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Oral Microbiology

Molecular evaluation of residual endodontic microorganisms after instrumentation, irrigation and medication with either calcium hydroxide or Septomixine

G Tang[1], LP Samaranayake[1], H-K Yip[2]

[1]*Oral Bio-Sciences and* [2]*Oral Diagnosis, Faculty of Dentistry, The University of Hong Kong, Hong Kong*

BACKGROUND AND OBJECTIVE: The correct choice of antimicrobial agents as inter-appointment medicaments is as important as the instrumentation and irrigation to remove pathogens from infected root canals. Calcium hydroxide [$Ca(OH)_2$] and framycetin sulfate (Septomixine) are common endodontic medicaments. Therefore, we evaluated the efficacy of either calcium hydroxide or Septomixine in eliminating residual intracanal bacteria, particularly *Actinomyces* spp., during inter-appointment interval in endodontic therapy using molecular methods.

METHODS: A total of 31 single-rooted teeth with primary root canal infections were studied immediately after opening the canals and subsequently after instrumentation, irrigation with sterile saline and 1-week medication with either $Ca(OH)_2$ ($n = 25$) or Septomixine ($n = 6$). Whole bacterial genomic DNA was isolated directly from samples and PCR with universal primers performed to detect total intra-canal bacteria. The variable regions of 16S rDNA of bacteria were amplified and labeled with digoxigenin for further hybridization to detect *Actinomyces* spp. A total of seven oligonucleotide probes specific for *A. bovis*, *A. gerencseriae*, *A. israelii*, *A. meyeri*, catalase-negative *A. naeslundii* (genospecies 1 and 2), catalase-positive *A. naeslundii* genospecies 2 and *A. odontolyticus* were used to detect *Actinomyces* spp. in 22 of 31 medicated root canals [$Ca(OH)_2$: $n = 17$; Septomixine: $n = 5$].

RESULTS: The PCR results showed that 25 of 31 examined canals were positively detected with residual microorganisms after instrumentation, irrigation with sterile saline and 1-week medication with either $Ca(OH)_2$ ($n = 20$) or Septomixine ($n = 5$). Thus, only six canals [$Ca(OH)_2$: $n = 5$, Septomixine: $n = 1$] were aseptic after treatment. Hybridization results showed higher detection frequency of both *A. odontolyticus* and *A. gerencseriae* after treatment. Significant correlation was found between exposed pulp before treatment and positive detection of *Actinomyces* spp., particularly *A. odontolyticus* on the second visit ($P < 0.05$).

CONCLUSION: The conventional, 1-week medication of either $Ca(OH)_2$ or Septomixine in endodontic therapy may not effectively inhibit residual bacterial growth in all root canals during inter-appointment intervals. Further investigations using, for instance quantitative real-time PCR analyses, are required to substantiate the present findings.

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Keywords: calcium hydroxide; framycetin sulfate; endodontic therapy; bacteria; *Actinomycetes*; polymerase chain reaction; oligonucleotide-DNA hybridization

Introduction

The main purpose of endodontic therapy is to eliminate infecting or contaminant bacteria from root canals. Therefore, the correct choice of antimicrobial agents for inter-appointment medication is as important as the instrumentation and irrigation of canals to remove the etiological pathogens. Of the different inter-appointment endodontic medicaments, calcium hydroxide is perhaps the most widely used (Stuart *et al*, 1991; Georgopoulou *et al*, 1993; Siqueira and Lopes, 1999; Peters *et al*, 2002a) and its antibacterial effect is mainly due to the release of free hydroxyl radicals (Siqueira and Lopes, 1999). Some *in vitro* studies have shown that calcium hydroxide is highly active against anaerobes and this activity was significantly higher than other commonly used intra-canal dressings, such as paramonochlorophenol (Georgopoulou *et al*, 1993) and formocresol (Stuart *et al*, 1991).

However, others have argued that calcium hydroxide may not be so effective in disinfecting complex dentinal systems *in vivo*, because proton donors within dentine,

Correspondence: Dr Hak-Kong YIP, Room 1A23, Faculty of Dentistry, The University of Hong Kong, 34 Hospital Road, Hong Kong. Tel: 852 2859 0286, Fax: 852 2547 0164, E-mail: hkyip@hkusua.hku.hk
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such as $H_2PO_4^-$, H_2CO_3 and HCO_3^-, could neutralize hydroxyl ions and keep the pH unchanged (Wang and Hume, 1988; Nerwich *et al*, 1993). Further, the necrotic tissue debris and/or cells could hamper the action of hydroxyl ions within the endodontic and dentinal milieu (Siqueira and Lopes, 1999). For example, some workers have found viable bacteria within the dentinal tubules even after extended period of medication with calcium hydroxide (Safavi *et al*, 1990; Weiger *et al*, 2002). Interestingly, Haapasalo *et al* (2000) found that the antimicrobial effect of calcium hydroxide could be neutralized *in vitro* by dentine powder.

The use of antibiotics as medicaments for endodontic therapy is also controversial. There is now a consensus that systemic delivery of antibiotics in the management of endodontic infections in otherwise healthy patients, either for therapeutic (Nagle *et al*, 2000; Sunde *et al*, 2002) or prophylactic purpose (Pickenpaugh *et al*, 2001) does more harm than good. However, the topical use of antibiotic-laced dressings for endodontic therapy remains controversial (Longman *et al*, 2000). Of the various antibiotic-containing dressings, Septomixine is one of the commonest commercial paste preparations available for inter-appointment medication (Abbott *et al*, 1990), although its clinical efficacy is little documented. The main components of Septomixine are framycetin sulfate and hydrocortisone acetate. Framycetin, a broad-spectrum aminoglycoside antibiotic, is active against many gram-negative aerobes and a few gram-positives, but inactive against fungi and most anaerobes (Egle, 1995). However, hydrocortisone essentially helps minimize inflammation and post-treatment pain (Negm, 2001).

Of the various bacteria involved in endodontic infections, gram-positive, pleomorphic, anaerobic *Actinomyces* spp. are commonly isolated from infected root canals (Sunde *et al*, 2000; Siqueira *et al*, 2002), unsuccessfully treated canals (Sjögren *et al*, 1997), as well as from refractory apical periodontitis cases (Sunde *et al*, 2002). Most *Actinomyces* spp. are considered lowly virulent, as they survive in human tissue for long periods without causing major morbidity. The main contribution of this genus to endodontic infections appears to be their ability to adhere to intra-canal surfaces that create a foundation for secondary colonizers, including anaerobes with high virulence, such as *Peptostreptococcus micros* (Peters *et al*, 2002b), *Prevotella intermedia* (Drucker *et al*, 1992) and *Fusobacterium nucleatum* (Jansen and van der Hoeven, 1997). Whereas some *in vitro* studies have revealed that calcium hydroxide is lethal for *Actinomyces* spp. (Stuart *et al*, 1991; Georgopoulou *et al*, 1993; Barnard *et al*, 1996), others however, have noted its limited microbicidal activity against these organisms *in vivo* (Peters *et al*, 2002a; Sunde *et al*, 2002). Due to these conflicting data, it would be instructive to evaluate the *in vivo* activity of calcium hydroxide as well as Septomixine against *Actinomyces* spp. using newly available molecular diagnostic tools.

Molecular detection techniques have been emerging as one of the most important diagnostic methods replacing the traditional cell culture technique, as the latter does not detect 'viable but non-culturable bacteria' in a 'dormant' state (Decker, 2001; Weiger *et al*, 2002). The latter is a reversible, intermediate state between active metabolism and death or cell-lysis, yet the dormant bacteria pose a threat of infections (Oliver, 1995). Thus, molecular evaluation of antibacterial efficacy of endodontic medicaments in comparison with the conventional agar culture technique should yield more accurate information.

Therefore, we employed (i) direct DNA extraction and PCR technique with universal primers to evaluate total intra-canal microorganisms, and (ii) PCR-based DNA-oligonucleotide hybridization to detect *Actinomyces* spp., before and after a 1-week medication with either calcium hydroxide or Septomixine. For hybridization technique, we used previously developed oligonucleotide probes specific for 16S rDNA belonging to the following *Actinomyces* spp., namely *A. bovis*, *A. gerencseriae*, *A. israelii*, *A. meyeri*, catalase-negative *A. naeslundii* (genospecies 1 and 2), catalase-positive *A. naeslundii* genospecies 2 (or previous *A. viscosus* serotype II) and *A. odontolyticus* (Tang *et al*, 2003).

Materials and methods

Patient selection

A total of 31 teeth in 27 patients otherwise in good general health (19 females and eight males), referred to the Prince Philip Dental Hospital, Hong Kong for root canal treatment, were selected for this study. All 31 teeth (22 incisors, two canines and seven premolars) were single-rooted, with primary root infection and, had not been subjected to previous endodontic treatment. The age of the 27 patients ranged from 17 to 73 years (mean age: 35 years; median age: 37 years). None of the subjects had taken antibiotics 3 months prior to this investigation, and none was prescribed systemic antibiotics during the investigative treatment period. Informed consent was obtained from all patients prior to the study that had approval from the institutional ethics committee.

First clinical sampling

On the first visit, the tooth was cleansed with pumice and a rubber dam was applied to isolate the operative area. The operative and surrounding areas were disinfected with 0.2% chlorhexidine gluconate (Panyu National Pharm. Co. Ltd, Guangzhou, China) (Sunde *et al*, 2000). An initial access cavity was prepared by removing bulk of the teeth or the restoration, using a sterile high-speed tungsten carbide bur (Beavers Dental Products Ltd, Morrisburg, Ontario, Canada). Before opening the pulp chamber, the access cavity was disinfected with 0.2% chlorhexidine gluconate. Another sterile low-speed bur (Maillefer, Ballaigues SA, Switzerland) was applied to open the pulp chamber. Afterwards, two sterile paper points (Diadent group Int'L, Chongiu, Korea) were inserted into the root canal consecutively, and each allowed remaining in the canal for 3 min. If the root canal was dry, sterile saline was introduced into the canal using a sterile syringe without overfilling the canal, before paper points were inserted into the canal. The paper points were

Table 1 Sequence of probes and universal primers specific for 16S rDNA (Tang *et al*, 2003)

Specificity	*Position*	*Sequence (5′–3′)*	*Genebank accession number*
Actinomyces bovis	196–213	tttttttttttttttttttcccaaccagaaaaagaac	X81061
A gerencseriae	170–188	tttttttttttttttttttcaaaaacaccaaacagtgc	X80414
A. israelii	109–127	tttttttttttttttttttcaaaaacaccacaaaagtg	M33912
A. meyeri	980–994	tttttttttttttttttttccgcacgcccagggc	X82451
A. naeslundii[a]	94–110	tttttttttttttttttttcaccaaaaaaggcgcca	M33911
A. odontolyticus	880–895	tttttttttttttttttttcagtgccgccgtgcat	M33910
A. viscosus[b]	163–179	tttttttttttttttttttcaaacctttcccaggcc	X82453
Universal probe	1020–1037	tttttttttttttttttttatggttgtcgtcagctc	X82453
Forward primer	70–90	gggtgagtaacacgtgagtaa	X82453
Reverse primer	1037–1054	cgagctgacgacaaccat	X82453

[a]The *A. naeslundii* probe targets catalase-negative *A. naeslundii* (genospecies 1 and 2).
[b]The *A. viscosus* probe targets *A. viscosus* (previous *A. viscosus* serotype I), as well as catalase-positive *A. naeslundii* genospecies 2 (previous *A. viscosus* serotype II).

removed from each canal and inserted into 200 μl sterile phosphate buffered saline (PBS, pH 7.2) in a 1.5-ml microcentrifuge tube, on ice, for genomic DNA isolation.

After the first sampling, the canals were enlarged with files to at least Size 35, irrigated with sterile saline and, medicated with either calcium hydroxide (Calasept, NordiskaDental, Angelholm, Sweden) ($n = 25$) or Septomixine (Septodont, St Maur Des Fosses, France) ($n = 6$). Afterwards, the pulp space was sealed with sterile cotton pellets and reinforced zinc oxide eugenol, intermediate restorative material (IRM; Caulk/Dentsply, Milford, DE, USA).

Second clinical sampling

After a 1-week period of medication, the patient returned for a second sampling that was performed as before with the operative area isolated with rubber dam. Again, the operative area was disinfected with 0.2% chlorhexidine gluconate, and the canal was accessed aseptically. The calcium hydroxide or Septomixine dressing was removed by rinsing with sterile saline. The second sampling was performed using the identical procedure described above. All 31 temporary restorations with IRM were found to be intact on the second visit, prior to the second sampling.

On both visits, the clinical signs associated with the treated teeth were noted. These clinical signs included pain and/or swelling, tenderness to percussion, sinus tract, presence of abscess, periapical radiolucency, cavitated/filled teeth, pulp exposure, abutment and traumatic history. All sampling as well as endodontic treatment was performed by the same operator.

Direct whole genomic DNA isolation

Each sample was transferred to the laboratory for total genomic DNA isolation within 30 min. Before DNA isolation, the tubes with paper points were vigorously vortexed for 30 s in order to ensure disaggregation of all bacteria into the PBS solution. Afterwards, the paper points were aseptically removed from the suspension and, the bacterial suspension (including the pulpal cellular matter) was pelleted by centrifugation for 10 min at 5000 *g*. Afterwards, the pellet was resuspended in 180 μl of buffer ATL supplied by QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). Subsequently, total bacterial genomic DNA was isolated, according to the QIAamp DNA Mini Kit protocol.

Polymerase chain reaction

A total of 62 whole genomic DNA samples obtained from 31 canals, before and after medication, were amplified with a pair of universal primers specific for 16S rDNA (Table 1). The PCR reaction were performed using GeneAmp PCR system 9700 (Perkin-Elmer, Foster City, CA, USA) as follows: pre-denaturation at 94°C for 5 min; denaturation at 94°C for 1 min; annealing at 57°C for 1 min; elongation at 72°C for 1 min; and a run of 25 cycles. Final elongation was at 72°C for 10 min. The final volume of each PCR reaction was 50 μl, which contained 1.5 mM of $MgCl_2$, 200 μM of each deoxynucleoside triphosphates (dNTP; GibcoBRL, Gaithersburg, MD, USA) or 5 μl PCR DIG labeling mix (Roche Molecular Biochemicals, Mannheim, Germany) containing 200 μM dATP, dCTP, dGTP each, 190 μM dTTP and 10 μM digoxigenin-11-dUTP, 0.5 μM of each primer, 1.5 unit of AmpliTaq Gold polymerase (Roche Molecular Systems, Branchburg, NJ, USA), and 1 μl of DNA template. The DNA content of unlabeled PCR products was used for evaluation of total bacteria and, digoxigenin-labeled PCR products for further detection of *Actinomyces* spp. with Southern hybridization.

Agarose gel electrophoresis of PCR for evaluation of total bacterial load

The PCR amplicons of 16S rDNA of mixed genomic DNA obtained from the first and second visits were compared. One percent (w/v) agarose gel was prepared and, 10 μl of PCR products were loaded. After electrophoresis and followed by ethidium bromide staining, the PCR profiles of the first and second visits of the same root canal were compared using gel visualization system (Bio-Rad Laboratory, Hercules, CA, USA).

Oligonucleotide-DNA hybridization for evaluation of Actinomyces spp.

Among the 62 PCR samples (obtained from 31 canals), 44 of them (obtained from 22 canals) were labeled with

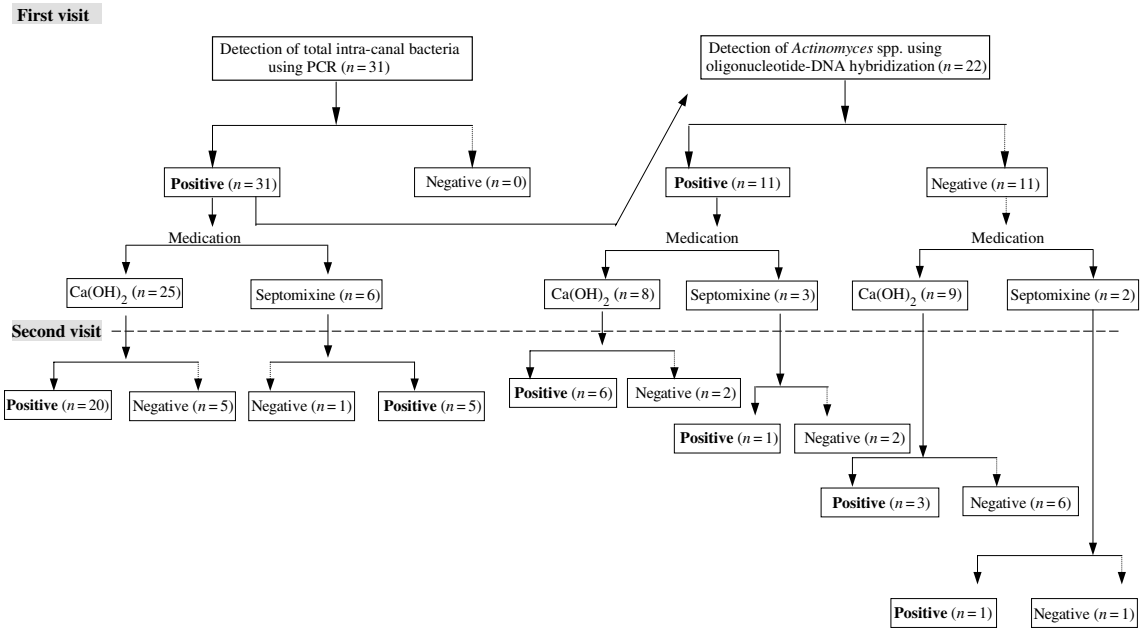

Figure 1 A flow chart illustrating the PCR and hybridization analyses of specimens used in the study and the results obtained

digoxigenin (Roche Molecular Biochemicals) for further hybridization detection. Of the latter 22 canals, 17 were medicated with calcium hydroxide and, the remainder with Septomixine (Figure 1). The oligonucleotide-DNA hybridization assay was performed using the Minislot^{TM} 10 and Miniblotter 28SL system (Immunetics, Cambridge, MA, USA) as per our previous study (Tang *et al*, 2003). In brief, probes were applied into the horizontal wells of the Minislot^{TM} 10, and cross-linked to the Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) by ultraviolet irradiation. The membrane was prehybridized in DIG Easy Hyb (Roche Molecular Biochemicals) at 40°C overnight, and then about 50–500 ng μl^{-1} digoxigenin-labeled PCR products in 100 μl of hybridization solution was loaded, and the hybridization was performed at 40°C overnight.

After hybridization, the membrane was washed, blocked using DIG Wash and Block Buffer Set (Roche Molecular Biochemicals). Prior to detection, the membrane was incubated in anti-Digoxigenin-AP, Fab fragments (1:5000) (Roche Molecular Biochemicals) and ultra-sensitive chemiluminescent substrate CDP-star (1:100) (Roche Molecular Biochemicals). Finally, a square of X-ray film (10 cm × 7.5 cm) (Amersham Pharmacia Biotech) was exposed to the membrane in a cassette in order to detect the hybrids.

Data analysis

Fisher's exact test and relative risk with 95% confidence interval (CI) were used to evaluate instrumentation, irrigation with sterile saline and 1-week medication with either calcium hydroxide or Septomixine on total intra-canal bacteria and *Actinomyces* spp. Fisher's exact test and odds ratio (OR) were used to evaluate the association between clinical signs obtained on the first visit and the presence of microorganisms on the second visit. The statistical analysis was processed with GraphPad InStat software (GraphPad InStat version 3.00 for Windows; GraphPad Software, San Diego, CA, USA). $P < 0.05$ and relative risk > 1 (or odds ratio > 1) with the whole of the 95% CI > 1 were considered statistically significant.

Results

Antimicrobial effects of Septomixine and calcium hydroxide

PCR was performed to evaluate the total intra-canal bacteria before and after instrumentation, irrigation with sterile saline and 1-week medication, and the obtained PCR profiles of the pre- and post-treatment visits compared. All the examined 31 root canals were positive for bacterial DNA on the first visit. On the second visit, the PCR results demonstrated that six of 31 canals were negative (19.35%) (calcium hydroxide: $n = 5$; Septomixine: $n = 1$) while the remainder were positive for PCR indicating the presence of bacteria despite instrumentation, irrigation and medication with either calcium hydroxide or Septomixine (calcium hydroxide: $n = 20$; Septomixine: $n = 5$) (Figure 2).

On statistical analysis, Fisher's exact test showed no significant difference in antimicrobial efficacy between calcium hydroxide and Septomixine, when used as endodontic medicaments ($P = 0.63$). It was further found that, compared with Septomixine, after medication with calcium hydroxide, the relative risk of positive detection of intra-canal bacteria was 0.96 with 95% CI from 0.64 to 1.44 (Table 2).

Antimicrobial effects of Septomixine and calcium hydroxide on Actinomyces spp.

The distribution of the examined *Actinomyces* spp. was evaluated in 22 cases (calcium hydroxide: $n = 17$; Septomixine: $n = 5$) that were PCR-positive for bac-

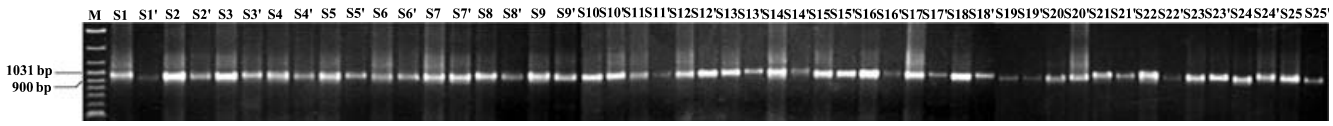

Figure 2 Evaluation of intra-canal total bacteria before (the first visit) and after instrumentation, irrigation with sterile saline and medication (the second visit). A total of 31 root canals were detected for bacteria on the first visit. On the second visit: 25 of 31 were positive [$Ca(OH)_2$: 20; Septomixine: 5] indicating the presence of bacteria despite the above treatment (S1–S25; S: first visit; S′: second visit)

Table 2 Comparison of residual endodontic microorganisms on the second visit

PCR evaluation of intra-canal bacteria	*Calcium hydroxide (n = 25) (%)*	*Septomixine (n = 6) (%)*	*Total (n = 31) (%)*
Negative detection	5 (16.13)	1 (3.22)	6 (19.35)
Positive detection	20 (64.52)[a]	5 (16.13)	25 (80.65)
Total	25 (80.65)	6 (19.35)	31 (100)

Fisher's exact test: antimicrobial efficacy between $Ca(OH)_2$ and Septomixine ($P = 0.63$).
[a]Compared with Septomixine, the relative risk of positive detection of intra-canal bacteria after medication with $Ca(OH)_2$ was 0.96 with 95% confidence interval from 0.64 to 1.44.

teria, both before and after treatment. The hybridization profiles of the detected *Actinomyces* spp. in the 22 pairs of samples are shown that in four of 22 samples (18.18%) (calcium hydroxide: $n = 3$; Septomixine: $n = 1$), *Actinomyces* spp. were not detected on the first visit, but the second visit. Very importantly, *A. odontolyticus* and *A. gerencseriae* were detected with higher frequency (9/22 *vs* 7/22; 2/22 *vs* 1/22, respectively) on the second visit than the first (Figure 3). Fisher's exact test showed no significant difference between the antimicrobial efficacy of calcium hydroxide and Septomixine on the examined *Actinomyces* spp. ($P > 0.05$, Table 3).

When the detection of residual *Actinomyces* spp. on the second visit and the associated clinical signs were analyzed, a statistically significant association was noted between pulp exposure prior to treatment and the presence of residual *Actinomyces* spp. (Fisher's exact test: $P = 0.04$), particularly *A. odontolyticus* ($P = 0.02$). Furthermore, the OR analyses also suggested that detection of residual *A. odontolyticus* (OR = 24.23, 95% CI: 1.10–532.42) and *A. meyeri* (OR = 34.20, 95% CI: 1.08–1080.2; Fisher's exact test: $P = 0.10$) after instrumentation, irrigation and 1-week medication might be related with pulp exposure before treatment. However, detection of residual *A. israelii* on the second visit marginally correlated with the stress of abutment teeth (odds ratio = 34.20; 95% CI: 1.08–1080.2; Fisher's exact test: $P = 0.10$). However, the wide 95% CI also suggests the necessity for further clarification of this issue with a larger clinical sample (Table 4).

Discussion

Calcium hydroxide has been extensively used as an inter-appointment medicament for endodontic therapy since 1920s and, its antimicrobial efficacy closely associated with the release and diffusion of hydroxyl radicals leading to a high alkaline microenvironment (pH 12.5). In order to exert optimal microbicidal effects, a high level of hydroxyl ions should penetrate all niches of the root canals, including the exposed dentinal tubules and their ramifications. A failure in complete penetration and/or buffering action of dentine against hydroxyl ions reaching optimal microbicidal pH (Nerwich *et al*, 1993)

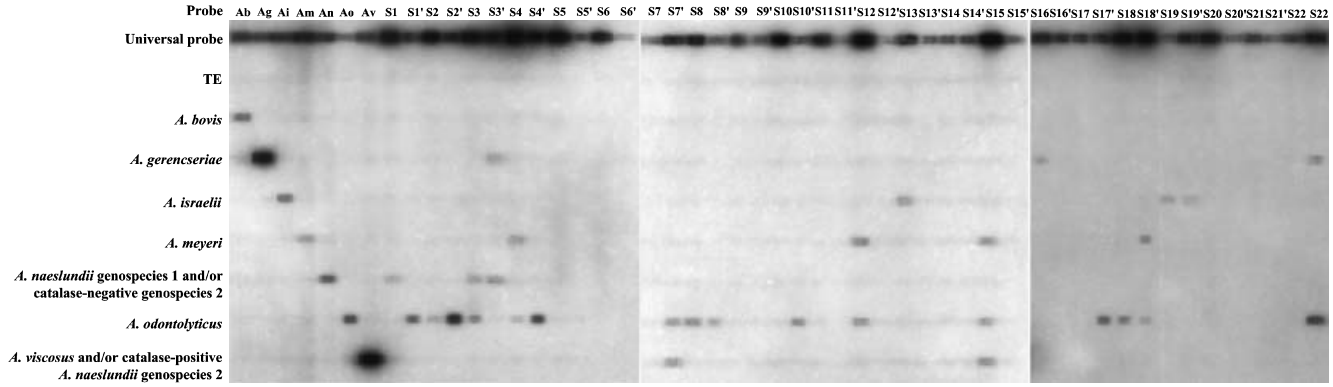

Figure 3 Distribution of the examined *Actinomyces* spp. in 22 cases before and after treatment ($Ca(OH)_2$: $n = 17$; Septomixine: $n = 5$). The horizontal lanes contained probes (TE: 10 mM Tris–HCl with 1 mM EDTA, pH 8.0). The vertical lanes contained digoxigenin-labeled PCR products of positive controls of American Type Culture Collection's (ATCC) reference strains and 22 pairs of clinical samples (S1–S22). Ab: *A. bovis* (ATCC13683); Ag: *A. gerencseriae* (ATCC23860); Ai: *A. israelii* (ATCC 10048); Am: *A. meyeri* (ATCC35568); An: *A. naeslundii* (ATCC12104); Ao: *A. odontolyticus* (ATCC17929); Av: *A. viscosus* (ATCC15987). Some canals were not positive for *Actinomyces* spp. on the first visit, but on the second visit. Very importantly, *A. odontolyticus* and *A. gerencseriae* were detected with higher frequency (9/22 *vs* 7/22; 2/22 *vs* 1/22, respectively) on the second visit than the first visit

Table 3 *Actinomyces* spp. distribution in 22 canals [$Ca(OH)_2$: $n = 17$; Septomixine: $n = 5$] before and after treatment (the number of canals medicated with Septomixine in the parentheses)

Specification	*First visit*	*Second visit*	*Only positive on the first visit*	*Only positive on the second visit*
Actinomyces spp.	11 (3)	11 (2)	4 (2)	4 (1)
A. odontolyticus	7 (1)	9 (1)	3 (1)	5 (1)
A. meyeri	3 (1)	1 (0)	3 (1)	1 (0)
A. israelii	2 (1)	1 (1)	1 (0)	0
A. naeslundii	3 (0)	2 (0)	2 (0)	0
An (C^{-ve})	2 (0)	1 (0)	1 (0)	0
An (C^{+ve})	1 (1)	1 (0)	1 (1)	1 (0)
A. gerencseriae	1 (1)	2 (1)	1 (1)	2 (1)
A. bovis	0	0	0	0

An (C^{-ve}): catalase-negative *A. naeslundii* (genospecies 1 and 2); *An* (C^{+ve}): catalase-positive *A. naeslundii* genospecies 2. Fisher's exact test: comparison of the antimicrobial efficacy between Septomixine and $Ca(OH)_2$ on *Actinomyces* spp. ($P > 0.05$).

could lead to potential bacterial reservoirs initiating secondary infection and failure of endodontic therapy.

Antimicrobial agents often function by disrupting basic biological processes, such as DNA, protein and cell-wall biosynthesis. The hydroxyl radicals released from calcium hydroxide lethally affect bacteria probably due to the following three mechanisms: (i) inducing splitting of DNA strands, consequently inhibiting DNA replication and disturbing cellular activity (Imlay and Linn, 1988; Nunoshiba *et al*, 1999); (ii) inducing lipid peroxidation, resulting in the destruction of phospholipids, components of the cellular membrane, and subsequent loss of unsaturated fatty acids and extensive membrane damage (Kohen and Shalhoub, 1994) and (iii) denaturation of proteins and disruption of cellular metabolism.

Framycetin (Septomixine), a member of aminoglycoside antibiotics that are used most widely against gram-negative enteric bacteria, belongs to protein synthesis inhibitors due to their irreversible binding to ribosome subunits. But before exerting their lethal effects on cells, aminoglycosides should penetrate the membrane, and this process involves both passive and active transportation. The initial event is passive diffusion via porin channels across the outer membrane and facilitated by antibiotics, e.g. β-lactams. The subsequent active transportation is, however, energy- and oxygen-dependent and cannot occur under anaerobic conditions, which suggests aminoglycosides may be ineffective against anaerobes without a synergistic drug (Egle, 1995). Therefore, in theory, Septomixine appears to be a less than satisfactory antibiotic for complex mixed infections with predominant anaerobes, as seen in endodontic infections.

Of the 31 examined canals in our study, only six (calcium hydroxide: $n = 5$; Septomixine: $n = 1$) were aseptic in term of the PCR findings, after instrumentation, irrigation with sterile saline and a 1-week period of medication and, the other 25 cases were positive for residual microorganisms. Further detailed studies, for instance with quantitative real-time PCR analyses may

Table 4 Detection of microorganisms on the second visit and associated clinical signs (odds ratio/95% confidence interval in the parentheses)

At the second visit	*Pain and/or swelling*	*Tenderness to percussion*	*Sinus tract*	*Abscess*	*Periapical radiolucency*	*Cavitated/filled teeth*	*Pulp exposure*	*Abutment*	*Traumatic history*	*Total*
Total microorganism	17 (1.06/ 0.16–7.07)	16[a]	7 (1.94/ 0.19–19.75)	7 (1.94/0.19–19.75)	21 (5.25/0.77–35.99)	16[a]	3 (2.02/0.09–44.43)	3 (2.02/0.09–44.43)	5 (3.49/0.17–71.99)	25
Actinomyces spp.	8 (1.44/ 0.29–7.22)	7[a]	2[a]	2[a]	9 (1.50/0.24–9.41)	7[a]	**3 (16.88/0.78–363.65)** *	1[a]	2 (1.26/0.18–8.97)	11
A. odontolyticus	7 (2.00/ 0.33–12.05	7 (2.00/ 0.33–12.05	1[a]	1[a]	8 (3.00/0.31–29.37)	6 (1.14/0.22–5.87)	**3 (24.23/1.10–532.42)*[b]**	0	2 (1.81/0.25–13.22)	9
A. gerencseriae	1[a]	1[a]	0	0	2 (1.67/0.07–38.8)	2 (3.11/0.14–70.75)	1 (13.50/0.59–306.52)	0	0	2
A. naeslundii	0	0	0	0	2 (1.67/0.07–38.8)	2	0	0	0	2
An (C^{-ve})	0	0	0	0	1[a]	1 (1.77/0.07–47.18)	0	0	0	1
An (C^{+ve})	0	0	0	0	1[a]	1 (1.77/0.07–47.18)	0	0	0	1
A. israelii	1 (1.54/ 0.06–41.11)	0	1 (9.40/ 0.34–256.21)	1 (9.40/0.34–256.21)	1[a]	0	0	**1 (34.20/1.08–1080.2)[b]**	0	1
A. meyeri	1 (1.54/ 0.06–41.11)	1 (1.54/ 0.06–41.11)	0	0	1[a]	1 (1.77/0.07–47.18)	**1 (34.20/1.08–1080.2)[b]**	0	0	1
Total	21	21	8	8	24	20	3	3	5	31

*Fisher's exact test: $0.01 < P < 0.05$.
[a]Odds ratio < 1.0.
[b]Both the odds ratio and the whole of the 95% confidence interval are greater than 1.

be necessary to evaluate whether the re-growth of intracanal bacteria during inter-appointment intervals has equaled or exceeded the original pre-treatment levels in the latter situation. In this study, the first sample was taken immediately after exposing the pulp chamber but prior to excavation of pulpal debris and, the second sample taken just after the medicaments were removed. Therefore, our data indicate that bacterial load in a majority of the canals (80.65%) could not be totally eradicated after instrumentation, irrigation with sterile saline and a 1-week period of medication.

In order to evaluate the efficacy of medicaments alone, we used sterile saline throughout for irrigating the instrumented canals, instead of the traditional sodium hypochlorite (Spångberg, 2002) or the more recently suggested chlorhexidine (Vianna *et al*, 2004). Thus, the high detection frequency of microorganisms after medication could be partly attributed to the rather bland irrigant we used in this study. It is well recognized that an ideal endodontic irrigant should be microbicidal in addition to having the ability to dissolve organic tissue remnants (Vianna *et al*, 2004). Obviously, sterile saline does not fulfill the above requirements except for its mechanical washing action of debris.

Interestingly, Peters *et al* (2002a) also found that using conventional microbiology technique of assessing the colony-forming unit(s) of cultivable bacteria in canals after a 4-week medication with calcium hydroxide, the mean number of viable bacteria were consistently higher than after instrumentation and irrigation with sodium hypochlorite, but lower than before treatment. It appears that the routine 1-week medication with either calcium hydroxide or Septomixine in a sealed pulp after instrumentation and irrigation may maintain, at most, the endodontic environment in a relative 'stable' status during the inter-appointment period. These two medicaments, however, could not further disinfect canals. Therefore, the clinical procedure of 1-week medication may be not very successful, comparing with instrumentation and irrigation.

There are two possible reasons for the failed inhibition of bacteria with calcium hydroxide: (i) rapid absorption of calcium hydroxide leading to a pH fall to neutral levels and (ii) bacteria that are pocketed from the action of the chemical by virtue of their survival and/or growth within dentinal tubules or their ramification and microleakage of temporary filling materials between appointments. The interim restorative endodontic material used in this study was zinc oxide eugenol IRM, resin-forced zinc oxide cement. It is one of the commonest interim endodontic restoration materials (Kazemi *et al*, 1994). The most important role of interim endodontic restorations is to prevent bacteria and their products re-contaminating pulp spaces. IRM has been reported to have less body penetration, compared with other interim restorative endodontic materials such as Tempit (Centrix Inc., Milford, CT, USA) and Cavit (Premier Dental Products Co., Philadelphia, PA, USA), but cannot prevent substantial marginal microleakage (Barkhordar and Stark, 1990; Kazemi *et al*, 1994). Spångberg (2002) indicated that the eugenol content of IRM may be able to provide a bacterial barrier but allow leakage of other liquid substances. Other studies, however, have showed that bacterial leakage could not also be prevented by IRM, even for a short period (from 2 to 49 days) (Siqueira *et al*, 2001). The latter provides another possible reason for the high level of detection of bacteria on the second visit in this study (25 of 31 canals).

A previous *in vitro* study has also shown that viable, but non-culturable *Streptococcus sanguinis* in root dentine using two-color fluorescent assay despite a 12-week regimen with calcium hydroxide and, *Enterococcus faecalis* in the dentine was not affected by calcium hydroxide (Weiger *et al*, 2002). The foregoing data suggest that some bacteria have the potency to survive within the dentinal tubules for prolonged period even under medication, and could lead to chronic and potential endodontic or periapical infections (Sjögren *et al*, 1997; Sunde *et al*, 2002).

The current data show a significant association between exposed pulp before treatment and positive detection of *Actinomyces* spp., particularly *A. odontolyticus* on the second visit. Further, odds ratio analyses also tend to suggest a correlation between pulp exposure and detection of *A. meyeri* on the second visit (Table 4). The persistence of viable bacteria in root canals or dentine after instrumentation, irrigation and medication could be due to the following: (i) Bacteria may ingress into an incompletely closed chamber through a periapical lesion, deep dentinal tubules or exposed pulp. (ii) Some endodontic pathogens are not lethally affected either by calcium hydroxide or Septomixine. For example, *E. faecalis* is not affected by calcium hydroxide (Weiger *et al*, 2002), and most anaerobes are resistant to Septomixine (Egle, 1995). (iii) There may be anatomical barriers where bactericidal components of either calcium hydroxide or Septomixine cannot penetrate to generate minimum inhibitory or bactericidal concentrations. For instance, Nerwich *et al* (1993) have shown differential diffusion rates of hydroxyl ions in cervical and apical root dentine. The pH of inner cervical root dentine peaked at 10.8 within hours after calcium hydroxide insertion, whilst apically, a plateau pH of approximately 9.5 was only reached 2 weeks after the dressing was in place; and the outer root dentine pH reached peak level of about 9.0 after 2–3 weeks.

The examined *Actinomyces* spp. within different root canals were variably affected by calcium hydroxide. Thus, *A. odontolyticus*, *A. gerencseriae*, *A. naeslundii*, and *A. meyeri* were detected at a higher concentration on the post-treatment visit from some canals. However, they were also partly or totally eliminated after medication in other cases (Table 3). There are reports of *in vitro* bactericidal efficacy of calcium hydroxide against *Actinomyces* spp. For instance, *A. viscosus*, *A. naeslundii* and *A. israelii* can be killed by 2% calcium hydroxide after 30-min exposure (Georgopoulou *et al*, 1993; Barnard *et al*, 1996). However, these *in vitro* findings do not directly correlate with our *in vivo* data.

However, it could be argued that the PCR and hybridization findings of our study do not necessarily

testify to the presence of viable bacteria in post-treatment canals and, they may reflect the molecular traces of remnants of the non-viable organisms that colonized the canals. While this may be the case in a few canals, the fact is that in this study, before medication, the canals were enlarged with files to at least Size 35 and irrigated with sterile saline. Thus, it is highly likely that majority bacteria or their traces would have been eliminated after instrumentation and mechanical irrigation with stile saline on the first visit. Secondly, hydroxyl radicals from calcium hydroxide also induce DNA degradation (Imlay and Linn, 1988; Nunoshiba *et al*, 1999). Therefore, the fact that intra-canal bacteria were detected by PCR in 25 of 31 canals after chemotherapy implies the persistence and growth of bacteria despite calcium hydroxide or framycetin sulfate.

In our study, we did not find significant difference in antimicrobial efficacy between calcium hydroxide and Septomixine either on (i) the total bacteria or, (ii) the examined *Actinomyces* spp. One reason for this could be the limited sample size particularly for Septomixine. As there are no previous studies comparing the antibacterial efficacy of these two agents within the endodontic milieu, further investigations, for instance using quantitative real-time PCR analyses are needed to confirm or refute our findings.

To conclude, the routine 1-week medication of either calcium hydroxide or Septomixine in endodontic therapy may not effectively inhibit residual intra-canal bacterial growth during inter-appointment intervals. As the use of oligonucleotide probes to detect *Actinomyces* spp. in intra-canal specimens appears to be successful, this method could be further extended to evaluate the presence of other common groups of intra-canal bacteria and their role in endodontic infections.

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