Original Article

Detection of Red complex bacteria, *P. gingivalis, T. denticola* and *T. forsythia* in infected root canals and their association with clinical signs and symptoms

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Abstract

Aim: This study aimed to investigate the association between endodontic clinical signs and symptoms and the presence of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* employing polymerase chain reaction (PCR). **Materials and Methods:** Microbial samples were obtained from 60 cases with necrotic pulp with primary teeth infections. DNA extracted from samples were analyzed for endodontic pathogens by using species-specific primers. **Results:** *P. gingivalis/T. denticola* were detected in 15 symptomatic teeth associated with periapical lesions. *T. forsythia/T. denticola* were found in 16 symptomatic teeth associated with pain and swelling. *P. gingivalis* was detected in 9 teeth which were associated with pain, 2 with tenderness on percussion, and 15 with periapical lesions. Statistically significant associations were found between *T. forsythia* as well as *T. denticola* in relation to clinical findings of pain and swelling. ($P < 0.05$). Red complex bacteria showed no statistical significant association with the presence of signs and symptoms. **Conclusion:** Prevalence of *P. gingivalis*, *T. denticola*, and *T. forsythia* suggested association of these bacteria with symptomatic infected pulp and periradicular diseases.

Keywords: *P. gingivalis*, red complex, *T. denticola, T. forsythia*

Introduction

Bacterial microflora in endodontic infections are affected by infectious origin, ecological niche, and host defense. The bacterial genera which are found in infected root canals include Gram-negative anaerobes such as *Prevotella, Veillonella, Porphyromonas; Gram-positive anaerobes such as Peptostreptococcus, Lactobacillus, Actinomyces; Gram-negative facultative such as Eikenella, Haemophilus* and Gram-positive *facultative* such as *Lactobacillus, Actinobacillus, Propionibacterium,* etc., These organisms produce clinical symptoms such as pain, swelling, tender on percussion, and sinus formation.[1]

Bacterial invasion of root canal often leads to an inflammatory cascade at the root apex that causes apical periodontitis. Root canal treatment aims to resolve the inflammation at the root apex by inactivation of these residing bacteria and the elimination of the endotoxins produced by the microorganisms. However, the complex root canal system and microorganisms residing in resilient biofilm communities impede sufficient cleaning, which can lead to persistent apical periodontitis.[2,3] An infected root canal comprises a unique microenvironment housing selective microflora. These organisms grow in planktonic forms or as aggregates as well as in biofilms. The microbial composition in

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the root canal system is an interesting area of research nowadays. Novel technologies such as immunological assays and molecular methods (PCR) methods were developed in recent years.[4]

Sequencing methods based on 16S rRNA are important tools for the identification of both non-cultivable and cultivable pathogenic bacteria. The 16S rDNA gene amplification from bacterial DNA extracted which is followed by cloning and gene sequencing helps in bacterial characterization. Though it is possible to detect genetic material from dead bacterial cells from extirpated dental pulp, the DNase produced by living microbes can degrade their DNA. Thus, bacteria detected in such cases represent endodontic pathogens in an acute infection. Bacterial population associated with primary endodontic infection is predominantly, Gram-negative bacteria though Gram-positive species such as *Peptostreptococcus stomatitis, Parvimonas micra, Eubacterium* spp. has also been detected in an earlier study.[5]

The present study was done to analyze association between clinical signs and symptoms and the presence of red-complex bacteria in an infected root canal.

Materials and Methods

A total of 60 symptomatic teeth were selected. Inclusion criteria for case selection included no previous treatment, necrotic pulp, or periapical periodontitis or abscess or wet canals. Ethical approval was obtained from the institutional ethical committee on 02/01/2019.

Collection of samples

Samples were collected using sterile paper points after exposure of root canals under rubber dam isolation. For the negative control group, samples were obtained by moistening 10 paper points in sterile normal saline. The paper points were thereafter transferred to Eppendorf tubes containing Tris-EDTA (ethylenediaminetetraacetic acid) (TE) buffer and were stored in −70°C for PCR.

Genomic DNA isolation

Isolation of genomic DNA was performed according to the protocol described by Brezezinska-Blaszezyk *et al.* (2018).[6] The paper points were then thawed on ice followed by vortexing for 3 min. Then, the paper points were removed and the samples were centrifuged at 200 rotation per minute (RPM) for 5 min. Pellets obtained were suspended in 100 µl of HCl buffer (pH 8.5). Microbial DNA was isolated using genomic mini-kit (A and A Biotech., Gdynia, Polana). The cells were lysed with proteinase K and DNA obtained was suspended in 100 µl of TE buffer (pH 7.4). The obtained DNA content was measured using a spectrophotometer.

Polymerase chain reaction methodology

The universal primers were designed as per 16S rRNA genes provided by GenBank, Primer Premier 5. These were specifically designed to target sequences unique toward the tested organisms. PCR primers used were:

Porphyromonas gingivalis

Forward primer: AGG CAG CTT GCC ATA CTG CG

Reverse primer: ACT GTT AGC AAC TAC CGA TGT (Bioserve India Pvt. Ltd.)

Treponema denticola

Forward primer: TAA TAC CGA ATG TGC TCA TTT ACA T

Reverse primer: TCA AAG AAG CAT TCC CTC TTC TTC TTA (Bioserve India Pvt. Ltd.)

Tannerella forsythysis

Forward primer: GCG TAT GTA ACC TGC CCG CA

Reverse primer: TGC TTC AGT GTC AGT TAT ACC T (Bioserve India Pvt. Ltd.)

The specificity of the probes was assessed using the BLAST program. DNA template (5 µl) was added to the PCR mixture with a final volume of 25 µl. Reaction mixture comprised 2.5 µl of 10X PCR buffer, 1 µl Mg^{2+}, 2 µl dNTP (deoxyribonucleotide triphosphate), 0.5 µl of forward and reverse primers each and 2.5 U of Taq polymerase.

PCR cycle

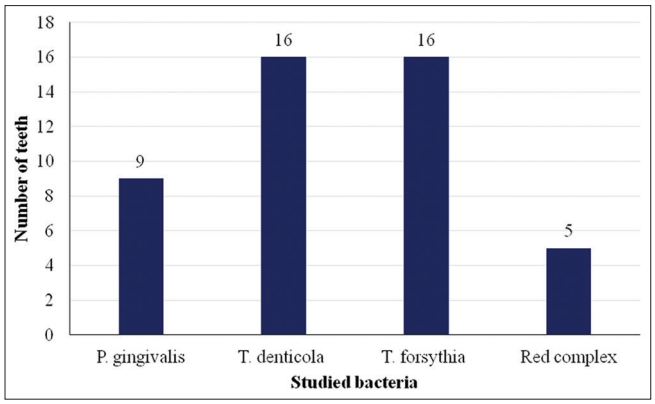
The PCR program cycle was run under 94°C for 4 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and finally, 72°C for 10 min. Visualization of bands was done under UV illumination using 1% agarose gel electrophoresis employing ethidium bromide.

Data analysis

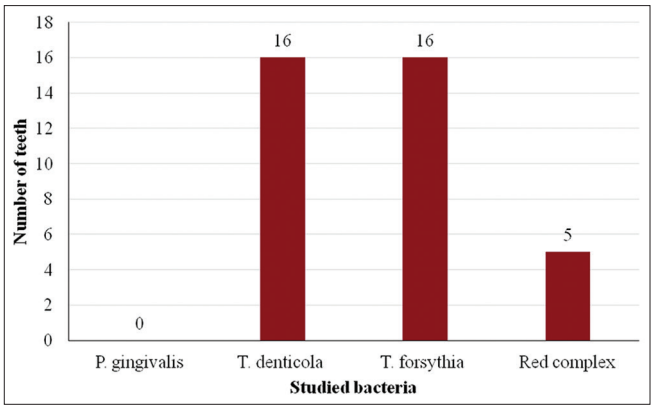
Data collected from each sample were recorded in Microsoft Excel Worksheet 2016 and analyzed using version 21.0 of the Statistical Package for Social Sciences (IBM Corporation, Armonk, New York, USA).

Table 1: Distribution of study samples according to clinical signs and symptoms and presence of *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia* individually or as a "Red complex"

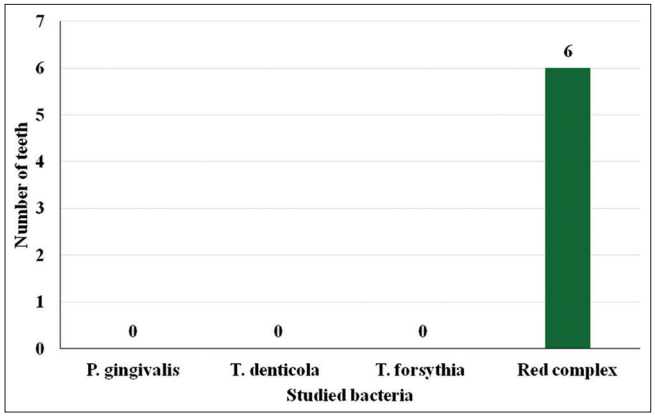
Clinical signs and symptoms	*P. gingivalis*	*T. denticola*	*T. forsythia*	Red complex
Pain	09	16	16	05
Swelling	-	16	16	05
Pus discharge	-	-	-	06
Tenderness on percussion	02	05	02	03
Periapical lesion	15	15	-	10



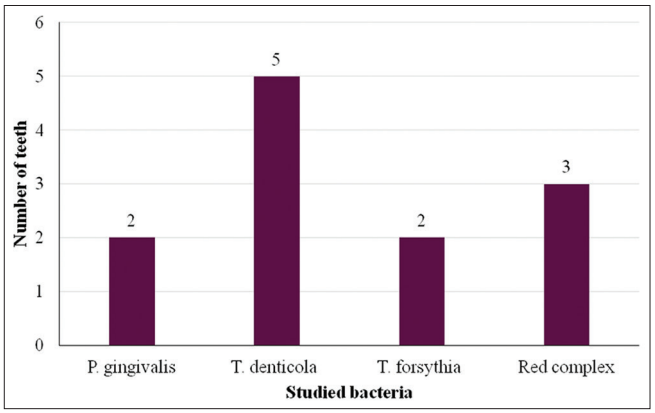
Graph 1: Studied bacteria and number of teeth with pain



Graph 2: Studied bacteria and number of teeth with swelling



Graph 3: Studied bacteria and number of teeth with pus discharge



Graph 4: Studied bacteria and number of teeth with tenderness on percussion

Results

Clinical data

Of the studied samples, 35 had pain, 29 had swelling, 06 had pus discharge, 08 were tender on percussion and 25 had periapical lesions. *P. gingivalis* was found in 09 teeth associated with pain, 02 associated with tenderness on percussion and 15 associated with periapical lesions. *T. denticola* was found in 16 teeth associated with pain and swelling, 05 with tenderness on percussion and 15 with periapical lesions. *Tannerella forsythia* was detected in 16 teeth associated with pain and swelling and, 02 teeth associated with tenderness on percussion. Red complex bacteria were detected in 05 teeth associated with pain and swelling, 06 with pus discharge, 03 with tenderness on percussion, and 10 with periapical lesions [Table 1 and Graphs 1-5].

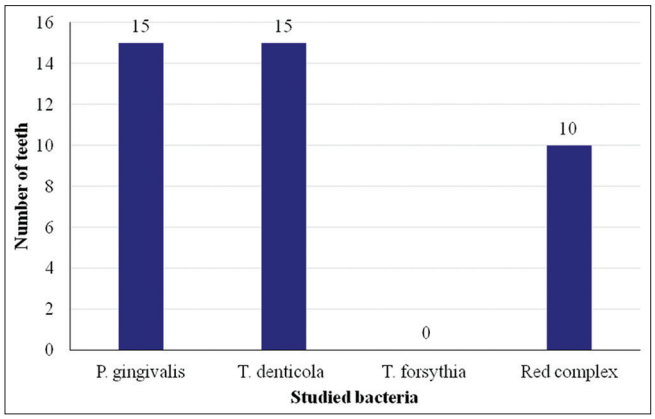
Statistically significant associations were observed between *T. forsythia* as well as *T. denticola* in relation to clinical findings of pain and swelling ($P < 0.05$). Other bacteria did not show any significant association with clinical findings. No bacterial DNA was detected in negative controls.

Discussion

Endodontic-origin microorganisms such as *P. gingivalis*, *T. denticola*, *T. forsythia*, and *Solobacterium moorei* have been linked with osteomyelitis, bacterial endocarditis, brain abscess, obesity, and preterm low-birth-weight.[5] Endodontic infections can progress to systemic infections that are characterized by fever and lymphadenopathy. This reflects the adaptations of microbes to different ecological niches varying from oxygenated to anaerobic environment.[7]

Endodontic flare-ups can occur due to the synergistic presence of *Fusobacterium nucleatum*, *Prevotella* spp., and *Porphyromonas* sp. by increasing the periapical inflammation.[8]

Apical periodontitis has a heterogeneous etiology due to interindividual variations in endodontic microflora. Molecular detection techniques such as species-specific PCR and checkerboard DNA-DNA hybridization assay can identify culture-difficult species from endodontic samples. 16S rRNA gene clone library analysis has revealed that nearly 45–50% of microbial taxa were not cultivable and, hence, was underdiagnosed. Cataloging of these bacterial species provides the 16S rRNA gene sequence.[9] The bacterial 16S rRNA comprises nine hypervariable regions that demonstrate uniform diversity in sequence among various bacterial species which can be used for bacterial identification. These conserved sequences can be used to design universal primers for bacterial DNA amplification.[10]



Graph 5: Studied bacteria and number of teeth with periapical lesions

PCR technique is more sensitive than traditional culture techniques for microbiological identification, especially in refractory cases.[11]

Gram-negative bacteria are found to predominate the microbial population in root canal infections. The lipopolysaccharides are the most important virulent factors which cause clinical symptoms and pathological changes in endodontic infections, due to inflammatory mediators such as IL-1α, IL-1β, TNF-α, PGE2, and matrix metalloproteinases. The microbial population in closed canals is anaerobic while in open canals, it is facultative. The bacterial community shows considerable interindividual variation, thus, influencing the treatment protocols as well.[12]

Gomes *et al.*[13] using nested PCR on necrotic pulp samples analyzed 46%, 38%, and 22% of positive cases of *P. gingivalis*, *T. denticola*, and *T. forsythia,* respectively.

P. gingivalis is a coccobacillus belonging to family Porphyromonadaceae, formerly it was known as "Bacteroides gingivalis."[14]

Seol *et al.*[15] have reported *P. gingivalis* in 22.5% of abscesses using multiplex PCR. In the current study, 15 teeth with periapical lesions were positive for this organism. Rocas *et al.*[16] have reported this organism in 70% of cases with endodontic abscesses.

Treponema are strict anaerobes, motile, Gram-negative and helically shaped bacterial rods.[17,18] Among all intra-oral *Treponema* spp, only *T. denticola*, *T. socransky*, *T. vincentii*, and *T. pectinovorum* can be readily cultivated.

In the present study *T. forsythia* as well as *T. denticola* showed significant association, only with clinical findings of pain and swelling however, these pathogenic bacteria were also found in teeth samples with tenderness on percussion and periapical lesions. The association of the red complex with clinical signs and symptoms was found to be non-significant.

Guven *et al.*[19] performed a study on pulpal samples collected from 20 primary molars with primary endodontic infection. Clinical signs included spontaneous pain, tenderness on percussion, swelling, and tooth mobility. This study found a positive and significant correlation between *T. denticola*, *P. gingivalis*, and *T. forsythia*. However, no statistically significant correlation was found between any of the clinical signs and any particular bacteria. This is a contrast to current study results.

Sanghavi *et al.*[20] conducted a study to evaluate the correlation between endodontic clinical signs and symptoms and the presence of red complex microorganisms such as *P. gingivalis*, *T. denticola*, and *T. forsythia* using PCR assay. It was concluded that *P. gingivalis*, *T. denticola*, and *T. forsythia* were prevalent in the examined samples that suggested their relationship to the etiology of periradicular diseases.

Treponema spp are highly fastidious, Gram-negative motile spirochetes found in periodontal pockets and root canals with primary infection. Noberga *et al.*[21] studied the prevalence of oral *Treponemas* in teeth with endodontic treatment failures and periapical pathologies using samples from 40 root canals using nested PCR technique. The study revealed a high incidence of *Treponema* spp in acute endodontic conditions indicating high pathogenicity.

In a study conducted by Foschi,[22] 56% of teeth with apical periodontitis showed a positive association with *T. denticola*.

Foschi *et al.*[23] performed a study to analyze the role of *T. denticola* as a mono-infection as well as a part of "red complex" infection in the causation of endodontic infections in 6–8 weeks old severe combined immunodeficiency mice. Study results demonstrated periapical bone resorption in *T. denticola* mono-infection when compared to the red complex.

Cardoso *et al.*[24] conducted a study to correlate the bacterial diversity and levels of endotoxins produced by bacteria found in primary endodontic infection with the volume of root canal determined by using cone-beam computed tomography. Culturable bacteria and endotoxins were detected in 100% of the root canal samples and larger canals hold higher levels of the bacterial population. The different clinical features were positively correlated with the presence of the bacterial population.

T. denticola and *T. forsythias* have been shown to predispose immunocompromised subjects such as in Down's syndrome to early-onset periodontitis.[25]

T. denticola has been associated with an increase in periodontal destruction, therefore, increasing tooth loss.[10] It is very difficult to isolate and identify *T. denticola* from clinical samples using culture technique, therefore, the 16S rRNA-based PCR method is used for determining their presence.[26]

P. gingivalis is an asaccharolytic, nonmotile Gram-negative organism requiring an anaerobic environment for growth. It is

a late colonizer and possesses numerous virulence factors such as lipopolysaccharides, capsular polysaccharide, fimbriae, and gingipains.[27]

Cao *et al.*[27] assessed 80 teeth with pulpal necrosis and primary endodontic infection using universal bacterial primers based upon 16S rRNA sequences. This technique demonstrated that both *P. gingivalis* and *P. endodontalis* showed significant association ($P < 0.005$ and $P < 0.05$, respectively) with the presence of sinus tracts along with abscesses.

Pattanshetty *et al.*[28] investigated the presence of selective anaerobic microorganisms in 100 primary root canals of symptomatic and asymptomatic non-vital teeth with periapical pathosis using multiplex PCR. It was concluded that significant differences exist in the bacterial composition between asymptomatic and symptomatic primary endodontic infections. *T. denticola* was present in 21 (42%) and 29 (58%) samples in the symptomatic and asymptomatic groups, respectively. *T. forsythia*, *P. gingivalis*, and *F. nucleatum* were significantly high ($P < 0.05$) in the symptomatic group, whereas *Prevotella intermedia* was significantly high ($P < 0.05$) in the asymptomatic group.

T. forsythia was first isolated at "Forsythe Institute" in subjects with progressive periodontitis. It was originally assigned to the genus "Bacteroides." It exhibits slow growth under fastidious requirements. Subgingival *T. forsythia* is most likely a source in endodontic samples.[21]

In a study by Lacevic *et al.*[29] it was observed that levels of *T. forsythia* in primary endodontic infection and in periodontal lesion were significantly decreased with the increase of patients age.

T. denticola was detected in 15/60 teeth samples with periapical lesions of which five were tender on percussion. The red complex was detected in 10% (6/60) samples taken from acute periradicular abscess samples. These observations are suggestive of the role of "red complex" in the pathogenesis of acute periradicular abscesses. These findings have been corroborated by Sanghavi *et al.*[30] using the multiplex PCR technique.

Siqueira and Rocas analyzed five stages of experimental tools for analyzing microorganisms found within acute apical abscesses: 1) culture techniques; 2) molecular tools such as PCR and checkerboard hybridization assay; 3) PCR in addition to cloning and sequencing of targeted amplicons; 4) PCR or DNA hybridization including reverse checkerboard hybridization, and 5) next-generation sequencing.[31,32]

These novel culture-independent methods using DNA amplification of 16S rDNA followed by cloning and sequencing have been used in determining the bacterial diversities.

Implications for clinical practice

Bacteria associated with endodontic infections trigger inflammatory responses in the immune cells, which in later stages of the disease, cause loss of both soft and hard tissue structures supporting the teeth. The red complex bacteria have been characterized as infectious agents of endodontic disease. Early diagnosis and identification of these microorganisms can help in treating such infections. Increased awareness of oral health and the identification of various disease-causing pathogens help to rule out the risk factors involved in oral diseases. Certain therapies have shown promising results that further needs evaluation in prospective clinical trials. Elimination of these pathogens from the site of infection remains a perplexing task, which demands the use of antibiotics.[33]

Conclusion

The high prevalence of *P. gingivalis*, *T. denticola*, and *T. forsythia* in the present study suggests that these bacteria can be correlated with etiopathogenesis of periapical abscesses. The red complex was detected in a few samples with endodontic signs. Further studies are required for identifying a relationship between root canal microbiota and periradicular diseases.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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