PCR-based detection of three anaerobic bacteria associated with endodontic-periodontic lesions in type-2 diabetic and nondiabetic subjects

Rakesh Rajeevan Nair, Moksha Nayak, Krishna Prasada L, Anoop V Nair, Drisya Soman, Hari Krishnan R
Department of Conservative Dentistry and Endodontics, KVG Dental College and Hospital, Sullia, Karnataka, Consultant Endodontist, Trivandrum, Department of Conservative Dentistry and Endodontics, Azeezia College of Dental Sciences and Research, Kollam, Department of Periodontics, PMS College of Dental Science and Research, Trivandrum, Kerala, India

Abstract
Aim: The aim of this study is to clinically isolate and detect three anaerobic bacteria associated with endodontic-periodontal lesions in type-2 diabetic and nondiabetic patients using polymerase chain reaction (PCR).

Materials and Methods: Sixty patients presenting endodontic-periodontal lesions were divided into two groups. Thirty patients with type-2 diabetics (Group 1) and 30 nondiabetic patients (Group 2) were evaluated for the presence of three anaerobic bacteria. Clinical examinations, periapical radiographs, and microbiological sampling from the canal system and periodontal pockets were performed. Qualitative evaluation of bacteria was performed using a multiplex PCR for Porphyromonas gingivalis and Prevotella intermedia. Statistical analysis was performed using Pearson’s Chi-square test and Fischer’s exact test.

Results: Enterococcus faecalis (73.3%) was the predominant bacteria isolated from the root canal in type 2 diabetic patients, followed by P. gingivalis (70%) and P. intermedia (36%) compared to 53.3%, 43.3%, and 23.3%, respectively, among nondiabetic patients. P. gingivalis (73.3%) was the predominant bacteria isolated from periodontal pockets in type II diabetic patients followed by P. intermedia 50% and E. faecalis 30% compared to 36.6%, 33.3%, and 30%, respectively, among nondiabetics. P. gingivalis was detected in the root canal and periodontal pocket in almost similar numbers (70% and 73%), respectively, among type-2 diabetics.

Conclusion: Detection of P. gingivalis, P. intermedia, and E. faecalis in both root canal and periodontal pocket samples confirm a viable pathway for the spread of infection through dual sites. Since in the present study, P. gingivalis was found to be present in similar numbers in dual sites among type 2 diabetic patients, importance should be given in treating such anaerobic bacteria in immune-compromised patients.

Keywords: Anaerobic bacteria; diabetic mellitus; endodontic-periodontic lesion; multiplex polymerase chain reaction; polymerase chain reaction

INTRODUCTION
The endodontic and periodontal systems are closely related, and the disease occurrence can overlap into each other. The communication routes between endodontic and periodontal tissues include apical foramen, lateral and accessory canals, inter-radicular canals, and the dentinal tubules.[1] The literature on microbiology of endodontic-periodontic lesion suggests a number of anaerobic, facultatively anaerobic, aerobic microorganisms, and viruses are involved in the pathogenesis of the periodontal and endodontic disease. Endodontic-periodontic lesions are responsible for >50% endodontic and periodontal diseases. This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

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of tooth mortality.[2,3] Hence, the axiom is to eradicate the microorganisms to increase the sustainability of the tooth.

The differential diagnosis of the endodontic-periodontal disease is difficult since they are caused by microbes, which are generally polymicrobial in nature.[4] Anaerobic microorganisms such as Porphyromonas gingivalis and Prevotella intermedia are the predominant bacteria involved in the pathogenesis of periodontal inflammation and periapical lesions.[5]

Success is considerably reduced in immunocompromised patients (68%).[6] India is the diabetic capital of the world, with a huge number of diabetic patients. Its rise in diabetic Mellitus is expected to be 69.9 million by 2025.[7] The diabetic host has shown to develop an increased periapical lesion size or serious infections in response to virulent root canal bacteria.[8]

Investigations on endodontic-periodontic lesions reveal increased rates of simultaneous detection of periodontal pathogens in root canals and vice versa.[2] However, there is a lack of evidence in the literature on the microbiology of endodontic-periodontic lesion in immunocompromised patients. Hence, the present study investigated three anaerobic bacteria associated with endodontic-periodontic lesions in non-diabetic and type-2 diabetic patients using polymerase chain reaction (PCR).

**Table 1: Study groups**

| Groups | Subgroups | Subjects |
|--------|-----------|---------|
| Group 1 (30 nos) | Sub group C- Root canal | Type II diabetic subjects |
| Group 2 (30 nos) | Sub group C- Root canal | Nondiabetic subjects |

**Table 2: Polymerase chain reaction conditions were set for the three bacteria**

| PCR conditions | P. gingivalis | P. intermedia | E. faecalis |
|----------------|--------------|---------------|------------|
| Initial denaturation | 95°C, 5 min | 95°C, 5 min | 95°C, 5 min |
| Denaturation | 95°C, 1 min | 95°C, 1 min | 95°C, 30 min |
| Annealing | 60°C, 1 min | 60°C, 1 min | 56°C, 1 min |
| Extension | 72°C, 1 min | 72°C, 1 min | 72°C, 1 min |
| Final extension | 72°C, 5 min | 72°C, 5 min | 72°C, 5 min |
| Storage | 4°C | 4°C | 4°C |

Table 3: Comparison between numbers of bacteria detected among groups

| Number of bacteria | Group 1 | Group 2 | Total |
|--------------------|---------|---------|-------|
| 0                  | 2 (6.7) | 6 (20.0) | 8 (13.3) |
| 1                  | 11 (36.7) | 14 (46.7) | 25 (41.7) |
| 2                  | 12 (40.0) | 8 (26.7) | 20 (33.3) |
| 3                  | 5 (16.7) | 2 (6.7) | 7 (11.7) |

Fisher’s exact test (P) 0.24 (NS)

NS: Not significant

**MATERIALS AND METHODS**

The root canal and periodontal pocket samples were collected from 60 patients between the age 30 and 60 years who had been referred for treatment to the Department of Conservative Dentistry and Endodontics, K.V.G. Dental College and Hospital, Sullia. The Ethical Committee of K.V.G. Dental College and Hospital, Sullia, approved the study protocol. The patients were informed of the study protocol, and written consent was obtained before the sampling procedure was performed. The patients were divided into two groups Table 1

- **Group 1 (30 Nos)** – Healthy Nondiabetic patients with endodontic-periodontic lesions
- **Group 2 (30 Nos)** – Type-2 diabetic patients with endodontic-periodontic lesions.

Patients with any systemic diseases for Group 1 and systemic diseases other than type-2 diabetes for Group 2 were excluded from the study. Besides teeth with root fracture, tortuous canal, developmental defects and that cannot be restored were also excluded.

**Diagnosis of endodontic-periodontal lesions and diabetic subjects**

Patients having the following clinical-radiographic factors were considered: periodontal probing depth >5 mm, boneloss, clinical attachment loss, presence of inflammation, bleeding on probing, irreversible pulpitis with chronic apical periodontitis. Each subject having two or more of the signs and symptoms were selected.

The patients in Group 2 had a random blood sugar level >200 mg/dl, fasting blood sugar level >126 mg/dl, 2 h post prandial blood sugar level >200 mg/dl, and glycosylated HB >6.5%.

**Sample collection from periodontal pockets**

The supragingival plaque was removed with an ultrasonic device and with gentle rotary brushing. The periodontal sites to be sampled were air-dried and isolated with cotton rolls and two sterile paper points ISO size 40 were inserted into the periodontal pocket, after 1 min, the paper points were removed and placed in T.E buffer and transported for PCR identification.
Table 4: Association between Enterococcus faecalis among groups in endodontic-periodontic lesions

| E. faecalis | Group 1, count (%) | Group 2, count (%) | Total, count (%) | Group 1, count (%) | Group 2, count (%) | Total, count (%) |
|------------|--------------------|--------------------|------------------|--------------------|--------------------|------------------|
| Absent     | Sub group C        | 8 (36.7)           | 14 (46.7)        | 25 (41.7)          | 21 (70.0)          | 42 (70.0)        |
|            | Sub group P        | 30 (100.0)         | 30 (100.0)       | 60 (100.0)         | 30 (100.0)         | 60 (100.0)       |
| Present    | Sub group C        | 22 (73.3)          | 16 (53.3)        | 38 (58.3)          | 9 (30.0)           | 18 (30.0)        |
|            | Sub group P        | 6 (20.0)           | 8 (26.7)         | 14 (23.3)          | 7 (23.3)           | 11 (18.3)        |
| Total      |                    | 30 (100.0)         | 30 (100.0)       | 60 (100.0)         | 30 (100.0)         | 60 (100.0)       |

Chi-square test: Chi square value (df)=0.62 (1), P=0.43 (NS)

Table 5: Comparison of presence of Porphyromonas gingivalis in periodontal pocket and root canal among groups

| P. gingivalis-Sub group P | Group 1 | Group 2 |
|---------------------------|---------|---------|
| Absent                    | 4 (13.3)| 10 (33.3)|
| Present                   | 6 (20.0)| 7 (23.3) |
| McNemar test              | 0.75 (NS)| 0.55 (NS)|

Chi-square test: Chi square value (df)=0.00 (1), P=0.00 (NS)

Table 6: Comparison of the presence of Prevotella intermedia in periodontal pocket and root canal among groups

| P. intermedia-Sub group P | Group 1 | Group 2 |
|---------------------------|---------|---------|
| Absent                    | 10 (33.3)| 16 (53.3)|
| Present                   | 9 (30.0)| 7 (23.3) |
| McNemar test              | 0.42 (NS)| 0.55 (NS)|

Sample collection from the endodontic site: (Moller’s criteria)

After access cavity preparation, root canal samples were collected as per Moller’s criteria using sterile paper points. In the case of multi-rooted teeth, roots with apical lesions were taken into consideration for sampling. If more than one root per tooth had an apical lesion, the widest and most permeable root canal adjacent to the lesion was sampled. Then, the paper points were transferred into the T.E buffer solution and immediately transported to the Department of Molecular Biology, Maratha Mandal Dental College and Hospital, Belgaum for PCR evaluation.

DNA extraction (modified proteinase-K method)

The samples were centrifuged at 5000 rpm for 5 min. The supernatant was discarded, and 500 microliter fresh T.E buffer was added and centrifuged for 3–4 min. The above procedure was repeated for 3–4 times with fresh T.E buffer. The supernatant was discarded, 50 μL lysis buffer I was added, vortexed and kept for 5 min, followed by addition of 50 μL Lysis buffer II and 10 μL proteinase-K (100 μg/ml) and vortexed vigorously. The mix was kept in a water bath for 2 h and later in a boiling water bath for 10 min. The DNA was extracted and stored at −20°C.

Polymerase chain reaction procedure

Multiplex PCR (mPCR) was performed for the identification of P. gingivalis and P. intermedia and a conventional PCR was performed to identify Enterococcus faecalis. [9,10]

The following PCR conditions were set for the three bacteria: Table 2.

Detection of amplified products

PCR amplification was performed, and the amplified products were subjected to electrophoresis through 2% Agarose gel containing 1× TAE. 20 μL of each amplified product were mixed with 3 μL of bromophenol blue loading dye, electrophoresis was performed at 25V for 2 h. The gel was visualized under UV light illuminator after staining with Ethidium bromide (0.5 μg/ml).

RESULTS

Microorganisms were isolated from 93.4% of the endodontic-periodontal lesions by the PCR method. In 6.6% of the samples, all the target species were negative using PCR. Among the type 2 diabetic patients (Group 1) 93.3% of the root canal and 80% of the periodontal pocket detected bacteria. Whereas among the nondiabetic patients, 80% and 76.7% of the root canal and periodontal pocket samples, respectively, detected bacteria.

E. faecalis (73.3%) was the predominant bacteria isolated from the root canal in type 2 diabetic patients followed by P. gingivalis (70%) and P. intermedia (36%) compared to 53.3%, 43.3%, and 23.3%, respectively, among nondiabetic patients. P. gingivalis (73.3%) was the predominant bacteria isolated from periodontal pockets.
in type II diabetic patients followed by P. intermedia 50% and E. faecalis 30% compared to 36.6%, 33.3%, and 30%, respectively, among nondiabetics. P. gingivalis was detected in the root canal and periodontal pocket in almost similar numbers (70% and 73%), respectively, among type 2 diabetics [Tables 3-6].

DISCUSSION

The periodontium and the pulp are closely interrelated and possess various communications between the pulp and the periodontium, such as accessory canals, lateral canals, interradicular canals, developmental grooves, and the dentinal tubules. The disease occurs in one of the tissues that can diffuse to another harboring microorganism common to both the sites.[1]

The microflora of primary endodontic infections are mixed with the predominance of obligate and facultative anaerobic bacteria; on the other hand, periodontal infections are dominated by aerobic and facultative anaerobic microorganisms. Endodontic-periodontic lesions are typically polymicrobial in nature, facultative anaerobes such as P. gingivalis, P. intermedia, and Fusobacterium usually dominate the bacterial flora. Moreover, studies have shown antagonistic and synergistic effects between different strains and species.[2-4] Infections of periodontal or endodontic origin can result in increased periodontal probing depths, localized gingival inflammation, or swelling, bleeding on probing, suppuration, fistula formation, tenderness on percussion, mobility, bone loss, and pain. Hence, the present study investigated the presence of P. gingivalis, P. intermedia and E. faecalis and the associated signs and symptoms in endodontic-periodontic lesions.

Infections are found to be the major cause of mortality among immunosuppressed patients. Type-2 diabetes mellitus is a syndrome characterized by abnormalities in carbohydrate, lipid, and protein metabolism that results from resistance of target tissue to its cellular metabolic effects.[11] By far the largest proportion of public health problem derives from type-2 diabetes, which accounts for >51 million population of India. The unique clinical and biochemical abnormalities in Indians, which include increased insulin resistance, greater abdominal adiposity, lower adiponectin, and higher high sensitive C-reactive protein levels, referred to as Asian Indians phenotype makes them more prone to diabetes.[7] Hence, the present study investigated endodontic-periodontic lesions among type 2 diabetic and nondiabetic patients.

In our study, mPCR was used to investigate the presence of P. gingivalis and P. intermedia as the primers of these two black pigmented anaerobes could be optimized for similar temperature cycles during PCR amplification. E. faecalis has been the predominant microorganism isolated from secondary endodontic infections using PCR.[8] PCR is a very complex process with very high sensitivity and specificity. mPCR is a modification of PCR intended to rapidly detect duplication or deletions in a large gene that can detect more than one species by optimization of primers and temperature cycles.[2] Various studies have shown investigated endodontic and periodontic microbiota using PCR methodology with varying results.[13] Hence, in the present study, PCR based detection was used to detect P. gingivalis, P. intermedia and E. faecalis from endodontic-periodontic lesions among type-2 diabetic and nondiabetic patients.

In the present study, among type 2 diabetic subjects, 93.3% of the root canals and 80% periodontal pockets contained microorganisms. More number of microbes were isolated from periodontal pocket and canal from type-2 diabetic patients (80%) compared to 76.7% in nondiabetic patients. A few studies have shown anaerobic bacteria to be prevalent in endodontic-periodontic lesions in a range of 45%–93%.[2-4]

In the present study, type-2 diabetic patients were more associated with E. faecalis (73%) P. gingivalis (70%), and P. intermedia (36%) in the root canal compared to nondiabetics (43.3%, 23.3%), and (53.3%), respectively, whereas P. gingivalis (73.3%) was the predominant bacteria followed by P. intermedia (50%) and E. faecalis (30%) in the periodontal pocket in type-2 diabetics compared to (36.6%), (33.3%), and (30.%), respectively, in nondiabetic patients.

P. gingivalis was the predominant bacteria isolated from the periodontal pocket samples among nondiabetics among the three bacteria tested. Our findings were similar to other reports where P. gingivalis was detected in a higher degree in the periodontal pocket of type-2 diabetic subjects compared to nondiabetic subjects.[13,14] Also, P. gingivalis was found to be present in both root canal and periodontal pocket in almost similar numbers among type-2 diabetic patients. Thorstenssen et al. investigated significantly more type-2 diabetic individuals harbored P. gingivalis compared to nondiabetics in the periodontal pocket. A few other studies using PCR have also shown similar results in nondiabetic patients.[2] P. gingivalis is a Gram-negative oral anaerobe that is shown to be involved in the pathogenesis of periodontitis. P. gingivalis can locally invade periodontal tissues and evade the host defense mechanisms. In doing so, it utilizes a panel of virulence factors that cause deregulation of the innate immune and inflammatory responses.[15]

The ability of P. gingivalis to cause adult periodontitis is determined by its virulence factors. Biofilm formation and
bacterial dipeptidyl peptidase IV (DPP IV) activity contribute to the pathogenic potential of \textit{P. gingivalis}. Furthermore, biofilm formation may enhance \textit{P. gingivalis} virulence through increased DPP IV activity. Studies have revealed the roles of gingipain R and gingipain K in the virulence of \textit{P. gingivalis}.

Foud et al. showed \textit{E. faecalis} to be present in 78\% of type 2 diabetic patients in nonvital teeth. Other studies have shown a prevalence of 81\%–93\% in nonvital teeth. The differences may be attributed to the case selection since the present study was investigated in endodontic-periodontic lesions. The probable reason for the increased amount of \textit{E. faecalis} in primary endodontic-periodontic lesion may be due to the interrelationships of both the periodontal and endodontic niches.

According to Isabelle Portenier et al. (2003), virulence factors of \textit{E. faecalis} play an important role in the bacterium’s pathogenesis which ranges from life threatening disease in compromised individuals such as bacteremia, septicemia, endocarditis and urinary tract infections to less severe conditions such as infections of the obturated root canal with chronic periapical periodontitis. Portenier et al. (2005) also gave a mechanism of alkaline tolerance of \textit{E. faecalis} which is responsible for its resistance against several disinfectants which was associated with a functioning cell wall associated proton pump, which drives protons into the cell to acidify the cytoplasm. \textit{E. faecalis} has also special capacities as endopathogen to invade dentinal tubules and adhere to dentin surface.

Vascular problems associated with diabetes mellitus also cause an increase in anaerobic infection, which may be attributed to reduced oxygen diffusion across the capillary wall. Infections become more severe and last longer because of neutrophil suppression and synergism of aerobic and anaerobic bacteria, attributed to anoxia. The vascular impairment fails to bring the cellular and humoral elements of the immune system to the area of injury. This might be the possible reason why \textit{E. faecalis} was more frequently associated with nonvital teeth of type-2 diabetic patients.

Diabetes has impaired defense mechanisms involving micro- and macro-vasculatures. The increased susceptibility to infection and reduced healing capacity with altered collagen metabolism may explain the increased occurrence of signs and symptoms in type 2 diabetic patients.

Thus, PCR based detection of three anaerobic bacteria associated with endodontic-periodontic lesions in type-2 diabetic and nondiabetic subjects showed an increased association between the detection of bacteria and type-2 diabetic subjects. All the samples showed a high detection rate in type-2 diabetics. \textit{P. gingivalis} was detected as the most frequently detected bacteria from the periodontal pocket followed by \textit{P. intermedia}. \textit{E. faecalis} was the predominant bacteria detected in root canal samples. All three target species of bacteria were shown to have an association with specific signs and symptoms. The present study also showed that in endodontic-periodontic lesions there was a high degree of simultaneous detection of all the target bacteria which point towards the polymicrobial interactions in such lesions and further emphasis the role of \textit{P. gingivalis, P. intermedia} and \textit{E. faecalis} in the pathogenesis of endodontic-periodontic lesion.

Type-2 diabetic patients are immune-compromised and hence more associated with microorganisms as well as signs and symptoms.

**CONCLUSION**

The detection of \textit{P. gingivalis, P. intermedia}, and \textit{E. faecalis} in both root canal and periodontal pocket samples in endodontic-periodontic lesions confirm an interrelation for the spread of infection through dual sites. The tested bacteria in type 2 diabetic subjects were highly prevalent and showed increased signs and symptoms indicating their immune-compromised state. Since in the present study, \textit{P. gingivalis} was found to be present in similar numbers in dual sites among type 2 diabetic subjects, importance should be given in treating such anaerobic bacteria in immune-compromised patients. A proper diagnosis and in-depth knowledge of the etiological factors associated with endodontic-periodontic lesion plays a vital role in the enhancement of the treatment outcome. Further, larger data and quantitative analysis should be the goal of future studies.

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**Conflicts of interest**

There are no conflicts of interest.

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