RESEARCH PAPER | Medical Science | Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

Spectroscopic Evaluation of Enterococcus Faecalis, And Streptococcus Species in Secondary Root Canal Infection of Type-2 Diabetic Patients

KEYWORDS: Enterococcus faecalis, Streptococcus spp. Type-2 Diabetes mellitus, Secondary root canal infection, FTIR spectroscopy.

***Dr.Grishma Florence Noronha**
Senior lecturer Department of Conservative dentistry and Endodontics A.B.Shetty memorial institute of dental science. *Corresponding author

Prof:Dr. Moksha nayak
Principal Department of Conservative dentistry and Endodontics K.V.G Dental college and Hospital, Kurunjibagh, Sullia

Dr. Ramya M.K
Senior lecturer Department of Conservative dentistry and Endodontics K.V.G Dental college and Hospital, Kurunjibagh, Sullia

Dr.JayanthNambiar
Senior lecturer Department of Conservative dentistry and Endodontics Century dental college Poinachi, Kerala

Dr.Moopilmidhunmohanan
Assistant dental surgeon District hospital Palakkad

ABSTRACT *Aim:- Detection and Identification of Enterococcus faecalisand Streptococcus species in secondary root canal infections of non diabetic and type-2 diabetic patients using spectroscopic signature development and evaluation.*

Material and methods:40 subjects grouped into 20 non diabetic and 20 type-2 diabetic subjects with secondary root canal infection were selected for the study. Root canal samples were collected and cultured using selective media to isolate Enterococcusfaecalis and Streptococcus mitis. The microorganisms obtained were subcultured, washed, and resuspended in phosphate buffered saline and directly analyzed using Transmission Fourier transform infrared spectroscopy (FTIR). The spectra obtained were correlated with reference spectrum library Statistical analysis was done using Student unpaired t test, Pearson's Chi-Square test and Fischer's exact test.

Result: Evaluation by FTIR spectroscopy detected type-2 diabetic subjects (85%) was associated with almost twice the amount of microorganism compared to non diabetic (45%). Enterococcus faecalis is the most predominant microorganism both in non diabetic(25.0%) and type-2 diabetic subjects (45.0%) followed by Streptococcus spp20.0% and 30.0% respectively.

A strong association was noted between Enterococcus faecalisand Streptococcus mitisin non diabetic subjects ($p<0.05$)

Conclusion:Enterococcusfaecalis, and Streptococcus sppwas significantly associated with type-2 diabetic subjects as identified usingFTIR. FTIR proved to be a reliable rapid and economic assessment tool for evaluation of endodontic pathogens.

INTRODUCTION:

Persistence or emergence of apical periodontitis in a root canal treated tooth is aremarkably widespread problem. Bergenholtz et al. reported a success rate of 78%and 94% in teeth with and without periapical pathologies.[1,2] A large body of scientific evidence indicates microorganisms as the major causative agents of endodontic failure characterized by one or just a few species, predominantly Gram positive micro-organisms.[3,4]

Type-2 Diabetes mellitus is one of the most common metabolic disorders. Diabetics with preoperative periradicular lesion have a significantlower chance of successful outcome compared with nondiabetic with a overallsuccess rate of 68%.[5, 6, 7]

Bacterial detection, identification, and classification are generally carried out using traditional methods based on biochemical or serological tests and the molecular methods based on DNA or RNA fingerprints.[3]Ideally, the need of the hour is to explore for a reliable, reagentless technique for rapid detection of threat agents, within complex matrixes.

Fourier transform infrared spectroscopy (FT-IR) and computational analysis had been used to characterize changes in nutrient conditions during the formation of microbial biofilms.[8] Hence it is prudent to construct a signature library and investigatespectroscopy-based detection, identification and confirmation of Enterococcusfaecalis and Streptococcus species in retreatment cases ofnondiabetic and diabetic subjects.

MATERIALS AND METHODS:

Patient population and clinical specimens

20 subjects with type 2 diabetes mellitus ranging from 40 to 60 years old diagnosed according to the criteria of the Expert committee on the Diagnosis and Classification of Diabetes Mellitus were included in the study group. An additional 20 subjects and sex matched patients ranging from 40 to 60 years who reported no history of diabetes and normal glucose tolerance served as control subjects. Written informed consent was obtained from each patient before inclusion in the study.Clinical and radiographic features were recorded for each patient.

Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

Clinical Sampling
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 guttapercha 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).

Microbiological methods:
The root canal samples were shaken in a vortex mixer for 60 seconds. After vortexing, 50µl of sample was plated onto selective culture media for identifying Enterococcus faecalis and Streptococcus species. Streaking of samples onto media was done using sterile wire loop (loop technique). Candle jar was used for incubation of Streptococcus mitisat 370C for 5 days. Selective media for Enterococcus faecaliswas incubated under aerobic conditionin an incubator at 37^0C for 48 hours.After incubation, the plate was biochemically analysed for growth and identification of bacteria using the colony morphology and gram staining.

Spectroscopic evaluation
The growth suspected to be Streptococcus mitis/Enterococcusfaecalisbased on phenotypic characteristics were subcultured on blood agar.

Preparation of bacterial deposit for transmission FTIR measurements:
Small amount of cells from isolated colonies were manually removed from blood agar surface with the help of sterile wire loops and transferred to cryotubes containing 1.0 mL of phosphate buffered saline (PBS). Cells were washed three times in PBS using centrifugation at 6,000 rpm for 6 minutes and were diluted in 1.0 mL PBS to a concentration of 10 (optical density = 1) and then frozen at 80^0C. The microorganisms obtained from processing were submitted to the spectroscopy /analytical test. Samples were directly analyzed using Liquid sampling cell accessory with circular Teflon spacer of 0.05 mm (volume-appr.60µl) thickness 1.0ml of the bacterial suspension was fixed in the Teflon spacer and air dried to remove any water content.

Transmission FTIR spectral acquisition:
The spectral images were collected on a Spectrum GX Series transmission FTIR spectrometer operating under Instrument software version 4.07 equipped with MIRTGS detector. A constant flow of dry air was used to purge the spectrometer, limiting spectral contributions from carbon dioxide and atmospheric water. Each spectral image was collected from a field of view of Calcium Flouride window, 41x23mm, and 4mm thick pair with a spatial resolution 4.00 cm-1. To enhance the signal to noise ratio, 32 scans were co-added (~1 min measurement time). Atmospheric vapor was automatically subtracted from the sample spectrum via instrument software version 4.07. A minimum of three spectral images from different location within each deposit was collected sequentially.

Mathematical processing of FTIR spectra
Spectral processing was carried out using instrument software version 4.07. Each of the 32 individual spectra comprising the 16 x16 spectral image acquired from thedetector was ratioed against the corresponding background spectrum in the spectral image recorded from a bare portion of the Teflon spacer. Among the resulting 32 absorbance spectra, only those spectra meeting predefined spectra quality criteria in terms of absorbance range and signal to noise ratio were retained. These spectral were then baseline corrected using a three- point correction (at 4000, 1780, and 950 cm-1, normalized to unit height of the amide I band, and mathematically transformed to first derivative spectra.

DATA ANALYSIS
Instrument software (version 4.07) was used for digital data processing. Each spectra incorporatedinto signature library was evaluated as a quality control procedure.

Confirmation procedure: Graphs were obtained for microorganism isolated from the positive culture sample. This graph was compared with the signature library spectrum of the same microorganism and analyzed using the instrument software and through human observation of sample morphology.

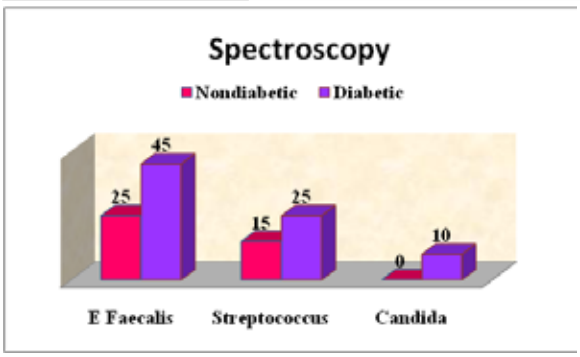
STATISTICAL ANALYSIS:
The data collected were typed onto a spreadsheet and statistically analysed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The results were statistically evaluated using student t test, chi square test and Fischer's exact test.

RESULTS:
In the present study, microorganisms were isolated from 37.5% (15/40) of selected teeth microbiologicallysampled from Group I and Group II by Spectroscopy. Spectroscopy yielded a total of 8 isolates in Group I. Enterococcus faecaliswas the most predominant microorganism isolated from 25% of root canal treated teeth followed by Streptococcus mitis(15%). Fischer's exact test showed no statistically significant association between the Microorganism and Group I as shown in table 1. In Group II a total of 16 isolates were recovered by Spectroscopy. Enterococcus faecalis(45%) was the most frequently isolated microorganism followed by Streptococcus mitis(25%). Fischer's exact test showed no statistical significant association between the Microorganism and Group II as shown in table 1.There was a no significant yet positive association of Enterococcusfaecalis,Streptococcus faecalis, in Group II when evaluated by Spectroscopy. Statistically high significant association was found between Streptococccusmitis and Enterococcus faecalisisolated from Group I (Non diabetic subjects) (p= 0.009), evaluated by Fisher's exact test.

Group	N	Enterococcus faecalis	Streptococcus faecalis
Group1 (Non diabetic patients) %	20 100%	5 25.0%	3 15.0%
Group2 (Diabetic patients)%	20 100%	9 45.0%	5 25.0%
Total Count %	40 100%	14 35.	8 20.0%

Table 1: Prevalence of Enterococcus faecalis, Streptococcus mitis, in Nondiabetic and diabetic subjects by spectroscopy

Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X



Graphical representation of prevelance of Enterococcus faecalis, Streptococcus mitis, in Diabetic and Non diabetic subjects by Spectroscopy method.

DISCUSSION:

The total percentage of Enterococcus faecalis, Streptococcus mitisrecovered from the retreatment cases were 35.0%, 25.0% respectively. Our results are in accordance with theauthors Waltimo et al., 1997, Sundqvist et al., 1998 Molander et al., 1998 whichconcluded the role of these microorganism in persistent cases of root canalinfection.[9] The predominance suggests resilience and ability of these microorganism to survive under sparsenutritional conditions.

The reason for this vulnerability to bacterial infections in type-2 diabetic subjects of developing more serious infections is because of disturbance in insulin uptake which could be explained by a generalized circulatory disorder due to accumulation of atheromatous deposits in the intimal tissues of the blood vessels lumen and development of a thickened basement membrane, with a resultant inadequate blood supply to regions of injury. Moreover, it enhances bacterial multiplication with ultimate cell death and apoptosis and clearance of leukocytes with arrest of polymorphonyclear leukocyte recruitment. 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.[10]

Our findings indicate that overall Enterococcus faecalis(35.0%) was the most frequently isolated pathogen present in retreatment cases which corroborates with the studies of Moller (29.0%), Molander et al (47%), Sundqvist et al (38%) evaluated by culture and P. chiara et al (39.1%) when evaluated by PCR.[11]Secondary root canal infections are about 9 times more likely to harbor E. faecalisthan cases of primary infections.[12]

Interestingly in the present study Enterococcus faecalis(45.0%) was the mostpredominant virulent organism in type 2 diabetic subjects identified by spectroscopywhich is in accordance with Fouad et al where there was a positive association ofEnterococcus spp (33%) when evaluated in six subjects of diabetics by PCR. [13, 14]Enterococcus faecalisis a facultative anaerobe and vascular problems indiabetics causes an increase in anaerobic bacteria which could be one of the reasonfor its increased prevalence of E.faecalisin diabetic subjects.[15]

Isabelle Porternier and his co authors (2003) have studied Enterococci to possess a number of virulence factors that permit adherence to host cells and extracellular matrix, facilitate tissue invasion, effect immunomodulation and cause toxin-mediated damage. [16]

In the present study evaluation of Streptococcus species by spectroscopy detected total 30.0% and 20.0% in type-2 diabetic and nondiabetic subjects of retreatment cases which is in accordance to study by Moller (16%), Molander et al(20.0%), Sundqvist et al (25.0%) and Siqueira&Rocas (23%) in non diabetics.[3] In contrary Fouad et al in his study on necrotic pulp showed very high significant association of Streptococcus spp(40%) when evaluated in 5 diabetic subjects using PCR.[17] However no such high statistical significance was observed in the present study. This may be due to the fact that different type of infections have different microorganism and streptococcus is moreassociated with primary infection than secondary infection.

There was a strong association in nondiabetics between Streptococcus mitisand Enterococcus faecalisinsecondary root canal infection (p=0.01) evaluated by spectroscopy. The present study showed association ofE. faecalisand S.mitisassociated with poor restoration, tenderness on percussion and sinus tract. However it cannot be confirmed in this study if it was the presence of those associations that initiated the clinical features or vice versa.[18] The influence of Streptococcus spp. on Enterococcus faecalisbiofilm formation have been studied by Hoogenkamp et al. and found that significantly more Enterococcus faecalisviable cells were found in biofilms in presence of Streptococcus sp.[19]Possibly, such microcommunities may provide resistance toward antimicrobial procedures which makes it mandatory to remove these virulent bacteria during root canal treatment.[20]

CONCLUSION

For the first time spectroscopic signature graphs were obtained from endodontic pathogen in secondary root canals. FTIR spectroscopy has the potential to provide sensitive, noninvasive, real-time identification of species. More of virulent organisms were associated with type 2 diabetics when evaluated with FTIR spectroscopy with Enterococcus faecalisbeing more predominant. Hence high powered studies and combining this technique with molecular and culture technique is probably the future approach in evaluation of microbial diversity.

Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

REFERENCE

Peciuliene VR. Maneliene E, Balcikonyte et al. Microorganisms in root canal infections:areview.Stomatol. Baltic Dent.max. facial Jn.2008; 10:4-9. | 2. Gorni GM, Gagliani MM. The Outcome of Endodontic Retreatment: A 2-yr. Follow-up JOE 2004; 30(1):1-4. | 3. Figdor D, Sundqvist.G. A big role for the very small- understanding the endodontic microbial flora.Aust Dent J 2007; 52(1):38-51. | 4. SiqueiraJr F J, Rocas I J. Clinical Implications and Microbiology of Bacterial Persistence after Treatment Procedures, J Endod 2008; 34:1291- | 1301. | 5. Huizing MM, Rothman RL. Addressing the diabetes pandemic: A comprehensive approach. Indian J Med Res 2006; 124:481-4. | 6. Mohan V, Sandeep S, Deepa R. Epidemiology of type 2 diabetes: Indian scenario. Ind J Med Res 2007; 125(3): 217 – 30. | 7. Fouad AF, Burleson J. The effect of diabetes mellitus on endodontic treatment outcome: data from an electronic patient record. J Am Dent Assoc. 2003; 134(1):43-51. | 8. Davis R, Mauer LJ. Fourier tansform infrared (FT-IR) spectroscopy: A rapid tool for detection and analysis of foodborne pathogenic bacteria. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. 2010:1582-94. | 9. Waltimo TM, Sen BH, Meurma JH, Orstavi D, Haapasalo MP. Yeasts in apical periodontitis.Crit Rev Oral Biol Med. 2003; 14(2):128-37. | 10. Bender IB, Bender AB. Diabetes mellitus and the dental pulp. J Endod. 2003 Jun; 29(6):383-9. | 11. SiqueiraJFJr, Rôças IN. Diversity of endodontic microbiota revisited. J Dent Res. 2009; 88(11):969-81. | 12. Baumgartner JC, Siqueira JF, Xia T, Rocas IN, Geographical differences in bacteria detected in endodontic infections using polymerase chain reaction, J Endod 2004;30:141-4. | 13. Fouad AF, Zerella J, Barry J, SpangberglS. Molecular detection of Enterococcus species in root canals of therapy-resistant endodontic infections. Oral Surg Oral Med Oral Pathol Oral RadiolEndod 2005; 99:112-8. | 14. Fouad AF. Diabetes Mellitus as a Modulating Factor of Endodontic Infections. J Dent. Edu. 2003(4):459-67. | 15. Bender IB, Bender AB. Diabetes mellitus and the dental pulp. J Endod. 2003 Jun; 29(6):383-9. | 16. Portenier I, Waltimo TMT, Haapasalo M. Enterococcus faecalis- the root canal survivor and'star'in post treatment disease, Endod Topics2003; 6: 135-9. | 17. Fouad AF, Barry J, Caimano M, et al. PCR-based identification of bacteria | in endodontic infections. J Clin Microbiol2002; 40:1491-501. | 18. Gomes BP, Lilley JD, Drucker DB. Associations of endodontic symptoms and signs with particular combinations of specific bacteria.IntEndod J 1999; 29: 69-75. | 19. Wang Q. Q, Zhang C. F, Chu C. H, Zhu X. F. Prevalence of Enterococcus faecalisin saliva and filled root canals of teeth associated with apicalperiodontitis. Int J Oral Science. 2012; 4(1): 9–23. | 20. Chavez L.D, Svensater.G, Dahlen.G, Bergenholtz.G. Streptococci from root canals in teeth with apical periodontitis receiving endodontic treatment. Oral Surg Oral Med Oral Pathol Oral RadiolEndod 2005;100: 232-241. |