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"Molecular Insights into *Porphyromonas Gingivalis* Infection in Periodontitis and Rheumatoid Arthritis"

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Abstract: P. gingivalis is a Gram-negative anaerobe and a key pathogen in periodontitis due to its potent virulence factors. Though normally part of the oral microbiome, it is capable of triggering systemic inflammation and has been implicated in several non-oral diseases, including RA, cardiovascular disease, Alzheimer's disease, diabetes, and cancer. This study focussed in detecting *Porphyromonas gingivalis* (*P. gingivalis*) in subgingival plaque samples from patients with Periodontitis and/or Rheumatoid arthritis (RA) using polymerase chain reaction (PCR).A total of 200 subjects were recruited and divided equally into four groups: (1) RA with periodontitis, (2) RA without periodontitis, (3) periodontitis without RA, and (4) healthy controls. Subgingival plaque was collected using sterile curettes. Genomic DNA was extracted from the samples and analyzed using polymerase chain reaction (PCR) to detect the presence of *Porphyromonas gingivalis* (*P. gingivalis*).The bacterium was detected in significantly higher counts in periodontitis patients, regardless of RA status. Mean colony-forming unit (CFU) counts were 70 ± 40 in RA with periodontitis and 60 ± 42 in periodontitis-only patients. P. gingivalis was absent in healthy controls. The findings reinforce the role of P. gingivalis in periodontal disease and suggest a potential link to RA pathogenesis, given its higher prevalence in RA patients. This study demonstrates the effectiveness of PCR in detecting P. gingivalis and highlights the need to consider its systemic implications, especially in individuals with RA.

Keywords: Periodontal disease, Polymerase chain reaction, Porphyromonas gingivalis, Rheumatoid arthritis

Introduction

Porphyromonas gingivalis is a gram negative , obligate anaerobe bacteria that typically thrive in subgingival sites within the human oral cavity. This bacteria is the part of oral microbiome but can become highly destructive due to its specialized virulence factors. Together with *Tannerella forsythia* and *Treponema denticola*, it forms the "red complex", a group of highly pathogenic bacteria strongly associated with severe periodontal disease (Mysak et al., 2014).Chronic periodontitis is an inflammatory condition which affects the dental plaque and the soft tissues which supports the teeth. It is one of the most prevalent oral health problems globally, with a detrimental impact on quality of life. The inflammatory response mediated by neutrophils in periodontitis leads to the destruction of the periodontal ligaments, gingiva, alveolar bone, and cementum. Some of the key invasive pathogens associated with periodontitis include *P.gingivalis, Aggregatibacter actinomycetecomitans* and *Fusobacterium nucleatum*. (Gasmi Benahmed et al., 2022). *P. gingivalis* is a part of the complex oral microbiota, which includes over 500 different bacterial species. This organism plays a role in the development of periodontitis, a chronic inflammatory disease of the gums. Although *P. gingivalis* is a bacterium of the oral microbiome, which possesses some specialized virulence factors which enable its proliferation and ultimately become highly destructive within periodontal lesions (Mysak et al., 2014).

In recent time, *P. gingivalis* has received substantial attention due to its strong link with periodontal disease. *P. gingivalis* demonstrates a dual lifestyle at periodontal sites, colonizing both the subgingival plaque biofilm and invading gingival epithelial cells. *P. gingivalis* gets benefits from inter-bacterial cross-feeding and metabolic interplay, which creates a supportive environment within the polymicrobial periodontal biofilm. Additionally, *P. gingivalis* can enter and exit gingival epithelial cells, allowing for persistent infection in the gingival tissues.. Recent research indicates that *P. gingivalis* is also associated with various non-oral diseases such as inflammatory bowel disease, cancer, cardiovascular diseases, alzheimer's disease, rheumatoid arthritis, diabetes mellitus, premature birth, and non-alcoholic hepatitis (Zhang et al., 2023).

Periodontal diseases, caused by pathogens like *P. gingivalis* and *A. actinomycetemcomitans*, are commonly observed in RA and have been implicated in disease pathogenesis by inducing citrullination (Gómez-Bañuelos et al., 2019). Periodontal disease appears to play a key role, as the periodontal pathogen *Porphyromonas gingivalis* (*P. gingivalis*) can induce protein citrullination, triggering the production of anti-citrullinated protein/peptide antibodies (ACPAs), which are highly specific biomarkers for Rheumatoid arthritis. *P. gingivalis* has been shown to trigger or

worsen arthritis in animal models through mechanisms such as inducing citrullination, NETosis, osteoclastogenesis, and Th17 inflammatory responses, ultimately causing bone damage and systemic inflammation (Perricone et al., 2019). As per meta-analysis, the relationship between *P. gingivalis* exposure and the risk of rheumatoid arthritis once searched via multiple databases and which includes 28 epidemiological studies, the results of which showed a significant increase in the RA risk among subjects which were exposed to *P. gingivalis* infection. Interestingly, the periodontal pathogen *P. gingivalis* has been implicated in the generation of autoantibodies in RA, suggesting a potential biological link between the two conditions (de Molon et al., 2019). The findings highlighted the potential which targeted *P. gingivalis* and its associated pathways as a therapeutic approach for RA (Ahmadi et al., 2023).

Detecting key periodontal pathogens, such as *Porphyromonas gingivalis*, is crucial for assessing the risk, severity, and appropriate treatment of periodontal disease. Although bacterial culture has long been considered the "gold standard" for microbial identification, it has significant limitations. These include being time-consuming, labor-intensive, and having low sensitivity mainly due to the slow growth rates and specific cultivation requirements of many oral pathogens. To overcome these challenges, several alternative detection methods have been developed, including immunoassays, DNA probe assays, and polymerase chain reaction (PCR) techniques (Boutaga K et al.,2003). Among these, PCR stands out for its higher sensitivity and specificity in identifying periodontal pathogens.In recent years, PCR-based detection targeting the bacterial 16S ribosomal RNA (16S rRNA) gene has gained prominence. This molecular approach is transforming our understanding of the subgingival microbiome, revealing a broader spectrum of bacterial species without the biases inherent in culture-based techniques. The present study aims to detect *P. gingivalis* in individuals with chronic periodontitis showing varying degrees of tissue destruction, using a PCR-based assay (De Lillo A et al.,2004). Additionally, it seeks to evaluate the correlation between the presence of *P. gingivalis* and clinical periodontal parameters.

Materials and Methods

Isolation of *Porphyromonas gingivalis*

Previous studies have identified dental plaque as a primary source of *Porphyromonas gingivalis* (*P. gingivalis*). In the present study, subgingival dental plaque samples were collected from human patients as the presumptive source of *P. gingivalis*. Sampling was done through Gracey curette No. 5/6 (Hu-Friedy, Chicago, USA) from patients visiting dental hospitals in and around the Nagpur region, India. To cultivate *P. gingivalis*, blood

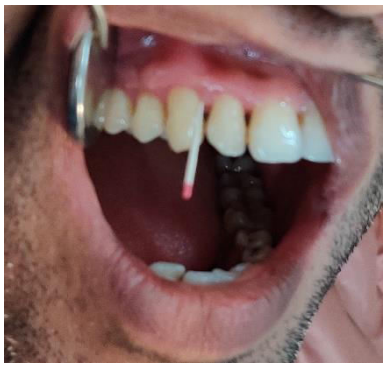
agar was employed as a selective medium. The composition of the blood agar (Himedia) was : Tryptone: 14.000 g, Peptone: 4.500 g, Yeast extract: 4.500 g, Sodium chloride: 5.000 g, Agar: 12.500 g. Samples were inoculated on blood agar plates using the streaking method and incubated at 37°C for 24 hours under anaerobic conditions, maintained using an anaerobic gas pack jar.

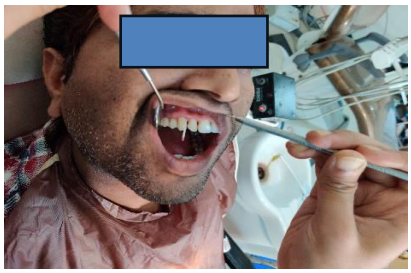
This study hypothesized that *P. gingivalis* contributes to the development of periodontitis and may further exacerbate rheumatoid arthritis (RA) in affected individuals. Given the bacterium's suspected involvement and associated virulence factors, a detailed investigation was warranted.

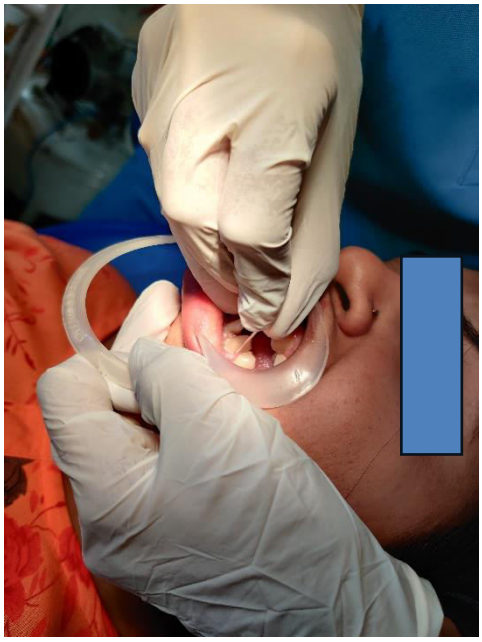
A study was conducted on 200 patients were included in the study, all of them provided with informed consent. The patients were categorised into four groups:

- Group A: RA with periodontitis (positivity confirmed)
- Group B: RA without periodontitis
- Group C: Periodontitis without RA
- Group D: Healthy controls

Following incubation, the presence of *P. gingivalis* was assessed based on the appearance of black-pigmented colonies on the blood agar plates (as shown in Fig. 1A). Further confirmation was performed via Gram staining, which revealed characteristic rod-shaped, Gram-negative bacterial morphology.







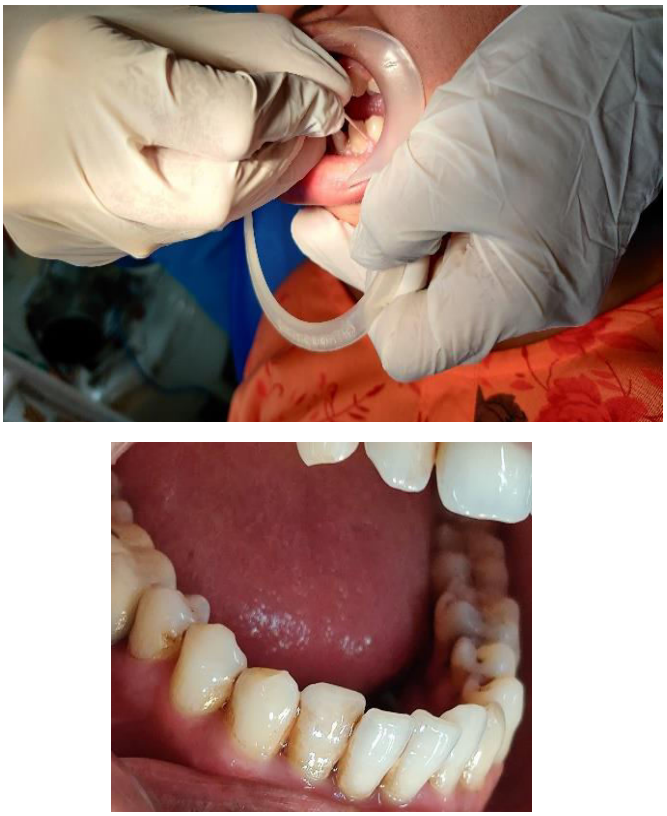


Fig. 1: Collection of samples from the patients as per groups.

DNA Extraction and Molecular Analysis

The subgingival plaque samples were subjected to molecular analysis at the Molecular Laboratory . DNA extraction was carried out using a simplified alkaline lysis method. Briefly, 50 µL of Tris–HCl buffer containing the plaque sample was incubated at 65°C for 15 minutes. After incubation, the samples underwent centrifugation at 10,000 rpm for 5 minutes, subsequently the supernatant was discarded.

The resulting cell pellet was resuspended in 25 µL of 0.5 N NaOH, taking care to avoid the formation of air bubbles, thereby ensuring efficient cell lysis and DNA release. Incubation of suspension was done for 30 minutes at room temperature. Neutralization was achieved by adding 25 µL of 1 M Tris buffer (pH 7.5), followed by vortex mixing. The final reaction volume was brought to 550 µL by adding 450 µL of sterile distilled water, and mixed thoroughly.

Polymerase Chain Reaction (PCR) for Detection of *P. gingivalis* (16S rDNA)

PCR amplification targeting the 16S rDNA region specific to *P. gingivalis* was performed in a 50 µL reaction volume. The primers used were:

- Forward primer (P1) : 5′-AAG CAG CTT GCC ATA CTG CG-3′
- Reverse primer (P2) : 5′-ACT GTT AGC AAC TAC CGA TGT-3′

The thermal cycling conditions were as follows;

- DNA denauration at 94° C (5 minutes)
- 35 cycles of:
    - Denaturation at 94°C for 1 minute
    - Annealing at 50°C for 1 minute
    - Extension at 72°C for 1.5 minutes
- Extension (Final) at 72 ° C for 7 minutes

The expected amplicon size was 404 base pairs (bp). Amplified PCR products were analyzed via 1.5% agarose gel electrophoresis and visualized under ultraviolet (UV) light after staining with ethidium bromide. Gel images were documented for further analysis.

Agarose Gel Electrophoresis

A horizontal gel electrophoresis apparatus (GIBCO-BRL, USA) was used for DNA analysis. DNA samples were mixed with loading dye in a 5:1 (sample: dye) ratio and loaded alongside a standard 1 kb DNA ladder. Post-electrophoresis, gels were stained with 0.5 µg/mL ethidium bromide and viewed under a UV transilluminator. Band sizes were estimated by comparing sample migration to the 1 kb molecular marker. The diagnostic band for *P. gingivalis* was expected at approximately 127 bp.

Statistical Analysis

The experimental data were collected in triplicate to ensure accuracy and reproducibility. Graph Pad Prism software was used for Statistical analysis of all the outcomes. Results were expressed as mean± standard deviation. Depending on the nature of the data set, comparisons were made using either analysis of variance (ANOVA) or Student's t-test. Data was considered Statistically significant for p value < 0.05.

Results

The present study was carried on total of 200 subjects, which were divided equally into four groups:

- **Group A**: Patients with rheumatoid arthritis (RA) and periodontitis
- **Group B**: Patients with RA but without periodontitis
- **Group C**: Patients with periodontitis but without RA
- **Group D**: Healthy controls

All participants were provided with informed consent before sample collection. Sampling procedures were conducted in accordance with the methodology outlined earlier and illustrated in Figure 1.

The present study was carried out on 200 subjects, distributed into four groups included patients with rheumatoid arthritis and periodontitis, patients with RA but without periodontitis, patients with periodontitis but without RA, and healthy controls. Sampling was conducted after obtaining consent from the participants, using the standard procedures described in the methodology and depicted in Figure 1.

The current study suggested a higher prevalence of *Porphyromonas gingivalis* (*P. gingivalis*) among patients suffered with rheumatoid arthritis (RA), regardless of the presence of concurrent periodontitis. In contrast, healthy control group was devoid of *P. gingivalis* . However, the bacterium was also present in patients with periodontitis who did not have RA, indicating its strong association with periodontal disease as well. These findings, summarized in **Table 1**, support the hypothesis that *P. gingivalis* may be linked not only to periodontal pathology but also to systemic inflammatory conditions such as RA.

Table 1 – PG count recorded with samples tested for RA with periodontitis (positively confirmed), RA without periodontitis, periodontitis with RA and healthy control once sampling inoculated on blood agar plate.

	RA with periodontitis (Positivity confirmed) (n = 50)	RA without periodontitis	Periodontitis with RA	Healthy control
Total positivity Colonies per sample	(n = 50) 100 % 60 ± 42	(n = 40) 80 % 15 ± 12	(n = 50) 100 % 70 ± 40	(n = 50) 100 % 0.0 ± 0.00

As shown in **Table 1**, a notable association was observed between *P. gingivalis* (PG) presence and rheumatoid arthritis (RA), both in patients with and without periodontitis. Remarkably, PG was detected in 80% of RA patients who did not present with clinical signs of periodontitis, although the colony-forming unit (CFU) counts in these samples were relatively low (15 ± 12 CFU/sample). In comparison, patients with confirmed periodontitis whether or not they had RA exhibited significantly higher PG counts, averaging 60 ± 42 and 70 ± 40 CFU/sample, respectively. *P. gingivalis* was notably absent in all the healthy control samples, consistent with expectations.

Furthermore, as illustrated in **Figure 2**, PG colonies exhibited characteristic black, mucoid pigmentation when cultured on blood agar under anaerobic conditions. These

colonies were successfully maintained through regular sub-culturing, confirming the culturability and stability of the isolate. This makes PG a promising candidate for further investigation into its virulence factors and its potential role in the pathogenesis of RA.

Figure. 2. *P. gingivalis* black pigmented colonies on Blood agar plates

Species-level identification of the *P. gingivalis* isolates was confirmed through partial sequencing of the 16S rRNA gene. The obtained sequences were aligned and analyzed using the BLASTN program against the NCBI nucleotide database. High sequence homology with reference *P. gingivalis* strains confirmed the identity of the isolates. Phylogenetic analysis based on the 16S rRNA gene sequences further validated the classification of the isolates within the *P. gingivalis* clade, as depicted in **Figure 3**. This molecular confirmation supports the culture-based identification and underscores the reliability of the isolates for further genomic and pathogenicity studies.

select all 5 sequences selected GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Porphyromonas gingivalis strain 14 16S ribosomal RNA gene, partial sequence	Porphyromona...	1626	1626	100%	0.0	100.00%	894	PP464152.1
Porphyromonas gingivalis strain HG66 genome	Porphyromona...	1626	6504	100%	0.0	100.00%	2441780	CP007756.1
Porphyromonas gingivalis strain 7 16S ribosomal RNA gene, partial sequence	Porphyromona...	1626	1626	100%	0.0	100.00%	902	PP464151.1
Porphyromonas gingivalis ATCC 33277 DNA, complete genome	Porphyromona...	1626	6504	100%	0.0	100.00%	2354886	AP009380.1
Porphyromonas gingivalis ATCC 33277 16S ribosomal RNA, partial sequence	Porphyromona...	1626	1626	100%	0.0	100.00%	1475	NR_074234.1

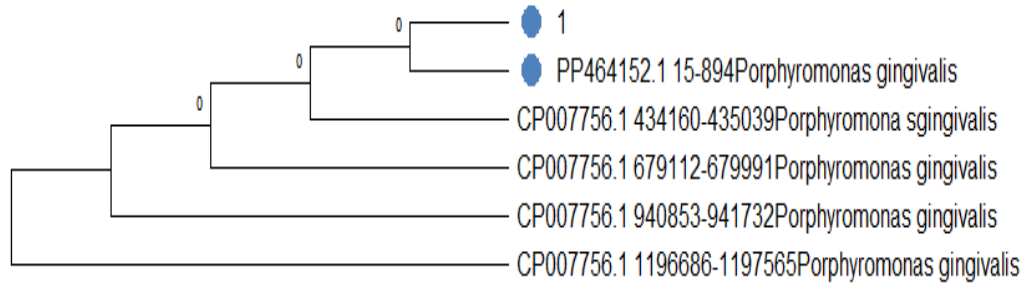


Figure 3: Isolate identified by 16S rRNA gene homology as *Porphyromonas gingivalis* showcased with BLAST homology and Phylogeny

Discussion

Periodontitis is a multifactorial disease that is associated with loss of the supporting tissues (i.e., periodontal ligament and alveolar bone) around the tooth (Malgikar S et al.,2016). It is widely accepted that the disease occurs as a result of subgingival plaque with specific bacteria, particularly Gram-negative anaerobes (Riggio MP et al.,1996). The use of clinical parameters in sample site selection, particularly PPD measurements, is likely to enhance the chance of detecting pathogenic bacteria. Several diagnostic methods can be used to detect bacterial species that have been identified as periodontal pathogens, including *P.gingivalis*. These methods include bacterial culture, enzymatic assays, immunoassays, nucleic acid probes, checkerboard DNA–DNA hybridization and PCR (Loomer PM 2004). The culture method is considered the gold standard (reference method) while determining the utility of a new microbial diagnostics in periodontics. Culture methods rely on detecting viable organisms and require immediate processing of samples to maximize bacterial survival and ensure accurate results.. The method can presumptively identify periodontal pathogens only in conjunction with biochemical tests, such as sugar fermentation and analysis of bacterial enzymatic activities (Riggio MP et al.,1996). Nucleic acid-based methods, such as use of DNA probes and PCR, are quicker and more consistent than anaerobic cultivation. These are exclusively used in the microbiological diagnosis of subgingival plaque samples of patients with progressive forms of periodontitis.

In the present study detailed relation of *P.gingivalis* with Periodontitis and with Rheumatoid arthritis (RA) has been put forward. *Porphyromonas gingivalis* is one of over 500 bacterial species that resides in the oral cavity (Mysak J et al.,2014). It is a Gram-negative and a black pigmented oral anaerobe that is involved in implication of periodontitis which also produces varoius virulence factors that causes directly or indirectly destruction to periodontal tissues by modulating the host inflammatory response (How KY et al.,2016). In the present study it has been strongly postulated that

periodontitis is linked with RA and possible causative agent is *P.gingivalis.* Thus, we got the success to sample most of the *P. gingivalis* from the RA positive patients as compared to patients reported with only periodontitis and obviously lower when compared to control. According to Zhang et al., (2021) periodontal disease brings about damage to periodontal support structure and the responsible *P.gingivalis* linked to produce outer membrane vesicles which is strongly linked with RA also. It is also been noted that PG able to involve in generation of ACPA in RA patients thus confirms the direct link between RA and PG (de molon et al., 2019). In the present study also, we have confirmed the association of PG with RA.

Loesche et al , suggested that healthy subjects either do not exhibit *P. gingivalis* or exhibit the bacterium in low numbers. Takeuchi and Missailidis also found similar results in their study. These findings are slightly lower than that of Griffen and Amano who showed the presence of *P. gingivalis* in 40 and 36.8% of healthy subjects respectively. Faghri detected *P. gingivalis* in 25% of healthy subjects. None of the healthy subject (0.00%) was tested positive for *P. gingivalis* in present study.. The present study reveals the casual link of *P. gingivalis* with Periodontitis and Rheumatoid arthritis; however, more extensive studies are advocated to correlate the periodontopathogens in other systemic diseases as well as to evaluate the pathogenic and nonpathogenic strains of *P. gingivalis*. Study also aimed to detect frequency of *Porphyromonas gingivalis* in patients suffering from both the diseases using PCR method.

Conclusion:

Porphyromonas gingivalis is strongly associated with chronic periodontitis and its detection frequency positively correlates with the severity of periodontal destruction and Rheumatoid arthritis In the present study *Porphyromonas gingivalis* found to be responsible for dental plaque and further linked successfully to rheumatoid arthritis once the prominent number of PG isolated from RA patients. In all, the present work highlights the isolation and screening of *P. gingivalis* from subgingival plaque samples from patients of respective groups exhibiting haemolytic ability and its molecular identification through PCR method . Further studies should be done for analysis of genomic data, which makes it possible to explore the patterns of gene expression of these bacteria and thus better define the pathogenesis of the disease. Integration of this information provides the basis for proactive approaches for prevention, diagnosis, and treatment of periodontal disease which control the onset of Rheumatoid arthritis.

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Conflicts of Interest:
The authors reveal no conflicts of interest concerning the work reported in this article.

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