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ORIGINAL ARTICLE

Quantitative Analysis of Candidate Endodontic Pathogens and Their Association with Cause and Symptoms of Apical Periodontitis in a Sudanese Population

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ABSTRACT

Objective: To investigate the prevalence of key endodontic pathogens and their association with the clinical features and the cause of apical periodontitis.

Methods: The study population included patients referred to Khartoum Dental teaching Hospital, Sudan for endodontic treatment. Samples were collected from single-rooted teeth carious or traumatised teeth with clinical and radiographic evidence of apical periodontitis. The endodontic pathogens *Porphyromonas endodontalis, Fusobacterium nucleatum* and *Treponema denticola* were quantified by real time polymerase chain reaction (qPCR). The prevalence of each species was identified at both a low detection threshold (>50 bacteria) and a high detection threshold (>1000 bacteria).

Results: 75 patients (mean age 30.1 yrs SD 10.1) were included in the analysis. The most prevalent bacterium at both the low and high threshold was *F. nucleatum* followed by *T. denticola* at the low threshold and *P. endodontalis* at the high threshold. There was no association with symptoms at the low detection threshold, but at high threshold *P. endodontalis* was associated with swelling, adjusted odds ratio (OR), 9.32 95%CI 1.11- 78.66, P=0.04. All species were more prevalent in apical periodontitis due to caries only at the low detection threshold, OR=5.01 (P=0.006) for *T. denticola*; 4.84 (P=0.01) for *F. nucleatum*; and 3.62 (P=0.03) for *P. endodontalis*.

Conclusion: There was a high prevalence of the *F. nucleatum, T. denticola* and *P. endodontalis* in apical periodontitis associated with caries. None of these bacterial were associated with pain but the presence of *P. endodontalis* at high levels was associated with swelling.

Keywords: Apical periodontitis, endodontic bacteria, real-time PCR, symptoms

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HIGHLIGHTS

- Key endodontic pathogens are more prevalent in apical periodontitis due to caries than trauma.
- The level of bacteria is important for association with the cause and symptoms of apical periodontitis.
- *P. endodontalis* at high detection threshold is associated with swelling.

INTRODUCTION

The main cause of apical periodontitis is persistent microbial infection within the root canal system. The disease usually follows death of the dental pulp secondary to trauma or a deep carious lesion, where bacteria and their products diffuse into the periapical region to induce an inflammatory response, tissue damage and subsequent development of periapical infection. Clinically the presentation of apical periodontitis can vary from acute symptomatic to chronic asymptomatic infection that is only identified during routine clinical or radiographic examination.

The root canal system offers an ideal environment for the establishment of a mixed polymicrobial community. Varied groups of Gram-positive and Gram-negative bacteria have been isolated in primary endodontic infections including species that belong to the phyla Bacteroidetes, Spirochaetes, and Fusobacteria (1). Candidate endodontic pathogens belonging to these phyla such as *Porphyromonas endodontalis*, *Treponema denticola* and *Fusobacterium nucleatum* have been consistently identified in primary endodontic infections (2-5), but whether their prevalence is similar

in cases where apical periodontitis is due to caries or trauma is not clearly understood. Furthermore, the relationship between these bacteria and the signs and symptoms of apical periodontitis, although previously studied, remains controversial. Some of these bacteria were reported to be associated with symptomatic apical periodontitis (6, 7), however, the same species have been found in similar frequencies in asymptomatic cases (8, 9). It has been proposed that in addition to the presence of a given pathogenic species, other factors may contribute to symptomatic apical periodontitis (10). Indeed, studies using semi-quantitative approaches including reverse capture-checkerboard DNA-DNA hybridisation revealed that presence of different types and load of bacteria, might explain the development of symptoms (11, 12). The quantity of specific virulent species or strains are considered important for community pathogenicity (13) and therefore, quantification to determine the level of certain key species may be useful in identifying the link between these species and the signs and symptoms of apical periodontitis (12).

Absolute quantification requires sensitive quantitative methods such as real-time qPCR (12, 14). Using this method and applying specific detection thresholds, previous studies have shown that the prevalence of periodontal pathogens varied considerably between different types of periodontitis, depending on the threshold applied (15). The majority of published culture and molecular studies have focused on the prevalence of endodontic bacteria, with limited studies on quantification of endodontic bacterial load (12). Although some previous studies have used qPCR to study endodontic pathogens, the focus was not on the absolute quantification of the bacteria present (16-18). This study, therefore, used real-time qPCR to quantify the key endodontic pathogens *P. endodontalis, T. denticola*, and *F. nucleatum* in apical periodontitis. The prevalence of these species was also found to differ in different geographical locations (19). To our knowledge, there is no study to date that has reported on the prevalence of primary endodontic pathogens among Sudanese. This study, therefore, aims to investigate the association of these species with the cause and symptoms of apical periodontitis in Sudanese patients with primary endodontic infections. A subsidiary aim was to investigate whether using different detection threshold for the species studied affect their association with the cause and symptoms of apical periodontitis.

MATERIALS AND METHODS

Study population

The study participants were recruited from adult patients referred to the Department of Conservative Dentistry at Khartoum Dental Hospital for root canal treatment. The study was approved by the Faculty of Dentistry, University of Khartoum Health Research Ethics Committee (HREC assigned number 2/2008), and all participants provided informed written consent.

All clinical examinations were performed by an experienced endodontist (S.A.A). Teeth that were eligible for inclusion had a visual inspection for caries (primary or secondary) and signs of periapical infection, including swelling and/or presence of a sinus tract. Teeth with history of trauma were included if they were intact, with no clinical evidence of caries or exposed pulp space. The periodontal assessment was performed by measuring probing pocket depth using a Williams periodontal probe. Sensibility testing, using ethyl chloride (Gebauer, Cleveland, OH, USA), was carried out to assess pulp vitality. Percussion and palpation tests were also performed for all teeth included in the study. An intraoral periapical radiograph using the parallel cone technique was obtained and examined under standard illumination to assess the root canal and periapical area. The history of self-reported pain and pain on percussion including severity and duration was recorded. All patients completed a medical history questionnaire.

Inclusion and exclusion criteria

Teeth were included in the study if they were single-rooted with non-vital pulp evidenced by a negative response to sensibility testing using ethyl chloride, had radiographic evidence of apical pathology, and had no previous root canal treatment. The study also included teeth which, had no periodontal pockets that were deeper than 4 mm. Subjects were excluded if their medical history included pregnancy, diabetes, immunosuppression, a requirement for antibiotic cover for routine dental treatment, or if they had received antibiotics in the 3 months prior to examination.

Sample collection

Samples were collected from each tooth under strict aseptic conditions as previously described (7, 12). Initially, all carious defects or coronal restorations, if present, were removed. The teeth were cleaned with pumice and isolated using a rubber dam. The rubber dam and tooth were disinfected with 2.5% sodium hypochlorite solution. Sterile round burs were used for the coronal access into the pulp chamber. The rubber dam and tooth were initially cleaned with 30% hydrogen peroxide and then disinfected with 2.5% sodium hypochlorite solution, which was deactivated with sterile 5% sodium thiosulfate to minimise interference with sampling. The samples were collected from the root canal using size 15 H-type file and paper points (Dentsply, Ballaigues, Switzerland) as previously described (7). A file was inserted 1 mm short of the radiographic apex as determined by means of an electronic apex locator (C-Root 1, COXO, Guangdong Province, China). Subsequently, a sterile paper point was inserted into the root canal to the established working length for 1 minute. The paper point was then immediately placed into an Eppendorf tube containing 150 µl Tris buffer (pH 7.6). Two paper points samples were collected per canal and the tube was placed on ice, transferred within minutes to the laboratory, and stored at -80°C until required.

Bacterial DNA extraction

The bacterial DNA was isolated using a DNA purification kit (DNeasy, Qiagen, Hilden, Germany) as previously described (15). The frozen samples were permitted to thaw at room temperature and centrifuged at 500 rpm for 15 minutes. Proteinase K at a concentration of 1 mg/mL was added and samples incubated at 37°C for 1-2 hours. Following that, the samples were processed for DNA extraction as per the manufacturer's protocol. The purity and concentration of the extracted DNA

TABLE 1. Sequences of primers used in this study. All primers have previously been validated (20, 21)

Bacteria	Forward primers	Reverse primers
T. denticola	5'CTTCCGCAATGGACGAAAGT3'	5'CAACCTTTCGGCCTTCTTCA3'
P. endodontalis	5'GCTGCAGCTCAACTGTAGTCTTG3'	5'TCAGTGTCAGACGGAGCCTAGTAC3'
F. nucleatum	5'ACCAGCGTTTGACATCTTAGGAATG3'	5'AGCCATGCACCACCTGTCTTTAG3'

were determined with a Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA).

Quantitative real-time PCR (qPCR)

Real-time qPCR was carried out using specific, previously validated primers for *P. endodontalis, T. denticola*, and *F. nucleatum* (20, 21) (Table 1). DNA isolated from pure cultures of *P. endodontalis* (NCTC13058), *T. denticola* (ATCC 35405) and *F. nucleatum* (NCTC10562) was used to create the standard curves. PCR reaction was made of 5 µl SYBR Green and Rox (Sigma, UK), 3.9 µl nuclease-free water, 0.1 µl of primers at a final concentration of 5 pmol/µl and 1 µl DNA from samples or standards. PCR was carried out on Mx3005P qPCR System, (Agilent Technologies, Cheshire, UK) with running conditions as previously described (15). The standard curve generated for each bacteria was used for quantification based on PCR default detection threshold. Further analysis was carried out using a low detection threshold (≥than 50 bacteria/sample) and a high threshold (≥1000 bacteria/sample. The presence of 50 bacteria was the minimum required for the sample to be considered positive (22).

Statistical analysis

Pearson's Chi-Square was used to assess the difference in bacteria prevalence. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to quantify the association of the specific bacteria with the causes (caries vs trauma) and the clinical signs and symptoms (pain vs swelling) at each detection threshold. Statistical Package for the Social Sciences (SPSS), version 22, was used for statistical analysis, with $P<0.05$ considered statistically significant.

RESULTS

Seventy-five patients, with an average age of 30.1 years (range 16-53 years), were included in the study. The majority, 49 (65.3%), were males and 26 (34.7%) were female. A diagnosis of apical periodontitis due to trauma was made in 45 (60%) subjects and due to caries in 32 (40%). Pain was a complaint reported by 34 (45.3%) and swelling was present in 41 (54.7%) of the subjects. There were 15 patients (20%) who had both pain and swelling.

TABLE 2. Prevalence, mean (±SD) and range of the bacterial species investigated

Bacteria	Prevalence n (%)	Mean (±SD)	Range
P. endodontalis	22 (29.3)	$1.107x10^3$ ($3.020x10^4$)	$0-3.02x10^4$
F. nucleatum	43 (57.3)	$1.2667x10^4$ ($4.8548x10^4$)	$0-3.72x10^5$
T. denticola	32 (42.7)	$7.12x10^2$ ($2.580x10^3$)	$0-1.58x10^4$

The most prevalent species was *F. nucleatum*, which was identified at the low detection threshold (>50 bacteria/sample), in 43 (57.3%) of the samples followed by *T. denticola* in 32 (42.7%) and *P. endodontalis* in 22 (29.3%) (Table 2). At a low threshold, all 3 species were identified in 18 (24%) of the samples. The prevalences were much lower when the high detection threshold (>1000 bacteria/sample) was applied. *F. nucleatum* remained the most prevalent and was identified in 24 (32%) of the samples followed by *P. endodontalis* in 10 (13.3%) with *T. denticola* in 8 (10.7%).

Associations of bacteria with symptoms of apical periodontitis

At the low detection threshold, there was no association with symptoms for any of the bacterial species (Table 3). However, at the high detection threshold, the prevalence of *P. endodontalis* was increased in patients with swelling, P=0.016 (Table 3). The identification of *P. endodontalis* at high levels showed a significantly increased association with swelling with an odds ratio of 9.32, 95%CI 1.11-78.66, P=0.04 after adjustment for age and gender. None of the bacteria were associated with pain history at the high detection threshold.

Associations of bacteria with the cause of apical periodontitis

Of the bacterial species investigated, they were more prevalent at the low detection threshold in samples from apical periodontitis in carious teeth compared to teeth with a history of trauma, *P. endodontalis* (P=0.007), *F. nucleatum* (P=0.022) and T. *denticola* (P=0.0006) (Table 4). The odds ratios, which remained significant after adjustment for age and gender, ranged from a high of 5.01 for *T. denticola* through 4.84 for *F. nucleatum* to a low of 3.62 for *P. endodontalis* (Table 4).

DISCUSSION

Using sensitive quantitative real-time PCR assay, this study showed that candidate endodontic pathogens; *P. endodontalis, F. nucleatum*, and *T. denticola* were detected in Sudanese patients with apical periodontitis. *F. nucleatum* was the most prevalent and was identified in 57.3% of the samples followed by T. denticola (42.7%) and *P. endodontalis* in 29.3%. These results differ from those reported for Brazilians, where *T. denticola* and *P. endodontalis* were the most prevalent species, but similar to the South Korean population with a high prevalence of *F. nucleatum* (23).

All three species were more prevalent in teeth with endodontic involvement related to caries compared to trauma. None of the bacterial species were associated with pain history, but *P. endodontalis*, at the high detection threshold, was found to be associated with swelling. The association of these bacteria with signs and symptoms of apical periodontitis has previously been studied using different methods and with

TABLE 3. Prevalence of bacterial species by symptoms of apical periodontitis. Low detection threshold >50 bacteria. High detection threshold >1000 bacteria. Comparisons made using Chi-square test. Odds ratios corrected for age and gender *P<0.05

	All (n=75) n (%)	Swelling (n=41) n (%)	P-value	Odds ratio (95% CI)	P-value	Pain (n=34) n (%)	P-value	Odds ratio (95% CI)	P-value
P. endodontalis									
Low threshold	22 (29.3%)	14 (34.1%)	0.31	1.65 (0.58-4.71)	0.35	9 (26.5%)	0.62	0.88 (0.30-2.52)	0.80
High threshold	10 (13.3%)	9 (22.0%)	0.016*	9.32 (1.11-78.66)	0.04*	5 (14.7%)	0.75	1.14 (0.29-4.52)	0.85
F. nucleatum									
Low threshold	43 (57.3%)	21 (51.2%)	0.24	0.58 (0.23-1.49)	0.26	23 (67.6%)	0.10	2.41 (0.90-6.40)	0.08
High threshold	24 (32.0%)	11 (26.8%)	0.29	0.57 (0.21-1.53)	0.26	14 (41.2%)	0.12	2.27 (0.82-6.30)	0.12
T. denticola									
Low threshold	32 (42.7%)	20 (48.8%)	0.24	1.74 (0.65-4.60)	0.27	16 (47.1%)	0.48	1.80 (0.66-4.95)	0.25
High threshold	8 (10.7%)	5 (12.2%)	0.64	1.59 (0.34-7.36)	0.55	3 (8.8%)	0.64	0.72 (0.15-3.36)	0.67

TABLE 4. PPrevalence of bacterial species by cause of apical periodontitis. Low detection threshold >50 bacteria. High detection threshold >1000 bacteria. Comparisons made using Chi-square test. Odds ratios corrected for age and gender. *P<0.05. **P<0.01

	Prevalence in caries (n=30) n (%)	Prevalence in trauma (n=45) n (%)	P-value	Odds ratio (95% CI)	P-value
P. endodontalis					
Low threshold	14 (46.7%)	8 (17.7%)	0.007**	3.62 (1.12-11.77)	0.03*
High threshold	6 (20.0%)	4 (8.9 %)	0.16	3.17 (0.67-15.16)	0.15
F. nucleatum					
Low threshold	22 (73.3%)	21 (46.7%)	0.022*	4.84 (1.44-16.32)	0.01*
High threshold	13 (43.3%)	11 (24.4%)	0.09	2.46 (0.80-7.54)	0.12
T. denticola					
Low threshold	20 (66.7%)	12 (26.7%)	0.0006**	5.01 (1.60-15.70)	0.006**
High threshold	4 (13.3%)	4 (8.9%)	0.54	2.78 (0.50-15.38)	0.24

conflicting reports. Applying detection thresholds for the number of bacteria present showed that *P. endodontalis* associated with symptoms at a high detection threshold. These results concur with a recent study that found the frequency (presence/absence) of bacteria was not associated with symptoms but applying a semi-quantitative approach it was shown that *P. endodontalis*, at levels $>10^5$, was significantly more prevalent in abscess than in asymptomatic cases (12). This supports previous views that the levels of specific virulent species or strains are important for pathogenicity (13). *P. endodontalis* is a black pigmented Gram-negative rod that, because of its high prevalence and virulence factors, is considered a key pathogen in apical periodontitis. In agreement with this study, Cao and associates (24) found *P. endodontalis* to be more prevalent in teeth with sinus tract and abscess formation.

The finding that *F. nucleatum* and *T. denticola* were not associated with symptoms disagrees with previous reports that showed an association between these bacteria and symptomatic apical periodontitis (11, 7, 24). *F. nucleatum* was the most frequently detected bacteria in our samples, in line with its role as a 'bridging' microorganism in biofilm formation. However, the association of *F. nucleatum* with symptoms is still open to debate. Using a closed-ended reverse-capture checkerboard approach targeting 50 candidate endodontic pathogens Rôças et al (9) found no difference in the prevalence of *F. nucleatum* in symptomatic and asymptomatic cases. Results of conventional PCR assay (4), showed a positive but not statistically significant association of *F. nucleatum* with symptoms. Most recently, F. nucleatum was found to be one of the prevalent bacteria in asymptomatic apical periodontitis but semi-quantitative data demonstrated no association at levels of $>10^5$ (12).

T. denticola is also a key endodontic pathogen that has previously been shown in many studies to be associated with acute endodontic infections (25, 18). Although detected in over 40% of subjects in this study it was not associated with symptoms. *T. denticola* is one of the red-complex bacteria and likely to exert its effect in a consortium. Further studies investigating the co-existence of red complex and other bacteria, their interactions, and their association with symptoms at different thresholds of detection is warranted.

There are no studies to date that directly compared the microbial composition of apical periodontitis lesions in traumatised and carious teeth. Earlier investigations on the microbial com-

position of traumatised teeth using culture methods showed a high prevalence of strict anaerobic bacteria (26). Subsequently, *F. nucleatum* was found in 30% of cases of necrotic pulps of traumatized teeth (27). In a more recent study, using a combination of culture and molecular approaches *P. endodontalis* and *F. nucleatum* were detected in 33% and T. denticola in 13% of cases (28). The discrepancy in the prevalence reported in this study could be attributable to detection methods, but likely that geographical differences affect the prevalence of certain bacteria as previously reported (19).

Dental caries is known to be a risk factor for the development of apical periodontitis (29), and therefore the high prevalence of *P. endodontalis, F. nucleatum,* and *T. denticola* in apical periodontitis is not unexpected. These bacteria are detected in carious dentine in teeth with pulpitis and their presence is associated with inflammatory degeneration of the dental pulp (21). Untreated deep caries ultimately results in pulp space infection and the creation of a complex multispecies biofilm that leads to the development of apical periodontitis (21).

Identification of specific bacteria and their association with features of apical periodontitis is important to help design appropriate antimicrobial strategies. For instance, high prevalence and abundance of *F. nucleatum, T. denticola,* and *P. endodontalis* in apical periodontitis related to caries suggest that in such teeth efficient disinfection is required. Chemo-mechanical instrumentation and calcium hydroxide-based dressing were shown to significantly reduce the number of microorganisms including, *F. nucleatum* and black pigmented rods in teeth with apical periodontitis (30). In traumatised teeth undergoing revascularisation procedures, Nagata and colleagues (28) found that dressing the canal with triple antibiotic paste and calcium hydroxide equally reduced *F. nucleatum, T. denticola,* and *P. endodontalis.*

It was a limitation of the study that the number of subjects recruited was small. As a consequence, it is likely that the power of the study, particularly when analysing the results for the high detection threshold cut off, was low. This resulted in the rather wide confidence intervals related to the odds ratio calculations. In addition the samples in this study were collected using paper points, which, although used routinely in endodontic microbiology sampling, may result in differential samples acquisition in dry compared to wet canals. Nevertheless, the data do add to the body of knowledge in the area and provided evidence for the first time on the prevalence of key endodontic pathogens in a Sudanese population.

CONCLUSION

The results of this study demonstrated a high prevalence of the endodontic pathogens *F. nucleatum, T. denticola,* and *P. endodontalis* in apical periodontitis. All the bacterial species investigated, were more prevalent at the low detection threshold in samples from apical periodontitis in carious teeth compared to teeth with a history of trauma. None of these bacterial species were associated with pain but the presence of *P. endodontalis* at high levels was associated with swelling.

Disclosures

Conflict of interest: All authors declare no conflict of interest in relation to this article.

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