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Molecular identification and quantification of bacteria from endodontic infections using real-time polymerase chain reaction

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Introduction: It was the aim of the present study to evaluate root canal samples for the presence and numbers of specific species as well as for total bacterial load in teeth with chronic apical periodontitis using quantitative real-time polymerase chain reaction (PCR).

Methods: Forty adult patients with one radiographically documented periapical lesion were included. Twenty teeth presented with primary infections and 20 with secondary infections, requiring retreatment. After removal of necrotic pulp tissue or root canal filling, a first bacterial sample was obtained. Following chemo-mechanical root canal preparation a second sample was taken and a third sample was obtained after 14 days of intracanal dressing with calcium hydroxide. Analysis by real-time PCR enabled the quantification of total bacterial counts and of nine selected species.

Results: Root canals with primary infections harbored significantly more bacteria (by total bacterial count) than teeth with secondary infections ($P < 0.05$). Mean total bacterial count in the retreatment group was 2.1×10^6 and was significantly reduced following root canal preparation (3.6×10^4) and intracanal dressing (1.4×10^5). Corresponding values for primary infections were: 4.6×10^7, 3.6×10^4, and 6.9×10^4. The numbers of the selected bacteria and their detection frequency were also significantly reduced.

Conclusion: Root canals with primary infections contained a higher bacterial load. Chemo-mechanical root canal preparation reduced bacterial counts by at least 95%.

Key words: bacteria; chlorhexidine; endodontic infection; *Enterococus faecalis*; real-time polymerase chain reaction; sodium hypochlorite

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The bacterial etiology of apical periodontitis is well accepted and the bacterial composition of endodontic infections is the focus of current research (25). The number of bacterial species found in endodontic infections is updated constantly (14). It is generally acknowledged that in most cases where endodontic treatment fails, the failure is the result of treatment procedures not having met a satisfactory standard for control and elimination of infection (17). Modern endodontic treatment procedures aim to eliminate the microorganisms during root canal preparation and disinfection. Earlier studies showed that endodontic preparation and antibacterial irrigation with sodium hypochlorite could remove approximately 50% of bacteria from the root canal and that bacteria could reliably be eliminated from the canal after antimicrobial dressing (30). Intracanal medication between appointments has been unequivocally shown to contribute to a favorable outcome in treating endodontic infections (21). Follow-up studies examining the outcome of endodontic therapy revealed a very high success rate when a

negative bacterial culture was a prerequisite before root filling (29), although there is clear evidence that a negative culture does not correlate with a bacteria-free root canal system (23). In cases of retreatment, specific species may survive disinfection and antimicrobial dressing, so that enhanced measures have to be taken. A microbiological diagnostic test method to characterize the bacterial infection could advance the outcome of endodontic therapy. New molecular detection methods offer the possibility of detecting new species and enable a more precise analysis. Whereas for periodontal infections methods based on quantitative polymerase chain reactions (PCR) are already well-established diagnostic tools (2, 4, 11, 12, 36), there is only limited information on their use in endodontics (24, 34, 35).

Therefore, it was the aim of the present study to evaluate root canal samples for the presence and levels of specific species as well as for the total bacterial load in teeth with chronic apical periodontitis using quantitative real-time PCR.

Material and methods

A total of 40 adult patients (aged 21–77 years; 18 men, 22 women), who had been referred to the Department of Operative Dentistry and Periodontology at the University of Bonn for root canal treatment or retreatment, were included in the study. Each patient presented with one single- or multi-rooted tooth that showed radiographic evidence of chronic apical periodontitis. We included 28 single-rooted teeth with one root canal and 12 multi-rooted teeth in this study. Two multi-rooted teeth had two canals and 10 had three canals. Any fillings present were carefully checked for their marginal adaptation. If necessary, restorations were replaced before root canal treatment or retreatment was initiated. No patient had received antibiotic treatment during the previous 3 months and teeth were free of caries at the time of endodontic treatment. All patients had been informed about the study and had given their informed consent. The study was conducted in full accordance with the declared ethical principles (World Medical Association Declaration of Helsinki, version VI, 2002) and had been approved by the Ethical Board of the University Hospital Bonn.

Patients were divided into two groups according to the clinical diagnosis (Fig. 1): primary infection – 20 untreated teeth with asymptomatic chronic apical periodontitis – and secondary infection – 20 root-filled teeth with asymptomatic chronic apical periodontitis that required retreatment. Single- and multi-rooted teeth were equally distributed between the two groups.

None of the selected teeth responded to sensitivity testing and none had periodontal pockets deeper than 4 mm. Teeth with primary infections had not received previous root canal treatment. All teeth with secondary infections had received endodontic treatment more than 2 years previously.

Each tooth was isolated with a rubber dam. The tooth and the surrounding field were cleansed with 30% hydrogen peroxide. The rubber dam was treated with 5% iodine tincture, which was inactivated with 5% sodium thiosulfate after 2 min (7). Surface swabbing of the decontaminated field was performed with a cotton pellet; this pellet was stored in brain–heart infusion broth for 24 h to check sterility. After preparation of the access cavity under cooling with sterile NaCl, coronal flaring was carried out with Gates–Glidden burs, sizes 2 and 4. The length of the canals was determined with an apex locator but as no consistently reliable readings could be obtained in all cases, the length was confirmed radiographically. Immediately after trephination and after determination of the tooth length the first bacterial sample was obtained using a sterile paper point (ISO 30; Roeko, Langenau, Germany). Each paper point was retained in position near the apex for 1 min. In multi-canaled teeth, one paper point sample was obtained from the root canal with the largest periapical lesion. Root canals were prepared using rotary instruments (Flexmaster, VDW, Munich, Germany).

As part of the chemo-mechanical root canal preparation, in each group 10 teeth were randomly assigned to irrigation with either 2% sodium hypochlorite or 0.1% chlorhexidine for 30 min. In addition, an ultrasonic activation (Cavitron®; Dentsply, Tulsa, OK) of the irrigation solution was performed for 1 min. A second bacterial sample was taken immediately after this treatment and the third sample was taken after a period of 14 days during which the canals were filled with a calcium hydroxide dressing (Calcicur®; Voco, Cuxhaven, Germany) placed with a syringe.

The paper points were placed in sterile DNA-free and RNA-free vials and sent to a specialized laboratory (Carpegen GmbH, Münster, Germany) within 24 h. They were stored at −20°C until further analysis. Validation experiments showed no significant variation in quantification results under these storage and shipping conditions. After thawing, the samples were immediately subjected to the automated process of meridol® Perio Diagnostics (GABA International, Basel, Switzerland) analysis and to a process for the detection of the endodontic pathogens. The real-time PCR-based analysis meridol® Perio Diagnostics, as well as the reactions for the detection of the endodontic pathogens, were developed and validated by Carpegen GmbH. Specificity had been verified using purified genomic DNA from several bacterial species (e.g. all species detected in this study, *Streptococcus mutans*, *Lactobacillus acidophilus*, *Lactobacillus casei*) and fungal species (various *Candida* spp., *Geotrichum*, *Aspergillus* spp.) as well as with human DNA. No cross-reactivity to non-target species was observed, even between closely related species, such as *Prevotella intermedia* and *Prevotella nigrescens*, or *Porphyromonas gingivalis* and *Porphyromonas endodontalis*. DNA sequencing of PCR amplicons obtained from positive real-time PCR patient samples (at least 10 samples per species), revealed in each case the corresponding species-specific sequence, demonstrating the specificity of the reactions even on complex templates.

The main validated test parameters of the diagnostic system were as follows.

1. The detection limit within a patient's sample for each of the periodontal pathogens was 100 bacteria and for the endodontic pathogens it was 50 bacteria.
2. The linear range for quantification was seven orders of magnitude for each pathogen.
3. The coefficient of variation was 15%.

The testing system meridol® Perio Diagnostics detects and quantifies six bacterial species (*Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum* ssp., *P. gingivalis*, *P. intermedia*, *Tannerella forsythia*, and *Treponema denticola*) as well as the total bacterial load. In addition, as putative endodontic pathogens, *Enterococcus faecalis*, *Peptostreptococcus micros*, and *P. endodontalis* were investigated. The bacterial genomic DNA was isolated and purified using the AGOWA® mag DNA Isolation Kit Sputum (AGOWA GmbH, Berlin, Germany). The protocol followed the manufacturer's instructions with minor changes to adjust the procedure to the use of automated isolation with a pipetting robot (Tecan, Genesis Workstation; Tecan Schweiz AG, Switzerland). All primers and probes used in this study were designed to match

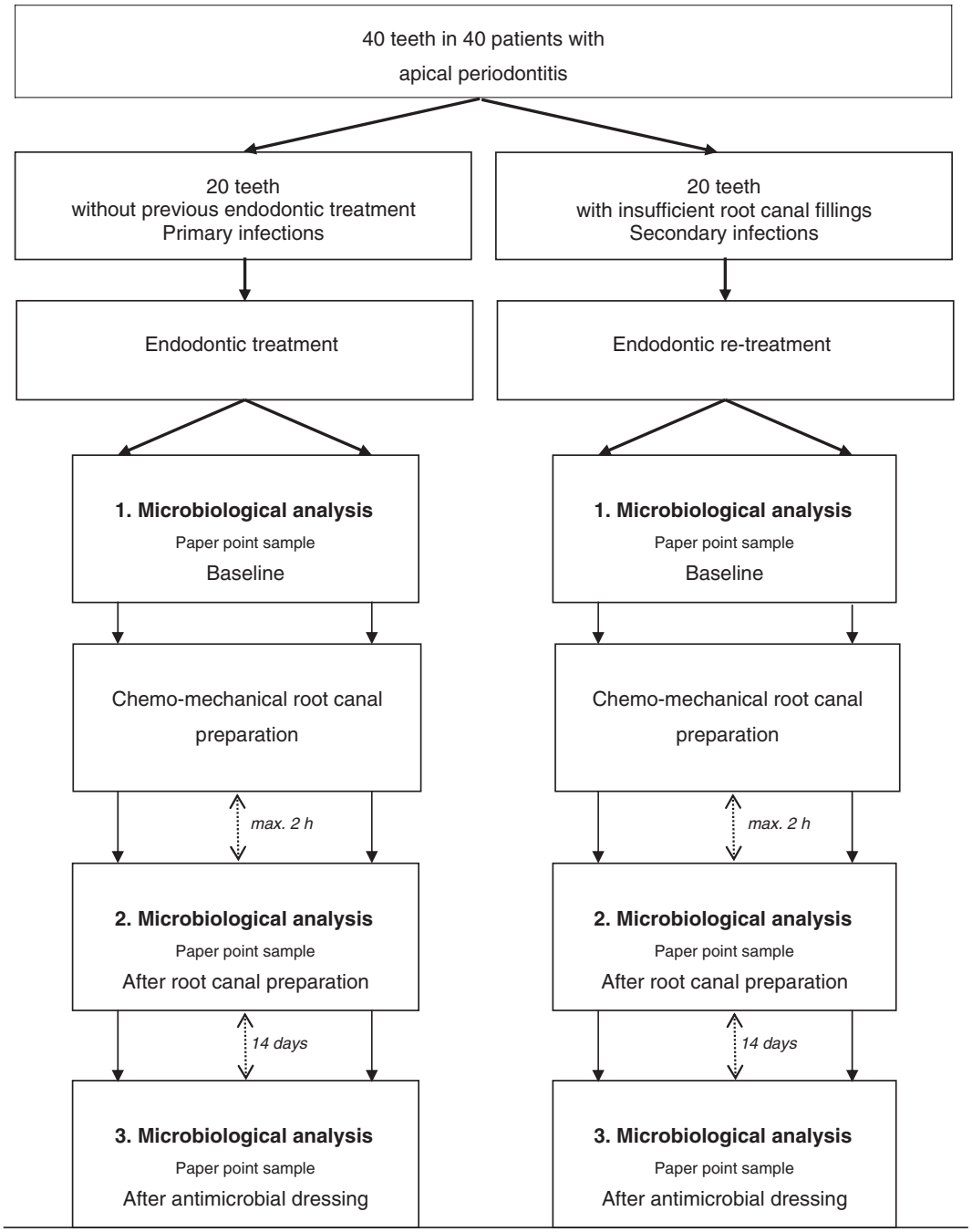

Fig. 1. Study design.

highly specifically to the ribosomal DNA (rDNA) of the bacterial pathogens. The exact primer and probe sequences were selected using PRIMER EXPRESS software (Applied Biosystems, Foster City, CA), which checks the primer and probe sets for their match to the guidelines that are recommended for real-time PCR with TaqMan® probes (melting temperature, secondary structure, etc.). The primers and probes were purchased from Applied Biosystems. Specificity was checked *in silico* with the 'basic local alignment search tool' (1) at the internet server of the National Center for Biotechnology Information, and by experiments as mentioned above. Real-time PCR was carried out with 2 µl of the isolated DNA as template in a reaction mixture containing the appropriate primer probe sets and the TaqMan® Universal PCR Mastermix. The PCR was carried out in a ABI 7900 HT (Applied Biosystems) real-time PCR cycler in 384-well plates. Cycling conditions were as follows: 10 min at 95°C (initial denaturation) followed by 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/elongation). Fluorescence data were collected at each denaturation and elongation step. Absolute quantification of the individual species as well as of the total bacterial load was carried out with the aid of serial dilutions of plasmid standards containing the appropriate DNA sequences of the amplicons. Each sample was tested for inhibition by an internal control reac-

tion. These samples were spiked with defined copy numbers of an artificial DNA sequence, which was coamplified and had to give a signal in the real-time PCR reaction within a defined range, bordered by the signals from the inhibition controls of the no-template control reactions (negative controls) and positive controls.

For statistical analysis, the SPSS 12.0 software (SPSS Inc., Chicago, IL) was used. A log-transformation of the bacterial counts was performed. The differences between total bacterial counts in the primary infection group and the secondary infection group were analyzed by a non-parametric test (Mann–Whitney). Differences in bacterial load before and after root canal preparation were analyzed using a Wilcoxon signed rank test. Association between the different bacterial species was assessed with the chi-squared test. Differences were considered as statistically significant at $P < 0.05$.

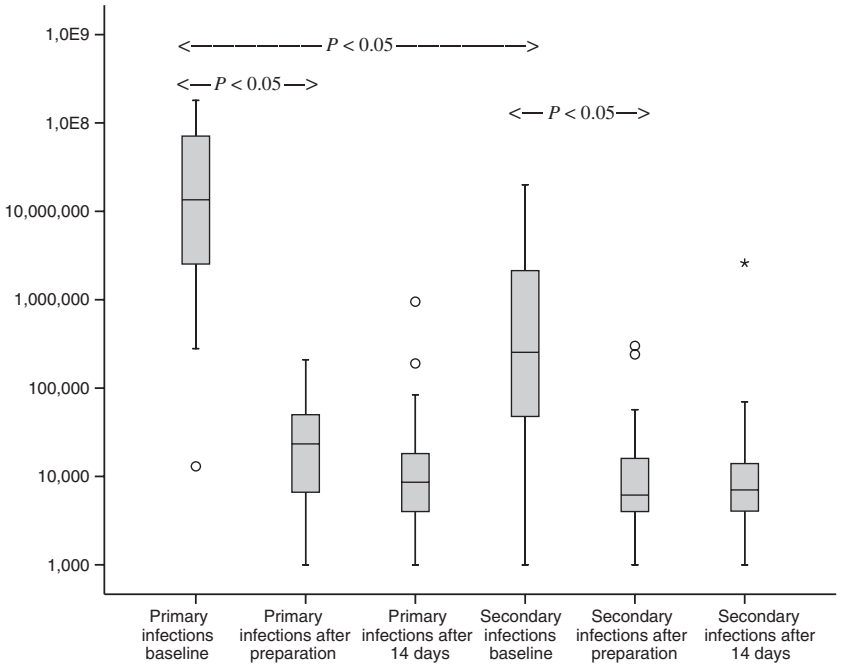

Fig. 2. Total bacterial counts (TBC) in root canals with primary infection and secondary infection at three sampling time-points. Box-plots show median, interquartile range between 25th and 75th percentiles, whiskers indicate maximum and minimum, outliers are shown as circles, $n = 20$ in each group. Compared to baseline TBCs were significantly reduced following irrigation and antimicrobial dressing ($P < 0.05$).

Results

Total bacterial counts

Root canals with primary infections harbored significantly more bacteria than teeth with secondary infections ($P < 0.05$) at baseline. Median total bacterial counts in the group with secondary infections amounted to 2.6×10^5 (min. 1.0×10^3, max. 2.0×10^7) and were significantly reduced following root canal preparation (6.4×10^3, min. 1.0×10^3, max. 3.0×10^5) and intracanal calcium hydroxide dressing (7.1×10^3, min. 1.0×10^3, max. 2.6×10^6). In contrast, the corresponding values for primary infections were: 1.5×10^7 (min. 1.3×10^4, max. 3.0×10^5), 2.3×10^4 (min. 1.0×10^3, max. 2.1×10^5) and 8.6×10^3 (min. 1.0×10^3, max. 9.5×10^5), respectively. This represents a median reduction of detectable bacteria in the group of secondary infections of 97.3% and in the group of primary infections of 99.9%. The specific results over time for both groups are presented in Fig. 2. There was a statistically significant reduction of bacteria from the first to the second sample and from the first to the third sample ($P < 0.05$) (Fig. 2). The use of an intracanal calcium hydroxide dressing did not lead to a further reduction of total bacterial counts in the root canals.

Selected bacterial species

P. micros and *P. endodontalis* were the most frequently detected species in both primary and secondary infections. Overall, *P. micros* was found in 22 of the samples at baseline (55%) and *P. endodontalis* was found in 14 of these samples (35%). The respective values for the other species were: *T. denticola* nine samples (22.5%), *T. forsythia* 12 samples (30%), *F. nucleatum* 11 samples (27.5%), *P. gingivalis* five samples (12.5%), and *P. intermedia* two samples (5%). *E. faecalis* was found five times in these samples (12.5%), whereas *A. actinomycetemcomitans* was never detected (Tables 1 and 2). In the group with no previous endodontic treatment the root canal systems showed a higher diversity of bacterial species. Even though the use of calcium hydroxide as an intracanal dressing for 14 days did not lead to a further

Table 1. Bacterial counts and frequency of detection (detection threshold: 100 bacteria) for selected bacteria in root canals (n = 20) with primary infections at baseline

	Median	Min/Max	Mean	SD	Positive samples
TBC	1.48×10^7	(1.30×10^4, 1.80×10^8)	4.64×10^7	6.20×10^7	20 (100%)
P. m.	2.95×10^3	($<5.0 \times 10^1$, 7.00×10^6)	7.14×10^5	1.72×10^6	13 (65%)
P. e.	6.25×10^2	($<5.0 \times 10^1$, 6.90×10^5)	8.87×10^4	1.83×10^5	10 (50%)
T. d.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 2.20×10^6)	1.53×10^5	5.02×10^5	7 (35%)
T. f.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 1.50×10^6)	1.53×10^5	4.15×10^5	8 (40%)
F. n.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 2.20×10^5)	1.90×10^4	5.28×10^4	7 (35%)
P. g.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 2.80×10^4)	2.29×10^3	7.01×10^3	2 (10%)
P. i.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 6.60×10^2)	1.13×10^2	5.8×10^1	1 (5%)
A. a	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 1.0×10^2)	$<1.0 \times 10^2$	0	0
E. f.	$<5.0 \times 10^1$	($<5.0 \times 10^1$, 6.70×10^4)	3.58×10^3	1.49×10^4	3 (15%)

TBC, total bacterial count; *P.m.*, *Peptostreptococcus micros*; *P.e.*, *Porphyromonas endodontalis*; *T.d.*, *Treponema denticola*; *T.f.*, *Tannerella forsythia*; *F.n.*, *Fusobacterium nucleatum*; *P.g.*, *Porphyromonas gingivalis*; *P.i.*, *Prevotella intermedia*; *A.a.*, *Actinobacillus actinomycetemcomitans*; *E.f.*, *Enterococcus faecalis*.

Table 2. Bacterial counts and frequency of detection (detection threshold: 100 bacteria) for selected bacteria in root canals (n = 20) with secondary infections at baseline

	Median	Min/Max	Mean	SD	Positive samples
TBC	2.65×10^5	(1.00×10^3, 2.00×10^7)	2.14×10^6	4.90×10^7	20 (100%)
P. m.	$<5.0 \times 10^1$	($<5.0 \times 10^1$, 5.10×10^5)	4.18×10^4	1.21×10^5	9 (45%)
P. e.	$<5.0 \times 10^1$	($<5.0 \times 10^1$, 1.20×10^6)	1.08×10^4	3.33×10^4	4 (20%)
T. d.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 2.40×10^5)	1.21×10^4	3.56×10^4	2 (10%)
T. f.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 7.90×10^5)	4.47×10^4	1.76×10^5	4 (20%)
F. n.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 3.30×10^3)	4.32×10^2	9.20×10^2	4 (20%)
P. g.	1.0×10^2	($<1.0 \times 10^2$, 1.50×10^5)	7.68×10^3	3.34×10^4	3 (15%)
P. i.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 1.20×10^5)	6.09×10^3	2.68×10^4	1 (5%)
A. a.	$<1.0 \times 10^2$	($<1.0 \times 10^2$, 1.0×10^2)	$<1.0 \times 10^2$	0	0
E. f.	$<5.0 \times 10^1$	($<5.0 \times 10^1$, 1.80×10^5)	9.79×10^3	4.01×10^4	2 (10%)

TBC, total bacterial count; *P.m.*, *Peptostreptococcus micros*; *P.e.*, *Porphyromonas endodontalis*; *T.d.*, *Treponema denticola*; *T.f.*, *Tannerella forsythia*; *F.n.*, *Fusobacterium nucleatum*; *P.g.*, *Porphyromonas gingivalis*; *P.i.*, *Prevotella intermedia*; *A.a.*, *Actinobacillus actinomycetemcomitans*; *E.f.*, *Enterococcus faecalis*.

reduction of the total bacterial counts, the detection frequency of individual bacterial species was further reduced (Fig. 3).

Studying the frequency of simultaneous detection of bacterial species at baseline, *P. micros* was found to be positively associated with *P. endodontalis*, *P. gingivalis*, *F. nucleatum*, and *T. forsythia* ($P < 0.05$, chi-squared test). *T. forsythia* and *T. denticola*, and *T. forsythia* and *F. nucleatum* were positively associated with each other ($P < 0.05$, chi-squared test). In addition, a significant association was shown between the species *P. endodontalis* and *P. gingivalis*, and between *P. endodontalis* and *F. nucleatum* ($P < 0.05$, chi-squared test) (Table 3).

Discussion

The present study demonstrated a high bacterial load in all the teeth with chronic apical periodontitis. The total bacterial counts in root canals with primary infections were significantly higher compared to those in teeth with secondary infections. Similar differences were also observed for the selected bacterial species. *P. micros* was the most frequently detected species in both groups. Both bacterial counts and their detection frequency could be significantly reduced by chemo-mechanical root canal preparation. The use of calcium hydroxide as an intracanal dressing for 14 days did not lead to a further reduction in the total bacteria counts, although the detection frequency of individual bacterial species was further reduced.

In the past, different methods have been employed to analyze the microbiota associated with endodontic infections. Culture methods were the only option until the development of molecular methods, particularly PCR-based techniques, which provide significant additional knowledge regarding the composition of the endodontic microbiota by allowing the detection of bacterial species that are difficult or even impossible to culture and of bacterial strains with a phenotypically divergent behavior (28). Real-time PCR has been shown to be a highly sensitive and specific assay for the identification and quantification of oral pathogens by using specific primers and a TaqMan probe (2, 4, 11, 12, 34–36). The real-time PCR method employed in the present study had been previously validated in comparison with standard anaerobic culture (12).

So far, only two studies have employed real-time PCR to evaluate the bacterial load of root canals containing necrotic pulp tissue during the different steps of endodontic treatment (24, 34). Vianna et al. (34) reported on teeth with primary infections and recorded initial bacterial loads that were of the same magnitude as the baseline total bacterial counts in the present study. In agreement with our data, bacterial loads declined significantly after chemo-mechanical preparation of the infected root canals. Mean reductions amounted to 96.6–99.9% and corresponded with the observed median reduction of 99.9% in the present study. Likewise, Sakamoto et al. (24), investigating teeth with primary infections, reported a mean reduction of detectable bacteria of 99.67% after chemo-mechanical preparation. One problem of molecular technologies when studying the efficacy of antimicrobial therapy is their ability to detect DNA from dead cells. However, the results of the present study, demonstrating over 99% reduction in bacterial counts, might question the relevance of this limitation in the context of this investigation. If real-time PCR had detected dead bacteria after antimicrobial therapy, the present results would be an underestimation of the true antimicrobial effect.

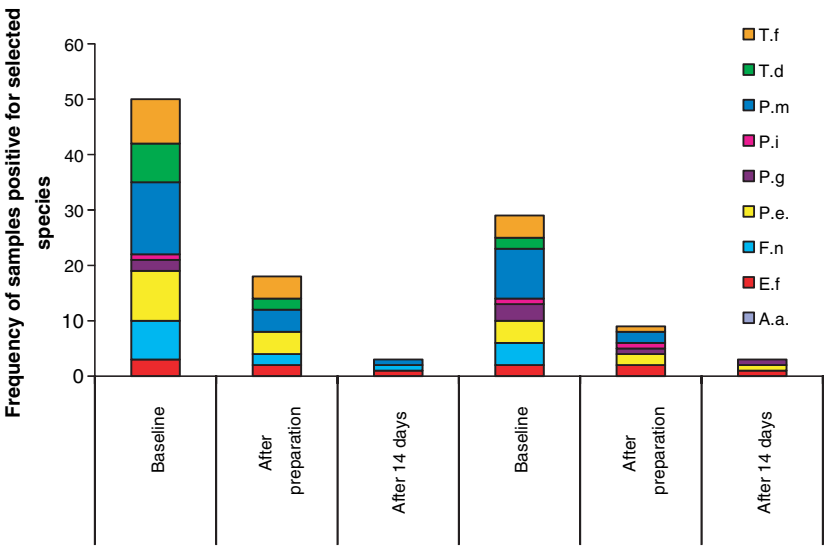

Fig. 3. Frequency of positive samples for selected species in groups with primary and secondary infections (T.f., *Tannerella forsythia*; T.d., *Treponema denticola*; P.m., *Peptostreptococcus micros*; P.i., *Prevotella intermedia*; P.g., *Porphyromonas gingivalis*; P.e., *Porphyromonas endodontalis*; F.n., *Fusobacterium nucleatum*; E.f., *Enterococcus faecalis*).

Table 3. Associations between species in the root canals at baseline

	E.f.	F.n	P.e	P.g.	P.i.	P.m.	T.d.	T.f.
E.f.	–	0.503	0.802	0.366	0.583	0.810	0.886	0.602
F.n.	0.503	–	**0.019**	**0.005**	0.372	**0.036**	0.656	**0.037**
P.e.	0.802	**0.019**	–	**0.024**	0.648	**0.004**	**0.001**	**0.001**
P.g.	0.366	**0.005**	**0.024**	–	0.100	**0.031**	0.316	0.118
P.i.	0.583	0.372	0.648	0.100	–	0.884	0.339	0.527
P.m.	0.810	**0.036**	**0.004**	**0.031**	0.884	–	0.119	**0.018**
T.d.	0.886	0.656	**0.001**	0.316	0.339	0.119	–	**0.001**
T.f.	0.602	**0.037**	**0.001**	0.118	0.527	**0.018**	**0.001**	–

Positive associations indicated in bold type, $P < 0.05$, chi-squared test; $n = 40$.
E.f., *Enterococcus faecalis*; *F.n.*, *Fusobacterium nucleatum*; *P.e.*, *Porphyromonas endodontalis*; *P.g.*, *Porphyromonas gingivalis*; *P.i.*, *Prevotella intermedia*; *P.m.*, *Peptostreptococcus micros*; *T.d.*, *Treponema denticola*; *T.f.*, *Tannerella forsythia*.

The selected bacterial species assessed in this study have previously been reported in infected root canals associated with primary as well as secondary infections. *E. faecalis* has been described in many studies as a very important species (16, 18, 32). *P. endodontalis* and *P. micros* are also considered as typical bacteria involved in endodontic infection (8, 10, 13). *T. forsythia*, *P. gingivalis*, and *T. denticola* (22), and *F. nucleatum* and *P. gingivalis* (21) have also been described as common endodontic bacterial pathogens.

It has been suggested that quantitative as well as qualitative differences may exist regarding the bacterial status of root canals with primary and secondary infection. In secondary infections, typically, one or two strains were found at rather low numbers (16). *E. faecalis* was found in a range of 0–77% and is the predominant species in most of the studies of secondary infection (18, 20, 27). In only three studies was *E. faecalis* not detected in persistent apical periodontitis (5, 10, 23). Pinheiro et al. (20) reported that the most frequently recovered bacterial genera from teeth with endodontic failure were *E. faecalis* (36.7%), followed by *Peptostreptococcus* spp. (23.4%), which were the most prevalent anaerobes found. Employing real-time PCR, *E. faecalis* was found to be three times more prevalent in refractory than in primary endodontic infections (35). Other studies have questioned the role of *E. faecalis* as the main species causing endodontic treatment failure (15, 37). The present investigation does not suggest that a specific infection with *E. faecalis* is associated with secondary infections, but it does suggest a complex bacterial etiology.

In the present study, *E. faecalis* was found in 10% of the canals in need of retreatment and *P. micros* was found in 45% of these cases. Peters et al. (19) investigated the combination of bacterial species in endodontic infections using culture methods. In 58 root canals, *P. micros* could be found in 29% and was one the most frequent species. *F. nucleatum* was found in 12% of the root canals. Both findings are in agreement with the present study, where *P. micros* was found most frequently and in 55% of the canals. In other studies *P. micros* was also the predominant species (8, 13). The present study found a statistically significant association between *P. micros* and the species *P. endodontalis*, *P. gingivalis*, *F. nucleatum*, and *T. forsythia*. The ecological niche of *P. micros* could be related to its wide range of peptidase activities, making amino acids and peptides available from serum glycoproteins (33). This selection advantage to assure nutrition may give *P. micros* a superior position among the pathogens of the endodontic microbiota. Other pathogens, like *F. nucleatum*, which have little or no proteolytic activity, can utilize this metabolism to secure their survival. The fact that in the present study *P. micros* and *F. nucleatum* were significantly correlated underlines this assumption, but further research is needed for confirmation.

The species of the so-called 'red complex' (22, 31), such as *T. forsythia*, *P. gingivalis*, and *T. denticola*, were simultaneously found in 5% of the cases. At least one species of this complex was detected in 40% of the cases, suggesting a high association of red-complex bacteria with endodontic infections. Moreover, a significant correlation between the species *T. forsythia* and *T. denticola* could be observed in the present study. Siqueira and Rocas (26) recorded *T. forsythia* in 59.1% of primary infections, and suggested that this species may play a major role in the pathogenicity of primary endodontic infection. In the present study *T. forsythia* was found in 40% of the cases, supporting this assumption.

With regard to disinfection procedures, sodium hypochlorite may still be considered the gold standard for endodontic irrigation, although chlorhexidine has been suggested as an alternative that shows an advantage against antimicrobial activity (6). Gomes et al. (9) reported that sodium hypochlorite at a concentration of 1.0% needed 20 min to inactivate *E. faecalis* completely but only 10 min at a concentration of 2.5%. In contrast, chlorhexidine required 30 s to eliminate *E. faecalis* at a concentration of 0.2%. More recently, Vianna et al. (34) reported the superior capacity of 2.5% sodium hypochlorite irrigation for reducing bacteria in comparison with 2% chlorhexidine gel, as demonstrated by real-time PCR. The results of the present study demonstrated that concentrations of 2% sodium hypochlorite and 0.1% chlorhexidine used for irrigation during chemo-mechanical root canal preparation were able to achieve a significant reduction of bacteria.

A complete elimination of bacterial infection with a one-visit treatment may be difficult to obtain so to accomplish a maximum reduction of bacteria before obturation, an inter-appointment antimicrobial dressing has been advocated (29). Calcium hydroxide is the most frequently used intracanal dressing and should prevent recontamination of the root canal system and eliminate residual bacteria (30). However, calcium hydroxide was not able to eliminate all bacterial species and especially *E. faecalis* may remain unaffected, as shown by culture studies (3). Interestingly, in the present study calcium hydroxide was not able to eliminate or reduce *E. faecalis* below the detection threshold in two of five root canals. Moreover, a calcium hydroxide dressing placed for 14 days failed to achieve a further reduction of total bacterial counts compared to values immediately after chemo-mechanical canal preparation. This is in agreement with the findings of Sakamoto et al. (24), who found no significant difference between postinstrumentation samples and the samples after medication with calcium hydroxide.

The novel method of quantitative real-time PCR appears to be a promising tool with which to characterize endodontic infection both qualitatively and quantitatively. It could be useful to detect selected bacterial species in root canal systems and to evaluate various disinfection protocols during endodontic therapy. Future studies are warranted to further explore the utility of this diagnostic method in endodontics. Within the limits of the present study, it is suggested that for teeth with either primary or secondary infections a thorough chemo-mechanical preparation is able to reduce the bacterial load of root canals by at least 95%.

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