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# COMPARATIVE MICROBIOLOGIC ANALYSIS OF SUBGINGIVAL PLAQUE SAMPLES IN TYPE II DIABETIC AND NON – DIABETIC PATIENTS WITH CHRONIC PERIODONTITIS BY POLYMERASE CHAIN REACTION

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## ABSTRACT

**Background:** Although Immuno inflammatory relationship between periodontal diseases and diabetes mellitus is acknowledged, the difference in putative periodontal microorganisms between diabetic and non diabetic individuals is not well established. **Aim:** To compare the prevalence of two putative periodontal pathogens namely ***Aggregatibacter actinomycetemcomitans*** and ***Porphyromonas gingivalis*** in Type II Diabetic and Non Diabetic patients with chronic periodontitis by Polymerase chain reaction

**Materials and Method:** Sixty subjects were selected from the Department of Periodontics, Tamilnadu Government Dental College and Hospital, Chennai – 03. 30 Type II diabetic patients with chronic periodontitis were categorized as Group I and 30 Non diabetic patients with chronic periodontitis were categorized as Group II based on American Dental Association classification 1997 and American Academy of Periodontology classification 1999. Two sites- 1 healthy site and 1 diseased site were chosen in each patient, Group I H, II H – healthy site samples and Group I D, II D- diseased sites samples. Subgingival plaque was collected, DNA isolation was done & the presence of ***A.actinomycetemcomitans*** & ***P.gingivalis*** DNA was determined by PCR. The PCR products were sequenced and confirmed. The data was statistically analysed. **Results:** ***A.actinomycetemcomitans*** was detected in 6.7 %, 6.7%, 13.3%, 10% in Groups I H, II H, I D, II D respectively. ***P.gingivalis*** was detected in 40%, 46.7%, 46.6%, 53.3% in Groups I H, II H, I D, II D respectively. When comparisons were made between Groups I H & II H and Groups I D & II D for the two organisms, no statistically significant difference was obtained

**Conclusion:** The present study shows no statistically significant difference in the prevalence of ***A.actinomycetemcomitans*** and ***P.gingivalis*** in Type II Diabetic and Non Diabetic patients with chronic periodontitis.

**Keywords**: Aggregatibacter actinomycetemcomitans, Porphorymonas gingivalis, Diabetes, Periodontitis, Polymerase chain reaction

## INTRODUCTION

Chronic inflammatory periodontal disease (periodontitis) is primarily an anaerobic Gram negative oral infection that leads to gingival inflammation, destruction of periodontal tissues, loss of alveolar bone and eventual exfoliation of teeth in severe cases [11]. Certain organisms within the microbial flora of dental plaque are the major etiological agents of periodontitis.

Traditional thinking / paradigms have maintained that periodontitis is an oral disease

and that the tissue destructive response remains localized within the periodontium. Whereas studies by **Cohen DW et al 1970[4]**, **Mattila KJ et al 1989[8]** have indicated that periodontitis may produce a number of alterations in systemic health.

Diabetes mellitus is a metabolic disorder characterized by altered glucose tolerance or impaired lipid and carbohydrate metabolism [1]. It has been suggested that a positive correlation exists between diabetes and periodontal destruction based on the fact that loss of periodontal attachment occurs more frequently and more extensively in moderately and poorly controlled diabetic patients than those under good control.

Diabetes mellitus influences prevalence and severity of periodontal disease. Although host immune inflammatory response plays an important role, it is the microflora that's proved to be the etiological agent in periodontitis

**AIM**

The aim of the present study was to compare the prevalence of two putative periodontal pathogens namely ***Aggregatibacter a**ctinomycetemcomitans*** and ***Porphyromonas gingivalis*** in Type II Diabetic and Non Diabetic patients with Chronic periodontitis by Polymerase chain reaction.

## MATERIALS AND METHOD

**SUBJECT SELECTION**

Sixty subjects were screened and selected from the out patient Department of Periodontics, Tamilnadu Government Dental College and Hospital, Chennai – 600 003. 30 Type II diabetic patients with chronic periodontitis were categorized as Group I (Study group) and 30 Non diabetic patients with chronic periodontitis patients were categorized as Group II (Control group). Within each group sub categorization was done as follows:

Group I H - 30 Healthy sites of Type II Diabetic patients with chronic periodontitis
Group I D - 30 Diseased sites of Type II Diabetic patients with chronic periodontitis
Group II H - 30 Healthy sites of Non Diabetic patients with chronic periodontitis
Group II D - 30 Diseased sites of Non Diabetic patients with chronic periodontitis

The criteria for selection of patients with Type 2 Diabetes was based on American Diabetes Association classification (1997)[1] and for Chronic periodontitis and Chronic periodontitis modified by Diabetes, the American Academy of Periodontology classification (1999)[2] was utilized.

**INCLUSION CRITERIA**

- Age 30 – 60 yrs, Either sex, At least 3 sites with PPD ≥ 7 mm, CAL >1mm, At least 2 sites with PPD ≤ 3mm, Type 2 Diabetic patients (Group I), Systemically healthy individuals (Group II)

**EXCLUSION CRITERIA**

- Patients with systemic disease other than Type 2 diabetes(Group I), Patients with systemic disease (Group II), Antibiotic therapy for the past 6 months, Smokers, Periodontal therapy for the past 1 year

Following Institutional Ethical Committee Approval, selection of subjects was done. Informed consent was obtained and a thorough medical and dental history was taken. Intra-oral examination was done using mouth mirror and William's periodontal probe. Periodontal evaluation was done by measuring the Plaque Index, the Gingival Bleeding Index, Probing Pocket depth( PPD) and Clinical Attachment level(CAL).

**Collection of subgingival plaque sample and polymerase chain reaction**

Two plaque samples were taken from the most diseased site and a healthy site from each patient with individual sterile Gracey curettes. The plaque was dislodged into a vial containing

200µl of sterile lysis solution (10mm tris, 1.0 mm EDTA , 1.0% Tris X – 100, pH 78), sealed and stored at -20$^{o}$C. Subgingival plaque samples was boiled for 10min, cooled to room temperature, centrifuged at 10,000 rpm for 3 min and the supernatant was stored at -20$^{o}$C till assay. 10µl of the supernatant was directly used as template in PCR.

**Primers utilized in this study**

**Aggregatibacter actinomycetemcomitans (Aa)**

Forward primer : 5' CAGCAAGCTGCACAGTTGCAAA – 3'

Reverse primer : 5' CATTAGTTAATGCCGGGCCG TCT – 3'

**(Kraig E et al / Infect Immun 1900, 58 : 920 – 929)**

**Porphyromonas gingivalis (Pg)**

Forward primer : 5' ATAATGGAGAACGCAGG AA -3

Reverse primer : 5' - TCTTGCCAACCAGTTCCA TTGC – 3'

**(Dickinson et al / J Bacteriol 1998 ; 170 ; 1658 – 1665**)

10 µl of the PCR master mixture was pipette into micro centrifuge tubes. 1.0 µl of forward primer, 1.0 µl of reverse primer, 3.0 µl of template DNA and 5.0µl of nuclease free water was added and mixed thoroughly. The micro centrifuge tubes were placed in thermocycler and cycling conditions were set. PCR was performed for 35 cycles of Denaturation at 95$^{o}$C for 1 min, Primer Annealing at 55$^{o}$C for 30 sec, Primary extension at 72$^{o}$C for 1 min, Final extension was 72$^{o}$C for 10 min. The PCR product was detected by 2% Agarose gel electrophoresis. After the completion of the electrophoresis, gel was taken to the transilluminator and observed under UV-light. (Biorad gel documentation)

The PCR product of 238 bp ***A.actinomycetemcomitans*** and131 bp ***P.gingivalis*** were given to **MWG – BIOTECH, GERMANY** for sequencing the PCR products by automated DNA sequencer. The data was collected and statistically analyzed.

**STATISTICAL ANALYSIS**

Pearson's chi square test was used to calculate the overall p value

The statistical package SPSS V18 (Statistical Package for Social Science, Version 18) was used for statistical analysis. In the present study, **< 0.05** was considered as the level of significance.

## RESULTS

In the present study ***A.actinomycetemcomintans*** was detected in 6.7% Type II diabetic patients with chronic periodontits -healthy sites (2 out of 30 healthy sites), 13.3% Type II diabetic patients with chronic periodontits -diseased sites(4 out of 30 diseased sites), 6.7% Non diabetic patients with chronic periodontits - healthy sites (2 out of 30 healthy sites) and10.0% Non diabetic patients with chronic periodontits -diseased sites(3 out of 30 diseased sites).

In the present study ***P.gingivalis*** was detected in 40% Type II diabetic patients with chronic periodontits - healthy sites (12 out of 30 healthy sites, 46.6% Type II diabetic patients with chronic periodontits -diseased sites( 14 out of 30 diseased sites), 46.7% Non diabetic patients with chronic periodontits - healthy sites(14 out of 30 healthy sites) and 53.3% Non diabetic patients with chronic periodontits -diseased sites (16 out of 30 diseased sites

## DISCUSSION

The clinical parameters used in this study were Gingival Bleeding Index, Plaque Index, Probing Pocket Depth and Clinical Attachment level similar to the study by Yuan K et al 2001[13.] Earlier studies employed curettes or paper points for subgingival plaque collection. Sampling by paper point is less invasive than by curette but may result in an underestimation of tightly

adherent bacteria in subgingival sites[5, 12].Hence in this study curettes were used for sample collection.

Although various diagnostic techniques are available to analyse the microbial population of subgingival plaque, PCR technique was opted as it can detect organisms of less than 100 cells[3]. In the present study, PCR procedure followed by Yuan et al 2001[13] was used. Mandel et al 1990[7] detected 7% *A.actinomycetemcomitans*, 13% *P. gingivalis* in diseased sites of NIDDM patients by culture. Zambon et al 1988[13] employed culture and ELISA assays and detected *P.gingivalis* in 75% NIDDM subjects. In the present study, 13.3% and 46.6% diseased sites in Type II Diabetic patients with chronic periodontitis and 10.0% and 53.3% of diseased sites in Non Diabetic patients with chronic periodontitis were positive for *A.actinomycetemcomitans* and *P.gingivalis* respectively.

Our microbiological data revealed higher detection rates when compared with the results of others (except Zambon et al). This may be due to the higher sensitivity of PCR, PCR is more sensitive than culture, immunofluroesence and DNA probes for which sensitivities are 2 x $10^2$, 2 x $10^5$, 2 x $10^4$, 2 x $10^3$ respectively[6,14]. In a PCR study by Yuan et al 2001[13], 6.7% and 64.8% of diseased sites in Type II diabetic subjects and 5.7% and 66.7% of diseased sites in Non diabetic patients were positive for *A.actinomycetemcomitans* and *P.gingivalis* respectively. The discrepancy in results, may be because a large sample size of 150 patients was chosen by Yuan et al 2001[13] when compared to the small sample of 30 patients chosen in this study. Also, different authors have analyzed the microorganism distribution in different population and races.

When comparing the prevalence rates of *A.actinomycetemcomitans* and *P.gingivalis*, the results in our study showed a lower detection rate for *A.actinomycetemcomitans* which is in concurrent to the study by Yuan K et al 2001[13]. This may be explained by the conclusion drawn from the studies by Rhodenburg JP et al 1990[9] , Slots J et al 1990[10] that the prevalence of *A.actinomycetemcomitans* was age related and decreased with increasing age. *A.actinomycetemcomintans* is more responsible for aggressive periodontitis whereas *P.gingivalis* contributes to chronic periodontitis. Since our subjects were all adults beyond 30 years of age, it is speculated that the contribution of *A.actinomycetemcomitans* to the periodontitis we examined was minimal. There was no statistically significant difference in the detection rates of the 2 tested microorganisms

The plausible reasons are

1) There was no difference in the contribution of the microbiological pathogens in patients with Type II diabetic patients with chronic periodontitis and in Non diabetic patients with chronic periodontitis.
2) The PCR assay is limited in the ability to differentiate large or small amounts of the same pathogen.
3) Other microflora rather than our targeted microorganism such as *P.intermedia*, *C.rectus* and *Capnocytophaga* species (since they are also regarded as important pathogen in periodontitis of NIDDM patients[13] )could be an etiological agent in Type II diabetic patients with chronic periodontitis and Non diabetic patients with chronic periodontitis.

## CONCLUSION

The search for the etiologic agent of periodontal diseases has been in progress for over a century. In this study it was found that the composition periodontal microflora in periodontal disease sites of Type II diabetic patients with chronic periodontitis was similar to that found in non diabetic patients with chronic periodontitis.

However, the significantly higher detection rate of ***P.gingivalis*** in diseased sites further confirms the possible pathogenic role of this bacteria for both groups studied. ***A.actinomycetemcomintans*** may not be a causative pathogen in Type II diabetic patients with chronic periodontitis and non diabetic patients with chronic periodontitis.

Also, PCR assay provides only a binary results i.e it detects the presence/absence of the microorganism and cannot differentiate positive result s quantitatively. Therefore the difference may exist in the quantitative composition of periodontal microorganism present in Type II diabetic patients with chronic periodontitis and non diabetic patients with chronic periodontitis. Hence further studies with a large sample size and diagnostic technique to quantitatively analyse the composition of microorganisms may be needed.


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**Table 1: Comparison of Distribution of Aa in Healthy Sites among Type II Diabetic and Non Diabetic patients with Chronic periodontitis**

| Aa | Diabetic Healthy n = 30 | | | | Non Diabetic Healthy n = 30 | | | |
|---|---|---|---|---|---|---|---|---|
| | Present | | Absent | | Present | | Absent | |
| | Count | % | Count | % | Count | % | Count | % |
| | 2 | 6.7% | **28** | **93.3%** | **2** | **6.7%** | **28** | **93.3%** |
| **P value** | **0.64 ( not significant )** | | | | | | | |

**Table 2: Comparison of Distribution of Pg in Healthy Sites among Type II Diabetic and Non Diabetic patients with Chronic periodontitis**

| Pg | Diabetic Healthy n = 30 | | | | Non Diabetic Healthy n = 30 | | | |
|---|---|---|---|---|---|---|---|---|
| | Present | | Absent | | Present | | Absent | |
| | Count | % | Count | % | Count | % | Count | % |
| | 12 | 40% | **18** | **16%** | **14** | **46.7%** | **16** | **53.3%** |
| **P value** | **0.301 ( not significant )** | | | | | | | |
| | | | | | | | | |

**Table 3: Comparison of Distribution of Aa in Diseased Sites among Type II diabetic and Non Diabetic patients with Chronic periodontitis**

| Aa | Diabetic Diseased n = 30 | | | | Non Diabetic Diseased n = 30 | | | |
|---|---|---|---|---|---|---|---|---|
| | Present | | Absent | | Present | | Absent | |
| | Count | % | Count | % | Count | % | Count | % |
| | 14 | 13.3% | **26** | **86.7%** | **3** | **10%** | **27** | **90%** |
| **P value** | **0.12 ( not significant )** | | | | | | | |

**Table 4: Comparison of distribution of Pg in diseased sites among Type II Diabetic and Non Diabetic patients with Chronic periodontitis**

| Pg | Diabetic Diseased n = 30 | | | | Non Diabetic Diseased n = 30 | | | |
|---|---|---|---|---|---|---|---|---|
| | Present | | Absent | | Present | | Absent | |
| | Count | % | Count | % | Count | % | Count | % |
| | 14 | 46.6% | **16** | **53.3%** | **16** | **53.3%** | **14** | **46.6%** |
| **P value** | **0.24 ( not significant )** | | | | | | | |

**Fig.1 : Distribution of Aa among Groups I H, II H**

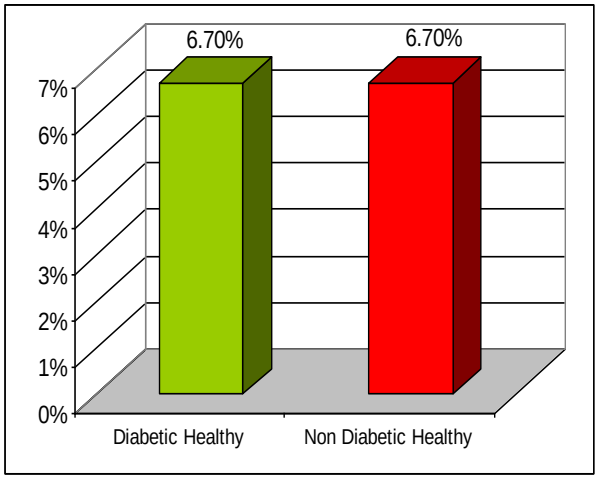



**Fig.2 : Distribution of Pg among Groups I H, II H**

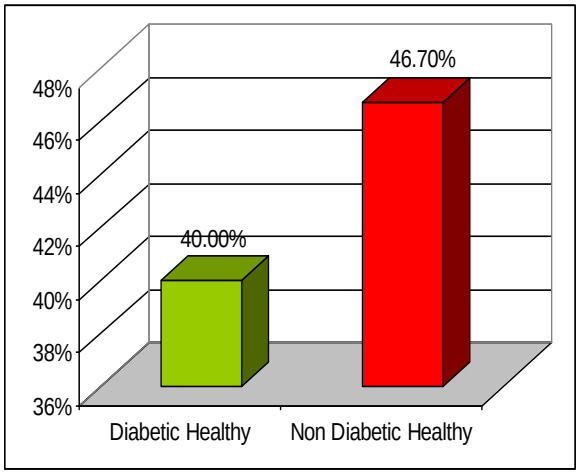



**Fig.3 : Distribution of Aa among Groups I D, II D**

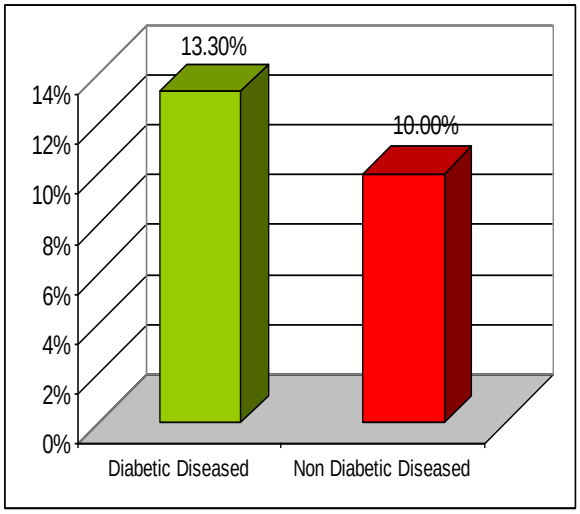
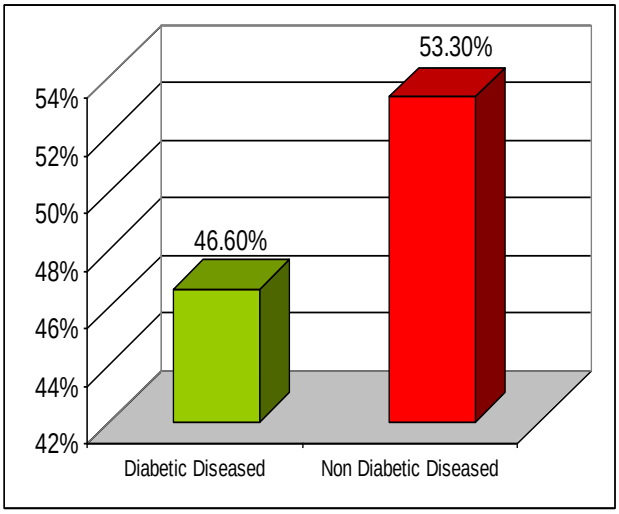
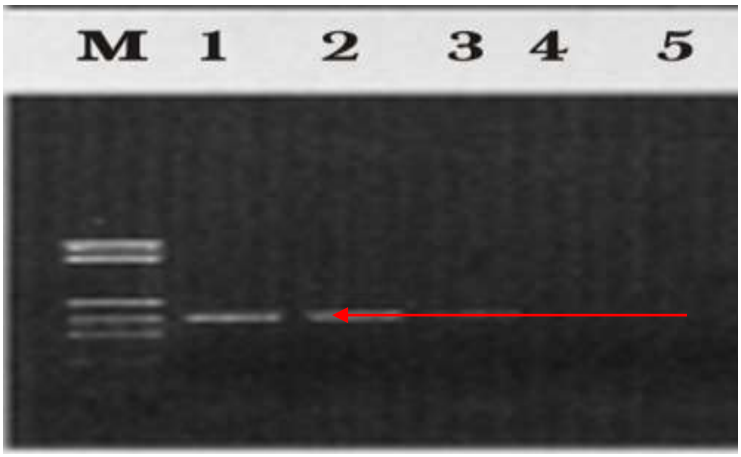
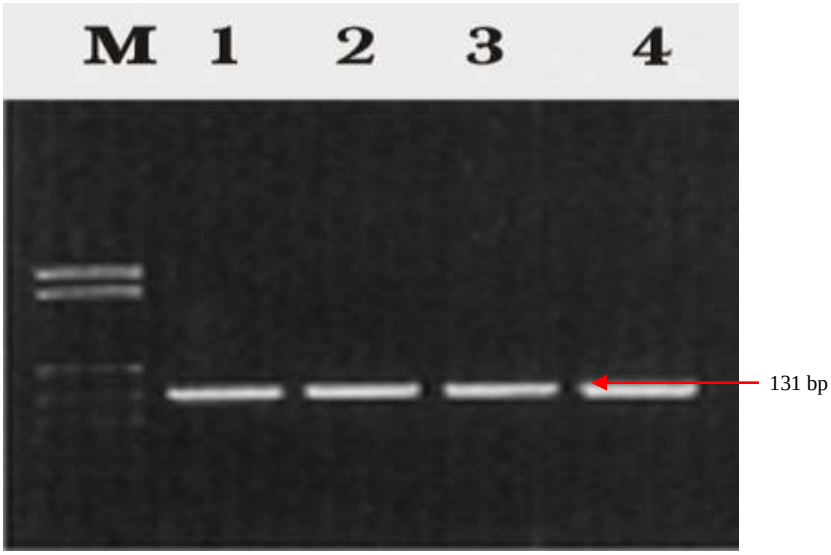



**Fig.4 : Distribution of Pg among Groups I D, II D**






**Photograph No.1: Electrophoresis showing the amplified product of *A.actinomycetemcomitans*M – 100 bp Ladder Lane 1, 2 – Positive for *A.actinomycetemcomitans* (238 bp) Lane 3, 4 – Negative for *A.actinomycetemcomitans* Lane 5 Blank Control**




**Photograph No.2: Electrophoresis showing the amplified product of *P.gingivalis* M – 100 bp Ladder ,Lane 1, 2, 3, 4 – Positive for *P.gingivalis* (131 bp)**