Comparison of Endotoxin Levels Found in Primary and Secondary Endodontic Infections

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Abstract

Introduction: This clinical study was conducted to compare the levels of endotoxins (lipopolysaccharides [LPSs]) found in primary and secondary endodontic infections with apical periodontitis by correlating LPS contents with clinical/radiographic findings. In addition, the presence of target gram-negative anaerobic bacteria was also investigated. **Methods:** Samples were taken from 15 root canals with primary infections and 15 with secondary infections by using paper points. The limulus amebocyte lysate assay was used to quantify endotoxins, and the polymerase chain reaction technique (16S rDNA) was used for bacterial investigation. **Results:** Endotoxins were detected in 100% of the root canal samples collected from primary (15/15) and secondary (15/15) infections with median values of 7.49 EU/mL and 3.96 EU/mL, respectively ($P < .05$). The median value of endotoxins found in the presence of clinical symptoms was significantly higher than in asymptomatic teeth with primary infections ($P < .05$). A positive correlation was found between endotoxin contents and a larger size of the radiolucent area (>3 mm) ($P < .05$). *Prevotella nigrescens* (10/15, 4/15), *Fusobacterium nucleatum* (5/15, 1/15), *Treponema denticola* (3/15, 1/15), and *Treponema socranskii* (5/15, 1/15) were detected in teeth with primary and secondary infections, respectively. *P. endodontalis* was present only in teeth with primary infections (5/15). **Conclusions:** Teeth with primary endodontic infections had higher contents of endotoxins and a more complex gram-negative bacterial community than teeth with secondary infections. Moreover, the levels of endotoxins were related to the severity of bone destruction in periapical tissues as well as the development of clinical features in teeth with primary infections. *(J Endod 2012;38:1082–1086)*

Key Words

Endodontic infection, endotoxin, limulus amebocyte lysate assay, root canal

Lipopolysaccharide (LPS), or endotoxin, is a major constituent of the outer cell wall of gram-negative bacteria (1) secreted in vesicles by growing organisms or released during disintegrations of bacteria after death (2). Endotoxin is one of the most important virulent factors involved in the development of periapical inflammation (3–5), activating immune-competent cells and leading to the release of a variety of proinflammatory mediators (5–8).

A primary endodontic infection is a polymicrobial infection caused predominantly by gram-negative anaerobic bacteria, especially *Prevotella*, *Porphyromonas*, *Treponema*, and *Fusobacterium* spp (6, 8–13). Clinical investigations of primary infections have found a correlation between endotoxins and the presence of apical periodontitis (3, 6, 14–18). Higher contents of endotoxins in root canals have been associated with the development of clinical signs/symptoms and a larger area of bone destruction (3, 6, 8, 14, 15).

Endodontic treatment failure is characterized by the presence of signs and/or symptoms of persistent or emergent apical periodontitis after treatment. Culture methods revealed the bacterial etiology of post-treatment apical periodontitis as being a gram-positive bacterial infection (19, 20). However, with molecular techniques, studies have also indicated the presence of *Porphyromonas*, *Prevotella*, and *Treponema* spp in post-treatment apical periodontitis (21–23). Currently, little information is provided in the endodontic literature regarding endotoxins in teeth with secondary/persistent endodontic infections (24). Moreover, no study compared the levels of endotoxins found in primary and secondary/persistent infections. Therefore, this clinical study was conducted to compare the levels of endotoxins found in primary and secondary/persistent endodontic infections with apical periodontitis by correlating their LPS contents with clinical/radiographic findings and to investigate the presence of target gram-negative anaerobic bacteria by using the polymerase chain reaction (PCR) (16S rDNA).

Materials and Methods

Patient Selection

A total of 30 patients who attended the Piracicaba Dental School, São Paulo, Brazil, were included in the present study, 15 needing primary endodontic treatment and 15 needing nonsurgical endodontic retreatment because of persistent or emergent apical periodontitis. A detailed dental history was obtained from each patient. Those who had received antibiotic treatment during the last 3 months or who had any general disease

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were excluded. The Human Research Ethics Committee of the Piracicaba Dental School approved the protocol describing the sample collection for this investigation, and all volunteer patients signed an informed consent form.

All the selected teeth were single rooted, showing the presence of 1 root canal and the absence of periodontal pockets deeper than 4 mm. None of the patients reported spontaneous pain. Teeth that could not be isolated with a rubber dam were excluded.

The failure of root canal treatment was determined on the basis of clinical and radiographic examinations. The reasons for retreatment were the presence of persistent apical radiolucent lesions; voids in or around the root canal filling; and persistent symptoms such as pain on palpation (POP), discomfort to percussion, and pain of the sinus tract (20). The quality of coronal restoration was evaluated in the present study (25).

The following clinical/radiographic features were collected for further analyses: tenderness to percussion (TTP), POP, exudation (EX), and the size of the radiolucent area > or ≤3 mm (SRA). Teeth with clinical symptoms were considered those with the presence of TTP and/or POP, whereas the asymptomatic ones were those showing no clinical symptomatology.

Sampling Procedures

All the materials used in this study were heat sterilized at 200°C for 4 hours, thus becoming apyrogenic. The method used for the disinfection of the operative field has been previously described (6, 8). The sterility of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it on blood agar plates, which were then incubated both aerobically and anaerobically.

A 2-stage access cavity preparation was made without the use of water spray but under manual irrigation with sterile/apyrogenic saline solution and by using a sterile/apyrogenic high-speed diamond bur. The first stage was performed to promote a major removal of contaminants, including carious lesions and restoration. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the protocol described previously. The sterility of the internal surface of the access cavity was checked as previously described, and all procedures were performed aseptically.

Sampling Procedures for Primary Endodontic Infections. Samples were collected from 15 single-rooted teeth with pulp necrosis and radiographic evidence of apical periodontitis. After the sterility of the access cavity was checked, a new sterile and apyrogenic bur was used followed by irrigation of the root canal access with sterile/apyrogenic water. The endotoxin sample was taken by introducing sterile pyrogen-free paper points (size #15; Dentsply-Maillefer, Ballaigues, Switzerland) into the full length of the canal (determined radiographically) and retaining them in position for 60 seconds. Next, the paper points were immediately placed on a pyrogen-free glass tube and frozen at −80°C for the limulus amebocyte lysate assay (LAL). For the microbial sample, the procedure was repeated with 5 sterile paper points. The paper points were pooled in a sterile tube containing 1 mL VMGA III transport medium and were immediately processed for DNA extraction to detect target bacteria using the molecular method (16S rDNA).

Sampling Procedures for Secondary Endodontic Infections. Samples were taken from 15 teeth that had been previously root filled and showed radiographic evidence of apical periodontitis. After the sterility of the access cavity was checked, a new sterile pyrogen-free bur was used with irrigation with sterile/endotoxin-free saline to access the canal. Root-filling materials were removed by rotary instrumentation (Gates-Glidden drills #5, 4, 3, and 2, Dentsply-Maillefer), a Hero-file #20.06 (MicroMega, Besançon, France), and K-files in a crown-down technique without the use of a chemical solvent accompanied by irrigation with a sterile/endotoxin-free solution. Next, endotoxin and microbial sampling procedures were performed as previously described.

Determination of Endotoxin Concentration (turbidimetric test LAL assay). The turbidimetric test (BioWhitaker, Inc, Walkersville, MD) using the LAL technique was used to measure endotoxin concentrations within the root canals according to the manufacturer's instructions. The test procedure used in the present study was performed according to Martinho et al (6, 8).

Bacterial Detection (PCR 16S rDNA). Reference bacteria strains from the American Type Culture Collection (ATCC) were used as follows: *Prevotella nigrescens* (ATCC 33099), *Porphyromonas endodontalis* (ATCC 35406), *Fusobacterium nucleatum* (ATCC 25586), *Treponema denticola* (ATCC 35405), and *Treponema socranskii* (ATCC 35536).

DNA Extraction. The bacterial DNA was extracted from endodontic samples as well as from ATCC bacteria and then purified with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration (absorbance at 260 nm) was determined by using a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE).

PCR Assay. The PCR reaction was performed in a thermocycler (MyCycler; Bio-Rad, Hercules, CA) at a total volume of 25 μL containing 2.5 μL 10× Taq buffer (1×) (Invitrogen, Eugene, OR), 0.5 μL deoxyribonucleoside triphosphate mix (25 μmol/L each deoxyribonucleoside triphosphate [ie, dATP, dCTP, dGTP, and dTTP]; Invitrogen), 1.25 μL 25 mmol/L $MgCl_2$, 0.25 μL forward and reverse universal primers (0.2 μmol/L; Invitrogen), 1.5 μL sample DNA (1 μg/50 μL), 1.5 μL Taq DNA polymerase (1 U, Invitrogen), and 17.25 μL nuclease-free water. Primer forward and reverse sequences as well as PCR cycling parameters are listed in Table 1. Negative controls corresponded to the reaction mixture without DNA. Either the positive or negative detection of gram-negative target bacteria species was based on the presence of clear bands of expected molecular size as shown in Table 1.

Statistical Analysis

The data collected for each case (clinical features and bacteria detection) were tabulated onto a spreadsheet and statistically analyzed by using SPSS for Windows (SPSS Inc, Chicago, IL). The Pearson chi-square test or 1-sided Fisher exact test was used as appropriate to test the null hypothesis that there was no relationship between endodontic clinical signs/symptoms or radiographic findings and the presence of a specific group of bacteria in the root canal samples. The comparison between the values of endotoxin concentrations found in primary and secondary infections was performed by using the Mann-Whitney U test. The correlation between clinical/radiographic findings and the median values of endotoxin concentration was analyzed by using either the Student's t test or the Mann-Whitney U test with $P < .05$ being considered statistically significant.

Results

Sterility samples taken from the external and internal surfaces of the crown and its surrounding structures, tested before and after entering the pulp chamber, showed no microbial growth. The following clinical/radiographic features were found in root canals with primary endodontic infections investigated: POP (6/15), TTP (7/15), EX (7/15), and an SRA >3 mm (7/15). Root canals with secondary endodontic infections showed POP (7/15), TTP (6/15), EX (7/15), and an SRA >3 mm (10/15).

TABLE 1. PCR Primer Pairs and Cycling Parameters Used for the Detection of Target Gram-negative Bacterial Species in Root Canals

Target bacteria	Primer pairs (5′–3′)	Amplicon size	Cycles
Universal (16S rDNA)	Forward: TCC TAC GGG AGG CAG CAG T Reverse: GGA CTA CCA GGG TAT CTA ATC CTG TT	466 bp	Initial denaturation at 95°C for 10 min and 40 cycles of 95°C for 10 s, 60°C for 10 s, and a final extension step at 72°C for 25 s
P. nigrescens	Forward: ATG AAA CAA AGG TTT TCC GGT AAG Reverse: CCC ACG TCT CTG TGG GCT GCG A	804 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
T. denticola	Forward: TAA TAC CGA ATG TGC TCA TTT ACA T Reverse: TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
P. endodontalis	Forward: GCT GCA GCT CAA CTG TAG TC Reverse: CCG CTT CAT GTC ACC ATG TC	672 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
T. socranskii	Forward: GAT CAC TGT ATA CGG AAG GTA GAC A Reverse: TAC ACT TAT TCC TCG GAC AG	288 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
F. nucleatum	Forward: AGT AGC ACA AGG GAG ATG TAT G Reverse: CAA GAA CTA CAA TAG AAC CTG A	645 bp	Initial denaturation at 94°C for 30 s and 30 cycles of 94°C for 30 s, 40°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min

Determination of Endotoxin Concentration (turbidimetric test LAL assay)

The standard curve for the detection of endotoxins fulfilled the criteria of linearity ($r = 1$). The LAL assay indicated that endotoxins were detected in 100% of the root canals with primary (15/15) and secondary (15/15) endodontic infections. Significantly higher levels of endotoxins were found in teeth with primary infections (median, 7.49 EU/mL; range, 0.25–289 EU/mL) compared with those with secondary infections (median, 3.96 EU/mL; range, 0.82–15.30 EU/mL; $P < .05$).

For Primary Endodontic Infections. The median value of endotoxin concentration found in the presence of clinical symptoms (19.10 EU/mL; range, 1.31–289 EU/mL) was significantly higher than that of asymptomatic teeth (3.50 EU/mL; range, 0.25–35.20 EU/mL; $P < .05$; Table 2). There was a difference between teeth with TTP (9.19 EU/mL; range, 1.31–289 EU/mL) and those without it (5.93 EU/mL; range, 0.25–35.20 EU/mL; $P < .05$; Table 2). A higher median value of endotoxin content was detected in teeth with an SRA >3 mm (26 EU/mL; range, 0.25–289 EU/mL) compared with those with a radiolucent area ≤3 mm (4.98 EU/mL; range, 1.31–115 EU/mL; $P < .05$; Table 2).

For Secondary Endodontic Infections. The endotoxin content was significantly higher in teeth with an SRA >3 mm (4.73 EU/mL; range, 0.08–15.30 EU/mL) compared with teeth with an SRA ≤3 mm (2.28 EU/mL; range, 0.44–8.04 EU/mL; $P < .05$; Table 2). No correlation was found between the levels of endotoxins and the presence of clinical symptomatology ($P > .05$). Table 2 shows the median and range values of endotoxins found in primary and secondary infections according to clinical/radiographic features.

Bacterial Detection (PCR 16S rDNA)

Bacterial DNA was detected in all root canal samples with primary (15/15) and secondary (15/15) endodontic infections as determined by the ubiquitous bacterial primers. No positive results were observed for the presence of bacterial DNA in negative control samples. At least 1 gram-negative bacterial species was detected in 93% (14/15) of the root canals with primary and 33% (5/15) with secondary infection. A combination of 2 or more gram-negative target species was detected in teeth with primary (8/15) and secondary (3/15) infections. *P. nigrescens* (primary, 10/15; secondary, 4/15), *F. nucleatum* (primary, 5/15; secondary, 1/15), *T. denticola* (primary, 3/15; secondary, 1/15), and *T. socranskii* (primary, 5/15; secondary, 1/15) were detected in the root canals, respectively. *P. endodontalis* was detected only in teeth with primary infections (5/15). Positive associations were found between

TABLE 2. Endotoxin Concentration (median and ranging values in EU/mL) Found in 15 Teeth with Primary Endodontic Infections and 15 Teeth with Secondary Endodontic Infection with Apical Periodontitis According to Clinical and Radiographic Features

	Primary infections		Secondary infections	
Total of endotoxins	7.49* (0.25–289.00)		3.96 (0.82–15.30)	
Clinical symptoms	Present	Absent	Present	Absent
Median values (EU/mL)	19.10*	3.50	3.26	3.96
Range values (EU/mL)	(1.31–289.00)	(0.25–35.20)	(0.08–15.30)	(0.44–9.27)
TTP	Present	Absent	Present	Absent
Median values (EU/mL)	9.19*	5.93	3.26	3.96
Range values (EU/mL)	(1.31–289.00)	(0.25–35.20)	(0.72–15.3)	(0.082–14.50)
POP	Present	Absent	Present	Absent
Median values (EU/mL)	6.535	9.19	2.23	4.73
Range values (EU/mL)	(1.31–19.10)	(0.257–289.00)	(0.08–15.30)	(0.44–14.50)
SRA	≤3 mm	>3 mm	≤3 mm	>3 mm
Median values (EU/mL)	4.98	26.00*	2.28	4.73*
Range values (EU/mL)	(1.31–115.00)	(0.25–289.00)	(0.44–8.04)	(0.08–15.30)

Present, teeth with TTP and/or with POP; Absent, teeth without TTP and/or POP.

*$P < .05$.

P. endodontalis and *T. denticola* (P = .003; odds ratio = 2.000, confidence bound = 0.899–4.452) as well as between *F. nucleatum* and *P. endodontalis* (P = .031, odds ratio = 13.000, confidence bound = 1.360–124.297) in teeth with primary infections. No statistically significant relationship was detected between the presence of any of these target bacteria species and clinical signs/symptoms.

Discussion

Analyses of the results indicated that teeth with primary endodontic infections contain higher levels of endotoxins and a greater incidence of gram-negative bacteria, showing different positive associations among gram-negative bacteria than teeth with secondary endodontic infections. Additionally, they indicated a correlation between higher contents of endotoxins and a larger area of bone destruction as well as the presence of specific clinical features found in primary infections.

The selection criteria for the investigation of *P. nigrescens*, *P. endodontalis*, *F. nucleatum*, *T. denticola*, and *T. socranskii* were based on the frequent occurrence of these species in endodontic infections (6, 8–13, 21–23) and their LPS toxicity (5, 7, 26, 27). Recently, a great interindividual variation in the composition of the apical microbiota has been disclosed by pyrosequencing analyses (28, 29).

The turbidimetric kinetic LAL assay indicated endotoxins in 100% of the root canals investigated, both in teeth with primary infections (6, 8, 17, 18) and those with secondary infections. This method, which is widely used for the analysis of primary infections (6, 8, 17, 18), was shown to be applicable to teeth with secondary/persistent infections, in particular because of its extreme sensitivity in detecting minute quantities of endotoxins.

In the present study, the median value of endotoxins found in teeth with primary infections was almost twice as high than in teeth with secondary infections. This finding is partially reflected by the microbiological findings regarding teeth with primary infections, which indicates a higher incidence of gram-negative bacterial species and a greater number of root canals with a combination of 2 or more of these species. The combination of gram-negative bacterial species indicates that different bacterial LPSs with different lipid A toxicity (30) can be involved in root canal infection and can even inhibit each other's antigenicity in the periradicular tissues. For instance, *P. endodontalis* seems to enhance the *F. nucleatum* toxicity (7).

The levels of endotoxins found in teeth with primary infections of 7.49 EU/mL is in accordance with previous investigations (6, 8, 17, 18) showing median values of endotoxins ranging from 7.49 EU/mL (6) to 9.19 EU/mL (17) as determined by the turbidimetric kinetic LAL test. Such variations can be attributed to the case selection, particularly regarding the presence or absence of clinical/radiographic features.

In contrast to teeth with primary infections, those with secondary infections indicated a median level of 3.96 EU/mL. Horiba et al (24) investigated endotoxins in teeth with post-treatment apical periodontitis and revealed a median value of endotoxins of 5.9 μg/mL, which was determined by the gel clot method (a semiquantitative test). However, it is not possible to compare the levels of endotoxins in both studies because of the methodologic differences (18).

Teeth with primary infections showing the presence of clinical symptoms contained higher levels of endotoxins than the asymptomatic ones, thus corroborating previous studies (3, 14, 15). However, there was no statistically significant correlation between higher levels of endotoxins found in teeth with secondary infections and the presence of clinical symptoms. Conversely, Horiba et al (24) reported a positive correlation between higher detection rates of endotoxins and symptomatic teeth with post-treatment apical periodontitis. Contrary to the present study, Horiba et al (24) found endotoxins in only 4 of 14 asymptomatic teeth, a figure possibly compromised by the semiquantitative analysis.

The presence of TTP in teeth with primary infections was related to higher levels of endotoxins, which is also corroborated by Jacinto et al (14) and Martinho and Gomes (15). Jacinto et al (14) reported a positive association between levels of endotoxins and the presence of POP after investigating 22 of 50 teeth with spontaneous pain, including an abscess of endodontic origin, which is an exclusion criterion in the present study. Such a finding was not found in the present study or elsewhere (6, 8, 15).

With respect to bone destruction in apical periodontitis, this study found higher levels of endotoxins in teeth with larger radiolucent areas, thus elucidating the role of endotoxins in the bone resorption present in apical periodontitis (6, 8). Typical results were shown by Schein and Schilder (3) and Horiba et al (24). It has long been known that oral bacterial LPSs are potent stimuli for proinflammatory cytokines involved in periapical tissue destruction (6, 8, 26, 27). Martinho et al (6) showed that higher levels of endotoxins were followed by an increased production of interleukin-1β in teeth with a larger area of bone resorption. Furthermore, Tang et al (31) indicated that *P. endodontalis* LPS has the ability to promote the expression of RANKL in osteoblast cells, which contributes to bone lesions.

It is worth pointing out that although *P. nigrescens*, *P. endodontalis*, *F. nucleatum*, *T. denticola*, and *T. socranskii* could not be found in 1 tooth with a primary infection or in 10 teeth with secondary infections using PCR analyses, endotoxins were still detected in 100% of the root canals investigated. Such a finding indicates that the endotoxins detected in these root canals might possibly have come from any of the species investigated but at such a low DNA concentration that it could not be detected by the PCR 16S rDNA method. In addition, the participation of other gram-negative bacterial species was not considered in the present study.

Despite the higher contents of endotoxins found in teeth with primary infections compared with teeth with secondary infections, endotoxins were still detected in all root canal samples. It might be argued that if endotoxins were detected at nanomolar levels by innate immune system receptors present on the macrophages (32), then they would be extremely strong stimulators of inflammatory reactions even at low concentrations because a single gram-negative bacterial cell contains $\approx 10^6$ of lipid A residues (the LPS domain responsible for its toxicity) (30). Therefore, special attention should be given for the establishment of protocols for the neutralization/elimination of them from the root canal infection.

Taken together, the positive correlations between higher contents of endotoxins and radiolucent areas found in teeth with primary and secondary infections, including the presence of specific clinical symptoms found in the former condition, suggest that the increased endotoxin levels in infected root canals may be associated with the severity of periapical disease as well as the development of specific clinical features in teeth with primary endodontic infections.

In conclusion, teeth with primary endodontic infections had higher contents of endotoxins and a more complex gram-negative bacterial community compared with teeth with secondary infections. Moreover, the levels of endotoxins were related to the severity of bone destruction in periapical tissues as well as the development of clinical features in teeth with primary infections.

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