

ORIGINAL ARTICLE

Molecular characterization of periodontal bacteria by 16S rRNA Sequencing in periodontitis patients

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ABSTRACT

Aim: To identify periodontopathogenic bacteria in periodontal pockets and subgingival plaque of patients with periodontitis in Karbala, Iraq, using 16S rRNA gene sequencing, the gold standard for molecular identification of microorganisms.

Materials and Methods: Molecular techniques based on 16S rRNA gene analysis provide reliable identification of bacterial species associated with periodontal infections. A crosssectional study was conducted with 26 patients, ranging in age from 20 to 50 years. The patients were excluded of the study if they had been on systemic antibiotics or periodontal therapy within the past three months. Every sample was aseptically collected with informed consent. The sterile paper points were used to take sample of the subgingival plaque, and they were inserted in the deepest periodontal pockets with a duration of 20-30 seconds. The samples were instantly frozen in sterile microtubes with transport buffer and frozen at -20 °C until further molecular analysis.

Results: The study population consisted of 65.38% females and 34.62% males. The most frequent clinical findings were gingival bleeding (55%) and inflammation (33%), with no significant differences observed between sexes. Molecular analysis revealed a high prevalence of *Porphyromonas gingivalis* (20/26) and *Tannerella forsythia* (18/26). *Veillonella parvula* was detected in 12 patients, while *Treponema denticola* was identified in 11 patients. *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum* were detected at lower frequencies, in 3 and 2 patients, respectively. Successful amplification of the 16S rRNA gene was confirmed by the presence of a ~1500 bp band in all samples.

Conclusions: Sequencing PCR is a powerful tool for the diagnosis of the subgingival microbiota in periodontitis, showing that *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* are the most frequently encountered pathogens in the sampled population. Moreover, the strong link between *Treponema denticola* and diabetes mellitus highlights the need to take into account systemic health factors when analysing microbiological data.

KEY WORDS: periodontal diseases, 16S rRNA, *Porphyromonas gingivalis*

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INTRODUCTION

Periodontitis is a long term inflammatory disease of tissues supporting the teeth, which is triggered by certain pathogenic microorganisms. It causes gradual degeneration of periodontal ligament and alveolar bone causing periodontal pocket formation, gingival recession, or both [1].

Metagenomics has made it possible to generate projects unimaginable to humanity in past decades, such as the Human Microbiome Project, which will allow us to analyze and understand the complex communities and interactions of the microorganisms in the oral cavity. Genomic techniques will also allow us to understand how the host's immune system interacts with the oral microbiota and explain its relationship to oral health or

disease [2]. This knowledge will lead dentists to make the oral microbiome a routine clinical tool for assessing their patients' susceptibility to certain oral diseases.

The mouth cavity has a set of surfaces, and each of them is covered with dense and heterogeneous populations of bacteria that create structured biofilms. Some of the members of these microbial populations are related to oral diseases such as dental caries and periodontitis that are among the most common bacterial infections, in humans [3].

There is mounting evidence that the oral microbiota happens to be at the centre stage in the pathogenesis of the two most common human oral diseases--dental caries and periodontal disease. These circumstances have been identified as some of the significant risk

factors of various systemic health conditions such as malignancies, diabetes mellitus, cardiovascular diseases, bacteremia, premature birth, and low birth weight. It is generally thought that oral microorganisms cause disease upon synergistic and cooperative interaction, the relationships between oral microbial species are of critical determinence concerning pathogenicity [4].

Understanding the oral microbiome is a complex task due to the wide variety of habitats within the oral cavity, which depends on oxygen concentrations, nutrient availability, temperature, exposure to immunological factors, and anatomical characteristics, the species of the genus (*Streptococcus*) are found in high proportions in soft tissues, saliva, and on the tongue. Species of the genus (*Actinomyces*) are found supragingivally and infragingivally, and in lingual fissures. Other bacteria, such as (*Veillonella parvula*) and (*Neisseria*), can be isolated from all oral habitats. Intracellular colonization can also occur in oral epithelial cells by bacterial complexes composed of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* [5]. Recent studies have shown that most oral microorganisms are cultivable; that the oral microbiome is much more diverse than previously thought; and that oral infections are polymicrobial in nature [6].

The human mouth cavity acts as a significant entry point to the environment and its viruses and bacteria and thus among the most highly colonized microbial habitats in the human body. It carries around six billion bacteria and possibly as many as 35 times the amount of viruses. It is due to the large population of bacteriophage communities in the oral cavity that the molecular diversity among bacterial populations is encouraged, and both the phages and their hosts continuously mutate in order to gain some evolutionary benefits [7].

Due to the peculiarities of primary oral ecosystems, and especially to the variability, heterogeneity, and quantity of the microbiota, there are numerous challenges in accurately determining its microbial composition. Up to 200 different species have been isolated in a single oral cavity over time; most of these appear to be transient, leaving only about 20 as resident species [8].

The role of the oral fungal microbiota has not been well established despite the comprehensive nature of the characterisation of the bacterial constituent of the oral microbiome. Fungus and bacteria are becoming increasingly considered to be significant results of interactions in oral health, with the synergistic relationship between *Candida albicans* and oral *streptococci* being an example of a fungus-bacteria interaction. Future research needs to combine research with pertinent disease models to mechanistically substantiate relationships in human research and determine fungal-bacterial

interactions that could be used to prevent and treat oral diseases [9].

AIM

The aim of the study was to identify periodontopathogenic bacteria in periodontal pockets and subgingival plaque of patients with periodontitis in Karbala, Iraq, using 16S rRNA gene sequencing, the gold standard for molecular identification of microorganisms.

MATERIALS AND METHODS

PATIENTS AND SAMPLES COLLECTION

Iraqi patients with periodontitis and those that had visited dental clinics in Iraq were used as study subjects and sampled to get periodontal samples. Periodontitis diagnosis was made on clinical periodontal parameters such as probing pocket depth, clinical attachment loss and radiographic indicators of alveolar bone loss. The patients were excluded of the study if they had been on systemic antibiotics or periodontal therapy within the past three months. Every sample was aseptically collected with informed consent. The sterile paper points were used to take sample of the subgingival plaque, and they were inserted in the deepest periodontal pockets with a duration of 20-30 seconds. The samples were instantly frozen in sterile microtubes with transport buffer and frozen at -20 °C until further molecular analysis.

A crosssectional study was conducted with 26 patients, ranging in age from 20 to 50 years. Of these 26 patients, 21 presented with aggressive periodontal disease and 5 with chronic periodontal disease.

Both types of periodontal disease were preceded by a series of symptoms, such as gingivitis, occlusal trauma, halitosis, and inflammation. The pocket depths in subjects with aggressive periodontal disease ranged from a maximum of 6 mm to a minimum of 4 mm. Similarly, in patients with chronic periodontal disease, pocket depths ranged from 8 mm to 2 mm.

The first step consisted of completing the designed periodontal record form. Subsequently, samples were taken for PCR testing, following this procedure.

DNA EXTRACTION

According to the instructions of the manufacturer, bacterial genomic DNA was extracted in the collected periodontal samples with the help of a commercial DNA extraction kit. Spectrophotometric analysis of the concentration and purity of the extracted DNA was measured and the DNA samples stored at -20 °C awaiting polymerase chain reaction (PCR) amplification.

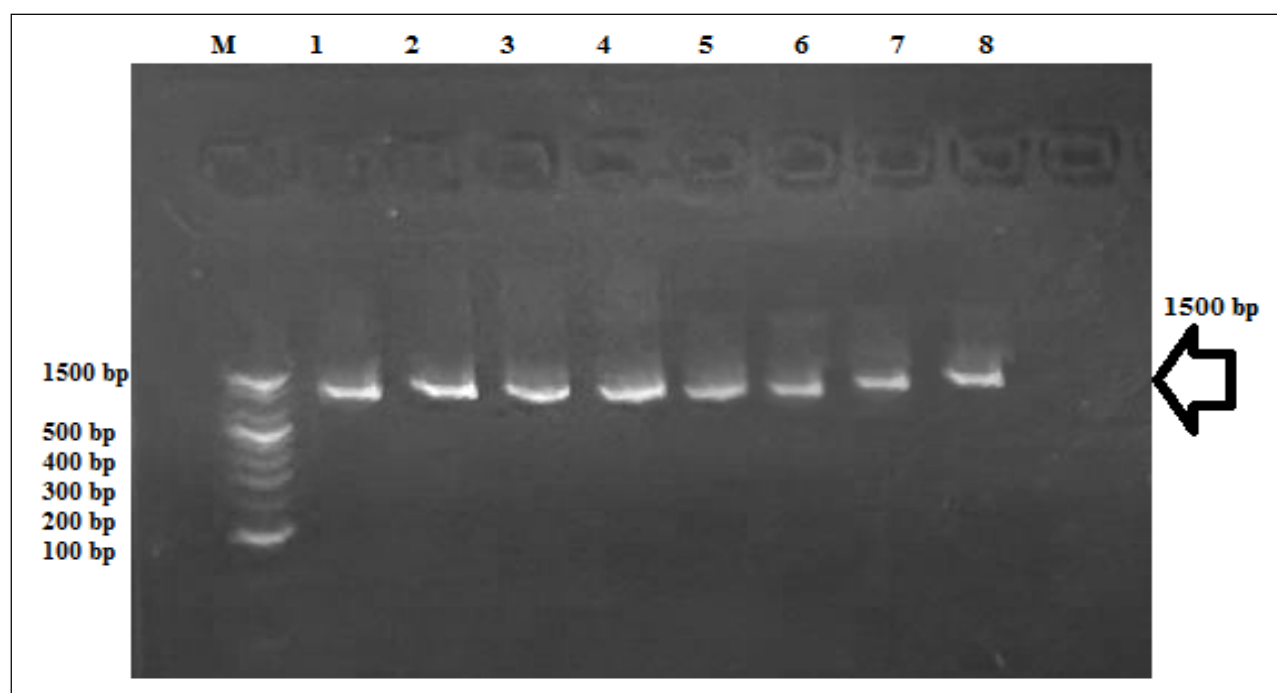


Fig. 1. Agarose gel electrophoresis showing PCR amplification of the bacterial 16S rRNA gene (~1500 bp) from periodontal samples. PCR products were resolved on a 1.5% agarose gel prepared in TBE buffer and run at 70 V for 1 hour. Lane M represents the DNA molecular weight marker, while lanes 1–8 correspond to clinical samples. All samples exhibited a clear band at approximately 1500 bp, indicating successful amplification of the 16S rRNA gene

Picture taken by the authors

PCR AMPLIFICATION OF 16S RRNA GENE SEQUENCING

Universal primers were used to amplify bacterial 16S rRNA gene which targeted about 1500 base pairs of the gene. The bacterial 16S rRNA gene was amplified using universal primers targeting approximately 1500 base pairs. The primer sequences used were as follows: Forward primer (27F): 5'-AGAGTTTGATCMTGGCTCAG-3'

Reverse primer (1492R): 5'-TACGGYTACCTTGTTACGACTT-3'[10]. PCR amplification was performed in a total reaction volume containing template DNA, primers, PCR master mix, and nuclease-free water. The amplification was carried out in a thermal cycler with an initial denaturation step 95C for 3 minutes, followed by 35 cycles of denaturation 95C for 30 second, annealing 56C for 30 second, and extension 72C for 60 second, and a final extension step 72C for 5 minutes.

The PCR products were analyzed by electrophoresis on a 1.0% agarose gel stained with an appropriate nucleic acid dye. A single DNA band of approximately 1500 bp indicated successful amplification. Thermal cycler amplification was performed under standard conditions, that is, initial denaturation, then repeated denaturation, annealing, and extension cycles, and finally a conclusion of the extension cycle. PCR products were separated by gel electrophoresis using agarose gel

and the presence of one band with about 1500 bp size was taken to suggest successful amplification.

DNA SEQUENCING

The purified PCR products were sequenced. Pure PCR products were sent back to a certified sequencing laboratory in the Republic of Korea using DHL courier service under controlled conditions. The Sanger sequencing used the same primers that were used in the PCR amplification.

BACTERIAL IDENTIFICATION AND SEQUENCE ANALYSIS

The received nucleotide sequences were revised and put together under the use of relevant bioinformatics software. The agreement sequences were compared to reference sequences in National Center of Biotechnology Information (NCBI) database with the help of BLASTn tool. The identification of bacterial species was done according to the best sequence similarity, and a similarity of 97% and above were deemed to be species level.

ETHICS

This work complies with the principles of the Declaration of Helsinki.

Table 1. Number and percentage of bacterium detection in patient with periodontitis

Bacterium	Female n (%)	Male n (%)
<i>Porphyromonas gingivalis</i>	12 (46.1%)	8 (30.7%)
<i>Tannerella forsythia</i>	11 (42.3%)	7 (26.9%)
<i>Veillonella parvula</i>	9 (34.6%)	3 (11.5%)
<i>Treponema denticola</i>	7 (26.9%)	4 (15.3%)
<i>A. actinomycetemcomitans</i>	2 (7.7%)	1 (3.8%)
<i>Fusobacterium nucleatum</i>	1 (3.8%)	1 (3.8%)

Source: compiled by the authors of this study

Table 2. Distribution of bacterial detection in periodontitis patients with and without diabetes

Bacterium	Diabetic Patients (n = 10) n (%)	Non-Diabetic Patients (n = 16) n (%)	χ^2 Value	p-value
<i>Porphyromonas gingivalis</i>	9 (90.0%)	11 (68.8%)	1.57	0.210
<i>Tannerella forsythia</i>	8 (80.0%)	10 (62.5%)	0.90	0.343
<i>Veillonella parvula</i>	6 (60.0%)	6 (37.5%)	1.27	0.260
<i>Treponema denticola</i>	7 (70.0%)	4 (25.0%)	5.20	0.023*
<i>Aggregatibacter actinomycetemcomitans</i>	1 (10.0%)	2 (12.5%)	0.04	0.848
<i>Fusobacterium nucleatum</i>	0 (0.0%)	2 (12.5%)	1.33	0.248

Source: compiled by the authors of this study

AI STATEMENT

The author states that no AI tools were used in writing this article.

RESULTS

The study included 26 patients with periodontitis. Sex distribution indicated that there was a high number of females (65.38%) than males (34.62%). In terms of clinical presentation, the most common results were gingival bleeding (55%), and gingival inflammation (33%). Under the analysis of the clinical findings based on the sex, there was no significant difference between the two sexes as similar clinical features dominated both among the female and the male patients.

Bacterial DNA analysis using PCR showed that some of the periodontopathogen species were present among the research subjects. *Tannerella forsythia* was found in 18 of 26 sample subjects and *Porphyromonas gingivalis* in 20 patients. The *Veillonella parvula* was identified in 12 subjects. Moreover, 11 patients also had *Treponema denticola*. The most common species was actinobacillus-actinomycetemcomitans with 3 patients and *Fusobac-*

terium nucleatum was the least common species which was found in 2 patients only.

Bacterial distribution analysis data were categorized by sex whereby *Veillonella parvula* was most frequently identified in female patients then *Tannerella forsythia*. *Porphyromonas gingivalis* was found in equal measures in male patients, with *Fusobacterium nucleatum* having the lowest prevalence Table 1.

Agarose gel electrophoresis demonstrated successful PCR amplification of the 16S rRNA gene in all periodontal samples, as evidenced by the presence of a single band at approximately 1500 bp (Fig. 1).

A total of 10 patients (38.5%) in this study were diagnosed with type 2 diabetes mellitus (DM) and 16 patients (61.5%) had no systemic disease. Table 2 shows the detection rates of the bacteria in diabetic and non-diabetic patients.

Using the Chi-square test, we found that the detection of *Treponema denticola* was significantly higher in diabetic patients (70.0%) than in non-diabetic patients (25.0%) ($p = 0.023$). While *Porphyromonas gingivalis* and *Tannerella forsythia* had a higher detection rate in diabetic patients (90.0% and 80.0%, respectively) than non-diabetic patients (68.8% and 62.5%, respectively), it

did not reach a level of statistical significance ($p > 0.05$). *Fusobacterium nucleatum* was only detected in non-diabetic patients (12.5%) and *Aggregatibacter actinomycetemcomitans* had similar detection rates in both groups

DISCUSSION

The current research study examined the bacterial profile of periodontitis in Iraqis through the use of PCR amplification and Sanger sequencing of the 16S rRNA gene. The results showed that periodontopathogenic bacteria have a heterogeneous distribution, which indicates the polymicrobial nature of periodontal disease, the study population was found to be more composed of female participants. This distribution can be showing variation in health seeking behavior and not actual sex differences in prevalence of diseases. The most frequent clinical findings were gingival bleeding and inflammation which are well-known predictors of active periodontal inflammation, as well as, as per the classical clinical presentation of periodontitis [11,12].

Traditional periodontal assessments, including probing pocket depth and radiography of alveolar bone destruction, measure the outcome of the disease, but don't directly address the causative agents or microbial infection, the cause of periodontal disease. This highlights the importance of sensitive and specific microbiological diagnostics to better understand the disease process and inform treatment [13].

In this study, we used sequencing PCR to profile the subgingival microbiota. Our findings on the high presence of "red complex" pathogens (including *P. gingivalis*) are consistent with their role as keystone pathways of disease dysbiosis [14].

Yet, the real power of our diagnostics is not only that it can detect, but it can also quantitate. As pointed out in the literature, qualitative testing has "limited clinical value" for periodontitis. Our quantitative PCR approach provides information on bacterial load, allowing a more sophisticated interpretation based on disease severity; this is an improvement on traditional colony culturing which may not detect important anaerobes [15].

Hundreds of different species have been isolated from the oral cavity, but only a few have been associated with periodontal pathogenic bacteria. Among these are *Porphyromonas gingivalis*, *Tannerella forsythensis* (*Bacteroides forsythus*), and *Actinobacillus actinomycetemcomitans*, although other *Gram-negative bacteria* also cause chronic and aggressive periodontitis [16].

Several methods exist for enumerating the number of bacteria present in subgingival plaque. These include bacterial culture, which has been the classic method par excellence for detecting and enumerating periodontopathogenic microorganisms [17,18]; immunofluorescence microscopy; and DNA probes. Unfortunately, these methods have certain limitations, such as a lack of specificity and limited sensitivity.

On the other hand, there is PCR, a simple technique that replicates fragments of RNA or DNA and has been developed following recent studies by Astorgia [19] in 2022, and Meurman et al. [20] in 2022. It is a rapid and relatively simple method that can detect bacteria even in low numbers. This method does not require live cells [21]. It offers high sensitivity and specificity in detecting bacteria in biological samples. Between 25 and 100 cells can be detected [22,23].

However, PCR has its limitations, including the possibility of false-positive results due to contamination [24]. The use of microbiological tests for diagnosing the different destructive forms of periodontal disease remains controversial. The limitations of this test, regarding the presence or absence of Pg and Aa, and its purpose of distinguishing subjects with aggressive periodontal disease from those with chronic periodontal disease, were clearly outlined in a review recently published by Mombelli et al. [25] in 2002. When diagnosing aggressive periodontal disease, we can often overlook a clear indicator of this type of periodontal disease, was not detected. For a correct microbiological diagnosis, we need a test with high specificity and sensitivity, such as the PCR test. Even if the presence of this bacterium is low, we must not discriminate, because its mere presence is a clear indicator of aggressive periodontal disease.

The data obtained regarding clinical history show that the most frequent findings were bleeding (55%) and inflammation (33%). However, in the study conducted by [26], which compared two groups-one with aggressive periodontal disease and the other with chronic periodontal disease-they concluded that, based on clinical history, aggressive periodontal disease presents with less inflammation and bleeding, but crevicular fluid is similar to that of chronic periodontal disease.

Regarding the PCR test results, where samples were taken from pockets 4 mm or larger, we found the presence of various bacteria: *Actinobacillus actinomycetemcomitans* (Aa), *Tannerella forsythensis* (Tf), *Prevotella intermedia* (Pi), *Porphyromonas gingivalis* (Pg), *Treponema denticola* (Td), and *Fusobacterium nucleatum* (Fn). In the studies conducted by Herrera et al. [27] in 2023 concluded that Aa is a typical bacterium of aggressive periodontal disease.

While Pg is more typical of chronic periodontal disease, it has been observed in patients with aggressive periodontal disease, as corroborated by studies conducted by Vandestein et al. [28] in 1984.

Diabetes mellitus is also known to affect the subgingival microbiota; thus, the present study investigated the effect of this comorbidity on the subgingival microbiota. We found that *Treponema denticola* was significantly more common in diabetic patients than in non-diabetic patients ($p = 0.023$). This observation is in agreement with earlier reports that have shown an increased prevalence of spirochetes, such as *T. denticola*, in diabetic patients with periodontitis. This finding

may be explained by changes in host immune responses caused by hyperglycemia, which may provide a suitable niche for certain anaerobic bacteria, while the prevalence of *P. gingivalis* and *T. forsythia* was higher in diabetic patients, these results were not significant [29]. This could be due to the limited number of diabetic patients in our study (n = 10), where smaller effect sizes might be undetected. Larger sample sizes are needed to help clarify the association between diabetes and the microbiome in periodontal disease [30]. In the study conducted by Takeuchi et al. [6], Pg and Td were detected, which, although more typical of chronic periodontal disease, can also appear in aggressive periodontal disease.

CONCLUSIONS

Sequencing PCR is a powerful tool for the diagnosis of the subgingival microbiota in periodontitis, showing that *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* are the most frequently encountered pathogens in the sampled population. Moreover, the strong link between *Treponema denticola* and diabetes mellitus highlights the need to take into account systemic health factors when analysing microbiological data, and the inclusion of molecular diagnosis into the planning of individualised periodontal treatment.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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