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ASSOSCIATION OF ANAEROBIC BACTERIA IN SUB GINGIVAL PLAQUE AND ORAL HEALTH OF WOMEN

Kanakam Elizabeth Thomas* and Dr. S. Savithri[1]

*Lecturer, Dept. of Microbiology,
[1]Professor Dept. of Microbiology SRM Dental College, Chennai, India.

***Correspondence for Author: Kanakam Elizabeth Thomas**
Lecturer, Dept. of Microbiology, SRM Dental College, Chennai, India.

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ABSTRACT

Aim: To determine the prevalence of periodontitis in women, covering comprehensively the entire life cycle of women, namely pre pubertal, pubertal, post pubertal, child bearing age, pregnancy and menopause and to confirm the identity of seven obligate anaerobes with Real Time PCR.

1. *Aggregatibacter actinomycetomcomitans*
2. *Porphyromonas gingivalis*
3. *Treponema denticola*
4. *Tannerrella forsythia*
5. *Capnocytophaga gingivalis*
6. *Fusobaacterium nucleatum*
7. *Prevotella nigerescens*

Material and Method: The periodontal status of each patient was determined with the help of a periodontition by calculating the CAL (Clinical attachment Level) and Probing depth. Sub gingival plaque was collected with the help of Wilsons probe. The plaque sample was subjected to Real Time PCR. ***Results:*** It was found that there was an increase in the percentage of periodontitis in women at Puberty, post puberty, pregnancy and at menopause. In our study we found that there is a higher prevalence of *Tannerella forsythia* in the current study population when compared to the other obligate anaerobes. ***Discussion and summary:*** In reports from American academy of Periodontology, it is pointed out that anaerobic bacteria like *Porphyromonas gingivalis*, *Prevotella* and *Tannerella forsythia* are the etiological agents for periodontitis. Similar results were found from studies done by Leonhardt, Jensen et al and Kamma et al,. In our study we found that *T.forsythia* was more prevalent when compared to the other anaerobic bacteria. The presence of these anaerobic bacteria in the subgingival plaque of our study population suggests the presence of periodontitis among women at puberty, pregnancy and at menopause.

KEYWORDS: *Porphyromonas gingivalis*, *Prevotella* and *Tannerella*.

INTRODUCTION

Oral Health of women has a strong influence on the overall health of her. Various studies around the world have pointed out women who had a weak oral health indicate her general health status. The etiological agent of oral diseases is mainly anaerobic bacteria that reside in the oral cavity of human beings. They play a major role in the formation of gingivitis and periodontitis.

Periodontitis

Periodontitis is a highly prevalent oral disease which is seen in 90% of the population. Periodontitis is defined as an apical extension of gingival inflammation, which involves the tissues supporting the tooth, including the periodontal ligament and the bone. A periodontal pocket is formed as a result of the destruction of the fiber attachment. The supra gingival plaque now invades into sub gingival area. This leads to multiplication of the anaerobic bacteria.[1-3]

TYPES OF PERIODONTITIS

Gingival Diseases

Milder form of periodontitis is known as gingivitis. Gingivitis can be of two types. Plaque Induced Gingivitis and Non-plaque Induced Gingivitis.

Aggressive Periodontitis

Aggressive type of periodontitis is usually seen in clinically healthy patients. Rapid detachment of the gums along with bone destruction is characteristic feature of this type of periodontitis. Other features include presence of *Aggregatibacter actinomycetomcomitans* and *Porphyromonas gingivalis* or either one. Higher concentrations of Prostaglandin E2 and Interleukin 1β are other feature of aggressive Periodontitis.[4]

Localized Aggressive Periodontitis
Onset of Localised Aggressive Periodontitis is around Puberty. There is a high antibody response at this stage. Attachment loss is seen at the site of two permanent teeth mostly around the incisors and or first molars.[5]

Generalised Aggressive Periodontitis
Generalised Aggressive Periodontitis is seen in patients above 30 years. Antibody response is very poor. Atleast three permanent teeth are affected other than the incisors.[5]

Chronic Periodontitis
There is a deep progression of lesion into the tissues. This condition is always associated with periodontal pockets. This type of periodontitis is a commonly seen in adults. Gingival hypertrophy or gingival recession, suppuration and bleeding on probing are the main symptoms of chronic periodontitis.

If less than 30% of the site is involved then it is classified as Localised chronic periodontitis. If more than 30% of the site is involved then it is classified as Generalized Chronic periodontitis. Depending on the severity of the infection they are classified into Slight Periodontitis, Moderate periodontitis and Severe periodontitis.[6]

Necrotizing Periodontitis
Necrosis of gingival tissues and alveolar bone is seen at this stage. The main symptoms are ulcerated papillae with bleeding and pain. This is accompanied by fever, malaise, halitosis and lymphadenopathy.

Necrotizing periodontitis is seen among Immunosuppressed and malnourished patient. The main difference between Necrotizing Ulcerative Gingivitis (NUG) and Necrotizing Ulcerative Periodontitis (NUP) is that in the former only the gingivitis is involved where as in the latter case the whole tissue and bones surrounding the teeth is involved.[7-8]

Porphyromonas gingivalis
These organisms are Gram negative anaerobic non motile bacilli. These bacteria are capable of producing black or brown pigment. These bacteria come under the group of Bacteroides. *P.gingivalis* is able to inhibit PMN migration across the epithelial cells. About 4-6% of the cultivable flora is constituted by pigmented species of anaerobic bacteria.[9] Various studies show that the presence of *P.gingivalis* is lesser in number or absent in healthy subjects, where as frequency of isolates increase in the case of destructive periodontitis.[10] Kawada points out that the presence of *P.ginigvlais* is directly proportionate to the pocket depth.[11] In another report by Kamma *et al* increasing frequency of isolates was reported in the case of deteriorating periodontitis and also in progressive periodontal disease.[12]

P.gingivalis is capable of elevating the immunological response in patients with different types of periodontitis.[13] In observations made by Ogawa *et al* showed that in the plasma cells from chronic periodontitis 5% antibodies belonged to the fimbriae of *P.gingivalis*. In many studies on the antibodies against the antigens of *P.ginigvalis* showed higher levels in some subjects with detached periodontal tissues but no in all. This suggests that *P.gingivalis* would have entered the periodontal tissues and would have initiated Periodontitis.[14]

Aggregatibacter actinomycetomcomitans
Aggregatibacter actinomycetomcomitans was earlier known as *Actinobacillus actinomycetomcomitans*. These bacteria are non motile Gram negative small rods. When grown on blood agar they are able to grow as star shaped centered small convex colonies. This was considered as an important periodontal pathogen due its high isolation rate from periodontic lesions both in aggressive periodontitis as well as from localized periodontitis.[15] *A.actinomycetomcomitans* is considered to be a part of the commensal flora of the oral cavity especially in the supragingival and gingival crevices. These bacteria are divided into 6 serotypes a-f among these serotypes a,b, and c are the predominant ones.[16]

The bacteria initially attach to the epithelial cells of the oral cavity and colonize. *A. actinomycetomcomitan*s has an adhesion and with the help of which, they attach to the carbhohydrate receptor present on the buccal cavity. Fimbriae along with the carbohydrate polymer attach to the hard surface. From the supragingival area they move to the subgingival environment. From here they can attach and invade the epithelial cell of the periodontal tissue and penetrate the connective tissues.[17]

Prevotella intermedia/Prevotella nigrescens
P.intermedia is the second most important black pigment producing Bacteroides which is a major etiological agent of periodontitis. *P.intermedia* is gram negative rods with rounded ends. These organisms are mainly isolated from (NUG) Necrtotizing Ulcerative Gingivitis.[18] According to Williams *et al Prevotella* sp. constitute about 30% of the cultivable oral microflora. About 10-20% of the isolated anaerobic bacteria is made up of the pigmented *Prevotella* species and the non pigmented strains constitute 30% from almost all cases of periodontitis.[19]

Treponema denticola
Treponema denticola is spirochete. They are Gram negative helically shaped anaerobic bacilli. These bacteria are also commonly present in periodontal pockets. *T.denticola* has been seen in elevated proportions from tissue biopsy from the affected area.[20]

Riviere *et al* used monoclonal antibody detect spirochetes that were present in supra and sub gingival plaque and those present in NUG. “Pathogen Related Oral Spirochetes” were mostly isolated from

periodontitis patients.[21] Spirochetes prolong wound healing and delays tissue remodeling after periodontal treatment procedure. Hence it is an ever healing wound that is seen during chronic periodontitis.[22]

Tannerella forsythia

T.forsythia are fusiform Bacteriodes. These are the third commonly present periodontal pathogen. These bacteria are very difficult to cultivate as it requires 7-14 days for the colonies to appear. They are gram negative spindle shaped bacilli and are highly pleomorphic bacteria.[23] The growth is enhanced by co cultivation along with *Fusobacterium nucleatum.* N-acetyl muramic acid enhances the growth and bacteria appear in short rods instead of pleomorphic.[24]

According to Hamlet *et al* adolescents whose gingiva harbored *T.forsythia* had had a greater risk for periodontal attachment loss when compared to whom this species was not detected.[25] It was also noted that those subjects who had colonization of *T.forsythia* had greater chances of loss of attachment, alveolar bone loss and tooth loss than those who do not have *T.forsythia*.[26]

Fusobacterium nucleatum

F.nucleatum a common bacteria that is frequently isolated from the periodontal pockets. They are spindle shaped gram negative bacilli. They comprise approximately 7-10% of the total isolate from the sub gingival plaque. These bacteria facilitate apoptotic cell death in both mononuclear and polymorphonuclear cells.[27]

There was a high titre of Immunoglobulin IgG and IgM against the Lipopolysaccharide of *F.nucleatum* in the serum of subjects with periodontitis. These bacteria are able to induce release of cytokines, oxygen radical and elastase from the leukocytes.[28]

Eikenella corrodens

E.corrodens is a capnophillic Gram negative small bacilli with blunt ends. *E. corrodens* is mostly isolated from sites of periodontal destructions. They are able to stimulate secretion of metalloproteinases and interleukin 6 and interleukin. These bacteria are mainly isolated from subjects with Localized Aggressive Periodontitis.[29]

Peptostreptococcus micros

These are Gram Positive anaerobic cocci. *Peptostreptococcus* is associated with oral infections. According to Tew *et al* level of serum antibodies is higher in Generalized Aggressive Periodontitis when compared to healthy subjects and Localized Aggressive periodontitis.[30]

MATERIAL AND METHOD

The samples for the study were collected from the Department of Periodontology and Oral Implantology, SRM Dental College and SRM Multi Specialty Hospital Ramapuram, Chennai. Microbiological analysis of the samples was done in the Dept. of Microbiology SRM Dental College, Ramapuram Chennai.

Specimen Collection

A total number of 430 subjects were taken up for the study. Women who are at puberty, pregnancy, fertile stage, menopause and women who are using Oral contraceptives were included in our study. Patients who had had antibiotics in a period of 6 months and who had less than twenty teeth were not included.

Patients with any current systemic diseases such as Downs syndrome, diabetics etc., which might influence either the oral microbial flora or host response or modify the progression of their periodontal disease were also excluded from the current study.

Ethical Clearance for the study was obtained from SRM University Ethical Committee. Ethics Clearance Number for the current study is *93/IEC/2011*.

Subgingival plaque from the patients were collected with the help of a Wilsons probe. The plaque samples were stored in Eppendorff vials with saline under -20^{0}C for Real Time PCR.

Periodontal status of the patients were determined by Clinical attachment Level (CAL) and the probing depth (PD) with the help of a periodontition. The subjects were categorized into those having normal ginigva, gingivitis and periodontitis.

REAL TIME PCR CONFIRMATION OF OBLIGATE ANAEROBES

Identification of the following seven obligate anaerobes was done by Real Time PCR method

1. *Aggregatibacter actinomycetomcomitans*
2. *Porphyromonas gingivalis*
3. *Treponema denticola*
4. *Tannerrella forsythia*
5. *Capnocytophaga gingivalis*
6. *Fusobaacterium nucleatum*
7. *Prevotella nigerescens*

The primers for the anaerobic bacteria were designed by Hayashi *et al* and Ashimoto *et al*.[31] SYBR(R)GREN JUMPSTART TAQ READY MIX MASTER CLEAR was obtained from BIOCORPORALS, Chennai, and from Bio Source and Surgicals, Chennai. Real Time PCR study was carried done at the Dept. of Human Genetics, Sri Ramachandra University, Chennai, India.

Chromosomal DNA Extraction by Phenol/Chloroform Extraction of DNA

Reagents Required

- Lysozyme, Tris -1M, EDTA – 0.5M, Sodium dodecyl Sulphate (SDS) (10%), Phenol, Chloroform, Isoamyl alcohol, Sodium acetate.

The collected sub gingival plaque samples were centrifuged at 7000 rpm for 5min.The supernatant was discarded. Lysis solution was prepared as follows
1 gm of lysosome powder was mixed with Tris HCL 1ml, EDTA 200 µl, TRITON600 µl andsterile water. Milli Q was added to make the solution up to 40ml and then vortexed for 5 min. 1ml of this solution was added to each sample and incubated for 30 minutes at 37˚C. 20ul of SDS was added to the sample, vortexed again and incubated for 30 minutes at 37˚C. To this, equal volume of phenol, chloroform and isoamyl alcohol was added and centrifuged at 10,000 rpm for 10 minutes and the supernatant was transferred to new Eppendorf tubes. 1ml of Chloroform and isoamyl alcohol was added to the new tube and centrifuged at 10000 rpm for 10 min and the supernatant was transferred to new tube and $1/10^{th}$ volume of sodium acetate. Double the volume of absolute alcohol was added and centrifuged again at 10,000 rpm for 10 minutes. The supernatant obtained was discarded and 500µl of 70% ethanol was added to the pellet and centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and the pellets were air dried. To the dried pellet, 30 µl of sterile water was added and stored at 4^{o}C for further analysis.

Qualitative Analysis of DNA

Reagent and buffer for agarose gel electrophoresis

- TAE Buffer (Tris-acetate EDTA Buffer) (50X) (pH 7.2), Ethidium Bromide (10mg/ml), DNA Sample Loading Dye (6X) and Agarose Low EEO

The quality of the DNA samples was checked in 0.8% agarose gel. 0.8g of agarose was dissolved in 100ml of 1X TAE buffer by boiling. The solution was allowed to become lukewarm followed by which ethidium bromide was added to a final concentration of 0.1mg/ml. The gel was poured on a gel-casting tray and allowed to solidify. The gel was placed in an electrophoresis tank with 1X TAE buffer. The samples were mixed with bromophenol blue dye and loaded on the gel. The gel was electrophoresed at 2 volts/cm and was visualized in a gel documentation system.

Quantification of the Bacteria using Real Time PCR

The following reagents were used in this analysis: SYBR Green master mix and Forward and reverse primers. The individual primers, specific to each bacteria, are tablulated in the Table….. Commercially available SYBR®GREEN PCR master matrix 5µl was added along with 0.5 µl of forward primer and reverse primer each, 2.0µl of Template DNA and 2.0µl of sterile water. 10 µl of the prepared mix was dropped in micro wells and was subjected to Real Time PCR analysis.

Protocol for Real Time PCR amplification

Real time assays undergo 40 cycles of amplification. In a real time PCR assay, a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold i.e. to exceed the background level. Ct levels are inversely proportional to the amount of target nucleic acid in the sample i.e. the lower the Ct level, the greater would be the amount of target nucleic acid in the sample. Ct values < 29 are indicative of abundance of the target nucleic acid in the sample. Ct values in the range of 30-37 are indicative of moderate amounts of target nucleic acid. Ct values between 38-40 are weak reactions and are indicative of minimal amounts of target nucleic acid which could represent either an environmental contamination or infection state. Such a state would require clinical correlation.

Primer pairs 5'--3' for different anaerobic bacteria[31]

	Name of the Anaerobic bacteria		Nucleotide sequence
1a	*Aggregatibacter actinomycetomcomitans*	F	AAA CCC ATC TCT GAG TTC TTC TTC
1b	*Aggregatibacter actinomycetomcomitans*	R	ATG CCA ACT TGA CGT TAA AT
2a	*Capnocytophaga gingivalis*	F	AGA GTT TGA TCC TGG CTC AG
2b	*Capnocytophaga gingivalis*	R	GGA CGC ATG CCC ATC TTT CAC CAC CGC
3a	*Porphyromonas gingivalis*	F	AGG CGA CTT GCC ATA CTG CG
3b	*Porphyromonas gingivalis*	R	ACT GTT AGC AAC TAC CGA TGT
4a	*Treponema denticola*	F	TAA TAC CGA AGC TCA TTT ACA T TCA AAG TCT CTG
4b	*Treponema denticola*	R	TGG GCT GCG A
5a	*Tannerrella forsythia*	F	GCG TAT GTA ACC TGC CCG CA
5b	*Tannerrella forsythia*	R	TGC TTC AGT GTG AGT TAT ACC T
6a	*Fusobaacterium nucleatum*	F	CTG AAC ATT GGA AAC TAT ATA GTA GAA CAA ACA AG
6b	*Fusobaacterium nucleatum*	R	GTC CTT CAT CGG CTC TTA CTA CCT AGG C
7a	*Prevotella nigerescens*	F	ATG AAA CAA AGG TTT TCC GGT AAG
7b	*Prevotella nigerescens*	R	CCC ACG TCT CTG TGG GCT GCG A
8a	Universal Primer	F	GATTAGATACCCTGGTAGTCCAC
8b	Universal Primer	R	TACCTTGTTACGACTT

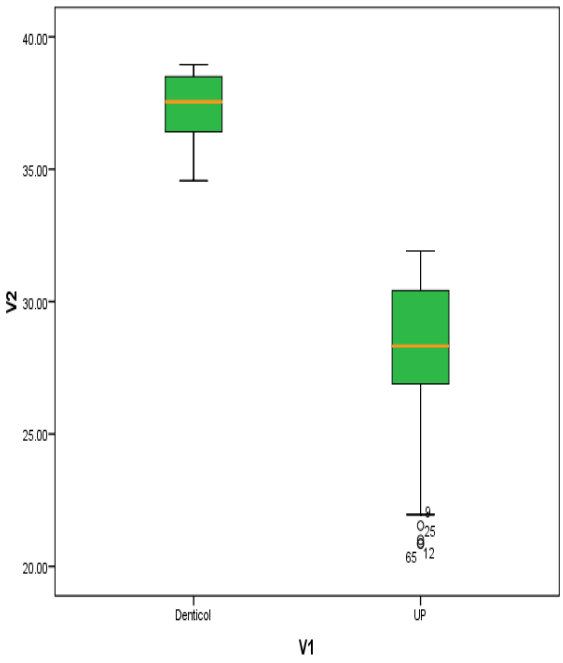
RESULTS

Real time PCR technique was employed to ascertain the presence of obligate anaerobes. Real time PCR with Universal primer was also employed for the semi quantification of the anaerobic bacterial load. The cycle threshold values of the obligate anaerobes were

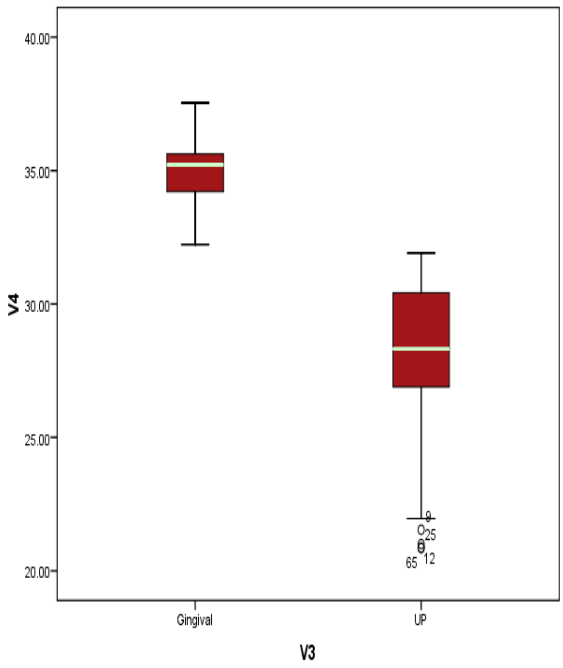
compared with the values of Universal primer, using *paired t test*. Seven anaerobic bacteria were found to have significant bacterial load.

Table 1: Comparison of the seven obligate anaerobes with Universal primer using paired *t test*

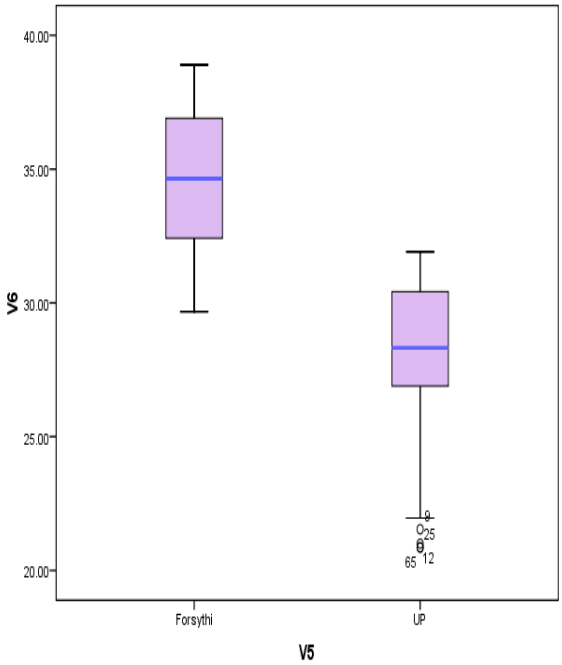
Name of Bacteria	Mean cycle threshold value	Std. Deviation	Paired t	*p value*
Treponema denticola	37.37	1.13351	39.639	0.00002
Universal Primer.	28.14	2.48393		
Porphyromonas gingivalis	34.94	.93251	27.389	0.00033
Universal Primer.	28.14	2.48393		
Tannerella forsythia	34.72	2.49218	21.386	0.00014
Universal Primer.	28.14	2.48393		
Prevotella nigrescens	37.59	.80071	41.657	0.00008
Universal Primer.	28.14	2.48393		
Agrregatibacter actineomycetomcomitans	37.80	1.04566	40.375	0.00026
Universal Primer.	28.14	2.48393		
Capnocytophagea gingivalis	36.33	.94834	33.568	0.00015
Universal Primer.	28.14	2.48393		
Fusobacterium nucleatum	35.04	1.33880	27.654	0.00012
Universal Primer.	28.14	2.48393		



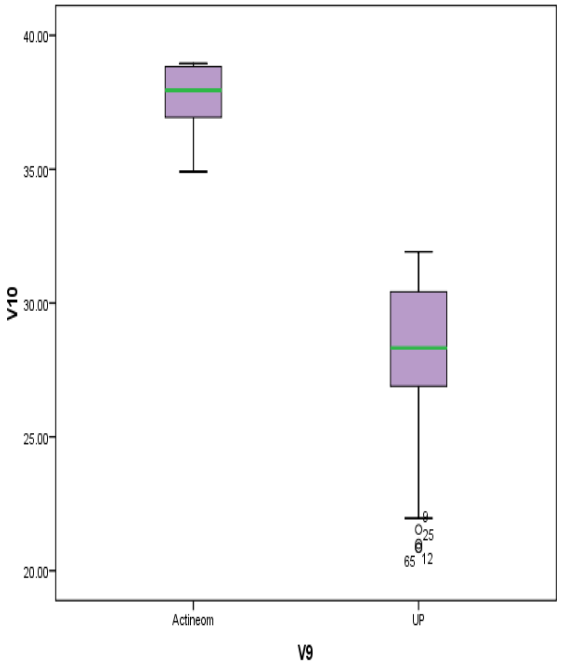
Box and Whiskers plot diagram for Universal PCR and *Treponema denticola*



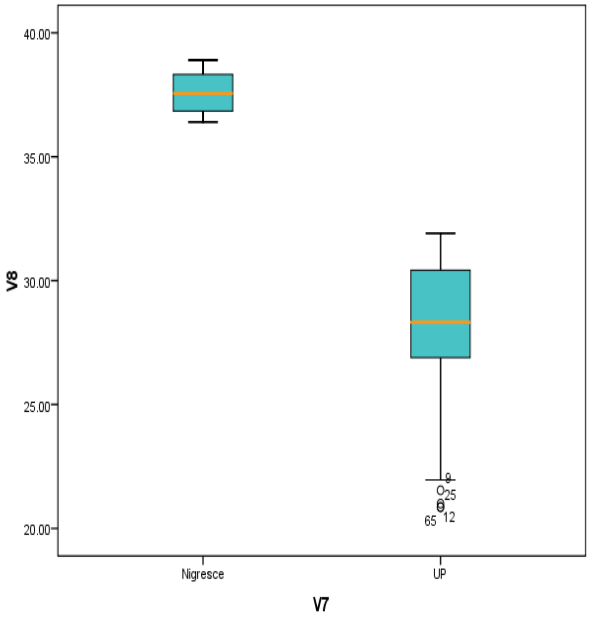
Box and whiskers plot diagram for universal PCR and *Porphyromonas gingivalis*



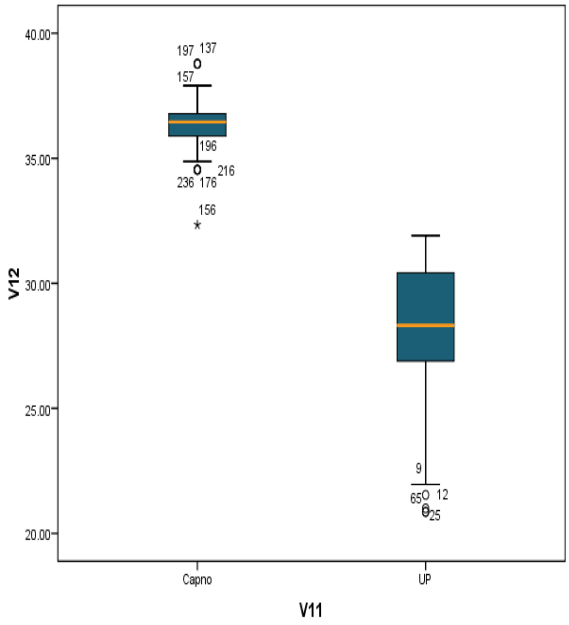
Box and Whiskers plot diagram for Universal PCR and *Tannerella forsythia*



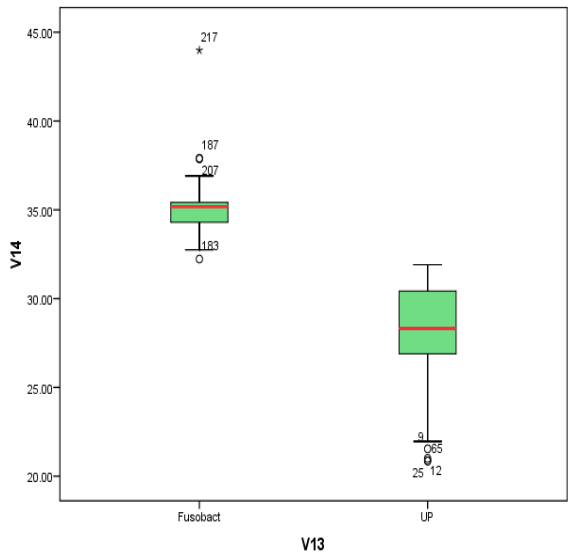
Box and Whiskers plot diagram for Universal PCR and *Aggregatibacter actinomycetomcomitans*



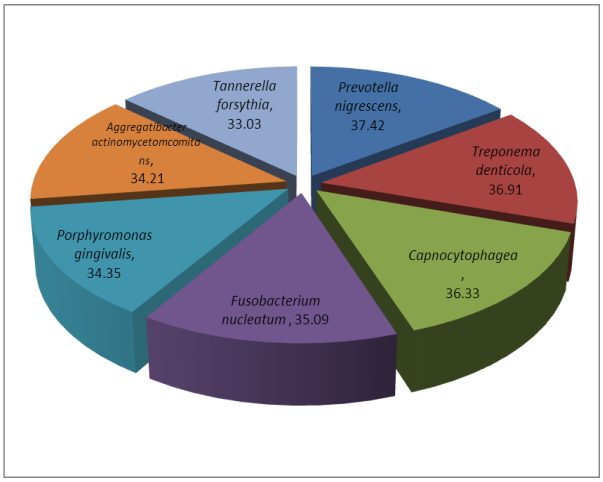
Box and whiskers plot diagram for universal PCR and *Prevotella nigrescens*



Box and whiskers plot diagram for universal PCR and *Capnocytophagea gingivalis*



Box and Whiskers plot diagram for Universal PCR and *Fusobacterium nucleatum*



Average cycle threshold values of obligate anaerobes

The average cycle threshold value indicates that *Tannerella forsythia* is found to be having the least cycle threshold value. This shows that *Tannerella forsythia* is in a higher proportion when compared with the other obligate anaerobes which were looked for. *Porphyromonas gingivalis* and *Fusobacterium nucleatum* showed an average cycle threshold value of 34.35 and 35.09.The highest average cycle threshold value is obtained for *Prevotella nigrescens.* The proportion of *Prevotella nigrescens* is found to be least in this study.

Comparison of median value for Universal primer and the cycle threshold values of the obligate anaerobes were plotted using Whisker box plot graph. From this it can be inferred that the Median of cycle threshold value of *Capnocytophagea gingivalis* was much higher than that of the Universal primer, when compared with the other anaerobic bacteria.

Presence of periodontitis at various stages in women

Table 2: Periodontitis among pubertal girls

Stage	n	Periodontitis present n	Percentage within the stage	Test of significance
Prepubertal	30	4	13	λ^2 19.477 p= 0.005
Pubertal	55	29	52.7	
Post pubertal	55	34	61.8	

An increase in the percentage of periodontitis is seen among pubertal and post pubertal stage when compared with pre pubertal subjects, which is statistically significant.

Table 3: Periodontitis among pregnant women

Stage of pregnancy	n	Periodontitis present Frequency (%)	Periodontitis not present Frequency (%)	Test of significance
Non pregnant women	60	16 (26.6)	44 (73.3)	λ^2 100.3846 p =0.0001*
First trimester	120	30 (32.5)	90 (75)	
Second trimester	120	105 (87.5)	15 (12.5)	
Post partum stage	120	78 (65)	42 (35)	

A comparative analysis of periodontitis within the various stages of pregnancy shows that the frequency of periodontitis is high among pregnant women in second trimester. During first trimester it can be noted that 30 out of 120 subjects had periodontitis. In second trimester there is an increase in the frequency of subjects with periodontitis to 105 out of 120. However during post partum stage, a decrease in periodontitis can be noted.

Table 4: Periodontitis among Menopausal women.

Stage	n	Periodontitis n(%)	Gingivitis n(%)	Normal gingival n(%)	Test of significance
Pre Menopausal	110	24(21.8%)	35(31.8)	51(46.3)	λ^2 25.33
Post Menopausal	110	88(80)	22(20)	---	p= 0.0001

DISCUSSION

Lynch and Beighton state that there is a progressive flora increase in the anaerobic bacterial flora during the progression of the disease and as a result the aerobes and the facultative anaerobes show a decrease.[32] As periodontitis develops, Moore and moore noted a shift in the balance of the normal microflora in the subgingival area.[33] In 2006, Daniluk et al noted that in the case of a healthy individual, the prevalence of Gram positive anaerobes such as *Streptococcus sp* along with some obligate gram positive anaerobes was present in a higher percentage. The Gram negative anaerobic bacteria that were consistently isolated from periodontic sites were *Porphyromonas gingivalis, Prevotella intermedia, Capnocytophagea,* some *Spirochetes* and *Aggregatibacter actinomycetomcomitans*.[2] In the current study we limited our findings to seven obligate anaerobes. In our study population however, we found that *Tanerrella forsythia* had a higher isolation frequency when compared to *Porphyromonas gingivalis, Prevotella intermedia* and *Capnocytophagea,*.

In our study, we found non pregnant women had less prevalence of periodontitis when compared with their pregnant counter parts. Within the pregnant category, an increase in periodontitis in the second trimester was present when compared with the First trimester and the post partum stages. Jensen *et al* had found a similar result. His opinion was that during pregnancy, the systemic levels of sex hormones showed an increase which can be correlated to the increase of some gram negative anaerobes in the oral cavity.[34]

American Academy of Periodontology consensus report 1996 has pointed out three species as the etiological agents for periodontitis. They are *Aggregatibacter actinomycetomcomitans, Porphyromonas gingivalis* and *Tannerella forsythia*. These organisms appeared to be responsible for bleeding on probing and in increasing the periodontal pocket depth.[35] In our study, the results of Real Time PCR showed the presence of the same above said obligate anaerobic bacteria in the subgingival plaque.

According to Leonhardt the putative pathogens such as *Porphyromonas gingivalis* and *Prevotella intermedia* were seen in 60% of the subgingival samples Apart from *Porphyromonas gingivalis* and *Prevotella intermedia, Tannerella forsythia* and *Aggregatibacter actinomycetomcomitans* showed higher prevalence when compared to other obligate anaerobes.[36] In contrast to Leonhardt we found that Porphyromonas gingivalis and *Prevotella* had a higher Cycle threshold value than *T. forsythia* suggesting a lesser prevalence for *P.gingivalis* and *Prevotella intermedia*.

Kamma *et al* studied moderate and minimal lesions seen in young adults with progressive periodontitis. They observed microbial complexes associated even with severe periodontitis. He was able to identify *Actinomyces* and *Streptococcus, Hemophilus, Capnocytophagea* and *Veillonella* from minor periodontitis condition.[37]

CONCLUSION

In the current study we noted that there is an increase in the presence of periodontitis among various stages of womenhood. This can be ascertained by noting the prevalence of the seven obligate anaerobes found in the sub gingival plaque from the study group. Even though there is a slight difference among these bacteria in our study, various other studies have pointed out the association of these bacteria with periodontitis. awareness among women about oral hygiene and routine dental checkup among women can be begun from school, in gynecologic sections by making oral check up mandatory during pregnancy and among elderly who have attained menopause.

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