Putative periodontal pathogens in persisting periodontal pockets of endodontic origin

Dhayanand John Victor, Sangeetha Subramanian, Prakash PSG, Deepika Rachel Samuel Raj

Abstract:
Background: The microbial profile of endodontically treated teeth, presenting with a persisting deep periodontal pocket, secondary to a primary endodontic lesion, draining through the gingival crevice, has received very less attention. This observational study was done to evaluate if these sites with persisting pockets of endodontic origin persist because they have acquired bacteria which are considered as putative periodontal pathogens. Materials and Methods: Subgingival plaque samples were collected from fifty patients diagnosed with a primary endodontic and a secondary periodontal lesion that persisted even after completion of the root canal treatment. Clinical parameters such as probing pocket depth, clinical attachment level, plaque index, fucation, and tooth mobility were recorded. Real-time polymerase chain reaction was used to determine the possible association between six bacteria, which are frequently associated with periodontal and endodontic lesions. Results: The mean cycle threshold value for *Treponema denticola* (Td) was found to be 33.74, and for *Enterococcus faecalis* (Ef), it was 34.39. With regard to clinical attachment loss, Td (*P* < 0.04) and *Parvimonas micra* (*P* < 0.05) had a significant correlation. Conclusion: Ef (92%) and Td (86%) were found to be most prevalent. *Porphyromonas gingivalis* and *Tannerella forsythia* were in minimal to nonexistent levels.

Key words: Persisting periodontal pocket, primary endodontic secondary periodontal lesion, real-time polymerase chain reaction

INTRODUCTION

The endodontic and periodontal systems are closely related wherein one system can express by-products of tissue destruction, into the other contiguous system, thereby confounding disease expression and the ability to treat these lesions.[1] The environmental dynamics of these differing ecologic niches determine the dominant microorganisms and their capability to modulate the persistence of disease. *Porphyromonas gingivalis* (Pg) and *Tannerella forsythia* (Tf) have been implicated as major pathogens in the etiology of periodontitis and are commonly isolated together, implying the existence of an ecological relationship between them.[2] *Treponema denticola* (Td) has also been considered as a major pathogen in periodontitis and is correlated with chronic periodontitis. These three bacteria, which were categorized together as the red complex have also been frequently isolated from infected dental pulps.[3-5] Rupf *et al.* noticed these putative periodontal pathogens in root canal infections and hypothesized that the low redox potential in the necrotic pulp favors the establishment of these obligate anaerobes within the root canal space.[4] *Enterococcus faecalis* (Ef) has been repeatedly identified as the species most commonly recovered from root canals undergoing retreatment, in cases of failed endodontic therapy, or in canals with persistent infection.[5,6] The root canal filled teeth, that present with a persistent deep periodontal pocket is one of the difficult clinical situations for the periodontists to treat. However, the microbial profile of such pockets has not been given appropriate attention.

The periodontal pathogens such as Pg, Tf, Td, and *Prevotella intermedia* (Pi) were often found in patients with persisting periapical lesions. Nevertheless, Ef and *Parvimonas micra* (Pm) are...
the two organisms frequently isolated from failing endodontic lesions. The microbial profile present in isolated periodontal pockets seems to differ from that of the pockets associated with periapical lesions.[4,5] Hence, this observational study seeks to assess whether these persisting pockets harbor the red complex bacteria and Prevotella intermedia along with evaluation for the presence of bacteria frequently associated with a failing endodontic lesion – Enterococcus faecalis and Parvimonas micra.

**MATERIALS AND METHODS**

Fifty patients from the Department of Periodontics and Department of Endodontics and Conservative Dentistry in our institution were recruited into a single group of 18–60 years. All the patients had a tooth which previously had undergone root canal treatment that was radiographically successfully obturated, with an associated deep periodontal pocket that was categorized as an endodontic periodontal lesion. Each patient was given a detailed verbal description of the purpose and nature of the study including clinical measurements and sample collection. After reading and signing the informed consent, patients were enrolled in the study. This study was ethically approved by the Institutional Scientific Committee and Ethical Review Board of the institution.

**Selection criteria**

**Inclusion criteria**

1. Systemically healthy patients diagnosed with an endodontic–periodontal lesion with clinical attachment loss >5 mm, with isolated attachment loss not extending to more than two surfaces on a tooth, associated with periodontal pocket extending beyond the apical third of root
2. Root canal treatment completed not <6 months and not more than 2 years at the time of examination
3. Radiographically dense root canal filling that is extending to the root apex.

Patients who had received periodontal therapy or antibiotics in the last 6 months, smokers or chronic alcoholics, patients who had endodontic failure due to root perforation and root fracture, and patients with incomplete obturation of the root canal space were excluded from the study.

The clinical parameters assessed were probing pocket depth, clinical attachment loss, plaque index, extent of destruction in the furcal region, and tooth mobility. Radiographically acceptable obturation was assessed by a radiodense, root canal filling that extends to the radiographic apex, with no evident voids in the root canal filling.

**Plaque sample collection**

Subgingival plaque samples were collected from the deepest pocket using sterile paper points (# 40, US Patent no.: 5,833,458) after wiping the site with a sterile cotton pellet to remove contamination by saliva. If the absorbent paper point was contaminated with blood, it was discarded. Samples were placed in Eppendorf tubes containing phosphate-buffered saline with a pH of 7.2 and stored in −80°C deep freezer. The samples were then analyzed for quantification using the real-time polymerase chain reaction (RT-PCR). The processing reagent, PCR reagents, and Master Mix Kit were obtained from Applied Biosystems, Warrington, UK.

**Evaluation of real-time polymerase chain reaction amplification**

In a RT-PCR assay, a positive reaction is detected by accumulation of a fluorescent signal. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the Ct level, the greater the amount of target nucleic acid in the sample). Real-time assays undergo 40 cycles of amplification.

1. Cts ≤29 are strong positive reactions indicative of abundant target nucleic acid in the sample
2. Cts of 30–37 are positive reactions indicative of moderate amounts of target nucleic acid
3. Cts of 38–40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.

**Statistical analysis**

Mean, median, and standard deviation were estimated for each of the six bacterial groups. Correlation of association between bacterial types was assessed using Tukey’s honestly significant difference post hoc test. Pearson’s correlation coefficient was used to correlate the presence of bacteria with clinical parameters. Chi-square test was applied to evaluate the relation of individual bacteria to furcation involvement. In the present study, P ≤ 0.05 was considered as the level of statistical significance.

**RESULTS**

The study analyzed six pathogens that were associated with the endodontic–periodontal lesion. The cycles of amplification that were required to detect the organisms tested were used to label the bacterial expression into three categories.

1. Score 1 – Ct value between 38 and 40
2. Score 2 – Ct value between 30 and 37
3. Score 3 – Ct value ≤29.

None of the organisms were present in high numbers. Only one sample revealed a strong expression of Pg. The only putative periodontal pathogen that was present in a high number in the samples evaluated was Td which was moderately expressed in 86% (n = 43) of the patients. However, the bacteria that was moderately expressed in the most number of patients (n = 46; 92%) was Ef [Table 1].

The mean Ct value for Td was 33.74, and for Ef, it was 34.39. All the other bacteria that were evaluated had a mean level of expression that was >37, indicating that there was an insignificant level of the bacterial nucleic acid that was being assessed [Table 2].

There was a highly significant difference in evaluating the presence of Pg with its association with Td, Ef, and Tf. A similar highly significant negative correlation was found when assessing the relationship of Pi with Td and Ef. A similar negative correlation was found between Td and Pm, Tf and Ef, as well as Pm and Ef [Table 3].

The presence of bacteria was correlated with pocket depth and clinical attachment level using the Pearson’s correlation
Victor, et al.: Periodontal pathogens of endodontic origin

Table 1: Quantitative assessment of organisms in plaque samples using real time-polymerase chain reaction

| Scores | Pg, n (%) | Pf, n (%) | Td, n (%) | Tf, n (%) | Pm, n (%) | Ef, n (%) |
|--------|-----------|-----------|-----------|-----------|-----------|-----------|
| Score 1 | 21 (42)   | 22 (44)   | 7 (14)    | 37 (74)   | 33 (66)   | 4 (8)     |
| Score 2 | 28 (56)   | 28 (56)   | 43 (86)   | 13 (26)   | 17 (34)   | 46 (92)   |
| Score 3 | 1 (2)     | 0         | 0         | 0         | 0         | 0         |

*n=50. Pg – Porphyromonas gingivalis; Pi – Prevotella intermedia; Td – Treponema denticola; Tf – Tannerella forsythia; Pm – Parvimonas micra; Ef – Enterococcus faecalis

Table 2: Mean cycle threshold levels for bacteria examined

| Bacteria | Mean±SD       |
|----------|---------------|
| P. gingivalis | 37.24±2.33 |
| P. intermedia | 37.68±1.98 |
| T. denticola | 33.74±2.99 |
| T. forsythia | 38.82±1.45 |
| P. micra | 37.95±2.26 |
| E. faecalis | 34.39±2.10 |

SD – Standard deviation; Pg – Porphyromonas gingivalis; Pi – Prevotella intermedia; Td – Treponema denticola; Tf – Tannerella forsythia; Pm – Parvimonas micra; Ef – Enterococcus faecalis

Table 3: Correlation of association between bacterial types present using Tukey’s honestly significant difference post hoc test

| Pairs       | Mean difference | P     |
|-------------|-----------------|-------|
| P. gingivalis |                 |       |
| P. intermedia | -0.44           | 0.920 |
| T. denticola | 3.49            | <0.001|
| T. forsythia | -1.58           | 0.006 |
| P. micra | -0.71           | 0.608 |
| E. faecalis | 2.84            | <0.001|
| P. intermedia |                 |       |
| T. denticola | 3.94            | <0.001|
| T. forsythia | -1.14           | 0.115 |
| P. micra | -0.27           | 0.991 |
| E. faecalis | 3.29            | <0.001|
| T. denticola |                 |       |
| T. forsythia | -5.07           | <0.001|
| P. micra | -4.20           | <0.001|
| E. faecalis | -0.65           | 0.692 |
| T. forsythia |                 |       |
| P. micra | 0.87            | 0.374 |
| E. faecalis | 4.42            | <0.001|
| E. faecalis | 3.55            | <0.001|

*P<0.05% is considered significant. P – Probability; Pg – Porphyromonas gingivalis; Pi – Prevotella intermedia; Td – Treponema denticola; Tf – Tannerella forsythia; Pm – Parvimonas micra; Ef – Enterococcus faecalis

DISCUSSION

It was estimated that over 50% of the endodontic failures were due to the merging of the endodontic and periodontal lesions by Chen et al.[9] Rupf et al. found that periodontal pathogens often accompany endodontic infections and concluded that periodontic and endodontic pathways are critical in determining whether the cases will be refractory to conventional endodontic or periodontal therapy.[11] The inflammatory by-products of pulpal origin may permeate through the apex or through smaller canals in the apical third of the root canal system and exposed dentinal tubules which in turn trigger an inflammatory vascular response in the periodontium. Among those are certain strains of bacteria and viruses which are encountered in periodontal inflammatory disease as well.[10-11] The current study has sought to evaluate the microflora of patients with a primary endodontic lesion and a secondary periodontal lesion and to evaluate if these isolated deep periodontal pockets harbor bacteria that are considered to play a significant role in periodontal disease.

This study has also sought to evaluate the association of probable periodontal pathogens, in these selected periodontal pockets, to pathogens that are frequently isolated from failing endodontic lesions. Two organisms, frequently identified from failing endodontic lesions, Ef and Pm, were selected to evaluate their levels in these pockets to assess the potential role they might have in these type of lesions.

The current study has found that Pg was not found in high levels in the subgingival plaque in all except one patient. Pg was not a significant part of the biofilm of patients with a primary endodontic lesion with a secondary periodontal lesion. This is significantly different from the bacterial profiles of deep periodontal pockets, as has been shown by the seminal cluster analysis by Socransky et al.[12] Tf apart from being a frequently isolated microorganism from deep pockets associated with chronic periodontitis has also been shown to be a frequent isolate from the failing endodontic lesion.[13] However, this study has shown that Tf was the least frequently isolated organism from these patients. Tf was not a significant part of the subgingival biofilm of the patients being evaluated. The current study found that 86% of the sites exhibited moderately strong levels of Td nucleic acid. The mean Ct level for Td was the lowest of all the microorganisms evaluated, indicating that Td was a highly significant organism in the subgingival biofilm of sites with a primary endodontic lesion and secondary periodontal lesion.

The most prevalent organism among the patients evaluated was Ef, which was present in moderately high numbers in about 92% of the patients. The mean Ct score for Ef was 34.39, with a standard deviation of 2.91. Further, the values indicate that Ef has a significant association with persisting pockets, associated with a tooth with a primary endodontic lesion. Its identification in all, but four of the patients, indicates that Ef is a very important organism in the pathogenesis of the persisting endodontic-periodontal lesion.

The role of Pi in the modulation of the inflammatory response, as well as its role as a co-aggregator, makes it an organism of importance in periodontal disease.[14] However, the current

Coefficient. With regard to probing depth, only Td had a significant correlation at P < 0.03, indicating a mildly significant association. When clinical attachment loss was correlated with the bacteria, a statistically significant correlation was found only for Pm and Td. The level of significance for Pm and Td was at P < 0.04 and P < 0.05, respectively, both of which were mildly significant. When assessed for plaque scores, furcation involvement, and tooth mobility, none of the bacterial types showed any significant correlations [Table 4].
study found that the levels of this organism were minimal to nonexistent in 44% of the sites. It was seen in moderately strong levels in the remaining 56%, with an arithmetic mean of 37.68, indicating that overall Pi was present in low levels. Pm apart from being an organism that is frequently isolated from the subgingival plaque of sites with chronic periodontitis has also been frequently isolated from periapical sites of teeth with failed endodontic treatment.[17] This study found that the persistent pockets that were secondary to a primary endodontic lesion harbored minimal to nonexistent levels of Pm in 66% of the sites tested. The study observed the greatest homogeneity between Ef and Td. About 82% of the patients expressed a homogeneous moderately strong Ct level for both Ef and Td. This finding of concurrently expressed high prevalence levels for Ef, and Td is unique in its presentation. Ef has distinctive virulence factors that can promote specific bacterial interaction with Td.

Ef is frequently found in association with unresolved lesions following endodontic treatment. It grows as a part of the biofilm on root canal walls and is also implicated in monoinfection in treated canals without synergistic support from other bacteria. These factors make it highly resistant to antimicrobial agents. It also possesses many survival mechanisms to live in unfavorable conditions such as low oxygen, high pH, or poorly nutrient environment. [16] Treponema species also has been found in failed endodontic treatment, particularly revealing a predominance of Td. Td possess several virulence determinants such as motility, low immunogenicity, invasion of host tissues, and secretion of proteolytic enzymes which allows them to colonize the sites rapidly, penetrate into tissue, and escape host defense systems. [17]

This study tested the hypothesis that endodontic–periodontal lesions that persist may harbor putative periodontal pathogens, therefore preventing the healing of these lesions following completion of endodontic treatment. The results of this study reject this hypothesis. Periodontal pathogens which are considered very important to periodontal disease progression, such as Pg and Tf, were identified in very low titers in these pockets. Td which is today considered a less significant periodontal pathogen[18] was, however, seen in significantly high prevalence.

This study has also been able to highlight the close relationship between Td and Ef within these sites. The distinctive bacterial characteristics to this lesion indicate that these endodontic–periodontal lesions are indeed unique not only because of their retrograde genesis but also because of their distinctive microflora. It requires to be further highlighted that, despite the high levels of prevalence of Ef and Td, these organisms were not present in significantly high titer within these pockets. Although Ef is present in the periradicular space, in small numbers, it seems to be potent enough to cause endodontic lesions to fail, and a similar role for Ef may be attributed the endodontic–periodontal lesions evaluated in this study. It is further probable that there are as yet unidentified organisms within this lesion. Open-ended bacterial techniques are possibly important, in these and various other periodontal lesions, to understand the entire spectrum of infection within these niches. Much more interrogation is required before we receive clarity as to the exact nature of the etiopathogenesis of these lesions.

CONCLUSION

This study has found that Ef and Td were more prevalent among the studied organisms in patients with persisting periodontal pockets of endodontic origin. However, organisms which are considered to be important periodontal pathogens, such as Pg and Tf, were found in minimal to nonexistent levels, quite unlike most sites with chronic periodontitis. These results also found that in all but one of the patient samples tested, none of the organisms evaluated were expressed strongly. It is, therefore, possible that there may be other organisms which are present in high titer. Further studies are required to comprehensively evaluate the microbial profile of these patients.

This study could have also used a more sensitive PCR technique to quantify the microorganisms evaluated. Ef has been shown to be a pathogen that is difficult to eliminate, even if present in
low titer. This may result in a requirement to modify treatment protocol for these patients. It has already been shown that the adhesions that are produced by Ef can potentiate the colonization of Td. This potential synergism in the pathogenesis of the lesion needs to be further explored. This study highlights, that this disease is unique in its presentation and therefore requires much greater attention henceforth.

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Conflicts of interest
There are no conflicts of interest.

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