recording Of DNA Bands.

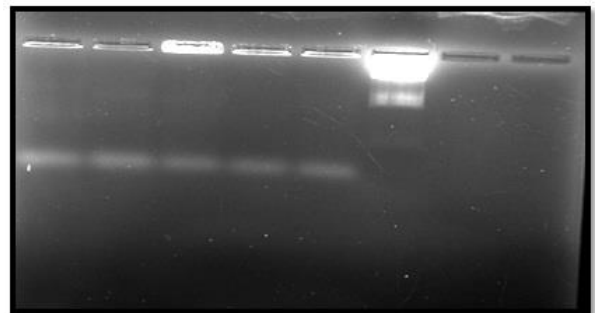


Fig 5: Positive Bands of three bacteria Pg, Tf and Aa.

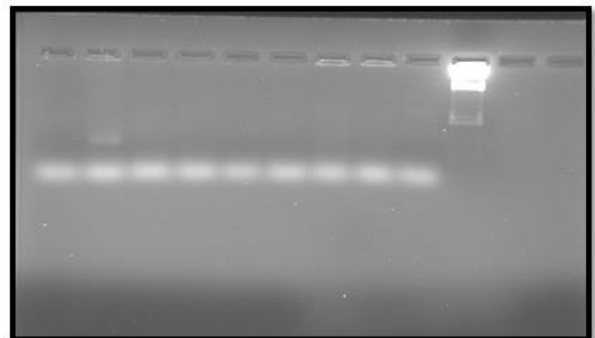
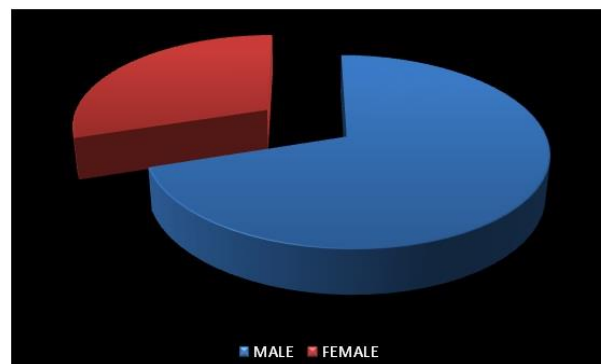
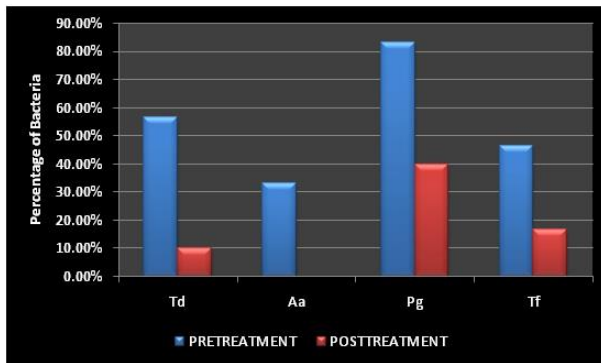


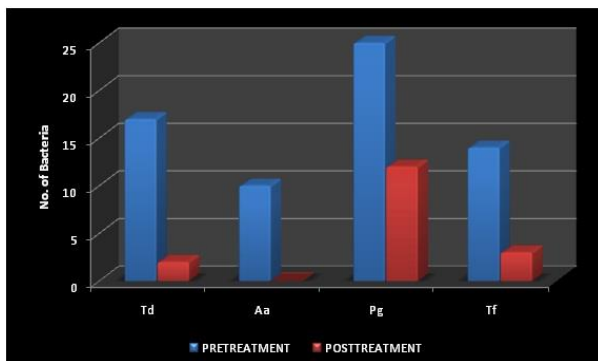
Fig 6: Positive bands for Td Bacteria.



Graph 1: Showing number of males and females in the study.



Graph 2: Showing comparison of prevalence of bacteria Pretreatment and Posttreatment.



Graph 3: Showing comparison of number of patients showing positivity for bacteria in pretreatment and Posttreatment.

plaque samples. The drawback of other methods is overcome by Polymerase chain reaction (PCR).

PCR is a powerful diagnostic tool that can detect even a small numbers of periodontal pathogens with a high degree of accuracy. It is extremely sensitive, being able to detect even one copy of the searched DNA target⁶. Mechanical therapies including scaling and root planing aim at improving clinical conditions by lowering the microbial load either by physical removal of plaque or by radical alteration of subgingival habitat⁷.

Present study was introduced with an aim to see the effect on the quantity of periodontal pathogens (P.gingivalis, T.denticola, T.Forsythia and A.actinomycetemcomitans) in saliva, preoperatively and postoperatively after non-surgical therapy.

Objectives:

- 1) To investigate whether saliva, can be used as diagnostic tool in detection of "Periodontopathogens".

- 2) To compare the quantity of four bacteria (T.d, T.f, A.a and P.g) present preoperatively with postoperatively in patients of chronic periodontitis.

Inclusion criteria:

- 1) Patient diagnosed with chronic periodontitis and having probing pocket depth ≥ 5 mm.
- 2) Age ≥ 35 years

Exclusion Criteria:

- 1) History of previous periodontal treatment in the past six months.
- 2) History of systemic diseases.
- 3) Patients on Anti-Psychotic therapy
- 4) Antibiotic and anti-inflammatory drugs taken in the past three months.
- 5) Smokers
- 6) Alcoholics
- 7) Pregnant and lactating females
- 8) Mentally retarded patients.

PROCEDURE

Unstimulated saliva of 30 chronic periodontitis patients was taken by spitting method. Sample of 0.5ml was collected preoperatively i.e before scaling and root planing with pocket depth more or equal to 5mm in eppendorf tube containing 1.5ml of tris-EDTA and the same procedure was repeated after scaling and root planing and sent to Maratha Mandal Dental College, Belgaum as per laboratory specification.

Steps for DNA extraction procedure (Figures 1 and 2)

- 1) Put 500µl of TE buffer and centrifuge it for 4 mins at 5000rpm
- 2) Discard the supernatant with micropipette
- 3) Put 500µl of TE buffer and centrifuge it for 4mins at 5000rpm
- 4) Discard the supernatant.
- 5) Put 500 µl of lysis 1 into the vial.
- 6) Centrifuge it for 4 mins at 5000rpm.
- 7) Discard the supernatant.
- 8) Put 500 µl of lysis 11 and 10µl of proteinase k into the vial.
- 9) Put these vials into hot water bath at 57 ° c for 2hrs.
- 10) Put it into boiling water for 100° c for 10mins.

11) Store it at 4° c till PCR procedure is done.

MASTER PRIMER

Two master primers were made. One of the three bacteria i.e. Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Tannerella forsythia and other primer was made for Treponema denticola.

S.no	Primer	Sequence(5'-3')	Length
1	(aaf)	ATTGGGGTTTAGCCCTG GTG	20Tm5 4
2	(bff)	TACAGGGGAATAAAAT GAGATACG	24Tm5 2
3	(pgf)	TGTAGATGACTATGGTG AAAACC	24Tm5 2
4	(C11R)	ACGTCATCCCCACCTTC CTC	20Tm5 6

Aaf primer- A.actinomycetemcomitans . A=Adenine
Bff Primer- T.forsythia. T=thymine
Pgf Primer- P.gingivalis. G=guanine
C11R primer- T.denticola. C=cytosine

20Tm54-20 base pairs with temperature melting is 54
24Tm52-24 base pairs with temperature melting is 52
24Tm52-24 base pairs with temperature melting is 52
20Tm56-20 base pairs with temperature melting is 56

POLYMERASE CHAIN REACTION

Multiplex PCR is a variant of PCR which enables simultaneous amplification of many targets of interest in one reaction by using more than one pair. It consists of multiple primer sets within a single PCR mixture to produce amplicons of various

Table 1: Amount of bacteria present pre- and post-treatment.

Bacteria	Pre treatment (n=30) Median (min-max)	Post treatment (n=30) Median (min-max)	P value	Significance (S)
Td	1000 (0-26000)	0 (0-18000)	0.001	S
Aa	0 (0-24000)	0 (0-0)	0.005	S
Pg	40000 (0-38000)	0 (0-61000)	0	S
Tf	0 (0-31000)	0 (0-26000)	0.088	NS

Table 2: Preoperative prevalence rate of all four bacteria

Bacteria	Preoperatively	Postoperatively
Td	56.6 %	10%
Aa	33.3%	0%
Pg	83.3%	40%
Tf	46.6%	16.6%

sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primers must be optimized to work correctly within a single reaction and amplicon sizes. That is their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

GEL ELECTROPHORESIS

Molecular weight markers are mixture of DNA with known molecular weights. The gel matrix acts like a sieve for the DNA molecules. The larger molecules will lag behind the smaller fragments as the DNA migrates through the gel and hence form a particular type of band pattern on the gel with which sample bands can be analysed.

Procedure for gel electrophoresis: For preparation of gel 1gm of agarose is mixed in 50ml of TAE buffer and then boiled with gentle shaking till homogenous clear solution is formed. It is then cooled for sometime followed by addition of 5µl ethidium bromide to the mix and gently shaken. The gel is then poured into tray (Figure 3) and a comb is placed into the gel allowing it to set for at least 20-30 minutes at room temperature. The comb is removed carefully and gel tray is placed in electrophoresis unit containing 200ml of TAE buffer. The gel should be completely submerged in the buffer. 5µl of loading dye is added into the amplified DNA product. 20µl of the amplified product is taken into the tray, an electrode is fixed

Table 3: Comparison of the amount of bacteria present pre- and post-operatively.

Bacteria	No. Of patients positive for respective bacteria (Pre-treatment)	No. Of patients positive for respective bacteria (Post-treatment)	Total no. Of patients who showed reduction in no. of bacteria (positive value)	P value	Significance (S)
Td	17	2	15	0.01	S
Aa	10	0	10	0.01	S
Pg	25	12	13	0.01	S
Tf	14	3	11	0.022	S

and the power supply is turned and adjusted to 16A gel for 2 hrs. It is removed and viewed on UV transilluminator (Figure 4). The result was documented the help of photography (Figures 5,6).

STATISTICAL ANALYSIS

Statistical analysis was done using Wilcoxon signed rank test. If T1 (Test criterion=the totals of negative values, as well as positive values obtained) value was higher than T-0.05, the difference between groups was taken as non-significant. Non-parametric test namely Mcnemar’s test was also applied to 2×2 contingency tables with a dichotomous trait.

RESULTS AND OBSERVATIONS

With application of Wilcoxon Signed Ranked Test, at 5% level of significance it was found that three bacteria namely T.d, P.g, A.a were found to show significant reduction after scaling and root planning whereas T.f did not show significant reduction after scaling and root planing (Tables 1-3). The study sample consisted of 30 patients. On pretreatment evaluation out of 30 patients T.d was positive in 17, A.a was positive in 10, P.g was positive in 25 and T.f was positive in 14 patients. On post treatment evaluation there was a reduction in number of initially positive cases in all the four bacteria. In case of T.d, number of patients were reduced from 17 to 2 (88.2%), in case of A.a patients were reduced from 10 to 0 (100%), in case of P.g positive patients were reduced from 25 to 12 (52%) and in case of T.f patients were reduced from 14 to 3(78.5%) (Graphs 1-3).

DISCUSSION

The initiation and progression of periodontal disease is attributed to presence of

elevated levels of pathogenic bacteria within gingival crevice⁸. Socransky et al and Mullally BH et al demonstrated that species such as Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Tannerella forsythia and Treponema denticola routinely occur together in the subgingival biofilm⁹. Microorganisms associated with periodontitis can be detected by various techniques like cultural techniques, immunofluorescence and ELISA. Bacterial culturing has been the classic diagnostic method widely used in the study of the composition of dental plaque and is still generally used as the gold or primary standard when determining the utility of a new microbial test in periodontal microbiology^{10,11}. However, culture techniques have many methodological problems when used in oral microbiology and attempts to culture anaerobic bacteria from periodontal pockets result in a significant underestimation of the quantity of these bacteria. Finally, the cultures require specific laboratory equipment, experienced personnels are labour is intensive, expensive, time-consuming and need a prolonged period before results can be obtained. According to Loesche et al¹², T.forsythia is uncultivable or extremely difficult to cultivate.

PCR is extremely sensitive, being able to detect even one copy of the searched DNA target and does not require rigorous conditions for transport of samples from the clinical department to the laboratory¹⁰. Riggio et al in¹³ reported that PCR is a powerful diagnostic tool that can detect low numbers of periodontal pathogens with a high degree of accuracy in subgingival plaque samples. It is rapid with results being available within hours of sample acquisition, cheaper and less labour intensive than conventional culture methods and permits many more samples to be easily screened at one time¹⁴.

For PCR, sample material like GCF, saliva and subgingival plaque have been used for detection of periodontopathogenic bacteria. Disadvantage of collecting subgingival plaque is that it has to be collected from all the sites where pockets are present and moreover it is difficult to collect as compared to saliva. In comparison to plaque and GCF the collection of saliva is easier. It establishes direct contact between four ecosystems, which are the important reservoirs of oral infection¹⁵. It is a suitable sample material for large scale oral microbiological studies utilizing PCR based assay which offer a labor minimizing technique^{16,17}.

CONCLUSION

Quantity of three bacteria (T.d, A.a, P.g) were reduced significantly after scaling and root planning as compared to T.f which was not significantly reduced. Above mentioned conclusion implies that, mechanical therapy like scaling and root planing is effective in treating chronic periodontitis. Also, saliva can be used as a diagnostic tool for detection of bacteria by using PCR analysis.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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