IMPACT OF PEPTOSTREPTOCOCCUS ON TYPE 2 DIABETES MELLITUS RELATED SECONDARY ROOT CANAL INFECTIONS

Moksha Nayak 1,*, Subbannayya Kotigadde 2, Harish Shetty 3, R.V. Vineet 4 and Beena Antony 5

Department of Conservative Dentistry & Endodontics, KVG Dental College and Hospital 1, Sullia- 574 239, Karnataka, India
Department of Microbiology, KVG Medical College & Hospital, Sullia 2- 574239, Karnataka, India
Department of Conservative dentistry & Endodontics, Yenepoya Dental College 3, Mangalore, Karnataka, India
Department of Conservative Dentistry & Endodontics, Sree Mookambika Institute of Dental Sciences 4, V.P.M. Hospital Complex, Padanilam, Kulasekharam, K.K. District, Tamil Nadu, India 629161
Department of Microbiology, Father Muller Medical College 5, Mangalore, Karnataka, India

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Correspondence to Author:
Moksha Nayak
Department of Conservative Dentistry & Endodontics, KVG Dental College and Hospital, Sullia-574 239, Karnataka, India
E-mail: prof.moksha@gmail.com

ABSTRACT:

Aim: The purpose of this study was to assess the impact of Peptostreptococcus on type 2 diabetes mellitus patients with secondary root canal infections by evaluating the prevalence of this bacterial species using culture and PCR technique and to correlate their association with specific endodontic signs and symptoms in Indian population.

Materials and method: 88 subjects scheduled for root canal retreatment were divided into two groups comprising of 44 subjects with history of type 2 diabetes mellitus (group 1) and 44 non-diabetic subjects as control (group 2). Root canal samples were collected as per Moller’s criteria using sterile paper points. The prevalence of Peptostreptococcus in secondary root canal infections was analysed using advanced culture technique and species-specific PCR technique. Statistical analysis was done using Student unpaired T test and Pearson’s Chi-Square test.

RESULTS: In group 1, Peptostreptococcus was detected in 52.2% and 54.5%, in contrast group 2 was associated with 27.3% and 31.8% of Peptostreptococcus using culture and PCR respectively, with statistically significant association of Peptostreptococcus with type 2 diabetes mellitus (p=0.014). There was strong association between pain and Peptostreptococcus in type 2 diabetic patients (p=0.012) as well as in non-diabetics (p=0.048). No statistically significant difference was observed in the detection of Peptostreptococcus using culture and PCR technique.

CONCLUSION: The study demonstrated that Peptostreptococcus has an impact in modulating the inflammatory and immunologic responses in secondary root canal infections in type 2 diabetic patients. Hence, pharmacotherapy for effective elimination of this potentially pathogenic microorganism can have a beneficial effect on the prognosis of root canal retreatments.

INTRODUCTION: Type 2 diabetes mellitus formerly called non-insulin dependent diabetes mellitus or adult onset diabetes is a disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency (WHO).

This common metabolic disorder is a risk factor for development of large or debilitating periapical infection as well as resistance to pharmacotherapy 1. It has been estimated that the global burden of type 2 diabetes mellitus for 2010 is 285 million people which is projected to increase to 438 million by 2030; a 65 % increase. Similarly, for India this increase is estimated to be 58%, from 51 million people in 2010 to 87 million in 2030 2. The acute exacerbation of periapical lesions is due to an increase in inflammatory microbiota persisting in the root canal and dentinal tubules of diabetic patients 3.
Root canal system harbours about $10^{10}$ bacteria mainly consisting of a complex polymicrobial population. Peptostreptococcus is a gram positive strict anaerobe frequently isolated from root canal infections. The success of endodontic treatment (87%-95%) is directly influenced by elimination of microorganisms in infected root canals. Secondary infection or failure of root canal treatment occurs when treatment procedures have not met a satisfactory standard for control and elimination of infection.

Baumgartner et al. observed that the cell walls of gram positive bacteria contain peptidoglycans and lipoteichoic acids, which influence inflammatory reactions and enhance the pain modulation as well as contribute to increased insulin resistance and poor glycemic control in diabetic patients. Tayler et al proposed that metabolic control is important not only in the pathogenesis and progression of diabetes mellitus but also in the high susceptibility to infectious diseases, as evidenced by a 2 to 5 fold higher risk for apical periodontitis. Conversely, the risk is reduced by effective pharmacological control of hyperglycemia.

The main objective of the present study was to evaluate the impact of Peptostreptococcus in type 2 diabetes mellitus patients with secondary root canal infections identified using advanced culture technique and species specific PCR and to correlate their association with endodontic signs and symptoms.

**MATERIALS AND METHOD:**

**Clinical material:** Patients were selected from those who attended the Endodontic Clinical Section of the Department of Conservative Dentistry & Endodontics, K.V.G Dental College & Hospital, Sullia, Karnataka, India for endodontic retreatment. The Ethical Committee of K.V.G Dental College & Hospital, Sullia approved the study protocol. The subjects were informed of the study protocol and written consent was obtained before the sampling procedure was performed. 88 patients requiring endodontic retreatment were selected for the study and were divided into two groups:

**Group 1:** 44 patients reporting history of type 2 diabetes mellitus with age ranging from 35 to 75 years. The inclusion of these patients under type 2 diabetes category was based on the criteria put forth by the Expert Committee on Diagnosis and Classification of Diabetes Mellitus.

**Group 2:** 44 patients with no history of diabetes mellitus and normal glucose tolerance served as controls.

A detailed medical and dental history was obtained from each patient. Patients who were on antibiotic treatment during the last 3 months, pregnancy and lactation, immunocompromised individuals, patients with teeth that cannot be isolated with rubber dam, calcified canals, tortuous canals, root fracture and teeth with developmental defects were excluded from the study. Failure of root-canal treatment was determined on the basis of clinical and radiographic examinations. The following features were recorded for each patient: tooth type, presence or absence of coronal restoration, pain, sinus tract, tenderness to percussion.

**Sampling procedure:** Aseptic techniques were used throughout the root canal sampling procedure as proposed by Moller. After plaque removal and rubber dam application, the operative field was cleaned using 30% hydrogen peroxide and disinfected with 2.5% sodium hypochlorite solution. Endodontic access was completed with a sterile high speed carbide bur until the root canal filling was exposed. After completion of the endodontic access, the tooth clamp and adjacent rubber dam were once again disinfected with 2.5% sodium hypochlorite. The sodium hypochlorite solution was then inactivated using sterile 5% sodium thiosulphite. Coronal gutta percha was removed using sterile Gates-Glidden burs and the apical material was retrieved using K-type and Hedstrom files. Root canal filing was always performed without the use of clinical solvents. Working length estimation was done with a radiograph using a small sterile file. To obtain microbial samples, a sterile 20 K-file was used to agitate canal contents for 1 minute.

Two or more sterile paper points (ADA products-Mynol, Milwaukee, WI, USA) were placed in the root canal for 60 seconds and then immediately transferred to two sterile 5 ml tubes (Eppendorf AG, Hamburg, Germany) containing 3ml of reduced transport fluid(RTF).
One Eppendorf tube was transferred to the Neurobiology Laboratory; National Centre for Biological Sciences (Tata Institute of Fundamental Research, Bangalore) for PCR analysis and the other tube was transferred immediately to Department of Microbiology, Father Muller Medical College, Mangalore for culturing.

**Bacterial culture method:** The root canal samples were shaken in a vortex mixer for 60 seconds. After vortexing, 50μl of sample was plated onto nutrient blood agar media for identifying *Peptostreptococcus*. Streaking of samples onto media was done using sterile wire loop (loop technique).

Anaerobic culturing technique was used for *Peptostreptococcus*. Incubation was done under anaerobic condition at 37°C for 7 days.

After incubation, the plate was biochemically analysed for growth and identification of bacteria using the colony morphology and gram staining. For *Peptostreptococcus* colonies are usually small, 1-2 mm or less in diameter, circular, convex, smooth, translucent to opaque and non-haemolytic. Gram staining shows *Peptostreptococci* as spherical shaped cells that occur in pairs, chains, tetrads or irregular clumps as shown in **Fig. 1**.

**FIG. 1: BACTERIAL CULTURE TECHNIQUE**

**DNA extraction:** The root canal samples were thawed to 37°C for 10 minutes and homogenized by vortex mixing for 1 minute. The paper points were removed, and the microbial suspension was washed 3 times with 200μL of ultrapure water by centrifugation at 2500g for 2 minutes.

After the final wash, the pellets were resuspended in 200μL of ultrapure water, boiled for 10 minutes in a water bath, quickly chilled by placing on ice at 4°C for 5 minutes, and centrifuged at 9000g to remove unbroken cells and large debris.

The supernatant was then collected and used as the template for PCR amplification. Reference DNA from several species was also extracted to serve as positive control for the taxon-specific primers used or to evaluate the specificity of the primers.
PCR Procedures: Aliquots of each sample (1 ml) were centrifuged at 13,000g for 10 minutes. The resulting pellets were washed with 500μL of phosphate-buffered saline, and placed in 200μL of TE buffer (10mM Tris-Cl pH 7.5, 1 mM EDTA). The DNA concentrations in test samples and the reference sample were determined by Nano Drop 1000 spectrophotometric measurement by absorbance at 260 nm. Serial 10-fold dilutions of known concentration of reference DNA of the target species were processed to determine PCR assay sensitivity. The lowest DNA concentration that resulted in a positive PCR product was regarded as indicative of the sensitivity of the assay. Primer specificity was further tested against reference DNA. Table 1 represents the PCR primers used in the present study.

TABLE 1: PCR PRIMERS USED

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Annealing Temperature (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptostreptococcus</td>
<td>AGA GTT TGA TCC TGG CTC AG ATA TCA TGC GAT TCT GTG GTC TC</td>
<td>60</td>
<td>207</td>
</tr>
</tbody>
</table>

PCR amplification was performed in volumes of 25μl containing 1x PCR/Mg++ Buffer (Boehringer Mannheim, Indianapolis, IN, USA), 0.5 U Taq DNA polymerase (Boehringer Mannheim), 0.4 μM of primer pair and 10 ng of template. Negative controls consisting of ultrapure water instead of sample were included with each batch of samples analyzed.

Amplification was performed in a DNA thermal cycler Gene Amp® PCR system (Applied Biosystems) programmed for 94°C (5min), followed by 30 cycles with adequate annealing temperature of 60°C, then 72°C (5min) to allow the completion of DNA extension. Amplification products were compared by electrophoresis in 1% agarose gel in 1x TBE (1M Tris, 0.9 M boric acid, 0.001 M EDTA, pH 8.4) buffer (Gibco BRL, Life Technologies, Ltd., Bethesda, MD, USA), with ethidium bromide (0.5 μg/ml), and photographed on a UV light transilluminator (Kodak Digtal Science System 120). Gene Ruler® DNA Ladder Mix (Fermentas GmbH, Germany) served as the molecular weight marker. The identity of each band was determined by visual comparison with a molecular weight ladder. Reactions were deemed positive in the presence of bands of the appropriate size. PCR amplification of reference genomic DNA of *Peptostreptococcus* using the species-specific primers resulted in a single band of the expected size. Fig. 2 represents the specific amplicons obtained after PCR amplification.

FIG. 2: DEPICTS REPRESENTATIVE PEPTOSTREPTOCOCCUS-SPECIFIC AMPICONS OBTAINED AFTER PCR AMPLIFICATION

Statistical Analysis: The data collected were typed onto a spreadsheet and statistically analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The results were statistically evaluated using Student unpaired T test and Pearson chi-square test.

RESULTS: The prevalence of *Peptostreptococcus* was 52.3% in group1 and 27.3% in group2 using culture technique, whereas PCR showed its prevalence to be 54.5% in group1 and 31.9% in group2 as shown in Table 2 and Fig. 3.
TABLE 2: PREVALENCE OF PEPTOSTREPTOCOCCUS IN GROUP 1 AND GROUP 2 USING CULTURE AND PCR METHOD

<table>
<thead>
<tr>
<th>Method</th>
<th>Peptostreptococcus</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 count %</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>%</td>
<td>52.3%</td>
<td>47.7%</td>
</tr>
<tr>
<td>Group 2 count %</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>%</td>
<td>27.3%</td>
<td>72.7%</td>
</tr>
<tr>
<td>PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>%</td>
<td>54.5%</td>
<td>45.5%</td>
</tr>
<tr>
<td>Group 2</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>%</td>
<td>31.9%</td>
<td>68.1%</td>
</tr>
</tbody>
</table>

Chi-square test showed significant association of Peptostreptococcus with type 2 diabetics using culture (p = 0.014) and PCR (p = 0.026).

TABLE 3: CLINICAL SIGNS AND SYMPTOMS

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Group 1 (n=44)</th>
<th>Group 2 (n=44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tooth type</td>
<td>Single root</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Multiroot</td>
<td>31</td>
</tr>
<tr>
<td>Coronal restoration</td>
<td>Present</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>24</td>
</tr>
<tr>
<td>Percussion sensitivity</td>
<td>Absent</td>
<td>33</td>
</tr>
<tr>
<td>Sinus tract</td>
<td>Absent</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>29</td>
</tr>
<tr>
<td>Pain</td>
<td>Absent</td>
<td>15</td>
</tr>
<tr>
<td>Secondary caries</td>
<td>Present</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>19</td>
</tr>
</tbody>
</table>

Patients in group 1 received HbA1c test to determine the degree of their glycemic control and were grouped according to Oglesby criteria (2006). The level of glycemic control and endodontic signs and symptoms were correlated as shown in Table 4.

TABLE 4: GLYCOXYLATED HAEMOGLOBIN ASSESSMENT

<table>
<thead>
<tr>
<th>Criteria</th>
<th>No. of patients (n=44)</th>
<th>Pain</th>
<th>Percussion sensitivity</th>
<th>Coronal restoration</th>
<th>Sinus tract</th>
<th>Secondary caries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Good control (7.5-8.5%)</td>
<td>35</td>
<td>24</td>
<td>11</td>
<td>28</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>Fair control (8.5-9.5%)</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Poor control (&gt;9.5%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Chi-square test showed no significant difference between level of glycemic control and endodontic signs and symptoms.

Pain was noticed in 68.2% (30 of 44 subjects) in diabetic group and 63.7% (28 of 44) in non-diabetic group. Statistically significant association between pain and Peptostreptococcus was observed in diabetics (p=0.012) as well as in non-diabetics (p=0.048) by Chi-square test as shown in Fig. 4. No statistical correlation was found between tenderness to percussion, sinus tract, and...
absence of coronal restoration with the presence of *Peptostreptococcus*.

**FIG. 4: ASSOCIATION OF PEPTOSTREPTOCOCCUS WITH PAIN IN GROUP 1 (DIABETIC) AND GROUP 2 (NON-DIABETIC)**

**DISCUSSION:** In the present study patients with diabetes mellitus who had failed previous root canal treatment were selected as they may develop more serious infections in response to virulent root canal bacteria. Patients with age ranging from 35 to 75 years were selected in the study. In the recent years, there is a shift in age of onset of type 2 diabetes to a younger age. Health and Lifestyle Survey (1993) suggested prevalence of type 2 diabetes to be 3.1% in individuals aged over 15 years, and 6.5% in those over 75 years. India leads the world with largest number of diabetic subjects due to certain unique clinical and biochemical abnormalities in Indians which include increased insulin resistance, greater abdominal adiposity *i.e.*, higher waist circumference despite lower body mass index, lower adiponectin and higher high sensitive C-reactive protein levels.

Type 2 Diabetes mellitus has a complex pathogenesis characterized by abnormalities in carbohydrates, lipid and protein metabolism that results from target tissue resistance to its cellular metabolic effects. Genetic and environmental factors may influence the risk of diabetes as shown in **Fig. 5**.

**FIG. 5: PATHOGENESIS OF TYPE II DIABETES**

Studies by Thoretson *et al* (1996), Winkelhoff *et al* (2002) and Ciantar *et al* (2005) revealed that ecology of oral flora changed in diabetic patients to a more virulent microbial profile compared to non-diabetics as shown in **Fig. 6**.
Glycosylated haemoglobin assay is an important diagnostic tool for type 2 diabetes. HbA1c reflects average plasma glucose over the previous eight to 12 weeks. An HbA1c of 6.5% is recommended as the cut point for diagnosing diabetes. It can be performed at any time of the day and does not require any special preparation such as fasting. These properties have made it the preferred test for assessing glycaemic control in patients with diabetes. Grouping patients with type 2 diabetes by glycemic control, Oglesby et al. (2006) found diabetes related complications to be 16% and 20% lower for patients with good control compared with fair and poor control, respectively.

In this research study, 9 diabetic patients had fair glycemic control compared to 35 diabetic patients with good glycemic control. Peptostreptococcus was identified in fair glycemic control patients (100%) however the good glycemic control patients showed only 32% Peptostreptococcus. This throws light that as the glycemic control reduces, there is increase in infection and more chance for identifying microorganisms. Larger lesions were associated with diabetic subjects (10%) compared to smaller size lesions in the non-diabetics.

Follow-up studies by Molander et al. on root canal retreatment with preexisting periapical lesions report an overall success rate of 66% which is further reduced in patients with history of diabetes mellitus due to significantly reduced healing following endodontic therapy. This may be explained by the fact that diabetes mellitus alters many functions of the immune system and is associated with delayed healing and compromised immune responses as shown in fig. 7.

Peptostreptococcus was present in 52.3% of type 2 diabetic cases and 27.3% of non-diabetic cases using culture, whereas PCR identified it in 54.5% and 31.9% of type 2 diabetic and non-diabetic patients respectively. There was a strong association between type 2 diabetes mellitus and presence of Peptostreptococcus supporting the role of this microorganism in the immunomodulation and inflammatory response in the secondary root canal infections in type 2 diabetic patients. In non-diabetic patients studies by Riggio et al have identified Peptostreptococcus species in a number of oral infections including root canal infections and endodontic abscesses.

**Periapical lesion involves activation of the broad axis of innate immunity through upregulation of proinflammatory cytokines from monocytes and polymorphonuclear leucocytes. Inappropriate secretion of these cytokines, characterizes a dysregulated immune response that leads to destruction of periapical tissues in the presence of bacterial biofilm.**
There was a strong association between presence of *Peptostreptococcus* and pain in both diabetic and non-diabetic cases. This has been well demonstrated by Pinheiro et al., Gomes et al., Ercan et al. Siqueira et al. 4, 7, 22. *Peptostreptococcus* is a gram-positive anaerobic coccus containing peptidoglycan and lipoteichoic acid as cell wall component, which can influence inflammatory reactions and enhance the pain modulation especially in diabetic patients due to altered immunologic status 24. *Peptostreptococcus* was not associated with any other clinical features in the present study. This can be explained by the findings of Gomes and Pinheiro et al. that potential complex interactions of species resulting in characteristic clinical features cannot be achieved by individual species alone 4, 7.

*Peptostreptococcus* is a slow-growing bacterium with high resistance to antimicrobial drugs 25. However, Brooke et al. found that they are susceptible to beta-lactam antibiotics 26. Infections caused by *Peptostreptococcus* are synergistic whereby there is mutual induction of sepsis enhancement, increased abscess inducement and enhancement of growth of the bacterial components in diabetic patients 27.

When *Peptostreptococci* and other anaerobes predominate, aggressive treatment of acute infection can prevent chronic infection. When the risk of anaerobic infection is high, proper antimicrobial prophylaxis may reduce the risk. However, in certain situations especially in diabetic patients pharmacotherapy with antimicrobials often does not eradicate anaerobes mostly due to antibiotic resistance 26.

Cultivation using artificial growth media has been the ‘gold standard’ diagnostic test in infectious diseases 28. The main advantage of culture method is their broad range nature, which makes it possible to identify a great variety of microbial species in a sample and to detect only viable microorganisms 29. Although the study protocol utilized an expert microbiologist for identification of microorganisms, it is noted that interpretation of results from culturing methods is based on characteristics observed in known and reference strains, which are subject to biases of interpretation 30. Some strains within a given cultivable species can be uncultivated.

One of the reasons for such unculturability can be related to the viable but noncultivable (VBNC) state that some bacteria can develop as a survival strategy when faced with adverse environmental conditions, including starvation. In this state, bacteria escape detection by conventional culturing methods but are still alive, metabolically active and able to exert pathogenicity 4.

Moreover, for the detection of microbes, culturing has low sensitivity and specificity when compared with molecular methods 31.

Although latest molecular based identification system (PCR) are designed to detect molecular DNA, a limitation is that it cannot distinguish between DNA from viable and dead cells and it is unclear whether the results from PCR method represent authentic living flora or rather a historical record of microorganisms that have entered but not survived in the canal 32. Hence, combining molecular and culture technique is probably the best approach available to provide comprehensive information about the role of virulent pathogens associated with endodontic infections.

The present study utilized culture and species specific PCR for identification of only one of the most common endodontic pathogen. However, further research using larger sample size and a broad range molecular microbiological method for identifying more number of endodontic pathogens may be necessary to demonstrate a significant association between diabetes mellitus and endodontic infections.

**CONCLUSION:** *Peptostreptococcus* has a high impact on type 2 diabetes mellitus with secondary root canal infections. *Peptostreptococcus* was significantly more associated with symptomatic cases of type 2 diabetes mellitus, suggesting a role of this microorganism in modulating the inflammatory and immunologic responses in secondary root canal infections in type 2 diabetic patients as well as its level in root canal infections can have an influence on the pain threshold of patient.

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