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Advances in Molecular Diagnostics of Red Complex Bacteria: The Role of PCR in Periodontal Pathogen Detection and Clinical Implications

Vidhi Gaur[1], Ohm Varia[1], Rakesh Patel[2], Priyanka Patel[3*], Preeti Kumari[4], Dev Patel[5]

[1]Dissertation Trainee, Department of Biotechnology and Bioengineering, Institute of Advanced Research, Gandhinagar, Gujarat, India
[2]Periodontist, Niva Dental Clinic, Ahmedabad, Gujarat, India
[3]Director, Department of Research, Rapture Biotech, Ahmedabad, Gujarat, India
[4]Research Associate, Department of Research, Rapture Biotech, Ahmedabad, Gujarat, India
[5]Research Intern, Department of Research, Rapture Biotech, Ahmedabad, Gujarat, India

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***Corresponding author:** Priyanka Patel
Director, Department of Research, Rapture Biotech, Ahmedabad, Gujarat, India

Abstract **Review Article**

Periodontal diseases, characterized by chronic inflammation and progressive destruction of supporting tooth structures, are closely linked to the red complex bacteria—*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*. These pathogens significantly contribute to disease development, making their accurate detection essential for effective diagnosis and treatment. Traditional diagnostic methods, though widely used, often lack sensitivity and reliability, highlighting the need for molecular diagnostics to improve clinical outcomes. The advent of polymerase chain reaction (PCR) has transformed periodontal pathogen detection, offering exceptional sensitivity, specificity, and rapid identification. Advanced PCR variants enable precise bacterial quantification, species differentiation, and comprehensive microbial profiling, significantly enhancing diagnostic accuracy. The integration of PCR into routine periodontal diagnostics allows for early intervention, personalized treatment strategies, and improved disease monitoring. Future advancements, such as point-of-care PCR systems, next-generation sequencing, and artificial intelligence-driven analysis, hold the potential to further refine molecular diagnostics, paving the way for more efficient and targeted periodontal disease management.

Keywords: Red Complex Bacteria, Molecular Detection, Dental Pathogens, *Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia.*

1. INTRODUCTION

Periodontitis emerges as a significant global health issue, possibly standing as the most prevalent chronic infectious disease affecting the human population. This intricate, multifactorial, arising from a diverse group of microorganisms, is characterized by the deterioration of the supportive tissues surrounding the teeth (Newman *et al*., 2018), (Gugale *et al*., 2024). Based on the findings of the Global Burden of Disease Study (2016), periodontitis ranks as the 11th most common condition globally, with an estimated prevalence of 20% to 50%. It significantly impacts quality of life, causing issues with chewing, aesthetics, and self-confidence, resulting in 3.5 million years lived with disability (YLD) during 2016. The global burden of periodontal diseases has increased by 57.3% between 1990 and 2010, a trend expected to continue due to longer life expectancy and greater retention of natural teeth in aging populations (Nazir *et al*., 2020).

The "red complex" bacteria—*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*—are highly pathogenic species directly related with periodontitis. These Gram-negative anaerobes contribute to the shift from a healthy oral microbiota to a dysbiotic state, promoting disease progression by colonizing subgingival biofilms, disrupting commensal bacteria, and resisting host immune responses, leading to tissue destruction and tooth loss (Coffey *et al*., 2016). Timely and accurate diagnosis is essential for effective periodontal therapy, enabling early intervention to prevent disease progression and related systemic complications. Since periodontitis is influenced by diverse bacterial communities and host responses, precise pathogen identification is crucial for targeted treatment. Advances in molecular diagnostics, such as PCR, have greatly improved the detection and quantification of key periodontal pathogens, enhancing diagnostic accuracy, guiding personalized treatment

plans, and helping manage systemic conditions like diabetes, where periodontal infections can worsen systemic inflammation (Shaikh *et al*., 2023).

2. Red Complex Bacteria in Periodontal Diseases

2.1 Pathogenicity and Virulence Factors of Red Complex Bacteria

The red complex bacteria play a crucial role in periodontitis progression through their synergistic interactions and adaptation to anaerobic conditions in deep periodontal pockets. Their virulence factors include enzymatic activity, structural components, metabolic products, and immune evasion mechanisms (Dahlen *et al*., 2019). Researchers have proposed that P. gingivalis plays a central role as a keystone pathogen in the development of periodontal disease. *P. gingivalis* impairs innate immunity in several ways that could alter the composition of the biofilm, triggering a destructive alteration in the normally homeostatic host-microbial balance (Chigasaki *et al*., 2021). *P. gingivalis* produces gingipains that degrade host proteins and disrupt immune responses, while *T. forsythia* secretes PrtH proteases that aid in adhesion and immune evasion. *T. denticola* generates trypsin-like proteases that degrade tissue and immune molecules. Structural components such as the fimbriae and capsule of *P. gingivalis* enhance adhesion, biofilm formation, and resistance to phagocytosis. Additionally, metabolic byproducts like hydrogen sulfide, ammonia, and volatile sulfur compounds contribute to cytotoxicity, inflammation, and tissue damage. Immune evasion is further facilitated by the degradation of host defence molecules and destruction of immune cells. *T. denticola* utilizes its motility to invade tissues, while *P. gingivalis* and *T. forsythia* produce enzymes that weaken epithelial barriers, enabling bacterial infiltration. Collectively, these factors contribute to the colonization, persistence, and pathogenicity of red complex bacteria in periodontitis (Dahlen *et al*., 2019).

2.2 Association with Periodontal Tissue Destruction and Systemic Diseases

Greater serum antibody levels to periodontal pathogens reflect their systemic dissemination, thereby resulting in their vascular and hepatic activation (Mahendra *et al.*, 2019). Red complex bacteria are highly pathogenic and central to periodontal tissue destruction and systemic complications. Through virulence factors like gingipains, they degrade host proteins, disrupt immune responses, and induce chronic inflammation, leading to gingival detachment, alveolar bone loss, and periodontal pockets. They manipulate immune pathways, such as Toll-like receptors and complement systems, promoting microbial persistence and dysbiosis. Beyond oral health, *P. gingivalis* enters the bloodstream, promoting inflammation and atherosclerosis, increasing myocardial infarction risk. In diabetes, chronic infection worsens systemic inflammation by releasing IL-6 and TNF-α, impairing insulin signalling. *P. gingivalis* also promotes protein citrullination in RA, generating autoantigens. In COPD, periodontal bacteria contribute to pulmonary inflammation via neutrophilic pathways. Persistent inflammation from *P. gingivalis* and *T. forsythia* is linked to gastrointestinal and oesophageal cancers. The systemic impact of these bacteria highlights the need for effective periodontal management, as controlling infections may reduce disease risks and improve overall health (Bourgeois *et al*., 2019).

2.3 Challenges in Identifying These Bacteria Using Traditional Diagnostic Methods

Identifying red complex bacteria such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* using traditional diagnostic methods presents several challenges. Culturing these anaerobic bacteria is time-consuming and often unsuccessful due to specific growth requirements, while semi-quantitative methods like culturing and immunochromatographic techniques lack precise bacterial quantification, leading to inaccurate assessments. Traditional methods also suffer from limited sensitivity, potentially missing low-abundance bacteria, and poor specificity, making it difficult to differentiate pathogenic from non-pathogenic strains. Inconsistent sampling further affects detection reliability, and these methods fail to capture the complex interactions within the oral microbiome. Molecular techniques, such as multiplex real-time PCR, offer improved sensitivity, specificity, and rapid quantification, addressing many of these limitations (Lochman *et al*., 2019).

3. Molecular Detection Techniques for Red Complex Bacteria

3.1 Overview of Diagnostic Methods

3.1.1 Traditional Methods: Culture-Based Techniques

Traditional culture methods have been widely used for detecting microorganisms in endodontic infections. However, these methods present challenges, particularly in identifying anaerobic bacteria. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, known together as "Red complex" bacteria, are difficult to isolate from root canals because they require specific growth conditions. Their black pigmentation, a key characteristic, is associated with haemolysis on blood agar, but this trait may be lost under suboptimal conditions, making detection more difficult (Tiwari *et al*., 2020).

3.1.2 Limitations of Traditional Methods

Culture-based techniques are not only time-consuming but also lack sensitivity and specificity in detecting anaerobic bacteria, particularly those that are non-cultivable or exist in biofilms. Studies have indicated that 16S rRNA-based sequencing techniques are essential for identifying cultivable as well as uncultivable pathogenic bacteria. Furthermore, while it is possible to detect genetic material from dead bacterial cells, DNase produced by living microbes can degrade

bacterial DNA, complicating the identification process (Tiwari *et al*., 2020). Additionally, traditional culture methods have several inherent limitations, including the need to preserve bacterial viability, difficulty in detecting low levels of microorganisms, effort-intensive procedures. These challenges make culture-based techniques less effective compared to modern molecular methods (Atieh *et al*., 2008).

3.2 Evolution of Molecular Techniques

3.2.1 Early Molecular Methods

Molecular techniques have significantly improved the detection of periodontal pathogens. Early molecular approaches, such as DNA-DNA hybridization, provided a way to identify microbial species that could not be cultured using conventional methods (Tiwari *et al*., 2020). Dot-blot hybridization of the amplified dental plaque was used to detect small amounts of highly pathogenic bacteria in periodontal patients (Surdu *et al*., 2017). These techniques enabled the detection of bacterial communities in periodontal and endodontic infections and provided insight into microbial diversity (Tiwari *et al*., 2020).

3.2.2 Introduction of PCR and Its Variants in Microbial Diagnostics

The introduction of polymerase chain reaction (PCR) revolutionized microbial diagnostics through the targeted amplification of DNA, enhancing the accuracy of bacterial identification. PCR-based techniques, including 16S rDNA-directed PCR, have been widely used to detect *P. gingivalis, T. denticola,* and *T. forsythia* in root canals harbouring infection. In a study that utilized target-specific primers, *P. gingivalis and T. denticola* were identified in symptomatic teeth exhibiting periapical lesions, while *T. forsythia and T. denticola* were associated with pain and swelling. The high sensitivity of PCR has enabled researchers to establish correlations between the presence of "Red complex" bacteria and clinical symptoms such as tenderness on percussion, periapical lesions, and pus discharge, reinforcing the importance of molecular diagnostics in periodontal and endodontic microbiology (Tiwari *et al*., 2020).

4. Role of PCR in Red Complex Bacteria Detection

4.1 Principle and Process of PCR

4.1.1. Overview of PCR steps: Denaturation, Annealing, and Extension

The Polymerase Chain Reaction (PCR) involves three primary stages: denaturation, annealing, and extension. In the denaturation phase, the double-stranded DNA is exposed to high temperatures, generally around 94–95°C, causing it to separate into individual strands. During the **annealing** step, the temperature is lowered to an optimal range (typically between 50–65°C), enabling the primers—short DNA sequences designed to match the target region—to attach to their complementary sites on the single-stranded DNA. Finally, in the extension phase, the temperature is raised to approximately 72°C, enabling the thermostable DNA polymerase enzyme, such as Taq polymerase-functions to build a new DNA strand by adding deoxynucleotides (dNTPs) complementary to the template strand. These steps are repeated in cycles, often 30 or more, leading to exponential amplification of the target DNA (Jones NL, 2002).

4.1.2 Specificity and Sensitivity Advantages

With the development of PCR techniques, especially real-time PCR, have allowed for more accurate quantification of bacterial DNA due to its high sensitivity and specificity. PCR is highlighted as superior to traditional methods like microbiological cultures because it can detect even non-cultivable bacteria efficiently, thus overcoming limitations such as time consumption and low detection accuracy (Tiwari *et al*., 2020).

4.2 PCR Applications

4.2.1. Identification of Red Complex Bacteria in Subgingival Plaque

Red complex bacteria, including *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, play a critical role in the onset and development of periodontitis. PCR is extensively utilized to identify these bacteria in subgingival plaque due to its sensitivity and specificity. In a study analyzing periodontal health, bacterial DNA was extracted from plaque samples collected using paper points, followed by amplification through reverse transcription PCR (RT-PCR). This technique allowed precise identification of these pathogens in plaque samples from periodontitis patients, highlighting their significant association with poor oral hygiene and increased probing depths. The study confirmed that the presence of red complex bacteria correlates strongly with clinical markers of inflammation, such as the Papillary Bleeding Index (PBI), underscoring their pathogenic role in periodontal disease (Khoirowati *et al*., 2023).

4.2.2. Quantitative PCR (qPCR) for Assessing Bacterial Load

Quantitative PCR (qPCR) is a robust method for measuring bacterial load, providing insights into the severity of periodontal infections. By employing specific primers for *P. gingivalis*, *T. denticola*, and *T. forsythia*, qPCR quantifies bacterial DNA in subgingival plaque samples. This method revealed that elderly patients with periodontitis exhibited significantly higher bacterial loads compared to adults, particularly for *P. gingivalis*. The correlation between higher Oral Hygiene Index-Simplified (OHI-S) scores and increased bacterial quantities reinforces the role of poor oral hygiene in exacerbating bacterial colonization (Khoirowati *et al*., 2023). qPCR thus serves as a valuable tool for detecting and quantifying specific bacterial species in subgingival biofilms, which may aid in periodontal risk assessment, determining optimal therapy, and evaluating treatment outcomes (Decat *et al*., 2012).

4.2.3. Multiplex PCR for Simultaneous Detection of Multiple Pathogens

The multiplex variant of PCR can be used for the simultaneous amplification of two or more loci in the same reaction (Seol *et al*., 2006), (Estrela *et al*., 2010). Multiplex PCR is a powerful technique that enables the parallel detection of several pathogens at once within a single reaction, making it a valuable tool for studying complex microbial communities. A study on chronic periodontitis utilized this method to identify the red complex bacteria from subgingival plaque samples. The species-specific primers targeting the 16S rRNA genes of these bacteria allowed precise identification. The reaction, conducted using a multiplex PCR kit, successfully amplified DNA from pooled plaque samples collected from deep periodontal pockets (≥5 mm probing depth). Post-PCR analysis using gel electrophoresis confirmed the presence of these pathogens either individually or in combination. This approach highlighted the prevalence and cooperative interaction of red complex bacteria in periodontal pockets, emphasizing the significance of multiplex PCR in understanding microbial interactions in chronic periodontitis (Nayak *et al*., 2018).

4.3 Advanced PCR Variants

4.3.1. Real-time PCR

Real-time PCR is highlighted as an efficient and sensitive molecular diagnostic tool capable of detecting and quantifying periodontal pathogens like *Porphyromonas gingivalis* and *Treponema denticola*. It operates by amplifying specific DNA sequences while simultaneously monitoring the process through fluorescence, providing both qualitative and semi-quantitative data. The technique demonstrated 100% specificity and high sensitivity for detecting target pathogens, making it particularly valuable in analyzing hard-to-culture bacteria. Real-time PCR results showed strong correlation with commercial tests like Micro-IDent®, confirming its robustness as a diagnostic tool in microbiological studies (Kuret *et al*., 2024). Recently, RTPCR assays have been developed for several oral bacterial pathogens, providing better detection tools for oral pathogens (Boutaga *et al*., 2005), (Boutaga *et al*., 2006).

4.3.2. Digital PCR and Its Advantages in Detecting Low-Abundance Pathogens

Digital PCR (dPCR) improves sensitivity and precision by dispersing the sample into numerous micro-scale reactions, amplifying DNA at the single-molecule level. This compartmentalization allows for absolute quantification and the detection of rare sequences even in a background of abundant wild-type DNA, which is particularly beneficial for identifying low-abundance pathogens. Despite its advantages over qPCR, such as bypassing the need for standard curves and greater resistance to inhibitors, dPCR remains limited in high-throughput settings due to higher costs and labor-intensive workflows. Its application in infectious disease diagnostics, like early sepsis detection, highlights its superior accuracy and ability to identify pathogens missed by traditional methods (Mirabile *et al*., 2024).

5. Diagnostic Accuracy and Clinical Utility

5.1 Sensitivity and Specificity of PCR in Detecting Red Complex Bacteria

Multiplex real-time PCR has demonstrated high sensitivity and specificity for detecting periodontal pathogens, including red complex bacteria (Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia). In a study conducted by Ozbek *et al*., species-specific TaqMan oligos and probes were designed to target conserved genomic regions, ensuring no cross-amplification and maintaining high amplification efficiency. The assay successfully amplified bacterial DNA according to the template concentrations used, demonstrating its reliability in detecting these periodontal pathogens. Furthermore, the optimized multiplex PCR strategy enabled the simultaneous detection of multiple species in subgingival plaque samples, highlighting its diagnostic precision in distinguishing red complex bacteria from other oral microbiota (Ozbek *et al*., 2010).

5.2 Integration of PCR in Clinical Diagnostics and Treatment Planning

The application of multiplex real-time PCR in periodontal diagnostics offers a rapid and sensitive method for profiling and quantifying bacterial species related to periodontal diseases. The red complex bacteria, known for their strong association with periodontitis, were detected at significant levels in diseased sites, reinforcing the importance of molecular diagnostics in clinical practice. Traditional culture methods often fail to isolate these anaerobic pathogens effectively, but PCR circumvents this limitation by providing accurate identification and quantification. The ability to detect microbial shifts in periodontal pockets enables clinicians to make informed treatment decisions, tailoring therapeutic approaches based on the microbial composition. Additionally, the study suggests that real-time PCR could serve as a valuable tool in assessing treatment efficacy by monitoring changes in bacterial loads before and after intervention (Ozbek *et al*., 2010).

6. Future Perspectives and Innovations

Point-of-care (POC) PCR diagnostic tools represent a significant advancement in rapid molecular diagnostics, enabling on-site testing with minimal manual intervention. The developed PCR system utilizes cheap 3D-printed components, readily available electronics, and motor systems to perform automated sample preparation and rapid PCR within 20 minutes. This system automates the entire workflow, including DNA extraction, separation, purification, and amplification, with sample preparation completed in less than 5 minutes. The customized thermocycler facilitates fast thermal ramp cycles, achieving 40 PCR cycles in approximately 13.8 minutes despite large sample

volumes. Its compact, low-cost design enhances portability, making it suitable for emergency POC testing of pathogenic bacteria, where rapid and reliable diagnostics are crucial (Lee *et al*., 2021).

The integration of PCR with next-generation sequencing (NGS) has revolutionized microbiome profiling, offering high-resolution taxonomic assignments down to the genus and species levels. This is achieved through advancements in sequencing technologies, such as the data analysis pipelines like DADA2, Phyloseq, and METAGENassist and 16S rRNA. These tools enable the accurate detection and characterization of complex bacterial communities, overcoming limitations associated with traditional methods. The ability to merge high-quality paired-end reads enhances the resolution and reliability of microbial diversity assessments, making this approach invaluable in clinical and research settings (Shahi *et al*., 2019).

Artificial intelligence (AI) plays a pivotal role in enhancing periodontal diagnostics through the interpretation of complex data, including PCR-derived information. AI models, particularly algorithms based on machine learning have demonstrated high predictive accuracy in identifying periodontitis by analyzing diverse datasets, including genetic markers and microbial profiles from PCR tests. These models can process large volumes of data efficiently, uncovering patterns that may not be evident through traditional analysis. By integrating patient demographics, medical history, and periodontal examination findings with PCR data, AI-driven tools can provide personalized risk assessments and improve diagnostic precision. Despite the promising potential, the reliability of these models requires further validation, especially considering the variability in data collection and analysis protocols (Polizzi *et al*., 2024).

CONCLUSION

The advent of PCR has revolutionized the molecular diagnosis of red complex bacteria, offering unparalleled sensitivity and specificity in detecting periodontal pathogens. Its ability to identify these bacteria at early stages enhances diagnostic precision, allowing for timely intervention and improved periodontal disease management. Traditional diagnostic methods, though historically valuable, fall short in accuracy and efficiency, reinforcing the necessity of molecular diagnostics for superior clinical outcomes. As PCR technology continues to evolve, integrating advanced variants such as qPCR, digital PCR, and multiplex PCR into routine clinical workflows can further streamline periodontal diagnostics. Future research should focus on the development of cost-effective, rapid, and portable PCR-based point-of-care testing systems to enhance accessibility in diverse healthcare settings. Additionally, the incorporation of next-generation sequencing and artificial intelligence in microbial analysis promises to refine diagnostic accuracy and facilitate personalized treatment strategies. Emphasizing the clinical adoption of these advancements will be pivotal in mitigating the global burden of periodontal disease and its systemic implications.

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