"IDENTIFICATION OF *Porphyromonas gingivalis* IN PERIODONTALLY HEALTHY ADULTS AND CHRONIC PERIODONTITIS PATIENTS USING POLYMERASE CHAIN REACTION."

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LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL FORM
P. gingivalis	*Porphyromonas gingivalis*
PCR	Polymerase chain reaction
PlI	Plaque index
PPD	Pocket Probing Depth
CAL	Clinical Attachment Level/Loss
ANOVA	Analysis of variance
AAP	American Academy of Periodontology
ml	Milliliter
mm	Millimeter
µl	Microliter
mg	Milligrams
DNA	Deoxyribonucleic Acid
bp	base pairs

ABSTRACT

INTRODUCTION: Periodontitis is a complex, multifactorial, polymicrobial infection is characterized by the deterioration of the supportive tissues surrounding the teeth. Amongst the microbiota, *Porphyromonas gingivalis*, identified as a keystone pathogen, has been strongly implicated in the etiology of chronic periodontitis.

AIM: Identification of *Porphyromonas gingivalis* in periodontally healthy adults and chronic periodontitis patients using polymerase chain reaction.

MATERIALS AND METHODS: This study conducted at the Outpatient Department of Periodontics, KLE V.K. Institute of Dental Sciences, involved 60 patients aged between 30 to 50 years based on specific inclusion criteria. Participants were categorized into periodontally healthy subjects (Group 1), moderate periodontitis patients (Group 2), and severe periodontitis patients (Group 3). Participants were recruited using a convenience sampling method ensuring informed consent. Plaque index, probing depth, and clinical attachment level were measured. Plaque samples collected were cultured and analyzed for black-pigmented colonies. After biochemical characterization including Gram staining, indole, catalase, and sugar fermentation tests, DNA isolation was performed. Samples showing bands suggestive of bacterial DNA presence underwent PCR analysis, utilizing species-specific primers for accurate detection and confirmation of *P. gingivalis.*

RESULTS: The age and gender showed no significance in prevalence of *P. gingivalis* ($p < 0.05$). *P. gingivalis* was not detected in periodontally healthy patients, however, it was found in 10% of moderate periodontitis patients and 50% of severe

periodontitis patients. Its prevalence was significantly higher among severe periodontitis patients ($p = 0.0001$). No significant differences were observed between moderate and severe periodontitis patients, nor between healthy and moderate periodontitis patients ($p=1.0000$). Severe periodontitis patients with positive *P. gingivalis* samples exhibited significantly higher mean PPD scores (8.90 ± 0.99 vs. 7.50 ± 0.71, $p = 0.0073$) and CAL scores (9.20 ± 1.03 vs. 7.10 ± 1.10, $p = 0.0022$).

CONCLUSION: The prevalence of *P. gingivalis* did not exhibit any significant association with age and gender. However, its presence was notable in both moderate and severe periodontitis patients, with a higher prevalence observed in severe periodontitis patients. PCR analysis revealed a significant increase in the detection frequency of *P. gingivalis* with deeper periodontal pocket depth and increased clinical attachment loss, indicating a correlation between *P. gingivalis* with the severity of periodontal destruction.

KEYWORDS: Chronic periodontitis, culture, periodontally healthy, polymerase chain reaction, *Porphyromonas gingivalis*

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INTRODUCTION

Periodontitis emerges as a significant global health issue, possibly standing as the most prevalent chronic infectious ailment affecting the human population. This intricate, multifactorial, polymicrobial infection is characterized by the deterioration of the supportive tissues surrounding the teeth.[1-3] It is defined as "an inflammatory disease of the supporting tissues of the teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both."[1]

The oral cavity is a highly intricate ecosystem. Throughout the years, researchers have amassed considerable data suggesting the participation of a minority of bacteria residing in the subgingival niche in initiating and progressing periodontal disease.[4] Leeuwenhoek initially described subgingival plaque, revealing a wide diversity of bacterial compositions.[5] The subgingival plaque is estimated to harbour over 700 distinct bacterial species,[6,7] with a potential 10 to 30 species assuming a more pivotal role in the pathogenesis of periodontal diseases.[8] Research has revealed a significant qualitative and quantitative contrast between individuals with periodontal health and those with periodontitis.[9] The prevailing microbiota isolated from teeth and the gingival sulcus of periodontally healthy subjects, primarily consist of Gram-positive bacteria, facultative anaerobic bacteria, and infrequently Gram-negative anaerobic rods. Conversely, as the severity of periodontal disease increases, Gram-negative anaerobic bacteria tend to predominate in the subgingival microflora.[10]

Included in this group of Gram-negative bacteria are "*Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia, Treponema species, Aggregatibacter actinomycetemcomitans, Selenomonas, Filifactor alocis,* and diverse *Synergistetes"* species, to name a few.[11] "*Porphyromonas gingivalis, Treponema*

denticola and *Tannerella forsythia*," collectively form the red complex. These species forming a part of the red complex exhibit significant predominance within the periodontal pocket and are closely associated with the progression of periodontal disease.[12-15]

Among the red complex, *"Porphyromonas gingivalis"* recognized as a keystone pathogen, plays a pivotal role in initiating a dysbiotic state that disturbs the balance of commensal microorganisms and induces persistent chronic inflammation,[2,8-10,12] and is strongly implicated in the etiology of "chronic periodontitis."[12,14,16] This Gram-negative, asaccharolytic, nonmotile, and obligately anaerobic bacterium forms black-pigmented colonies on blood agar plates, as outlined in the literature.[17,18] Recent research has also revealed the extensive array of virulence factors produced by "*P. gingivalis*," contributing significantly to tissue damage and complications seen in periodontal disease. Moreover, this pathogen is associated with a range of systemic diseases, such as atherosclerosis, coronary artery disease, preterm labour, and rheumatoid arthritis.[19] The identification of *"P. gingivalis"* is crucial as it indicates the potential risk, severity, and treatment approaches for the disease, therefore, making its detection imperative for necessary preventive and therapeutic interventions.

While "*P. gingivalis"* was identified in 85.75% of subgingival plaque samples obtained from "chronic periodontitis" patients,[20] limited research has been undertaken in India. Given the observed variations in oral microbe prevalence across geographic locations,[21] there arose a necessity to evaluate this prevalence. Various laboratory techniques have been devised and implemented for the identification and isolation of *P. gingivalis* from clinical samples.[22,23] While bacterial culture is commonly regarded as the "gold standard," its effectiveness is hindered by low sensitivity. This limitation

arises from the specific growth requirements of certain oral pathogens. To address these challenges, alternative methods such as DNA probe and PCR assays, immunoassays, have been developed for bacterial detection.[22] Among these alternatives, PCR stands out as the favoured method due to its ease of execution, high specificity, and sensitivity compared to bacterial culture.

Hence, a cross-sectional study was designed to detect and isolate "*P. gingivalis"* among individuals categorized as periodontally healthy as well as those diagnosed with moderate and severe "chronic periodontitis," who reported to the Outpatient Department of Periodontics at "KLE V.K. Institute of Dental Sciences in Belgaum," with the PCR technique employed to confirm the presence of "*P. gingivalis"* in all study groups.

AIM AND OBJECTIVES

Aim of the study:

Identification of "*Porphyromonas gingivalis"* in periodontally healthy adults and "chronic periodontitis" patients using polymerase chain reaction.

Objectives of the study:

1. Isolation of "*Porphyromonas gingivalis"* in the periodontally healthy subjects.
2. Isolation of "*Porphyromonas gingivalis"* in moderate periodontitis subjects.
3. Isolation of "*Porphyromonas gingivalis"* in severe periodontitis subjects.
4. To compare the prevalence of "*Porphyromonas gingivalis"* among periodontally healthy, moderate "periodontitis," and severe "periodontitis" groups
5. To evaluate and compare the association of "*Porphyromonas gingivalis"* and clinical periodontal parameters.

REVIEW OF LITERATURE

"Chronic periodontitis", a common oral condition, is marked by inflammation of the gingiva and the consequential deterioration of the supporting tissues resulting in tooth loss.[24] The role of microorganisms residing in the tooth biofilm and the host tissues elicits an immune-inflammatory response, leading to "periodontitis."[25] Various bacteria, particularly those of the red complex found in the subgingival environment, are recognized for initiating the onset of periodontal disease.[7]

Among these, "*Porphyromonas gingivalis"* plays a crucial role in the periodontal disease pathogenesis serving as a key pathogenic factor.[14]

Porphyromonas gingivalis:

History: Isolated initially in 1953 by K. G. Löe, *P.gingivalis*, a pivotal pathogen in periodontal disease, has been subsequently verified in studies for its association with the localized production of specific IgA and its involvement in recurrent periodontal infections.[26] Studies have demonstrated the bacterium's capability to retain and transfer plasmids, along with harbouring insertion sequence elements.[27] Its prevalence has been correlated with both natural and experimental gingivitis, exhibiting a substantial occurrence in both situations.

Previously known as Bacteroides gingivalis, the bacterium was reclassified, in 1994 as part of a new genus named Porphyromonas,[18] which is derived from the Greek word "porphyreos," denoting purple and "monas," signifying unit. This nomenclature, signifies a porphyrin cell, which refers to black discolouration of colonies on blood agar plates after 6 to 10 days due to the accumulation of heme.[28]

Taxonomy: Within the phylum Bacteroidota, "*Porphyromonas gingivalis"* is classified in the genus "*Porphyromonas"* and belongs to the Porphyromonadaceae

family. It is a nonmotile, anaerobic, Gram-negative, rod-shaped pathogenic bacterium. On blood agar, it exhibits the formation of black colonies.[29]

Morphological characteristics: Members of the *"P. gingivalis"* species are characterised as gram-negative, non-motile, asaccharolytic, obligatory anaerobic coccobacilli with diameters ranging from 0.5–0.8 to 1.0–3.5 m.[30] These bacteria form smooth, round colonies initially appearing white to cream-coloured on a blood agar surface. Over 6–10 days, colonies progressively darken from the edge towards the centre, attaining a deep red-to-black colour, in correlation with the protoheme concentration.

Biochemical properties: *"Porphyromonas gingivalis,"* an asaccharolytic and anaerobic species, thrives in environments with low oxygen tension but abundant nitrogenous substrates, making subgingival sites ideal. In this ecosystem, characterized by a low redox potential, the bacterium utilizes an electron transport system with protoheme and menaquinones as major carriers. Aspartate stimulates growth, and its catabolism via the succinate pathway has been demonstrated. Fumarate serves as an electron sink, and *"P. gingivalis"* can biosynthesize menaquinones in the absence of exogenous menadione.[29] Colonies displaying characteristics such as catalase negativity, indole positivity, nitrate reduction, carbohydrate non-fermentation, and lack of fluorescence under UV light are identified as *"P. gingivalis."*[19]

Virulence factors: In recent years, extensive research has yielded significant evidence regarding a wide array of virulence factors *"P. gingivalis"* produces. These factors contribute to inducing tissue damage and contributing to the observed complications in periodontal disease. These include "Outer membrane proteins, Capsule, Lipopolysaccharide (LPS), Fimbriae, Proteinases"[31]

Capsule: The existence of a capsule in *"P. gingivalis"* is recognized as a crucial virulence factor with antiphagocytic properties. The capsule provides heightened resistance against phagocytosis, enhances serum resistance, and reduces the chemotaxis of polymorphonuclear leukocytes (PMNs).

Outer membrane proteins: *"P. gingivalis"* harbours approximately 20 major proteins varying from 20 - 90 KDa. Research conducted in vitro has primarily focused on studying the impact of major outer sheath membrane proteins, released as outer membrane vesicles on various cells. Mihara and Holt [32-34] identified a protein termed fibroblast activating factor of 24 KDa, possessing strong stimulatory effects on human gingival fibroblasts during thymidine incorporation.

Fimbriae: Cellular appendages manifest in two types: type-specific fimbriae, which aid in interactions with other bacteria and mammalian cells, thereby contributing to adhesion, toxin delivery, and motility; and sex pili, which play a role in bacterial conjugation. These fimbriae 3-25 nm in diameter and 3-25 μm in length, are peritrichously arranged. Notably, these fimbriae possess chemotactic abilities, allowing them to detect host stimuli, potentially influencing the formation of inflammatory lesions and progression of periodontal tissue and bone destruction.[35]

Proteinases: One notable virulence trait of "*P. gingivalis*" is its ability to produce a wide array of proteolytic, hydrolytic, and lipolytic enzymes, a characteristic found across all known strains. Proteases associated with "*P. gingivalis*," such as thiol, trypsin, caseinolytic proteinases, and two peptidases known as gingipains, play crucial roles in peptide bond hydrolysis. Gingipains, specifically Lys- and Arg-proteinases, are cysteine proteinases. Additionally, "*P. gingivalis*" produces at least 40 different proteinases, including collagenase, which preferentially hydrolyzes collagen.[36] The discovery of genes encoding various enzymes such as collagenase, endothelin-converting-like enzyme, protease-hemagglutinin, broad-spectrum protease, dipeptidyl peptidase, and the reported protease periodontain emphasizes the enzymatic diversity within "*P. gingivalis.*" [37,38]

Lipopolysaccharide (LPS): Lipopolysaccharide (LPS), a critical component of the bacterial outer membrane, is a large molecule with a minimum size of 10 kDa.[39] It is essential in Gram-negative bacteria for maintaining structural and cellular integrity and regulating hydrophobic molecule entry and toxic chemicals. Absence of LPS, shows an impaired insertion and folding of outer membrane proteins.[40] LPS disrupts the host's innate immune response, interfering with leukocyte distribution around bacterial colonization. These are not frequently detected by innate host-defense system in contrast to other Gram-negative species.[42-44]

MICROBIOLOGICAL TESTS:

Culture methods: Culture methods are widely acknowledged as the gold standard for microbiological identification, serving as a reference against which other methods are evaluated. While it's not feasible to culture all microorganisms, this technique facilitates the identification of numerous potential periodontopathogenic microorganisms using both selective and non-selective media. One benefit of

culturing is the ability to assess antibiotic sensitivity. Nevertheless, bacterial culture methods have limitations, including their incapacity to detect low levels of microorganisms, high costs, labour-intensive protocols, extended turnaround times for results, and difficulties in cultivating particular bacterial species. In case of "*P. gingivalis,*" samples can be plated on Blood Agar enriched with hemin, Vitamin K, and sterile horse blood/serum, followed by incubation for 6-10 days in an anaerobic jar with a modified gas pack system (80% N_2, 10% CO_2, and 10% H_2).[45,19]

Enzymatic Assays: A method has been developed utilizing enzymatic assays to detect bacteria with trypsin-like enzymes. In this approach, a plaque sample containing bacteria is applied onto a strip of paper treated with the colorless substrate BANA,[46,47] which breaks resulting in blue-black color, with the intensity corresponding to the total number of organisms present. While this chairside test cannot differentiate the relative proportions of bacteria/detect other oral microorganisms, it has demonstrated a significant association with pocket depth in periodontal disease sites. However, its reliability as a diagnostic tool is questionable due to inconsistency in results.[48,49]

Immunoassays: Immunoassays utilize monoclonal/polyclonal antibodies that target species-specific antigens for bacterial identification. Various methods, such as ELISA, immunofluorescence microscopy, membrane and latex agglutination assays are employed for this purpose.[50-53] These approaches provide benefits such as affordability, quick results, partial quantification, and low detection threshold.[54-56] However, they do not enable the assessment of antibiotic sensitivity.

Nucleic acid probe: Nucleic acid probes play a crucial role in microbiological analysis of plaque in periodontal diseases. These probes, consisting nucleic acid sequences, bind to nucleic acid sequences on microorganisms. There are three types

of probes: whole genomic probes, randomly cloned probes, and synthetic oligonucleotide probes. Among these, oligonucleotide probes stand out for their high specificity and low cross-reactivity, targeting genes specific to a bacterial species. Nucleic acid probes surpass culture methods in sensitivity and can be employed even when microorganisms are non-viable, making them particularly beneficial for delayed plaque sample analysis.[57]

Polymerase chain reaction (PCR): A technique employed for efficient DNA replication. It permits the synthesis of numerous copies of small DNA samples, even those as minute as a single bacterium, facilitating subsequent analysis. PCR assays, when paired with synthesized 16S rRNA probes, facilitate identification and quantification of microorganisms in plaque samples. This technique exhibits remarkable sensitivity, capable of detecting virtually any microorganism present in a plaque sample. Real-time PCR, modification of original PCR technology, not only identifies specific microorganisms but also quantifies their abundance. PCR surpasses culture methods in sensitivity and doesn't necessitate viable microorganisms for analysis, presenting an advantage, especially in scenarios where plaque sample analysis might be delayed.[57]

In a study conducted by **Ingalagi et al. 2022**,[19] "*Porphyromonas gingivalis"* prevalence investigated using real-time polymerase chain reaction (PCR) and culture method from subgingival plaque samples of 400 individuals, equally divided into chronic periodontitis and healthy subjects. Results showed that 73% of samples were positive for "*P. gingivalis"* through culture, while PCR detected it in 75%. In "chronic periodontitis," culture identified "*P. gingivalis"* in 89.5%, compared to 54.4% in healthy individuals. RT-PCR demonstrated a 91.5% detection rate. The study

emphasized the importance of comparing different identification methods for understanding "*P. gingivalis"* prevalence in periodontal conditions.

Marín MJ et al. 2019,[45] conducted a study to validate multiplex real-time quantitative polymerase chain reaction. This assay aimed to simultaneously detect and quantify "*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*, and *Tannerella forsythia*" in subgingival plaque samples, comparing its efficacy with culture. The collected samples from patients undergoing periodontal therapy were subjected to m-qPCR and culturing targeting bacterial species. The findings demonstrated a high level of sensitivity in simultaneously quantifying *"A. actinomycetemcomitans, P. gingivalis,* and *T. forsythia"* with the tested m-qPCR assay compared to culture.

Kugaji MS et al. 2019,[58] analyzed the pivotal role of *P. gingivalis* as a pathogenic agent in periodontal disease. Utilizing SYBR Green assay, the study targeted the 16S rRNA species-specific region of "*P. gingivalis*." Results revealed a significant prevalence of "*P. gingivalis"* in 79.16% with pronounced differences among genders and age groups. The findings revealed a significant association of "*P. gingivalis"* with "chronic periodontitis," indicating its presence with disease severity

Kulkarni et al. 2018,[5] investigated "*Porphyromonas gingivalis*" presence in individuals with "chronic periodontitis" and those without disease. Employing polymerase chain reaction (PCR), the authors associated the presence of "*P. gingivalis*" with clinical parameters. Using a sterile curette, subgingival plaque samples were collected, DNA extraction and PCR was carried out for detection of "*P. gingivalis*". The results revealed prevalence of "*P. gingivalis*" in 60% of patients with pocket depths ≤5 mm and 93.33% in pocket depths ≥ 5 mm. Even a periodontally healthy subject exhibited presence of "*P. gingivalis.*" The study concluded that

frequency of "*P. gingivalis*" detection significantly increased with higher pocket probing depths and loss of attachment, thereby indicating its association with periodontal disease severity.

Kumavat RM et al. 2016,[59] carried out a study for detection of "*Porphyromonas gingivalis* and *Treponema denticola"* in "chronic periodontitis (CP) and aggressive periodontitis (AgP)" among the Indian population. Utilizing polymerase chain reaction (PCR), the study investigated these microorganisms' presence and their correlation with clinical periodontal severity. "*P. gingivalis* and *T. denticola"* were frequent in deeper periodontal pockets. The study revealed a significant correlation of "*P. gingivalis* and *T. denticola"* to periodontal disease severity.

Farias BC et al.'s 2012[60] investigation evaluated the presence of red complex pathogens ("*Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola")* and *"Aggregatibacter actinomycetemcomitans"* in "chronic periodontitis" patients. Analyzing 116 samples using polymerase chain reaction, the study found 46.6% "*P. gingivalis"* prevalence, 41.4% of "*T. forsythia"*, 33.6% of "*T. denticola*," and 27.6% for *A.a.* "*P.gingivalis* and *T. forsythia"* were seen to be more frequent in pockets ≥ 8 mm. The study concluded that red complex bacteria are associated with "chronic periodontitis," particularly in deep pockets, and their simultaneous presence may contribute to disease progression.

Abiko Y et al. 2010,[61] investigated subgingival flora in periodontal pockets to understand their correlation with "periodontitis." Using quantitative PCR on samples from 12 healthy and 28 "periodontitis" patients. A higher proportion of total obligate anaerobes in "periodontitis" patients, notably "*Tannerella forsythia, Porphyromonas gingivalis,* and *Eubacterium saphenum.*" The total "*Streptococci*" proportion was

found to be lower in patients with “periodontitis.” These findings showed an altered subgingival environment in “periodontitis,” favouring obligate anaerobes, with their proportion corelating with “periodontitis.”

Morikawa M et al. 2008,[62] conducted a study to improve PCR's practicality in diagnosing and treating periodontitis. They aimed to develop a more clinically feasible PCR-based technique. A multiplex “PCR” for five potential periodontopathic bacteria ("*Treponema denticola, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, and Tannerella forsythia*") and two non-periodontopathic species was established. The study involved 85 subjects undergoing supportive periodontal therapy. Results indicated higher detection frequencies of "*T. denticola* and *P. gingivalis*" associated with increased PPD and CAL. The multiplex PCR system demonstrated efficient detection, less time and labour-intensive than conventional PCR.

Atieh MA 2008,[63] conducted a meta-analysis to examine diagnostic precision of real-time “polymerase chain reaction (PCR)” in detecting "*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*" comparing it with bacterial culture. Five eligible studies were analyzed, revealing some heterogeneity. Real-time PCR showed high accuracy for "*A. actinomycetemcomitans* and *P. gingivalis.*" Diagnostic odds ratios were calculated, yielding values of 18.5 for A.a. and 40.47 for “*P. gingivalis*.” The review concluded that real-time PCR surpassed culture techniques in accuracy, but other factors such as cost, availability, and necessity for antibiotic susceptibility testing should be considered when selecting a microbiologic test.

Thiha K et al. 2007,[64] aimed to detect periodontopathic bacteria in "periodontitis" patients. They analyzed subgingival plaque and gingival tissue from "periodontitis" patients. Results showed "*P. gingivalis and T. forsythensis*" in 70% of samples, with "*A. actinomycetemcomitans*" more prevalent in LAgP (63%) compared to CP (16%) or GAgP (38%). Serotype c of *A.a.* was prominent in 50% of LAgP patients. Identification of "*S. oralis* and *S. sobrinus"* was minimal in plaque and tissue. Similar amount of "*P.gingivalis A.a.*, and *T. forsythensis*" were found in tissues among three groups, thereby concluding its role in periodontal tissue destruction.

Mayanagi et al. 2004,[65] conducted a study comparing 25 different bacterial species detection frequency in plaque samples from eighteen untreated "periodontitis" patients and 12 healthy participants. Genomic DNA extraction and PCR targeting the 16S rRNA genes were carried out for bacterial detection. Nine bacteria, including "*Eubacterium saphenum, Prevotella intermedia,* and *Treponema denticola*," seemed associated with "periodontitis," subjects, although showing no significance statistically ($P<0.05$). "*Mogibacterium timidum* and *P. gingivalis*" were identified in "periodontitis" patients than healthy group ($P<0.002$), with "*P. gingivalis"* exclusively found in "periodontitis" indicating a close association with periodontal disease severity.

Boutaga et al. 2003,[66] conducted a study to detect and quantify "*P. gingivalis"*. Analysing subgingival samples from "periodontitis" patients they utilized culture and PCR for "*P. gingivalis"* 16S rRNA gene amplification. Real-time PCR employing specific primers and a probe, successfully detected "*P. gingivalis"* even at 1 CFU, and was identified in 43% of samples by culture and 53% by PCR. This study concluded that real-time PCR, with its rapid and sensitive detection, complements quantitative culture results for "*P. gingivalis"* in subgingival plaque samples.

Ximénez-Fyvie et al. 2000,[67] compared supra and subgingival plaque in healthy and "periodontitis". They analyzed 2358 samples for 40 bacterial taxa through hybridization and DNA probes, alongside clinical assessment. Results showed higher DNA probe counts in "periodontitis" subjects' of "*P. gingivalis, B. forsythus, and T. denticola*.". The study highlighted differences in *Actinomyces* and orange, and red complex species levels between healthy and diseased plaque.

Lamont and Jenkinson (2000),[12] examined "*P. gingivalis,*" role as the major etiological agent in severe "periodontitis." They highlighted the organism's colonization process within the subgingival region, involving various adhesins like fimbriae, hemagglutinins, and proteinases for surface retention and growth. Once established, "*P. gingivalis"* engages in intercellular communication with both oral prokaryotic and eukaryotic cells, fostering biofilm formation, root dentin invasion, and gingival epithelial cells internalization. These interactions contribute to the bacterium's successful colonization of the periodontal region.

In their study, **Johansson and Kalfas in 1998,**[68] examined the cytotoxicity of "*Porphyromonas gingivalis"* proteins on gingival fibroblasts and epithelial cells. They utilized techniques like vital dye uptake, DNA synthesis measurement, and apoptosis evaluation, along with microscopic examination to detect morphological changes. The experiments included dialyzed culture supernatants and supernatant fractions obtained via isoelectric focusing. Observed cellular damage entailed cell rounding and detachment, which correlated with cysteine-dependent proteolytic activity and presence of gelatinolytic protein bands consistent with gingipains R and K. Heat treatment or cysteine proteinase inhibitors abolished the cytotoxic activity, indicating gingipains' role in degrading the intercellular matrix.

MATERIALS AND METHODS

SOURCE OF DATA

This cross-sectional study was carried out at the Department of Periodontics, "KLE V.K. Institute of Dental Sciences, Belgaum." The laboratory procedures were performed at "KLE University's Dr. Prabhakar Kore Basic Science Research Center, Belgaum."Ethical approval was obtained from the Ethical Committee, KLE V.K. Institute of Dental Sciences, Belgaum, before conducting the study.

The study included 60 participants aged between 30 and 50 years, reporting to the Outpatient department, Department of Periodontics, KLE V.K. Institute of Dental Sciences, Belgaum. The patients were recruited in the study using a convenience sampling method. A written informed consent form was signed by all the participants before commencing the study.

Criteria for group selection

The subjects were divided into three groups of 20 each.

- *Group 1* - 20 subjects with healthy periodontium.
- *Group 2* - 20 subjects with Moderate periodontitis having CAL of 3 to 4 mm and a pocket probing depth of 5-6mm
- *Group 3* - 20 subjects with Severe periodontitis having CAL of ≥5mm and a pocket probing depth of ≥7mm.

Inclusion criteria:

1. Systemically healthy subjects.
2. Subjects of 30-50 years.
3. Periodontally healthy group - Periodontally healthy subjects having sulcular depth of ≤ 3mm and no clinical attachment loss.
4. Subjects with moderate "periodontitis" (AAP 1999 Classification of periodontitis)[69] having CAL of 3 - 4 mm and PPD of 5 - 6 mm
5. Subjects with severe "periodontitis" (AAP 1999 Classification of periodontitis)[69] having CAL of ≥5mm and PPD ≥7mm.

Exclusion criteria:

1. The subjects who have undergone periodontal treatment or antimicrobial therapy in the last 6 months before sampling.
2. Subjects who have a habit of smoking or consuming tobacco in any form.
3. Pregnant women
4. Patients unwilling to participate in the study

CLINICAL ARMAMENTARIUM

- Mouth mirror
- Explorer
- Graduated William's periodontal Probe
- Tweezer
- Gracey Curettes
- Cotton rolls

- Kidney tray
- Disposable gloves
- Mouth mask
- Eppendorf tubes
- Transport media (Thioglycollate broth)

CLINICAL PARAMETERS:

The following clinical parameters were recorded:

1. Plaque Index - Loe's modification (1967)[70]
2. Pocket Probing depth
3. Clinical attachment level.

PLAQUE INDEX (PlI)

The Plaque Index (PlI) utilized in this study was originally formulated by Silness J and Loe H. in 1964, with subsequent modifications by Loe H. in 1967. Assessment of plaque accumulation involved examining teeth with a mouth mirror and dental explorer. Plaque levels were evaluated across different areas of teeth, including mesio-facial, facial, disto-facial, and lingual surfaces. (Fig. 5)

The teeth were airdried and examined visually. In cases where plaque was not visibly present, an explorer was used to inspect the surface. The explorer was carefully passed across the surface in the cervical third and near the entrance to the gingival sulcus. The following scores were given based on the amount of plaque present:

SCORE	CRITERIA
0	No plaque
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen only by running a probe, across the tooth surface.
2	Moderate accumulation of soft deposits within the gingival pocket, on the gingival margin, and/or adjacent tooth surface, which can be seen by the naked eye.
3	Abundance of soft matter within the gingival pocket and /or on the gingival margin and adjacent tooth surface.

Calculation of PlI:

PlI score for the area:

Each area (disto-facial, facial, mesio-facial, lingual) is assigned a score from 0 to 3.

PlI score for a tooth:

The scores from the four areas of the tooth are added and then divided by four.

PlI score for the individual:

The indices for each of the teeth are added and then divided by the total number of teeth examined. The scores range from 0 to 3.

Interpretation:

Excellent	0
Good	0.1 – 0.9
Fair	1.0 – 1.9
Poor	2.0 – 3.0

SULCULAR DEPTH, POCKET PROBING DEPTH (PPD) AND CLINICAL ATTACHMENT LEVEL (CAL)

In healthy subjects:

- Sulcus Depth was measured as the distance from gingival margin to the base of the gingival sulcus.
- CAL was measured as the distance from the CEJ to the base of the gingival sulcus.

In "chronic periodontitis" patients:

- PPD was measured as the distance from gingival margin to the base of the periodontal pocket.
- CAL was measured as the distance from the CEJ to the base of the periodontal pocket.[71] (Fig. 6)

PROCEDURE:

COLLECTION OF PLAQUE SAMPLES:

Plaque specimens were collected using a sterilized Gracey curette from the deepest probing depths within groups 2 and 3. Conversely, samples from the site with the highest plaque accumulation were collected from the control group (Group 1).

1. The sites were isolated using sterile cotton rolls and air-dried.
2. Supragingival plaque samples were collected from the site with the highest plaque accumulation, starting from the distal aspect of the tooth and gently inserting the curette into the gingival sulcus. (Fig. 7)
3. Subgingival plaque samples were collected from the site with the deepest pocket probing depth, using a sterile Gracey curette. (Fig. 8)
4. The collected plaque samples were immediately pooled in an Eppendorf tube containing 1 mL thioglycollate broth.
5. The samples were sent to the laboratory for further qualitative assessment to detect the presence or absence, and to isolate "*P. gingivalis.*"

LABORATORY ARMAMENTARIUM

- Eppendorf tubes
- Petri plates
- Inoculating Wire loop
- Micropipettes (10-1000 µl)
- Micropipette tips
- Electronic weighing machine
- Autoclave
- Laminar airflow
- Hot air-oven
- Anaerobic jar
- Anaerobic incubator/chamber
- Microscope
- Deep freezer (-20°C)
- Micro-centrifuge
- Gel electrophoresis unit
- Gel documentation unit
- Thermocycler

PREPARATION OF THIOGLYCOLLATE BROTH

For the preparation, HiMedia Enriched Thioglycollate broth was utilized. A solution with a fixed amount of 31.06 grams per 1000 mL of distilled water was prepared. The powder was accurately weighed using an electronic weighing scale, and distilled water was measured using a calibrated flask. Subsequently, the measured powder was carefully added to the distilled water, and the mixture was thoroughly swirled to ensure complete dissolution. The flask was securely packed with a cotton plug and paper, which was fastened using a thread. Additionally, autoclave indicator tape was affixed to the flask to indicate the completion of sterilization. The broth was sterilized by autoclaving at 15 pounds pressure (121°C) for 15 minutes to ensure its sterility and suitability as a transport medium. Following sterilisation, the prepared Thioglycollate broth was allowed to cool down to room temperature, and under aseptic conditions within a laminar airflow cabinet, 1 mL of the broth was carefully transferred into autoclaved Eppendorf tubes. These tubes were stored in the refrigerator for later use.

PREPARATION OF BLOOD AGAR

For the preparation, HiMedia Blood Agar Base (Infusion Agar) was utilized. A predetermined quantity of 40.00 grams per 1000 mL of distilled water was used for the preparation. The powder was weighed using an electronic weighing scale, while distilled water was measured using a calibrated flask. The measured powder was then added to the distilled water, and the mixture was thoroughly swirled until fully dissolved. Subsequently, the flask was sealed with a cotton plug and paper, and secured with a thread. An autoclave indicator tape was attached to the flask to confirm the completion of sterilization. The agar was sterilized by autoclaving at 121°C for 15 minutes to ensure its sterility.

Following sterilisation, the prepared agar was left to cool at 45-50°C. Under aseptic conditions within a laminar airflow cabinet, it was enriched with blood (5 mg/L), menadione (1 mg/L), and 1% sterile horse serum. This supplemented mixture was then poured into autoclaved petri plates before solidification, and refrigerated after solidification for further use. (Fig. 11)

INOCULATION OF PLAQUE SAMPLES

The pooled plaque samples collected from patients across the three groups were subjected to serial dilution in phosphate-buffered saline (PBS) using a 7-fold dilution method. In this process, a 100µL sample was diluted in 900µL of saline in the first tube. 100 µL aliquots from the first tube were transferred into subsequent tubes, maintaining the dilution ratio until the seventh tube was prepared. This was done within 20 minutes of sample collection under aseptic conditions.

Following serial dilution, the diluted samples were plated onto Blood agar plates employing a pour plate culture method. The plates were then placed in an anaerobic jar.

PREPARATION OF ANAEROBIC CONDITIONS

The inoculated blood agar plates were carefully transferred into an anaerobic jar. The jar was infused with a precise mixture of gases, comprising 85% nitrogen (N_2), 10% carbon dioxide (CO_2), and 5% hydrogen (H_2). To facilitate the removal of residual oxygen, a sachet containing palladium catalyst pellets was heated in a hot air oven and then placed inside the jar lid. This setup ensured efficient absorption of any remaining oxygen molecules. Additionally, methylene blue indicator tablets were placed within the jar to monitor the degree of anaerobiosis. Once all preparatory measures were completed, the anaerobic jar was securely sealed by tightening the clamp screw and

subsequently transferred into an anaerobic chamber, for 7-8 days, allowing for the optimal growth of *P. gingivalis* bacteria.

SUBCULTURING OF COLONIES

Following incubation for 7-8 days, the black colonies were observed. (Fig. 13) Under strict aseptic conditions within a laminar airflow cabinet, the identified black colonies were meticulously selected using a sterilized wire loop. The selected colonies were then transferred and plated onto freshly prepared blood agar plates using a streak culture technique. The newly inoculated plates were then incubated under anaerobic conditions for another 7-8 days. Upon completion of the incubation period, colony morphology was examined in detail. (Fig. 14) Furthermore, biochemical characterization assays were performed to confirm the identity of *P. gingivalis*. The identified pure colonies were transferred to the glycerol stock solution for later use.

BIOCHEMICAL TESTS TO CONFIRM *P. gingivalis*

Gram staining Procedure

A black colony was initially selected from the Petri dish, and with the help of an inoculation loop was transferred onto microscope slide with a drop of distilled water to assist in minimal colony transfer. Subsequently, the culture was evenly spread over a 15 mm diameter circle on a glass slide and gently dried over a flame to ensure optimal cell adhesion. For the procedure, HiMedia Gram's stain kit was used. Following fixation, a crystal violet stain was applied to the smear, left for 60 seconds, and then rinsed off with water to remove excess stain. Iodine solution was applied to the smear for 60 seconds to fix the dye, followed by rinsing with running water and shaking off excess water. The smear was then treated with a decolourizer until the blue dye stopped flowing. After washing the slide with tap water, the smear was

counterstained with safranin solution for 20 seconds, rinsed with water, and excess water was blotted. After air-drying the slide, it was examined under a microscope using oil immersion to assess smear distribution at 40x magnification, and then at 100x for comprehensive analysis. The characteristic morphology was observed. The samples showing gram-negative coccobacilli were selected for further biochemical tests.[72] (Fig. 15)

Indole Test Procedure

To confirm the presence of "*P. gingivalis*," a Spot Indole Test was conducted. A few drops of 1% p-dimethylaminocinnamaldehyde reagent were applied to apiece of filter paper until it was saturated. Using an inoculation loop, a portion of the identified black colony from the Petri dish was picked and rubbed onto the filter paper saturated with reagent. The reaction was observed and compared to positive and negative controls, "*E. coli*" MTCC 443 and "Proteus mirabilis" MTCC 425, respectively. A positive reaction, indicated by blue to blue-green color change in 2-3 minutes, indicating presence of "*P. gingivalis*." (Fig. 16)

Catalase Test Procedure

The Slide Method involved transferring a small portion of the identified black colony on a dry and clean glass slide with the help of a loop. A drop of 3% H2O2 was added to the slide, and oxygen bubble evolution was observed. "*Staphylococcus aureus"* MTCC 96 served as the positive control, while "*Enterococcus faecalis"* MTCC 439 was the negative control. A positive result, indicated by abundant bubbles, confirmed catalase activity. Conversely, the absence or presence of very few bubbles suggested a negative result for catalase activity, indicating the presence of "*P. gingivalis."* (Fig. 17)

Sugar Fermentation Test Procedure

A well-isolated black colony from the Petri dish was picked up using a sterile inoculating loop and inoculated into the broth. The tubes were incubated at 35 ± 2°C for 18 - 24 hours. Subsequently, observations were made for any colour change of the broth and presence of trapped air bubbles in Durham's tube. Positive fermentation was denoted by a colour change of the media from reddish-orange to yellow, while negative fermentation exhibited no alteration in colour remaining reddish-orange. "*E. coli"* MTCC 443 served as the positive control, and "*Proteus mirabilis"* MTCC 425 served as the negative control in the procedure. Therefore, the absence of colour change suggested the presence of "*P. gingivalis.*" (Fig. 18)

BACTERIAL DNA ISOLATION

Initially, the bacteria were pelleted by centrifugation at 7500 rpm - 10 minutes. Following this it was resuspended in 180 µL of 20 mg/µL lysozyme and incubated at 37°C - 30 minutes. Subsequently, 20 µL of Proteinase K and 200 µL of Buffer AL were mixed to suspension, further vortexed and incubated for next 30 minutes -56°C. After a brief centrifugation, 200 µL ethanol was added to mixture and vortexed - 15 seconds for removing droplets. The resulting solution was then poured into a mini spin column and centrifuged at 8000 rpm -1 minute. 500 µL Buffer AW1 was added, followed by centrifugation. Buffer AW2 was then incorporated and subjected to centrifugation at 14,000 rpm. Finally, 200 µL of Buffer AL was introduced to column, centrifuged - 1 minute at 8000 rpm.

PREPARATION OF GEL

To prepare 1% agarose gel, 0.5 grams of agarose was accurately weighed and added to an Erlenmeyer flask. 50 ml 1x TAE buffer was then added to the flask and the mixture was swirled to ensure thorough mixing. The agarose/buffer mixture was melted by heating it in a microwave, with intervals of 30 seconds. At intervals, the flask was removed from the microwave and the contents were swirled to ensure the complete dissolution of the agarose. This process was repeated until the agarose had completely dissolved. Following dissolution, 2 µL of ethidium bromide (EtBr) was added to the mixture. The agarose was allowed to cool to approximately 50°C. The agarose was carefully poured into the gel casting tray along with the comb. Further, the gel was left until it solidified for approximately 20-25 minutes. Following solidification, the well comb was removed, and the gel casting tray was placed into the gel box filled with 1x TAE buffer to load the samples.

SETTING UP OF GEL APPARATUS AND SEPARATION OF DNA FRAGMENTS

The isolated DNA was mixed along with the loading dye. Loading dye was used as a DNA marker to track the DNA. Running buffer (1x TAE) was then carefully added to cover the entire gel surface, using the same buffer as that used for gel preparation. The leads of the gel box were connected to the power supply. DNA samples, along with a 100 kb DNA ladder, were slowly loaded into the gel wells, and the lid was securely placed onto the gel box. The power supply was programmed to maintain a voltage gradient of 100V. Upon activation of the power supply, the negatively charged DNA molecules migrated towards the positive electrode (black to red). Gel electrophoresis was conducted until the dye line migrated approximately 75-80% of the gel length, with a typical runtime of about 45 minutes. This method

ensured the successful setup of the gel apparatus and the efficient separation of DNA fragments for subsequent analysis.

VISUALISATION OF DNA FRAGMENTS

After electrophoresis was completed the gel was carefully taken out from the gel box and was exposed to UV light transilluminator to visualize the DNA bands using gel documentation system. Photographs of the gel were taken to record the observed DNA fragments. The samples showing bands indicating the presence of bacterial DNA were further selected for PCR. (Fig. 19 A and 19 B)

POLYMERASE CHAIN REACTION (PCR)

PRINCIPLE:

PCR typically initiates with the extraction of DNA from a specimen. Through a process of heating, the double-stranded DNA separates into single-stranded forms, which serve as templates dictating the nucleotide sequence in vitro. The amplification process employs a DNA polymerase enzyme that necessitates a primer - known short oligonucleotide sequence corresponding to the border of the region to be amplified. To achieve amplified fragments of consistent length and in substantial quantities, a second primer, complementary to the opposite chain, is required to anneal (bind) to the template and flank the region of interest.

This amplification occurs over multiple cycles, typically ranging from 25 to 40. In each cycle, the process involves denaturation of the complementary chain, hybridization of the primer, and extension of the primer by the DNA polymerase. With each cycle, there is an exponential increase in the quantity of DNA. The temperature during the cycle plays a critical role in controlling the denaturation of the double chain and the stability of the hybridization between the template

fragment and the primer.

Subsequently, the sequenced DNA is detected and visualized through electrophoresis in an agarose gel, supplemented with ethidium bromide. This process allows for the observation and analysis of the amplified DNA fragments.

PROCEDURE:

All PCR reactions were conducted using a 30 µL reaction volume, with the reaction mixture prepared in PCR tubes. The primers employed were P1 (Forward): 5'AAG CAG CTT GCC ATA CTG CG 3', and P2 (Reverse): 5'ACT GTT AGC AAC TAC CGA TGT 3'. The PCR protocol began with an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C -1 minute, primer annealing at 50°C for 1 minute, and extension at 72°C - 1.5 minutes. A final extension step was carried out at 72°C for 7 minutes using a Thermal Cycler. The detection and relative size of DNA fragments from PCR products were determined by comparing it with standard 100 kb ladder and positive control. The anticipated amplicon size of 420 bp was analysed on 1.8% agarose gel under ultraviolet (UV) transillumination using GeneSys software. (Fig. 20)

STATISTICAL ANALYSIS

The data was entered in Microsoft Excel and subjected to statistical analysis using SPSS software, version 21; SPSS Inc., (Chicago, IL, USA). Shapiro-Wilk test was used to check if data was following normal distribution Thus, non-parametric tests were used since the data was not following normal distribution. A descriptive analysis was conducted. Intergroup comparisons were done by “Kruskal Wallis ANOVA.” “Chi-square” test was used to check the association between two

categorical variables. Pair-wise comparison of the study groups was carried out by "Mann - Whitney U" test.

A probability value (p-value) of <0.05 was considered to be statistically significant.

PHOTOGRAPHS

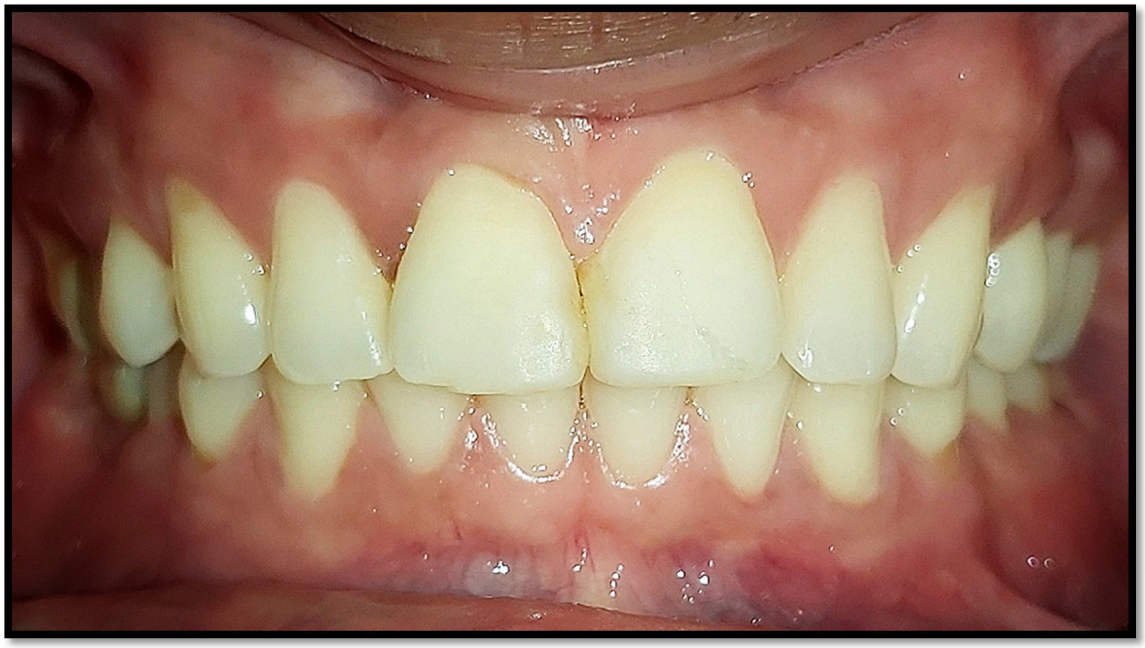


Fig. 1: Periodontally healthy subject

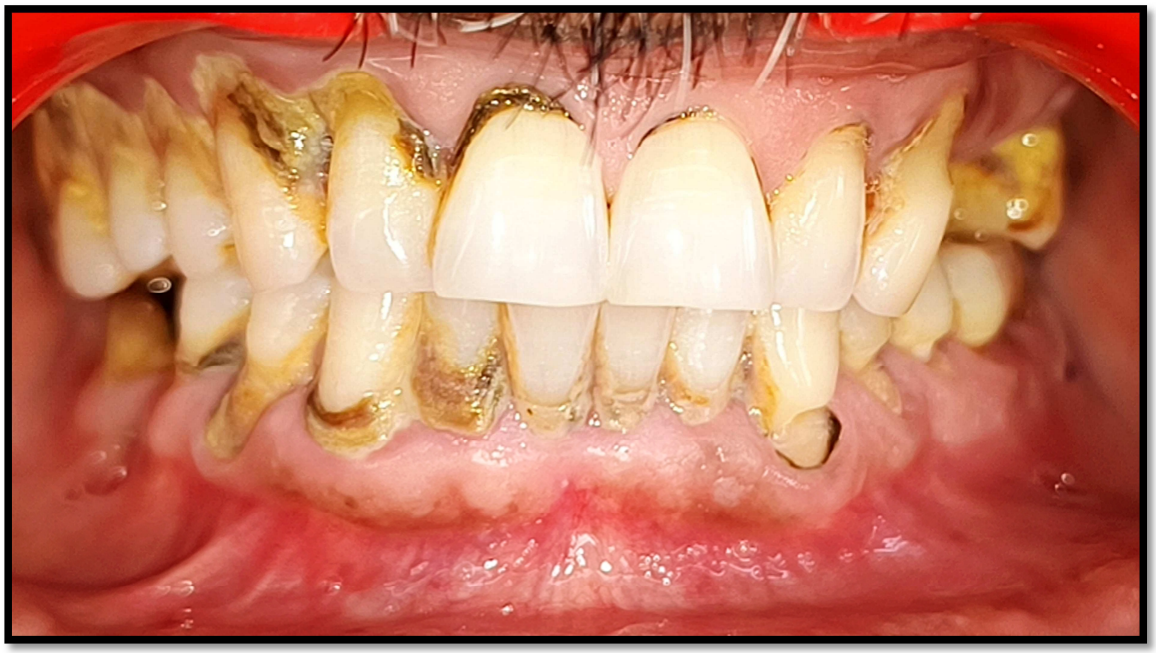


Fig. 2: Moderate periodontitis patient

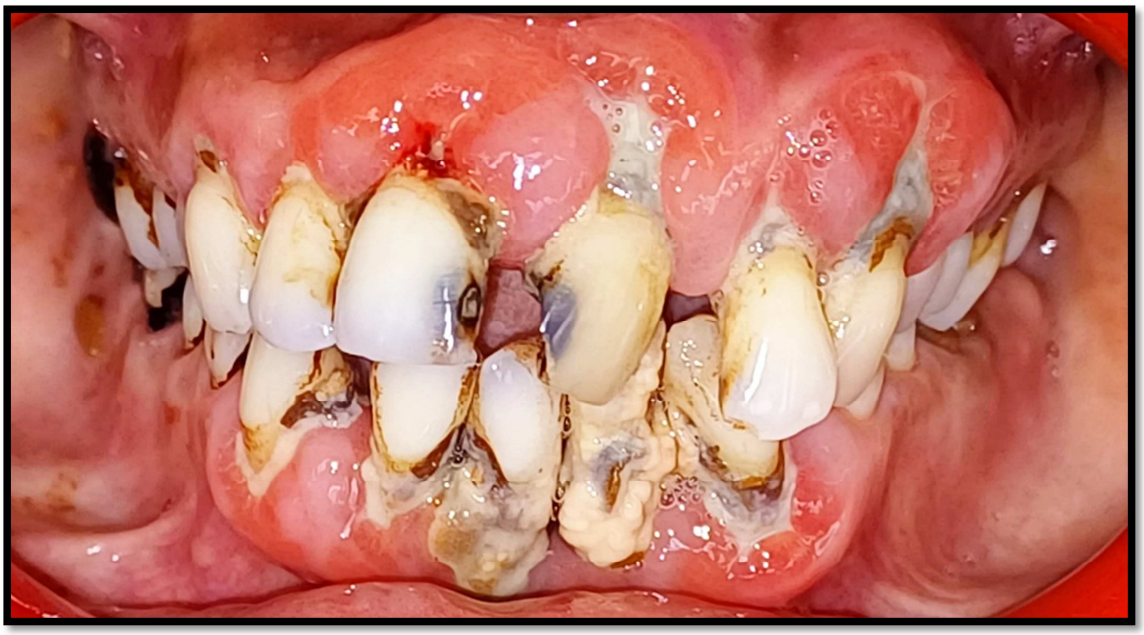


Fig. 3: Severe periodontitis patient

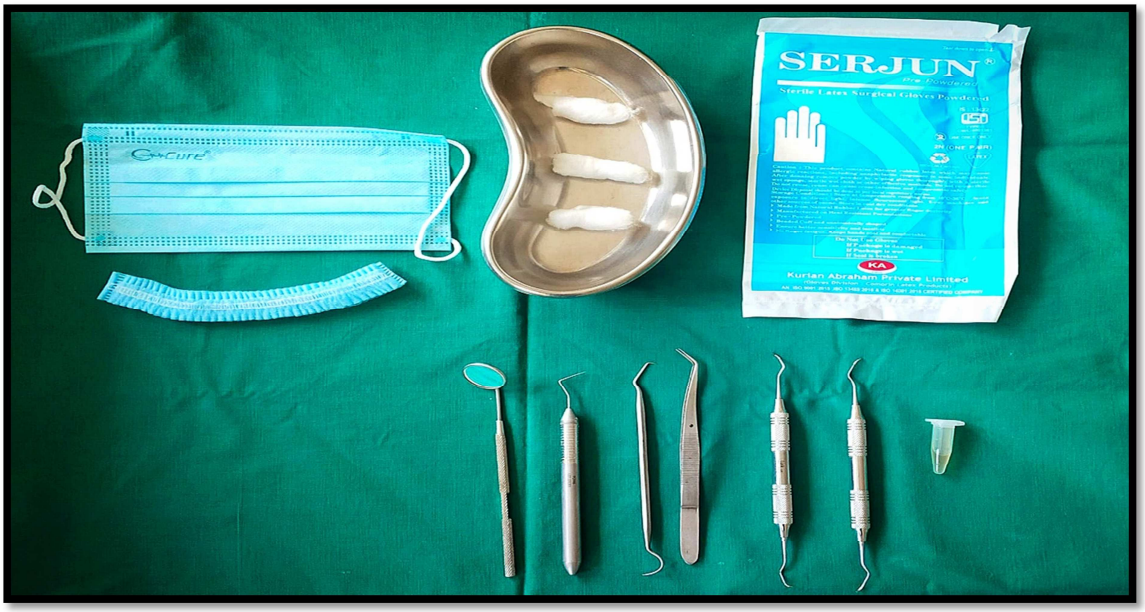


Fig. 4: Clinical Armamentarium

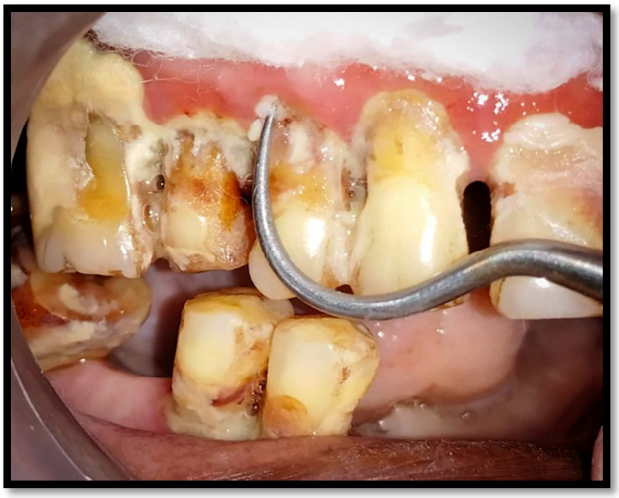


Fig. 5: Assessment of plaque accumulation

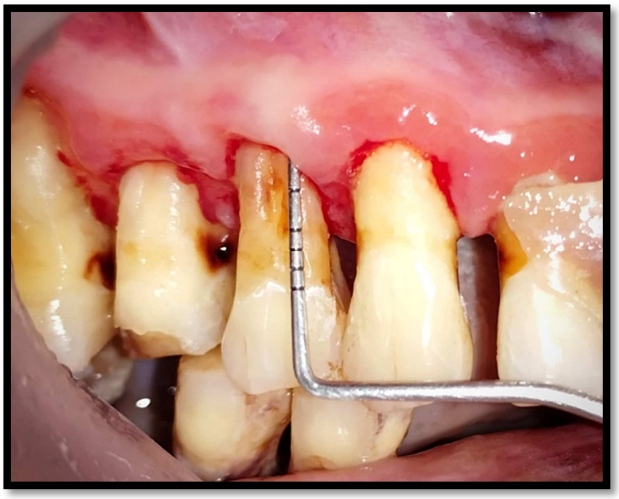


Fig. 6: Measurement of pocket probing depth and clinical attachment loss

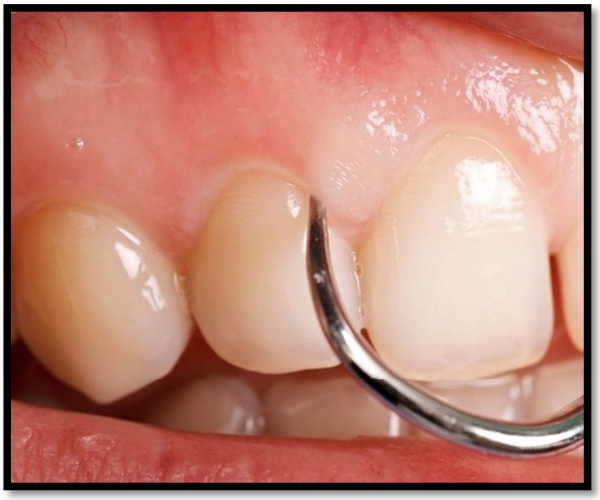


Fig. 7: Collection of supragingival plaque sample

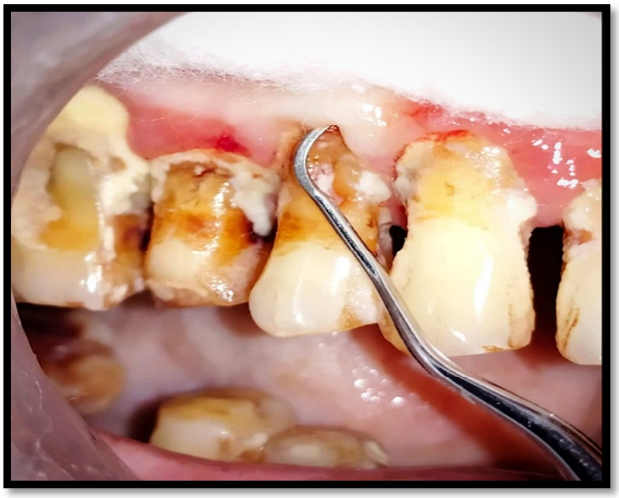


Fig. 8: Collection of subgingival plaque sample

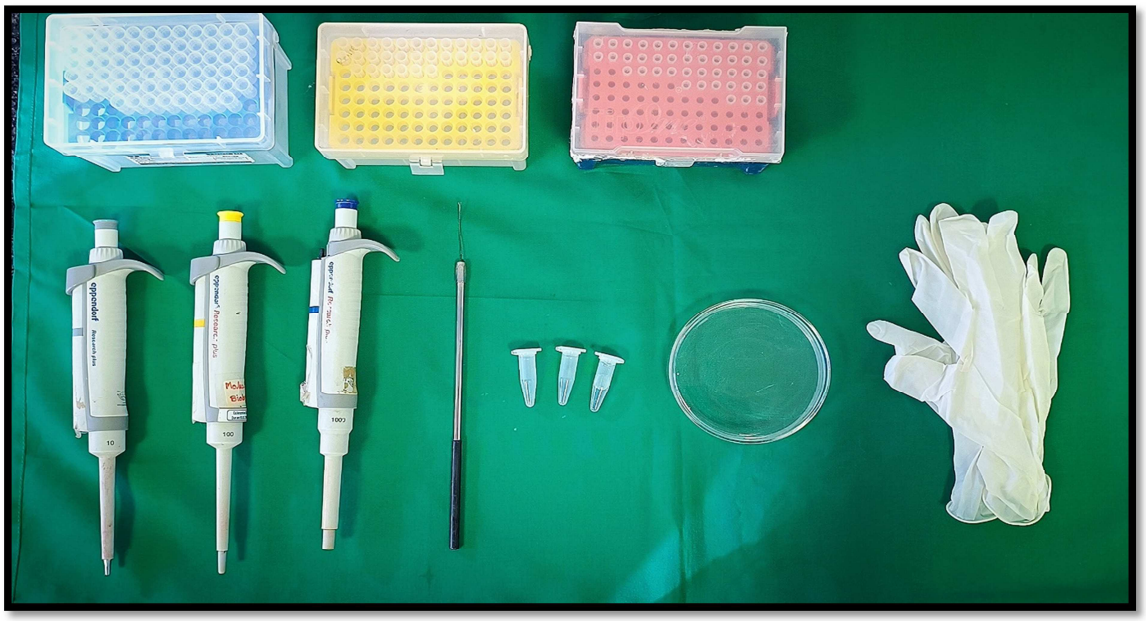


Fig. 9: Laboratory Armamentarium

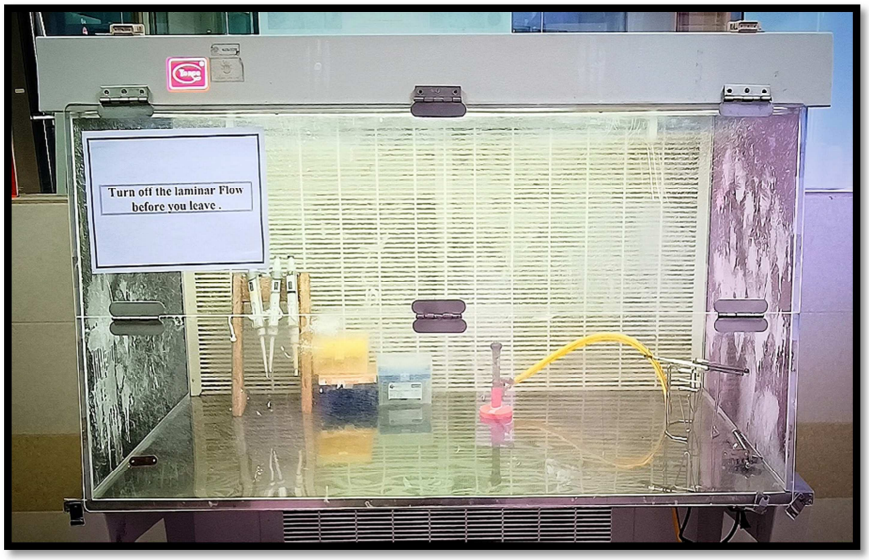


Fig. 10: Laminar air-flow cabinet

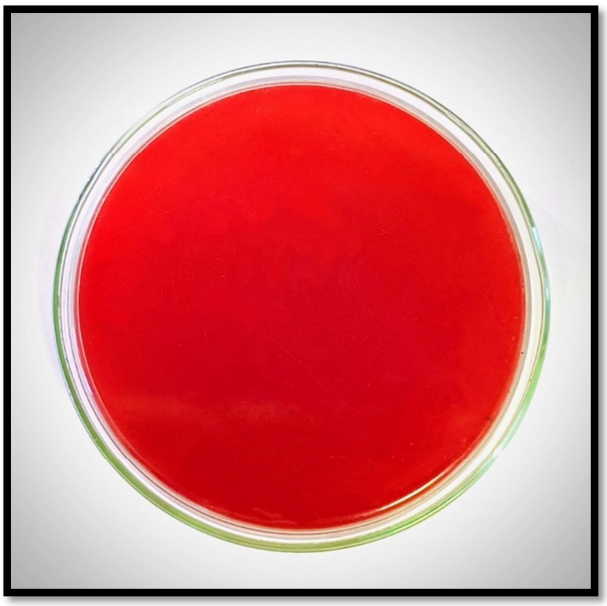


Fig. 11: Freshly prepared blood agar plate to culture plaque samples

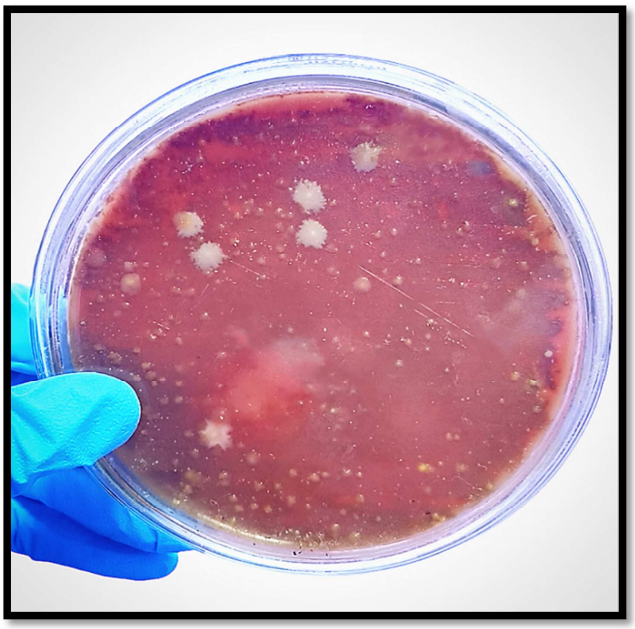


Fig. 12: Plaque culture: healthy sample (no black-pigmented colonies) on blood agar plate

BIOCHEMICAL TESTS

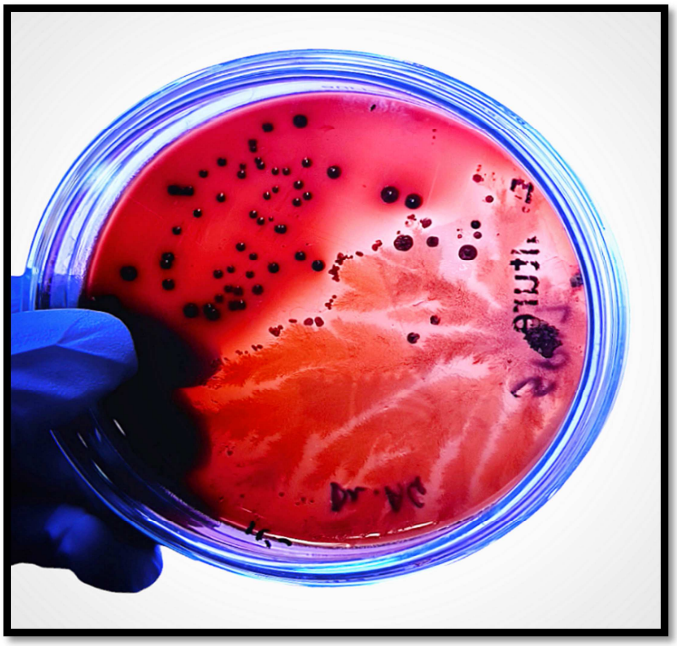


Fig. 13: Culture showing presence of black-pigmented colonies on blood agar plate

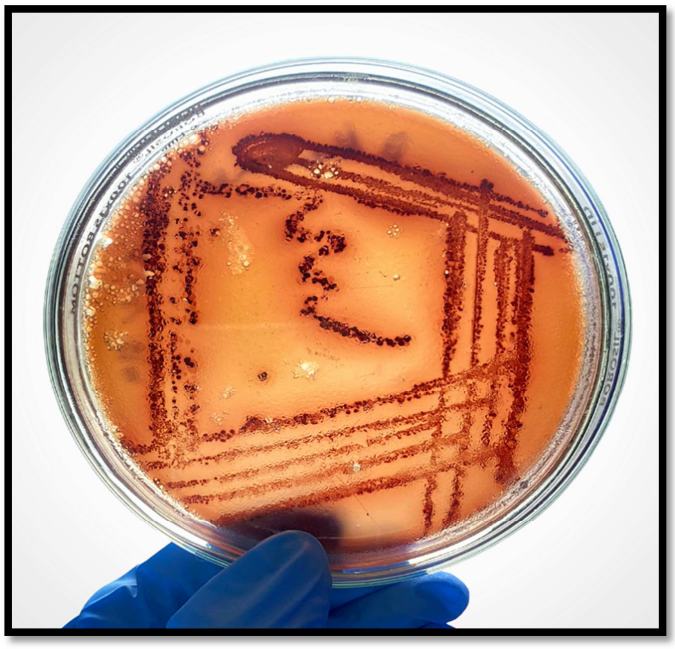


Fig. 14: Pure culture (black-pigmented colonies) on blood agar plate

VISUALISATION OF DNA

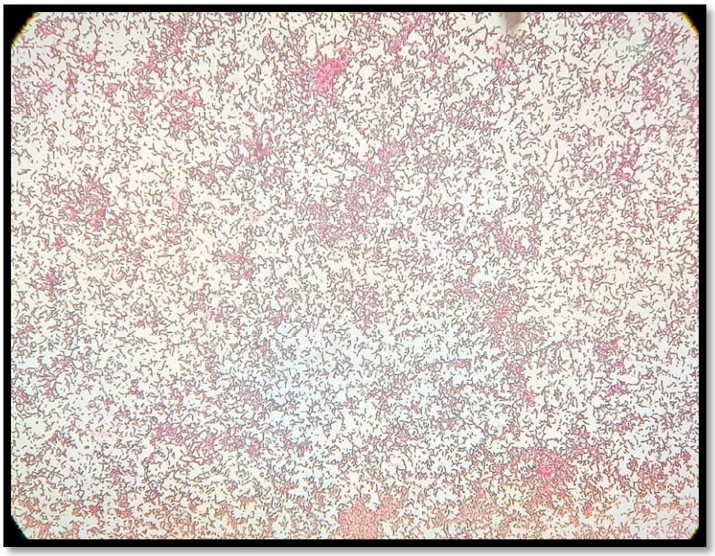


Fig. 15: Gram staining showing gram-negative coccobacilli

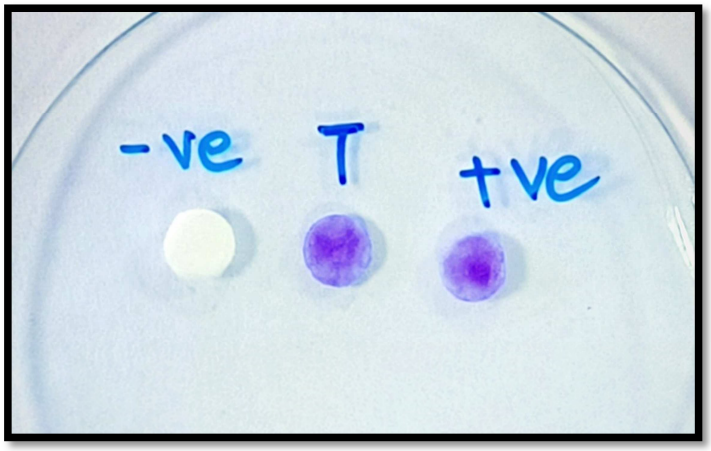


Fig. 16: Indole test showing blue colour change indicating presence of *P. gingivalis*

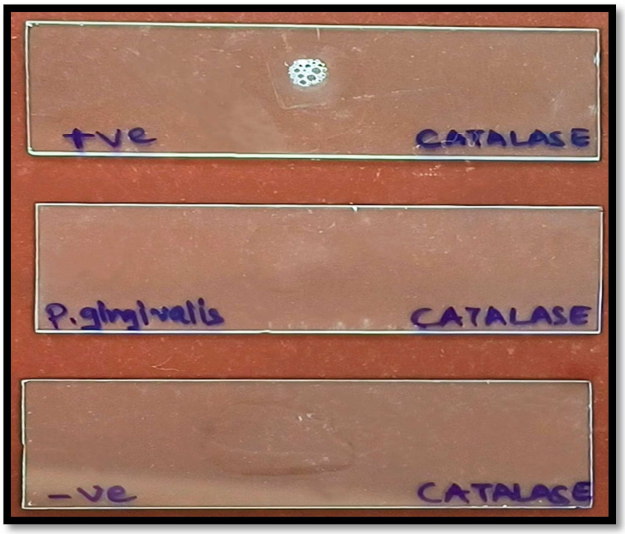


Fig. 17: Catalase test showing absence of bubbles indicating presence of *P. gingivalis*

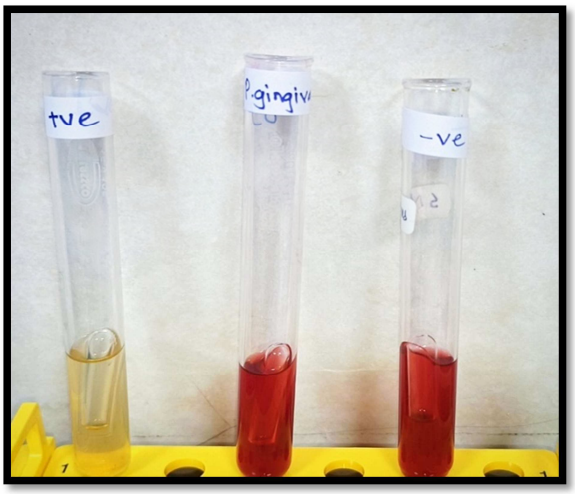


Fig. 18: Sugar fermentation test showing absence of colour change indicating presence of *P. gingivalis*

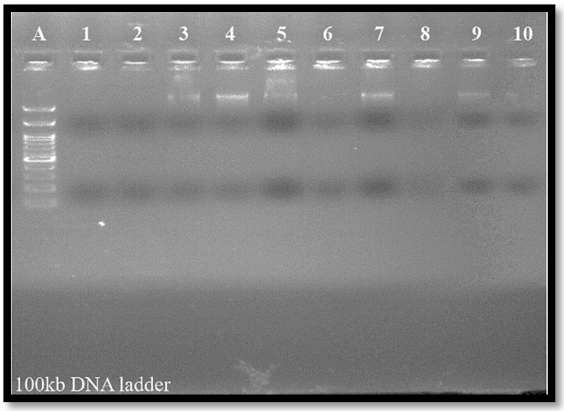


Fig. 19 A: Lane A showing 100kb DNA ladder
Lanes 3-5,7,9 and 10 showing significant bands, indicating presence of the DNA

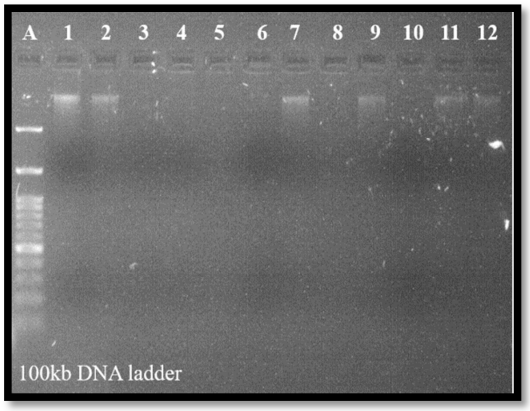


Fig. 19 B: Lane A showing 100kb DNA ladder
Lanes 1,2,7,9,11 and 12 showing significant bands, indicating presence of the DNA

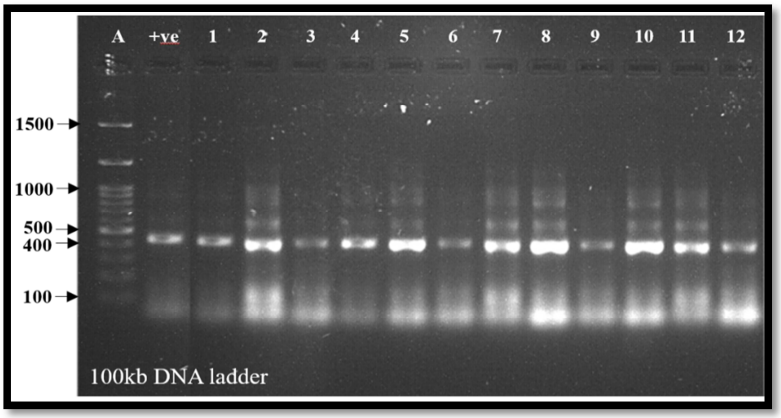


Fig. 20: Lane A showing 100kb DNA ladder
Lane +ve showing positive control (*P. gingivalis*)
Lanes 1-12 showing PCR products confirming the presence of *P. gingivalis*

RESULTS AND OBSERVATIONS

Table 1: Comparison of three groups by age:

Age groups	Healthy	%	Moderate Periodontitis	%	Severe Periodontitis	%	Total	%
30-34yrs	7	35.00	5	25.00	6	30.00	18	30.00
35-39yrs	6	30.00	2	10.00	4	20.00	12	20.00
40-44yrs	4	20.00	4	20.00	4	20.00	12	20.00
>=45yrs	3	15.00	9	45.00	6	30.00	18	30.00
Mean	37.75		41.60		39.25		39.53	
SD	5.61		7.29		6.38		6.55	
Total	20	100.00	20	100.00	20	100.00	60	100.00
Chi-square=5.3330, p=0.5020								

Observation:

The detailed distribution of study samples by age group is depicted in the above table.

A total of 60 subjects were included in the study, of which 20 individuals were classified as periodontally healthy, 20 were moderate periodontitis patients and 20 were severe periodontitis patients.

The mean age of the periodontally healthy subjects was 37.75 ± 5.61 years, while moderate periodontitis patients had a mean age of 41.60 ± 7.29 years, and severe periodontitis patients had a mean age of 39.25 ± 6.38 years.

However, no statistically significant differences were observed among these groups. (p=0.5020)

Table 2: Comparison of three groups with gender:

Gender	Healthy	%	Moderate Periodontitis	%	Severe Periodontitis	%	Total	%
Male	10	50.00	8	40.00	12	60.00	30	50.00
Female	10	50.00	12	60.00	8	40.00	30	50.00
Total	20	100.00	20	100.00	20	100.00	60	100.00
Chi-square=1.6000, p=0.4490								

Observation:

The detailed distribution of study samples by gender is depicted in the table above.

Among the 20 healthy patients, 10 (50.00%) were males and 10 (50.00%) were females. In moderate periodontitis group, 8 (40.00%) were males and 12 (60.00%) were females. In severe periodontitis group, 12 (60.00%) were males and 8 (40.00%) were females.

There was no statistically significant difference observed between male and female patients (p=0.4490)

Table 3: Comparison of three groups with Plaque scores by Kruskal Wallis ANOVA

Groups	Mean	Std.Dev.	Median	IQR	Mean rank
Healthy	1.04	0.35	1.10	0.65	16.70
Moderate Periodontitis	1.50	0.66	1.60	1.00	34.48
Severe Periodontitis	1.99	0.59	1.90	0.95	40.33
H-value	19.9320				
P-value	0.0001*				
Pair-wise comparisons by Mann-Whitney U test					
Healthy vs Moderate Periodontitis	P=0.0013*				
Healthy vs Severe Periodontitis	P=0.0001*				
Moderate Periodontitis vs Severe Periodontitis	P=0.031*				

*p<0.05

Observation:

The detailed distribution of study samples with Plaque scores is depicted in the table above.

The mean plaque score among periodontally healthy subjects was 1.04 ± 0.35, while moderate periodontitis patients had a plaque score of 1.50 ± 0.66, and severe periodontitis patients had a plaque score of 1.99 ± 0.59.

A highly statistically significant difference was observed in the plaque score between healthy and moderate periodontitis patients (P=0.0013*), as well as between healthy and severe periodontitis patients (P=0.0013*). Additionally, a statistically significant difference was observed between moderate periodontitis and severe periodontitis patients (P=0.031).

Table 4: Comparison of three groups with PPD scores by Kruskal Wallis ANOVA

Groups	Mean	Std. Dev.	Median	IQR	Mean rank
Healthy	1.90	0.79	2.00	1.50	10.50
Moderate Periodontitis	5.25	0.44	5.00	0.50	30.50
Severe Periodontitis	8.20	1.11	8.00	2.00	50.50
H-value	53.7130				
P-value	0.0001*				
Pair wise comparisons by Mann-Whitney U test					
Healthy vs Moderate Periodontitis	P=0.0001*				
Healthy vs Severe Periodontitis	P=0.0001*				
Moderate Periodontitis vs Severe Periodontitis	P=0.0001*				

*p<0.05

Observation:

The table above displays the detailed distribution of study samples with PPD scores.

Among periodontally healthy subjects, the mean PPD score was 1.90 ± 0.79, while moderate periodontitis patients had a PPD score of 5.25 ± 0.44, and severe periodontitis patients had a PPD score of 8.20 ± 1.11.

A highly statistically significant difference was observed in the PPD scores among the three groups (P=0.0001*)

Table 5: Comparison of three groups with CAL scores by Kruskal Wallis ANOVA

Groups	Mean	Std. Dev.	Median	IQR	Mean rank
Healthy	0.00	0.00	0.00	0.00	10.50
Moderate Periodontitis	3.85	0.49	4.00	0.00	30.50
Severe Periodontitis	8.15	1.50	8.00	2.50	50.50
H-value	55.4710				
P-value	0.0001*				
Pair wise comparisons by Mann-Whitney U test					
Healthy vs Moderate Periodontitis	P=0.0001*				
Healthy vs Severe Periodontitis	P=0.0001*				
Moderate Periodontitis vs Severe Periodontitis	P=0.0001*				

*p<0.05

Observation:

The table above displays the detailed distribution of study samples with CAL scores.

Among periodontally healthy subjects, the mean CAL score was 0.00, while moderate periodontitis patients had a CAL score of 3.85 ± 0.49, and severe periodontitis patients had a CAL score of 8.15 ± 1.50.

A highly statistically significant difference was observed in the CAL scores among the three groups. (P=0.0001*).

Table 6: Comparison of three groups with status of *P. gingivalis*

P.gingivalis	Healthy	%	Moderate Periodontitis	%	Severe Periodontitis	%	Total	%
Negative	20	100.00	18	90.00	10	50.00	48	80.00
Positive	0	0.00	2	10.00	10	50.00	12	20.00
Total	20	100.00	20	100.00	20	100.00	60	100.00
Chi-square=17.5000, p=0.0001*								
Between healthy vs Moderate Periodontitis, p=1.0000								
Between healthy vs Severe Periodontitis, p=0.0001*								
Between Moderate Periodontitis vs Severe Periodontitis, p=1.0000								

*p<0.05

Observation:

The table above provides a detailed distribution of the prevalence of *P. gingivalis* status among the three groups.

There was a significant association between *P. gingivalis* and its prevalence in different groups of patients. (p=0.0001). The prevalence of *P. gingivalis* in periodontally healthy subjects was 0%, compared to 10.00% in moderate periodontitis patients and 50.00% in severe periodontitis patients. A significantly higher prevalence of *P. gingivalis* was reported in severe periodontitis patients compared to periodontally healthy subjects and moderate periodontitis patients.

A highly statistically significant difference was observed between healthy and severe periodontitis patients (P=0.0001*).

However, there was no statistically significant difference observed between moderate periodontitis and severe periodontitis patients (p=1.0000), as well as between healthy and moderate periodontitis patients (p=1.0000).

Table 7: Comparison of age groups and gender with status of *P. gingivalis* in Severe Periodontitis

Profile	Negative	Positive	Total	Fisher exact P
Age group				
<=40yrs	8	4	12	0.1720
>=41yrs	2	6	8	
Gender				
Male	4	8	12	0.1720
Female	6	2	8	
Total	10	10	20	

Observation:

The table above presents a detailed distribution of the prevalence of *P. gingivalis* status among severe periodontitis patients, categorized by age and gender.

The number of positive samples for *P. gingivalis* in the age groups ≤ 40 years and ≥ 41 years was 4 and 6, respectively. The difference among the different age groups was not statistically significant ($p<0.05$).

Among *P. gingivalis* positive individuals, 8 were males and 2 were females. The difference among the different genders was not statistically significant ($p<0.05$).

Table 8: Comparison of status of *P. gingivalis* in Severe Periodontitis with PPD and CAL scores by Mann-Whitney U test

Variable	*P.gingivalis*	Mean	SD	Median	Mean rank	t-value	P-value
PPD	Negative	7.50	0.71	7.00	6.90		
	Positive	8.90	0.99	9.00	14.10	-2.6835	0.0073*
CAL	Negative	7.10	1.10	7.00	6.40		
	Positive	9.20	1.03	9.50	14.60	-3.0615	0.0022*

*p<0.05

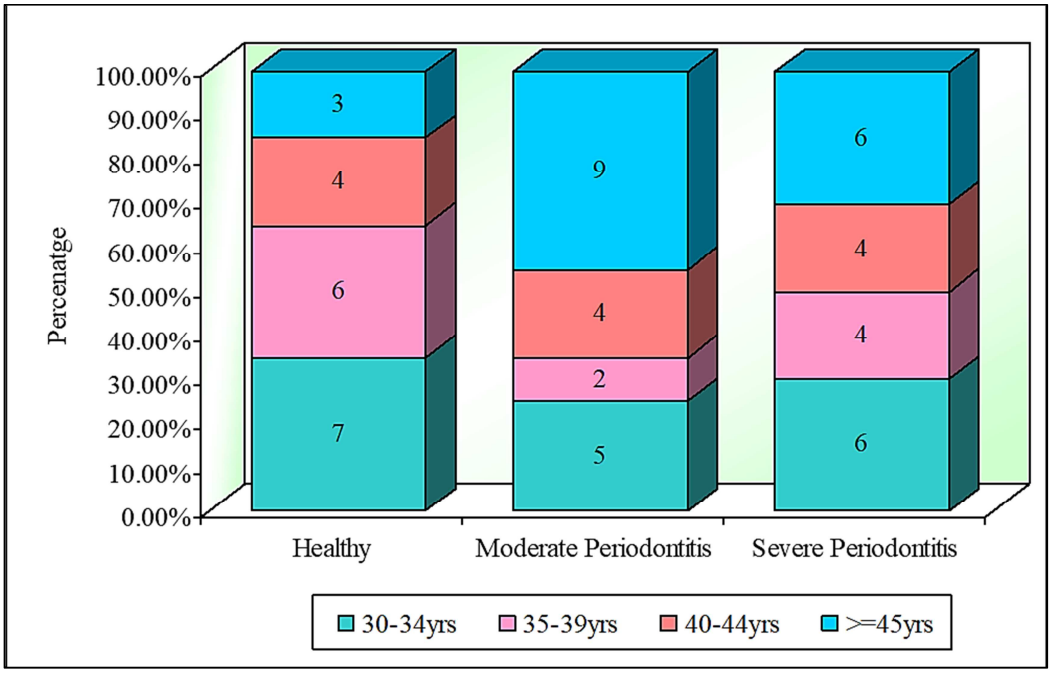
Observation:

The table above displays the detailed distribution of *P. gingivalis* status in severe periodontitis with PPD, and CAL scores.

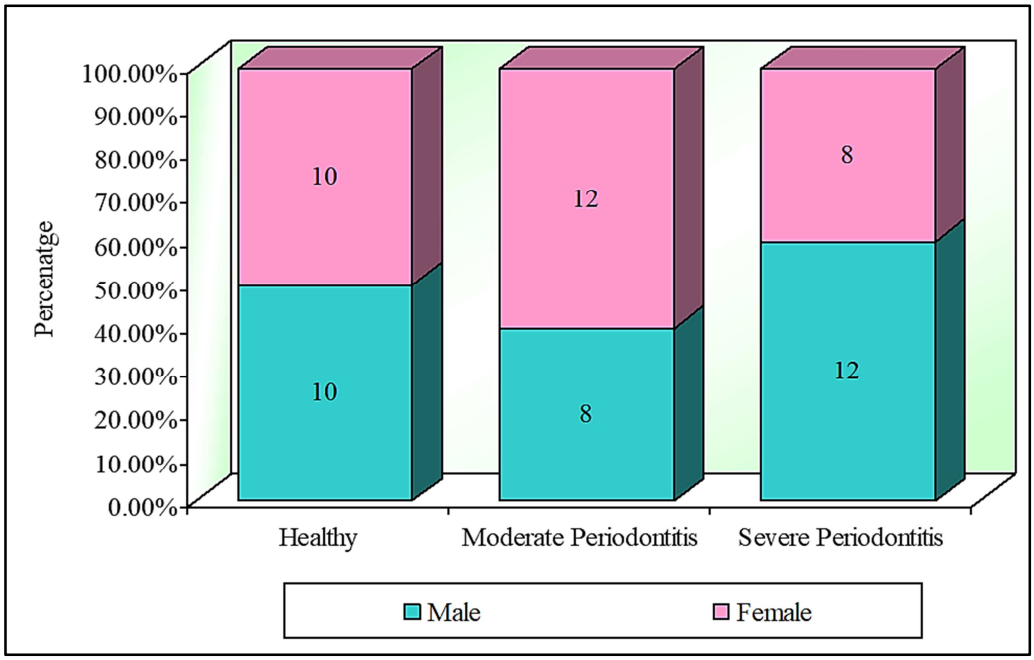
The mean PPD score in severe periodontitis patients was significantly higher in *P. gingivalis* positive samples 8.90 ± 0.99 than in negative samples 7.50 ± 0.71. A highly statistically significant difference was observed (p=0.0073*).

The mean CAL score in severe periodontitis patients was significantly higher in *P. gingivalis* positive samples 9.20 ± 1.03 than in negative samples 7.10 ± 1.10. A highly statistically significant difference was observed (p=0.0022*).

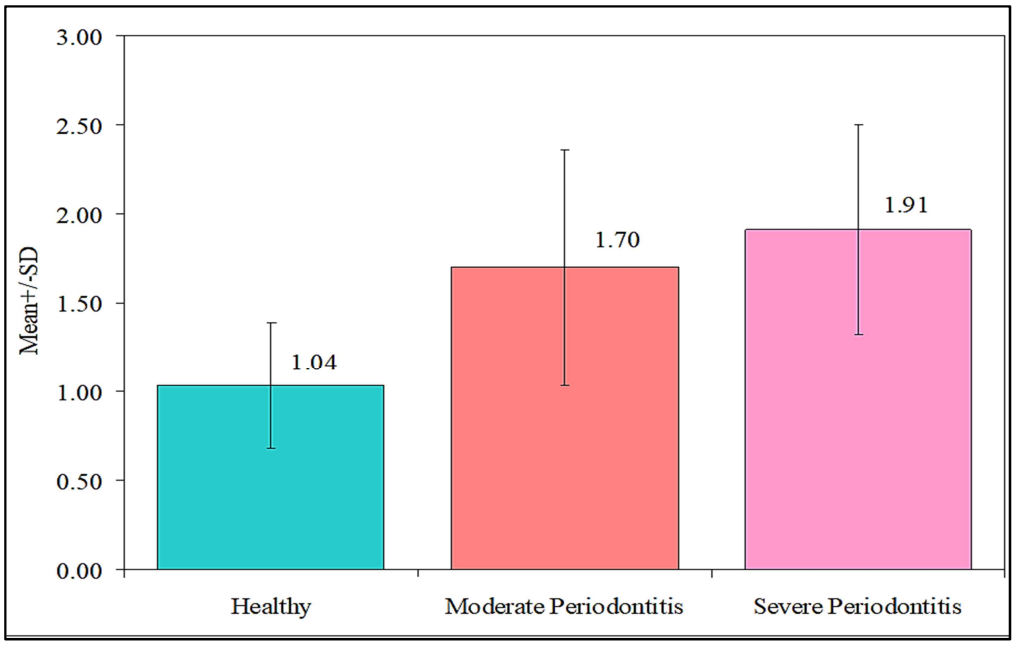
Graph 1: Comparison of three groups with age



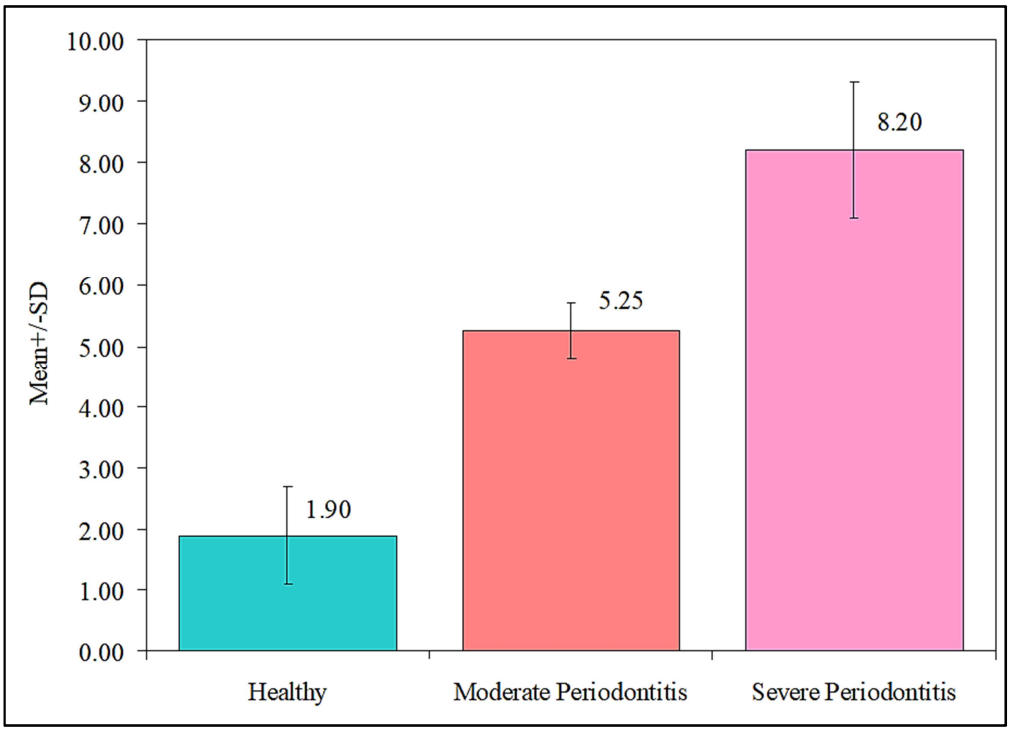
Graph 2: Comparison of three groups with gender



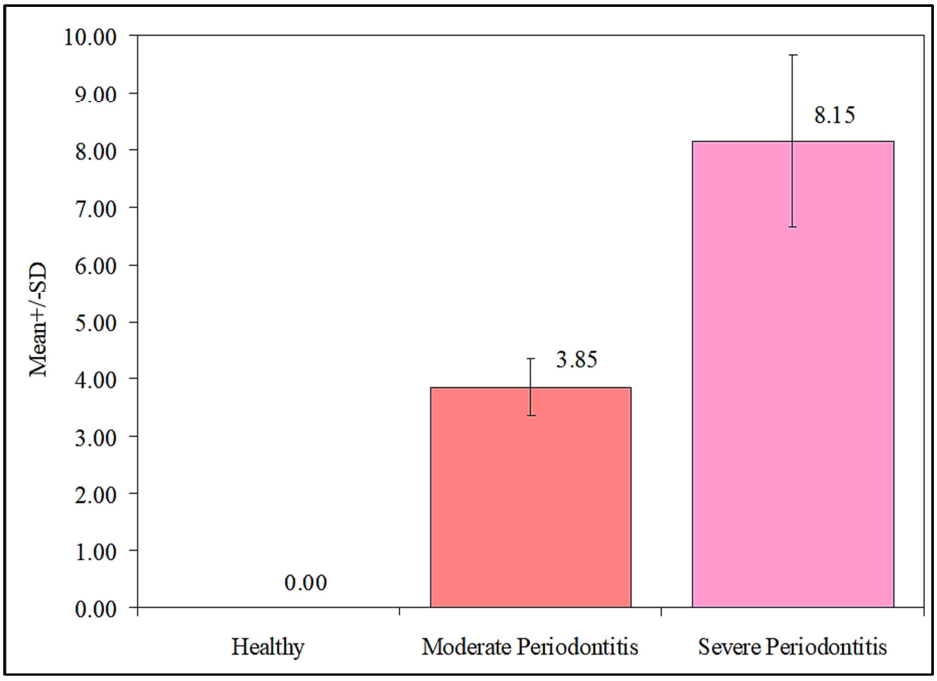
Graph 3: Comparison of three groups with Plaque scores



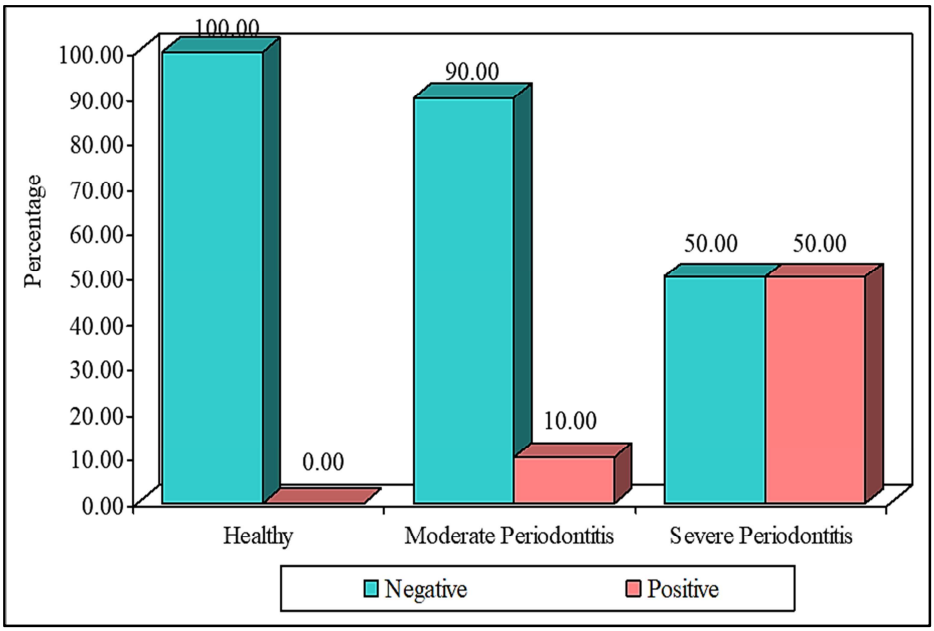
Graph 4: Comparison of three groups with PPD scores



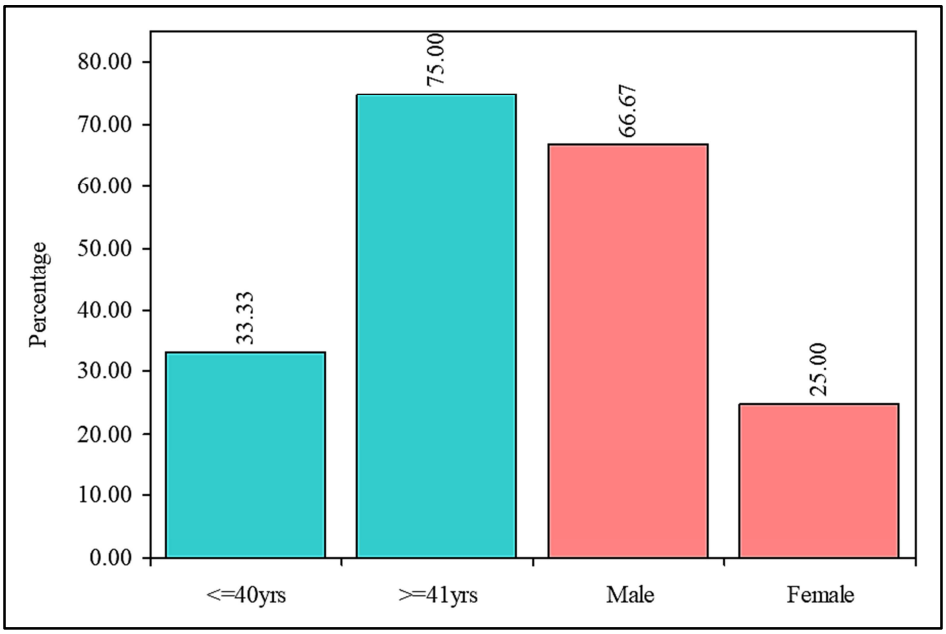
Graph 5: Comparison of three groups with CAL scores



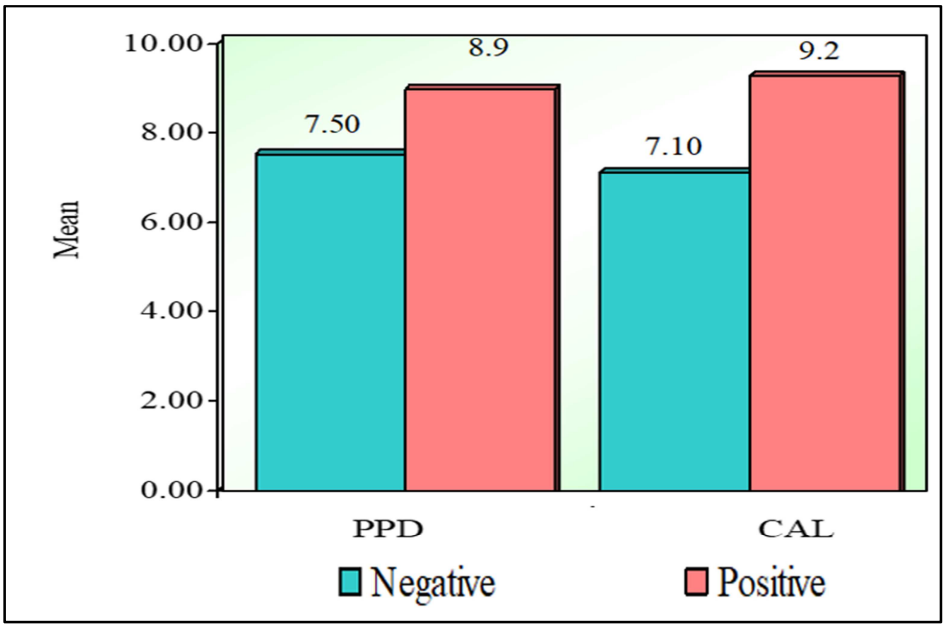
Graph 6: Comparison of three groups with status of *P. gingivalis*



Graph 7: Comparison of age groups and gender with status of *P. gingivalis* in Severe Periodontitis



Graph 8: Comparison of status of *P. gingivalis* in Severe Periodontitis with PPD and CAL scores



DISCUSSION

The prevailing understanding of "periodontitis" etiology considers three factors 1) a susceptible host 2) the presence of pathogenic species and 3) the absence or small proportion of beneficial bacteria.[73-76] The interplay between microbes in biofilm and host induce inflammatory/immune response in the host, resulting in "periodontitis."[25] Several bacteria, especially those within red complex found in the subgingival niche, initiate periodontal disease.[7] Among them, "*Porphyromonas gingivalis"* a key pathogen is recognized for its pivotal role in periodontal disease pathogenesis.[14]

Numerous studies have evaluated prevalence of "*P. gingivalis*" in "chronic periodontitis," patients revealing varying prevalence rates. Discrepancies may arise from differences in periodontal conditions, ethnic backgrounds, or geographic locations of subjects. "*P. gingivalis*" being an obligate anaerobe, acts as secondary colonizer, attaching to primary colonizers like "*S. gordonii* and *P. intermedia*" during dental plaque formation. Research by Bodet et al. (2006)[77] highlighted its collaboration with "*T. denticola* and *T. forsythia*" to form red bacterial complex, commonly found in advanced periodontal lesions. Moreover, variations in bacterial sampling, detection, and identification methods can also impact study outcomes.

Thus, given the limited data available on the distribution of "*P. gingivalis"* in the Indian population particularly in health and chronic periodontitis patients, a cross-sectional study was formulated to identify this microorganism in patients visiting the Outpatient Department of Periodontics at "KLE V.K. Institute of Dental Sciences in Belgaum." Plaque samples were cultured using a selective media (blood agar supplemented with menadione and sterile horse serum) to detect and isolate *P. gingivalis.* Samples indicating the presence of *P. gingivalis* underwent additional

confirmation via PCR, ensuring an accurate assessment of its prevalence in the study population.

In the current study, 60 subjects aged between 30 and 50 years were enrolled considering the inclusion criteria and categorised into three groups, each comprising 20 participants. Group 1 comprised periodontally healthy individuals, while Group 2 consisted of patients diagnosed with moderate periodontitis, and Group 3 included patients with severe periodontitis. The participants in this study were recruited using a convenience sampling method. Plaque score, PPD and CAL were assessed for all subjects. Plaque samples were then collected from Group 1 subjects at sites with the highest plaque accumulation, (Fig. 7) while for Groups 2 and 3, samples were obtained from sites with the deepest pocket probing depth, using a sterilized Gracey's curette. (Fig. 8) This targeted approach to sampling from sites with the deepest probing depths is particularly advantageous, as these sites are more conducive to the discovery of periopathogens.[78] The detection of black-pigmented colonies from plaque samples was conducted using a conventional culture method. Subsequently, to ensure accurate identification and differentiation from other black-pigmented anaerobic bacteria such as "*P. intermedia* and *P. nigrescans,*" which exhibit morphological similarities, a PCR confirmation was employed. This confirmation involved the utilization of species-specific primers to enhance the specificity and accuracy of detecting "*P. gingivalis"* within the plaque samples

The demographic characteristics of the study participants, including age group and gender distribution, were meticulously examined in our study. The mean age of periodontally healthy subjects was 37.75 ± 5.61 years, while moderate and severe periodontitis patients had mean ages of 41.60 ± 7.29 years and 39.25 ± 6.38 years, respectively; however, no statistically significant differences were observed

(p=0.5020). (Table 1; Graph 1). Similarly, among the healthy patients, there were 10 (50.00%) males and 10 (50.00%) females. In the moderate periodontitis group, there were 8 (40.00%) males and 12 (60.00%) females, while in the severe periodontitis group, there were 12 (60.00%) males and 8 (40.00%) females. Notably, no statistically significant difference was observed between male and female patients (p=0.4490). (Table 2; Graph 2). The demographic analysis revealed that neither age nor gender showed any significant correlation with periodontal health status or the presence of periodontal disease among the recruited individuals, thus, implying that age and gender do not play a significant role in differentiating between healthy individuals and those with periodontitis in our study. This observation aligns with prior research, such as the study by Morikawa Y et al. in 2008, which suggested that other factors like host-microbe interaction, genetics, and environmental influences could also contribute to this distinction.[62]

However, it's essential to acknowledge potential variations in demographic characteristics reported by other studies, such as the contrasting findings from the study by Kugaji MS et al. in 2019,[58] where older age groups (41-60 years) exhibited a higher prevalence of periodontal disease compared to younger age groups. In a systematic review by Shiau HJ et al. 2010,[79] it was highlighted that men exhibit a higher susceptibility to periodontal disease compared to women. This increased risk may be attributed to a more potent innate immune response in men, along with potential differences in the regulation of inflammation amplification and termination. The differences observed highlight the complex nature of periodontal disease epidemiology and the potential influence of various factors beyond age and gender on periodontal health status.

In the present study, a detailed comparison of plaque scores among three groups was conducted. Among periodontally healthy subjects, the mean plaque score was 1.04 ± 0.35, while moderate periodontitis patients exhibited a plaque score of 1.50 ± 0.66, and severe periodontitis patients had a plaque score of 1.99 ± 0.59. A highly statistically significant difference was evident in plaque scores between healthy individuals and both moderate and severe periodontitis patients (P=0.0013*). Additionally, a statistically significant difference was observed between moderate and severe periodontitis patients (P=0.031). Our results align with the concept initially proposed by Slots et al. in 1979, and further supported by Listgarten M.A. in 1988; revealing a higher bacterial load in diseased periodontal sites compared to healthy sites, implying a correlation between plaque accumulation and disease severity. This concept emphasizes the delicate equilibrium between the periodontal microbiota and the host, which is vital for maintaining periodontal health.[80,30] (Table 3; Graph 3)

Furthermore, a comprehensive analysis of periodontal pocket depth (PPD) scores was conducted across the three distinct groups in our study. Notably, periodontally healthy individuals exhibited a mean PPD score of 1.90 ± 0.79, whereas moderate periodontitis patients displayed a significantly higher PPD score of 5.25 ± 0.44. Severe periodontitis patients presented the highest PPD score of 8.20 ± 1.11. The difference in PPD scores among the three groups was highly statistically significant (P=0.0001*), indicating a clear distinction between PPD and the severity of periodontal disease across the study population (Table 4; Graph 4). In addition to PPD scores, clinical attachment level (CAL) scores were assessed to provide additional insights into periodontal health status and chronic periodontitis.

Periodontally healthy subjects displayed a CAL score of 0.00. Conversely, moderate periodontitis patients exhibited a CAL score of 3.85 ± 0.49, while severe

periodontitis patients presented a significantly higher CAL score of 8.15 ± 1.50. A highly significant difference in CAL scores was observed (P=0.0001*), thus, highlighting varying degrees of periodontal destruction corresponding to the severity of the disease. (Table 5; Graph 5)

These findings resonate with prior research conducted by Mdala I et al. in 2014,[81] which similarly identified an increase in PPD and CAL as key factors associated with the progression from periodontal health to chronic periodontitis. This emphasises the clinical significance of PPD and CAL scores in assessing periodontal disease progression.

Plaque samples collected from 60 subjects were cultured on supplemented blood agar plates. Following an incubation period of 7-8 days, only those samples displaying characteristic black-pigmented colonies (Fig. 13,14) and gram-negative coccobacilli (Fig. 15) upon gram staining were selected for further analysis. Subsequently, biochemical characterization was conducted, with samples exhibiting specific properties - positive indole, (Fig. 16) negative catalase, (Fig. 17) and negative sugar-fermentation reactions (Fig. 18) were chosen for DNA isolation. The isolated DNA underwent agarose gel electrophoresis, allowing visualization of bacterial DNA bands using a gel documentation system (GeneSys software). Samples demonstrating bands indicative of the presence of bacterial DNA were further processed for PCR analysis. (Fig. 19A, 19B) Utilizing a species-specific primer, PCR was conducted to confirm the presence of "*P. gingivalis*." The resulting bands of PCR products were compared to a standard control, with the visualization of a 420 bp band confirming the presence of *"P. gingivalis"* in the plaque samples. (Fig. 20) This meticulous methodology ensured accurate detection and confirmation of *"P. gingivalis"* within the study population

The presence of *P. gingivalis*, either alone or in combination with other oral pathogens, has long been implicated in the etiology of advanced periodontitis (Haffajee and Socransky, 1994[17]). Our study contributes to this understanding by demonstrating a clear association between "*P. gingivalis"* prevalence and the severity of periodontal disease. Our findings reveal a notable disparity in *P. gingivalis* prevalence among the different groups studied. While periodontally healthy subjects showed a complete absence of *P. gingivalis*, its prevalence increased substantially in patients with moderate periodontitis (10.00%) and was markedly higher in severe periodontitis patients (50.00%). Our results align with previous research indicating that the number of *P. gingivalis* tends to escalate significantly in sites affected by periodontitis while being undetectable in areas with subgingival health or plaque-associated gingivitis (Schmidt et al. 2014,[82] Mayanagi G. et al. 2004[65]). The substantial increase in "*P. gingivalis"* prevalence observed in severe "periodontitis" patients compared to both periodontally healthy and moderate "periodontitis" patients highlights its pivotal role in periodontal disease progression, thus emphasizing its importance as a key pathogen contributing to the pathogenesis of periodontal disease. Furthermore, our statistical analysis showed a highly significant difference in *P. gingivalis* prevalence between periodontally healthy and severe periodontitis patients (P=0.0001*), corroborating the substantial impact of *P. gingivalis* on disease severity. However, no significant differences were observed between moderate and severe periodontitis patients (p=1.0000), nor between periodontally healthy and those with moderate periodontitis (p=1.0000). These findings suggest that although *P. gingivalis* prevalence increases with the severity of periodontitis, other factors such as microbe-host interaction, genetics, and environmental influences may also contribute to disease progression, particularly in the transition from moderate to severe forms. (Table 6; Graph 6)

The distribution of *P. gingivalis* prevalence among severe periodontitis patients, categorized by age and gender, was outlined. The number of positive *P. gingivalis* samples in age groups ≤ 40 years and ≥ 41 years was 4 and 6, respectively, with no statistically significant difference observed ($p<0.05$). Similarly, among *P. gingivalis*-positive individuals, 8 were males and 2 were females, with no statistically significant difference detected between genders ($p<0.05$). These results suggest that age and gender are not determining factors in *P. gingivalis* prevalence among our study population. (Table 7, Graph 7)

Our findings are consistent with previous research conducted by Griffen et al. (1998)[83] and Claesson et al. (2023), which also observed no significant differences in *P. gingivalis* prevalence attributed to age. They noted that differences in *P. gingivalis* prevalence between healthy and periodontitis groups could not be solely attributed to age or sex, further supporting our observations regarding age and gender-related prevalence. These findings highlight the complexity of *P. gingivalis* colonisation patterns and their association with periodontal disease status. These findings suggest other factors such as genetic predisposition, oral hygiene practices, and systemic health status may play a more influential role.[84] By acknowledging the findings of previous studies and integrating them with our own, we advance our understanding of the multifactorial nature of periodontal disease pathogenesis. In contrast to the current study's findings, Savitt ED et al. (1991) observed an inverse correlation between *P. gingivalis* prevalence and subject age, noting higher levels in older individuals (30 years and older) compared to younger age groups. Similarly, Nayak et al. (2018) identified a statistically significant rise in red complex bacteria, including *P. gingivalis*, among individuals aged over 41 years, without any gender bias. These conclusions were based on the recognition that advancing age serves as a significant

host-related factor influencing oral microbial ecology. Consequently, changes in the oral microbial community over time may pose challenges to the oral and systemic health of older individuals.[85, 86] (Table 7, Graph 7)

In the present study, we examined the detailed distribution of *P. gingivalis* status among individuals with severe periodontitis, focusing on pocket probing depth (PPD), and clinical attachment level (CAL) scores. Table 8 provides an overview of these findings.

Our study revealed that severe periodontitis patients with *P. gingivalis*-positive samples exhibited a significantly higher mean pocket probing depth (PPD) score of 8.90 ± 0.99 compared to 7.50 ± 0.71 in patients with negative samples. The findings of our study align with previous research conducted by Torrungruang et al. 2009 and Abdulaziz et al. 2015, which highlighted a significant association between a higher prevalence of *"P. gingivalis"* and deeper pocket depth. This observation emphasizes that deep periodontal pockets provide an ideal environment for the growth and survival of *P. gingivalis*, contributing to its higher prevalence in severe periodontitis cases.[87,88] As an obligate anaerobe, primarily inhabiting subgingival areas, *P. gingivalis* relies on amino acid fermentation for energy production, a property crucial for its survival in a deep periodontal pocket, where sugar availability is limited. The findings of our study supporting this concept, revealed an increased frequency of *P. gingivalis* in deeper periodontal pockets. The statistically significant difference observed further supports the role of *P. gingivalis* in the progression and severity of periodontal disease (p=0.0073*). (Table 8, Graph 8)

Severe periodontitis patients with *P. gingivalis*-positive samples demonstrated significantly higher mean CAL score of 9.20 ± 1.03 compared to 7.10 ± 1.10 in those with negative samples, with a highly statistically significant difference observed

(p=0.0022*). These findings are consistent with previous research conducted by Christersson et al. (1992), Takeuchi et al. (2002), and Kulkarni et al. (2018). These studies have demonstrated a correlation between the detection frequency of *P. gingivalis* and CAL, indicating a propensity for increased prevalence of *P. gingivalis* in sites with greater CAL in chronic periodontitis patients. This indicates a potential association between *P. gingivalis* and the severity of attachment loss seen in severe forms of periodontitis. [89,90,5] (Table 8, Graph 8)

Our study findings indicate that age and gender did not significantly influence prevalence of "*P. gingivalis"* among severe "periodontitis" patients. However, the frequency of *P. gingivalis* detection significantly increased with greater plaque scores, periodontal pocket depth, and clinical attachment loss. Notably, while "*P. gingivalis"* was detected in both moderate and severe "periodontitis" patients, its prevalence was significantly higher in severe "periodontitis" patients. Therefore, the outcomes of our PCR analysis imply a strong correlation between "*P. gingivalis"* and deeper periodontal pockets as well as increased clinical attachment loss in individuals afflicted with severe periodontal disease

SUMMARY AND CONCLUSION

Chronic periodontitis is a multifactorial infection, and substantial evidence has demonstrated that *"P. gingivalis"* plays a crucial role as a keystone periodontal pathogen. Several studies investigating *"P. gingivalis"* prevalence in both periodontally healthy individuals and those with "chronic periodontitis" have yielded varying results. This variability may result from differences in periodontal conditions, geographic locations of study populations, as well as variations in bacterial detection methods.

Thus, our study aimed to identify *"Porphyromonas gingivalis"* in periodontally healthy adults and "chronic periodontitis" patients visiting the Outpatient Department of Periodontics at "KLE V.K. Institute of Dental Sciences in Belgaum," using polymerase chain reaction.

This cross-sectional study was conducted at "Outpatient Department of Periodontics, KLE V.K. Institute of Dental Sciences." 60 subjects, aged 30 to 50 years, were enrolled based on inclusion criteria and categorized into three groups: periodontally healthy, moderate "periodontitis" and severe "periodontitis" Recruitment utilized a convenience sampling approach, with informed consent obtained from all participants. Plaque index, PPD and CAL were assessed. Plaque specimen was obtained using a sterilized Gracey curette from sites with the highest plaque accumulation in Group 1 and at the deepest pocket probing depths in Groups 2,3. Collected plaque samples were cultured on supplemented blood agar plates, followed by a selection of those displaying black-pigmented colonies and gram-negative coccobacilli. Biochemical characterization and DNA isolation were conducted, followed by agarose gel electrophoresis for visualization of bacterial DNA bands. Samples demonstrating the presence of bacterial DNA underwent PCR

analysis using a species-specific primer to confirm "*P. gingivalis*" presence. Confirmation was achieved through comparison with a standard control, revealing the presence of *"P. gingivalis"* with a 420 bp band. This approach ensured accurate detection and confirmation of "*P. gingivalis.*"

Statistical analysis of the results was conducted using Chi-square, Mann-Whitney U test, and Kruskal-Wallis ANOVA, with a significance level set at $p < 0.05$.

Within the limitations of this study, the following conclusions were drawn:

1. *"P. gingivalis"* was found in none of the periodontally healthy subjects, 10% of those with moderate "periodontitis," and 50% of individuals diagnosed with severe "periodontitis." Notably, its prevalence was significantly higher among severe "periodontitis" patients. A highly significant difference with the increase in the plaque score was observed among healthy, moderate and severe "periodontitis" patients, underscoring the significance of plaque accumulation in periodontal disease progression.

2. Additionally, our findings suggest that age and gender do not show any significant difference in the prevalence of "*P. gingivalis"* among patients with moderate and severe "periodontitis."

3. The detection frequency of "*P. gingivalis"* increased significantly with the increase in periodontal pocket depth and increased clinical attachment loss. Thus, suggesting significant increase of "*P. gingivalis"* with the severity of periodontal destruction.

Therefore, the findings of our PCR analysis concluded the prevalence of "*P. gingivalis*" did not have any significance with age and gender. However, the findings revealed the presence of "*P. gingivalis"* in both moderate and severe periodontitis patients with a higher prevalence in severe periodontitis patients, co-relating the severity of "*P. gingivalis"* with severe "periodontitis."

LIMITATIONS

- The main limitation of the present study is its small sample size. Studies with larger sample sizes are necessary to provide a comprehensive understanding of the presence of "*P. gingivalis."*
- We first utilized culture to detect the presence of black colonies, which may have introduced contamination and affected the accuracy of results due to its low sensitivity. While PCR being more expensive than culture, its higher sensitivity could have potentially detected all bacteria if employed directly.
- The conventional PCR assay utilized in this study has the limitation of detecting only the presence or absence of "*P. gingivalis.*" However, it cannot quantify the amount of bacteria present. Thus, employing quantitative methods such as real-time PCR can be considered for additional insights into the correlation between the amount of bacteria and periodontal status.

FUTURE PERSPECTIVES

- To advance our understanding of periodontal disease, it is imperative to conduct comprehensive evaluations of virulent strains of "*P. gingivalis.*" This approach will provide valuable insights into the diverse roles played by these strains in the pathogenesis of periodontitis.
- Exploring more advanced and sensitive detection methods, such as next-generation sequencing (NGS) or metagenomic analysis, could improve the accuracy of identifying "*P. gingivalis"* in plaque samples.

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ANNEXURE 1: ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
KLE VK INSTITUTE OF DENTAL SCIENCES
A Constituent Unit of KLE Academy of Higher Education & Research
Accredited 'A' Grade by NAAC | Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
☎: 0831-2470362 | Web: http://www.kledental-bgm.edu.in
FAX: 0831-2470640 | E-mail:principal@kledental-bgm.edu.in

Sl. No. : 1584

CERTIFICATE

EC/NEW/INST/2021/2435
Research & Ethics Committee

This is to Certify that the synopsis titled

Identification of Porphyromonas gingivalis in periodontally healthy adults and chronic periodontitis patients using polymerase chain reaction

Submitted by

Dr. _____ *P. G. Student / Staff, Guided by* _____ *from Department of* Periodontics *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 3/4/24

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

MEMBER SECRETARY
Research & Ethical Committee
KLEVK Institute of Dental Sciences
BELAGAVI.

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLE VK Institute of Dental Sciences

ANNEXURE 2: BSRC REPORT

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI, KARNATAKA,
(Formerly Known as KLE University) (Deemed to Be University u/s 3 of the UGC Act, 1956)
DR. PRABHAKAR KORE BASIC SCIENCE RESEARCH CENTER (BSRC), BELAGAVI, KARNATAKA.
III Floor, V. K. Institute of Dental Sciences Campus, Nehru Nagar, Belagavi - 590 010, Karnataka – INDIA
E-mail: research@kledeemeduniversity.edu.in; Web: www.klepkbsrc.org; Phone: 0831- 2444444, Extn: 4122
GSTIN/UIN: 29AABTK0881E1ZN

Report

TITLE OF RESEARCH: "Identification of *Porphyromonas gingivalis* in periodontally healthy adults and chronic periodontitis patients using polymerase chain reaction."

NAME OF THE STUDENT:

NAME OF THE GUIDE:

NAME OF THE CO-GUIDE:

Objective parameters:

1. Identification of *Porphyromonas gingivalis* in the periodontally healthy, moderate and severe periodontitis subjects.
2. To evaluate the correlation of *Porphyromonas gingivalis* with clinical periodontal parameters.

Laboratory Methods for assessing microbiome:

1. **Method used:** Serial dilution and subculturing of pure colonies. Isolation of DNA and PCR amplification.
2. **Sample Quantity:** Plaque sample in 1ml of transport media (Thioglycolate broth)
3. **Sample Size:** 60 (20 in each group)

Lab Investigations done in BSRC:

All 60 samples were serially diluted and cultured on blood agar plates supplemented with menadione and horse serum.

Pure colonies were isolated and plated onto fresh blood agar plates.

Gram staining and biochemical tests were performed for all the positive colonies.

Further, the DNA was isolated from pure colonies and amplified using PCR.

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GSTIN/UIN: 29AABTK0881E1ZN

Results:

Healthy Patients (HP)

Sr. no	Sample Name	Age	Gender	Date of Sample collection	Results
1.	HP-1	49	M	09-10-2023	Negative
2.	HP-2	31	F	09-10-2023	Negative
3.	HP-3	36	M	09-10-2023	Negative
4.	HP-4	34	F	09-10-2023	Negative
5.	HP-5	38	M	09-10-2023	Negative
6.	HP-6	46	F	17-10-2023	Negative
7.	HP-7	30	F	17-10-2023	Negative
8.	HP-8	42	F	17-10-2023	Negative
9.	HP-9	33	M	17-10-2023	Negative
10.	HP-10	46	F	17-10-2023	Negative
11.	HP-11	35	F	26-10-2023	Negative
12.	HP-12	39	M	26-10-2023	Negative
13.	HP-13	30	M	07-11-2023	Negative
14.	HP-14	41	M	07-11-2023	Negative
15.	HP-15	34	F	07-11-2023	Negative
16.	HP-16	40	F	07-11-2023	Negative
17.	HP-17	32	M	07-11-2023	Negative
18.	HP-18	38	F	17-11-2023	Negative
19.	HP-19	37	M	17-11-2023	Negative
20.	HP-20	44	M	17-11-2023	Negative

All samples were Negative for *P.gingivalis* (No black colonies were observed).

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GSTIN/UIN: 29AABTK0881E1ZN

Moderate Periodontitis Patients (MP)

Sr. no	Sample Name	Age	Gender	Date of Sample collection	Results
1.	MP-1	50	M	05-05-2023	Negative
2.	MP-2	30	M	08-05-2023	Negative
3.	MP-3	44	F	17-05-2023	Negative
4.	MP-4	45	F	07-06-2023	Negative
5.	MP-5	45	M	15-06-2023	**Positive**
6.	MP-6	42	F	21-06-2023	Negative
7.	MP-7	33	M	08-07-2023	**Positive**
8.	MP-8	50	M	11-07-2023	Negative
9.	MP-9	48	M	20-07-2023	Negative
10.	MP-10	47	M	20-07-2023	Negative
11.	MP-11	44	M	08-08-2023	Negative
12.	MP-12	33	F	17-08-2023	Negative
13.	MP-13	30	F	04-09-2023	Negative
14.	MP-14	35	F	12-09-2023	Negative
15.	MP-15	50	F	03-10-2023	Negative
16.	MP-16	47	F	06-10-2023	Negative
17.	MP-17	30	F	10-10-2023	Negative
18.	MP-18	49	F	27-10-2023	Negative
19.	MP-19	42	F	02-11-2023	Negative
20.	MP-20	38	F	17-11-2023	Negative

Positive: Black colonies were observed

Negative: No black colonies were observed

2 samples were positive for *P.gingivalis*

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GSTIN/UIN: 29AABTK0881E1ZN

Severe Periodontitis Patients (SP)

Sr. no	Sample Name	Age	Gender	Date of Sample collection	Results
1.	SP-1	36	F	03-05-2023	Negative
2.	SP-2	31	M	10-05-2023	Positive
3.	SP-3	34	F	02-06-2023	Positive
4.	SP-4	40	M	28-07-2023	Negative
5.	SP-5	33	F	01-08-2023	Negative
6.	SP-6	36	F	21-08-2023	Negative
7.	SP-7	40	M	18-09-2023	Positive
8.	SP-8	34	F	21-09-2023	Negative
9.	SP-9	47	M	25-09-2023	Negative
10.	SP-10	49	M	29-09-2023	Positive
11.	SP-11	30	M	03-10-2023	Positive
12.	SP-12	31	F	06-10-2023	Negative
13.	SP-13	45	M	12-10-2023	Positive
14.	SP-14	48	M	18-10-2023	Negative
15.	SP-15	42	M	26-10-2023	Positive
16.	SP-16	42	F	31-10-2023	Positive
17.	SP-17	47	M	06-11-2023	Positive
18.	SP-18	32	F	21-11-2023	Negative
19.	SP-19	36	M	08-12-2023	Negative
20.	SP-20	43	M	12-12-2023	Positive

Positive: Black colonies were observed

Negative: No black colonies were observed

10 samples were positive for *P.gingivalis*

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GSTIN/UIN: 29AABTK0881E1ZN

Biochemical tests:

Sr. no	Sample Name	Gram Staining	Indole test	Catalase test	Sugar fermentation test	Overall Results for P.G
1.	MP-5	Gram Negative	Positive	Negative	Negative	Positive
2.	MP-7	Gram Negative	Positive	Negative	Negative	Positive
3.	SP-2	Gram Negative	Positive	Negative	Negative	Positive
4.	SP-3	Gram Negative	Positive	Negative	Negative	Positive
5.	SP-7	Gram Negative	Positive	Negative	Negative	Positive
6.	SP-10	Gram Negative	Positive	Negative	Negative	Positive
7.	SP-11	Gram Negative	Positive	Negative	Negative	Positive
8.	SP-13	Gram Negative	Positive	Negative	Negative	Positive
9.	SP-15	Gram Negative	Positive	Negative	Negative	Positive
10.	SP-16	Gram Negative	Positive	Negative	Negative	Positive
11.	SP-17	Gram Negative	Positive	Negative	Negative	Positive
12.	SP-20	Gram Negative	Positive	Negative	Negative	Positive

Remarks:

The results are satisfactory and relevant references have been followed.

Further the amplified product was sent to Eurofins lab for identification of Strain.

29/01/24
[Dr. Rubeena D. Nadaf]

KLE Academy of Higher Education and Research, Belagavi.
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29/01/24
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KLE Academy of Higher Education and Research
Belagavi-10, Karnataka, India.

ANNEXURE 3: BIOSTATISTICS CERTIFICATE

K L E VISHWANATH KATTI
INSTITUTE OF DENTAL SCIENCES
(A Constituent unit of KLE Academy of Higher Education & Research
(Formerly known as KLE University) Deemed-to-be-University u/s 3 of the UGC Act, 1956)
J.N.M.C. Campus, Nehru Nagar, Belagavi-590 010, Karnataka, India
Accredited 'A+' grade by NAAC (3rd Cycle) Placed in Category 'A' by MHRD (GoI)
☎: 0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail : principal@kledental-bgm.edu.in

Biostatistics Clearance Certificate

This is to certify that Biostatics aspect of the Dissertation/Research work of

Post Graduate Student, under the guidance of M.D.S, Ph.D.,

Professor, Department of Periodontics, entitled "**Identification of *Porphyromonas Gingivalis* In Periodontally Healthy Adults And Chronic Periodontitis Patients Using Polymerase Chain Reaction**" has been done under my guidance and completed satisfactorily.

Place: Belagavi
Date : 15·02·2024

Name & Signature of Biostatistician

Dr. S. B. JAVALI Ph.D.
Sr. Associate Professor in Statistics
Department of Community Medicine
USM KLE International Medical Programme
BELAGAVI-590010.

ANNEXURE 4: PLAGIARISM REPORT

Scientific Correspondence and Review Committee
KLE VK Institute of Dental Sciences
A Constituent Unit of KLE Academy of Higher Education and Research
(Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi - 590 010, Karnataka State
Accredited 'A' Grade by NAAC (2nd Cycle)
Placed in Category 'A' by MHRD (GoI)
☎: 0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail:principal@kledental-bgm.edu.in

Date : 2 . 04 . 2024 Serial No. : 161

PLAGIARISM CHECK REPORT

Name of the Applicant :
~~UG~~ / PG / Ph.D / Staff : POST GRADUATE
Batch & Year : 2021 - 2024
Department : PERIODONTICS

The soft copy of Research Work / Manuscript by ..... entitled

"IDENTIFICATION OF "PORPHYROMONAS GINGIVALIS" IN PERIODONTALLY HEALTHY ADULTS AND CHRONIC PERIODONTITIS PATIENTS USING "POLYMERASE CHAIN REACTION""

under the guidance of .....has been submitted for Anti-Plagiarism check to the Scientific Correspondence & Review Committee of KLE VK Institute of Dental Sciences using "Turn-it-in" software.

The scan has been carried out and the scanned output reveals a Similarity Index of ..... 7 .....%, which is **within / not within** the acceptable limits of 10% as per the UGC guidelines.

2/04/2024

Member Secretary
Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
KAHER-Belagavi

Chairman
Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
KAHER - Belagavi

ANNEXURE 5: CONSENT FORM

DEPARTMENT OF PERIODONTICS

KAHER'S KLE'S V.K. INSTITUTE OF DENTAL SCIENCES, BELAGAVI

CONSENT FORM

IDENTIFICATION OF *Porphyromonas gingivalis* IN PERIODONTALLY HEALTHY ADULTS AND IN CHRONIC PERIODONTITIS PATIENTS USING POLYMERASE CHAIN REACTION.

PRINCIPAL INVESTIGATOR: DR.

I, _____, aged _____ years have been informed about my involvement in the study.

- I agree to give my personal details like Name, Age, Gender, Residential Address, Previous and Present dental history and any other details if required for the study to the best of my knowledge.
- I will co-operate with the dentist.
- I will follow the instructions given by the dentist during study.
- I will visit the dentist as and when required for the study, at the given time and date.
- I permit the dentist to utilize the information given and results obtained from this study for presentation and publication without disclosing my identity.
- I have understood the nature of the study and permit the dentist to collect plaque samples.

- I will not claim any returns for co-operation in this study, even if it is being sponsored by any agency. I am participating with my own will and wish.
- If for any reason I am unable to participate in the study, for reasons unknown, I can withdraw from the study.
- In my full consciousness and presence of mind, after understanding all the procedures and related complications if any, in my vernacular language, I am willing and give my consent to participate in this study.

Date:

Address & Ph. No:

Signature:

DEPARTMENT OF PERIODONTICS

KAHER'S KLE'S V.K. INSTITUTE OF DENTAL SCIENCES, BELAGAVI

CONSENT FORM

IDENTIFICATION OF *Porphyromonas gingivalis* IN PERIODONTALLY HEALTHY ADULTS AND IN CHRONIC PERIODONTITIS PATIENTS USING POLYMERASE CHAIN REACTION.

PRINCIPAL INVESTIGATOR: DR.

मी _____, वय _____ वर्षे मला अभ्यासातील माझ्या सहभागाबद्दल माहिती देण्यात आली आहे.

- मी माझे वैयक्तिक तपशील जसे की नाव, वय, लिंग, निवासी पत्ता, पूर्वीचा आणि सध्याचा दंत इतिहास आणि माझ्या सर्वोत्तम माहितीनुसार अभ्यासासाठी आवश्यक असल्यास इतर कोणतेही तपशील देण्यास सहमत आहे.
- मी दंतवैद्याला सहकार्य करीन.
- मी अभ्यासादरम्यान दंतवैद्याने दिलेल्या सूचनांचे पालन करीन.
- दिलेल्या वेळी आणि तारखेला अभ्यासासाठी आवश्यक असेल तेव्हा मी दंतवैद्याला भेट देईन.
- मी दंतचिकित्सकाला माझी ओळख उघड न करता सादरीकरण आणि प्रकाशनासाठी दिलेली माहिती आणि या अभ्यासातून मिळालेले परिणाम वापरण्याची परवानगी देतो.
- मला अभ्यासाचे स्वरूप समजले आहे आणि दंतचिकित्सकाला प्लेकचे नमुने गोळा करण्याची परवानगी दिली आहे.

- मी या अभ्यासामध्ये सहकार्यासाठी कोणत्याही परताव्याचा दावा करणार नाही, जरी ते कोणत्याही एजन्सीद्वारे प्रायोजित केले जात असले तरीही. मी माझ्या इच्छेने आणि इच्छेने सहभागी होत आहे.
- कोणत्याही कारणास्तव मी अभ्यासात सहभागी होऊ शकलो नाही, अज्ञात कारणांमुळे, मी अभ्यासातून माघार घेऊ शकतो.
- माझ्या पूर्ण जाणीवेने आणि मनाच्या उपस्थितीत, माझ्या स्थानिक भाषेत सर्व प्रक्रिया आणि संबंधित गुंतागुंत समजल्यानंतर, मी या अभ्यासात सहभागी होण्यास तयार आहे आणि माझी संमती देतो.

तारीख:

पत्ता व फोन नंबर**:** स्वाक्षरी:

DEPARTMENT OF PERIODONTICS

KAHER'S KLE'S V.K. INSTITUTE OF DENTAL SCIENCES, BELAGAVI

CONSENT FORM

IDENTIFICATION OF *Porphyromonas gingivalis* IN PERIODONTALLY HEALTHY ADULTS AND IN CHRONIC PERIODONTITIS PATIENTS USING POLYMERASE CHAIN REACTION.

PRINCIPAL INVESTIGATOR: DR.

- **ನಾನು, _____, _____ ವರ್ಷ ವಯಸ್ಸಿನ, ಅಧ್ಯಯನದಲ್ಲಿ ನನ್ನ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆಯ ಬಗ್ಗೆ ತಿಳಿಸಲಾಗಿದೆ.**
- **ನನ್ನ ವೈಯಕ್ತಿಕ ವಿವರಗಳಾದ ಹೆಸರು, ವಯಸ್ಸು, ಲಿಂಗ, ವಾಸದ ವಿಳಾಸ, ಹಿಂದಿನ ಮತ್ತು ಪ್ರಸ್ತುತ ದಂತ ಇತಿಹಾಸ ಮತ್ತು ನನ್ನ ಜ್ಞಾನದ**
- **ಮಟ್ಟಿಗೆ ಅಧ್ಯಯನಕ್ಕೆ ಅಗತ್ಯವಿದ್ದರೆ ಇತರ ಯಾವುದೇ ವಿವರಗಳನ್ನು ನೀಡಲು ನಾನು ಒಪ್ಪುತ್ತೇನೆ.**
- **ನಾನು ದಂತವೈದ್ಯರೊಂದಿಗೆ ಸಹಕರಿಸುತ್ತೇನೆ.**
- **ಅಧ್ಯಯನದ ಸಮಯದಲ್ಲಿ ದಂತವೈದ್ಯರು ನೀಡಿದ ಸೂಚನೆಗಳನ್ನು ನಾನು ಅನುಸರಿಸುತ್ತೇನೆ.**
- **ನಾನು ದಂತವೈದ್ಯರನ್ನು ಅಧ್ಯಯನಕ್ಕೆ ಅಗತ್ಯವಿರುವಾಗ ಮತ್ತು ನಿರ್ದಿಷ್ಟ ಸಮಯ ಮತ್ತು ದಿನಾಂಕದಂದು ಭೇಟಿ ಮಾಡುತ್ತೇನೆ.**
- **ನನ್ನ ಗುರುತನ್ನು ಬಹಿರಂಗಪಡಿಸದೆ ಪ್ರಸ್ತುತಿ ಮತ್ತು ಪ್ರಕಟಣೆಗಾಗಿ ನೀಡಲಾದ ಮಾಹಿತಿ ಮತ್ತು ಈ ಅಧ್ಯಯನದಿಂದ ಪಡೆದ**
- **ಫಲಿತಾಂಶಗಳನ್ನು ಬಳಸಿಕೊಳ್ಳಲು ನಾನು ದಂತವೈದ್ಯರಿಗೆ ಅನುಮತಿ ನೀಡುತ್ತೇನೆ.**

- ನಾನು ಅಧ್ಯಯನದ ಸ್ವರೂಪವನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ ಮತ್ತು ಪ್ಲೇಕ್ ಮಾದರಿಗಳನ್ನು ಸಂಗ್ರಹಿಸಲು ದಂತವೈದ್ಯರಿಗೆ ಅನುಮತಿ ನೀಡಿದ್ದೇನೆ.
- ಯಾವುದೇ ಏಜೆನ್ಸಿಯಿಂದ ಪ್ರಾಯೋಜಿತವಾಗಿದ್ದರೂ ಸಹ, ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಸಹಕಾರಕ್ಕಾಗಿ ನಾನು ಯಾವುದೇ ಆದಾಯವನ್ನು
- ಕ್ಲೈಮ್ ಮಾಡುವುದಿಲ್ಲ. ನಾನು ನನ್ನ ಸ್ವಂತ ಇಚ್ಛೆ ಮತ್ತು ಆಶಯದೊಂದಿಗೆ ಭಾಗವಹಿಸುತ್ತಿದ್ದೇನೆ.
- ಯಾವುದೇ ಕಾರಣಕ್ಕಾಗಿ ನಾನು ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಸಾಧ್ಯವಾಗದಿದ್ದರೆ, ಅಜ್ಞಾತ ಕಾರಣಗಳಿಗಾಗಿ, ನಾನು ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆ ಸರಿಯಬಹುದು.
- ನನ್ನ ಪೂರ್ಣ ಪ್ರಜ್ಞೆ ಮತ್ತು ಮನಸ್ಸಿನ ಉಪಸ್ಥಿತಿಯಲ್ಲಿ, ಎಲ್ಲಾ ಕಾರ್ಯವಿಧಾನಗಳು ಮತ್ತು ಸಂಬಂಧಿತ ತೊಡಕುಗಳು ಯಾವುದಾದರೂ
- ಇದ್ದರೆ, ನನ್ನ ಸ್ಥಳೀಯ ಭಾಷೆಯಲ್ಲಿ ಅರ್ಥಮಾಡಿಕೊಂಡ ನಂತರ, ನಾನು ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಸಿದ್ಧನಿದ್ದೇನೆ ಮತ್ತು ನನ್ನ
- ಒಪ್ಪಿಗೆಯನ್ನು ನೀಡುತ್ತೇನೆ ದಿನಾಂಕ:

ವಿಳಾಸ ಮತ್ತು Ph. ಸಂಖ್ಯೆ: ಸಹಿ:

ANNEXURE 6: PROFORMA

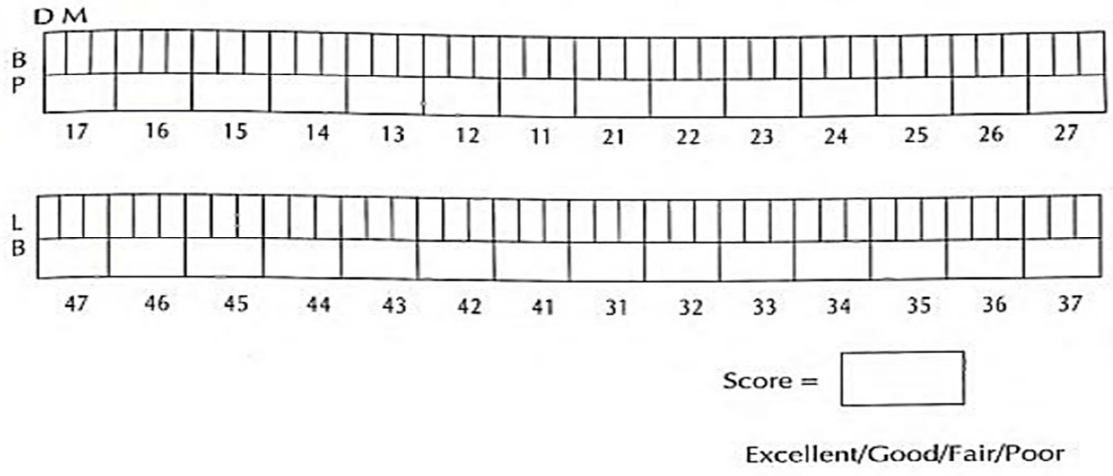
DEPARTMENT OF PERIODONTICS

KAHER'S KLE V.K. INSTITUTE OF DENTAL SCIENCES, BELAGAVI.

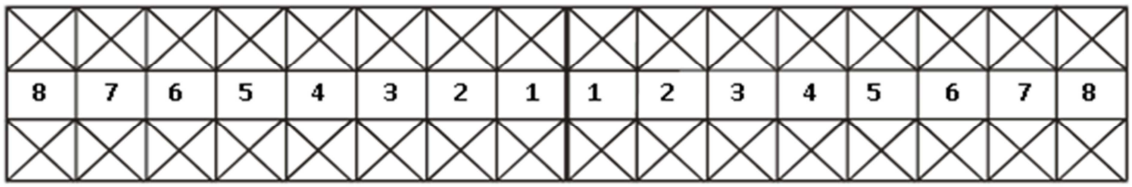
PROFORMA

IDENTIFICATION OF *Porphyromonas gingivalis* IN PERIODONTALLY HEALTHY ADULTS AND IN CHRONIC PERIODONTITIS PATIENTS USING POLYMERASE CHAIN REACTION.

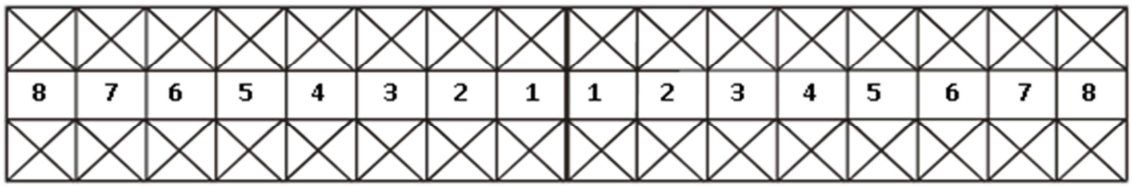
- Case No: OPD No:
- Name:
- Age: Sex: Occupation:
- Address:
- Chief Complaint:
- Dental history:
- **Plaque Index (PI)** (Silness J and Loe H, 1967):



- **Pocket Probing Depth (PPD):**



- **Clinical Attachment Level (CAL):**



ANNEXURE 7: MASTER CHART

Sr. No.	Group	Age	Gender	Plaque Score	PPD	CAL	Result
1.	Healthy	49	M	0.5	1	0	Negative
2.	Healthy	31	F	0.8	2	0	Negative
3.	Healthy	36	M	1.4	3	0	Negative
4.	Healthy	34	F	0.7	3	0	Negative
5.	Healthy	38	M	1.3	2	0	Negative
6.	Healthy	46	F	1.4	2	0	Negative
7.	Healthy	30	F	1.2	1	0	Negative
8.	Healthy	42	F	1.4	1	0	Negative
9.	Healthy	33	M	1.1	1	0	Negative
10.	Healthy	46	F	1	3	0	Negative
11.	Healthy	35	F	0.5	2	0	Negative
12.	Healthy	39	M	1.4	2	0	Negative
13.	Healthy	30	M	1.6	2	0	Negative
14.	Healthy	41	M	1.1	3	0	Negative
15.	Healthy	34	F	0.5	3	0	Negative
16.	Healthy	40	F	0.7	2	0	Negative
17.	Healthy	32	M	1.2	2	0	Negative
18.	Healthy	38	F	0.9	1	0	Negative
19.	Healthy	37	M	0.7	1	0	Negative
20.	Healthy	44	M	1.3	1	0	Negative
21.	Moderate Periodontitis	50	M	0.7	5	4	Negative

22.	Moderate Periodontitis	30	M	1	5	4	Negative
23.	Moderate Periodontitis	44	F	1.1	5	3	Negative
24.	Moderate Periodontitis	45	F	1.3	5	4	Negative
25.	Moderate Periodontitis	45	M	2.1	6	4	**Positive**
26.	Moderate Periodontitis	42	F	1.8	5	4	Negative
27.	Moderate Periodontitis	33	M	1.6	6	4	**Positive**
28.	Moderate Periodontitis	50	M	2.4	5	4	Negative
29.	Moderate Periodontitis	48	M	1.4	5	4	Negative
30.	Moderate Periodontitis	47	M	2.8	5	3	Negative
31.	Moderate Periodontitis	44	M	2.5	6	4	Negative
32.	Moderate Periodontitis	33	F	0.8	5	5	Negative
33.	Moderate Periodontitis	30	F	1.8	6	4	Negative
34.	Moderate Periodontitis	35	F	1.2	5	4	Negative
35.	Moderate Periodontitis	50	F	1.7	6	4	Negative
36.	Moderate Periodontitis	47	F	1.3	5	4	Negative

37.	Moderate Periodontitis	30	F	1.6	5	4	Negative
38.	Moderate Periodontitis	49	F	2.7	5	4	Negative
39.	Moderate Periodontitis	42	F	1.3	5	3	Negative
40.	Moderate Periodontitis	38	F	2.8	5	3	Negative
41.	Severe Periodontitis	36	F	1.4	7	6	Negative
42.	Severe Periodontitis	31	M	1.1	7	10	**Positive**
43.	Severe Periodontitis	39	F	2.8	8	9	**Positive**
44.	Severe Periodontitis	40	M	2.3	7	6	Negative
45.	Severe Periodontitis	33	F	1.7	9	7	Negative
46.	Severe Periodontitis	36	F	2.5	7	6	Negative
47.	Severe Periodontitis	40	M	1.9	8	9	**Positive**
48.	Severe Periodontitis	34	F	1.2	8	7	Negative
49.	Severe Periodontitis	47	M	1.1	7	6	Negative
50.	Severe Periodontitis	49	M	2.7	10	10	**Positive**
51.	Severe Periodontitis	30	M	2.2	9	9	**Positive**

52.	Severe Periodontitis	31	F	1.5	8	8	Negative
53.	Severe Periodontitis	45	M	2.5	9	10	**Positive**
54.	Severe Periodontitis	48	M	1.1	7	8	Negative
55.	Severe Periodontitis	42	M	1.6	10	8	**Positive**
56.	Severe Periodontitis	42	F	2.2	9	7	**Positive**
57.	Severe Periodontitis	47	M	1.5	10	10	**Positive**
58.	Severe Periodontitis	32	F	1.9	7	8	Negative
59.	Severe Periodontitis	36	M	2.1	8	9	Negative
60.	Severe Periodontitis	47	M	2.9	9	10	**Positive**