Cultivable microbial flora associated with persistent periapical disease and coronal leakage after root canal treatment: a preliminary study

V. Adib, D. Spratt, Y.-L. Ng & K. Gulabivala

Unit of Endodontology, Eastman Dental Institute for Oral Health Care Sciences, University College London, London, UK

Abstract

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Aim To identify the cultivable bacterial flora in root filled teeth with persistent periapical lesions and to locate their distribution within the root canal system using an *in vitro* sampling protocol.

Methodology Eight freshly extracted root filled teeth were collected from the Oral and Maxillo-facial Surgery Department, Eastman Dental Hospital. Seven teeth were associated with persistent apical periodontitis and also showed evidence of coronal leakage. Teeth were transferred to an anaerobic chamber immediately after careful extraction and sectioned transversely to give a crown (in all but one case) and two root segments (coronal and apical). Two samples were obtained from each segment, one from dentine and the other from the restoration or gutta-percha (GP) root filling (46 sites in total). The samples were dispersed, serially diluted and cultured on blood agar and fastidious anaerobic agar (with 5% defibrinated horse blood). The primary growth was subcultured to obtain pure isolates, which were identified by routine microbiological techniques and commercial enzyme tests.

Results A total of 252 strains were isolated from all the teeth. Of all the isolates, the most prevalent bacteria were Gram-positive facultative anaerobes 189/252 (75%) with staphylococci (48/252, 19%), streptococci (44/252, 17%), enterococci (20/252, 8%) and *Actinomyces* species (20/252, 8%) being found in most of the teeth (6/8, 6/8, 5/8, 5/8 and 7/8, respectively). Of the obligate anaerobes (17%), peptostreptococci (7%) were also present in most teeth (7/8). A statistical association between bacterial flora and site (crown/coronal/apical) or surface (dental/GP/restoration) could not be shown.

Conclusions The predominant group of bacteria in root filled teeth with persistent apical periodontitis and coronal leakage was Gram-positive facultative anaerobes of which staphylococci followed by streptococci and enterococci were the most prevalent.

Keywords: bacteria, endodontic retreatment, microbiology, root canal.

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Introduction

Many studies have evaluated the microbial flora associated with failed root canal treatment, the majority in the last 5 years (Sirén *et al.* 1997, Waltimo *et al.* 1997, Molander *et al.* 1998, Sundqvist *et al.* 1998, Peciuliene *et al.* 2000, 2001, Cheung & Ho 2001, Hancock *et al.* 2001, Rolph *et al.* 2001, Egan *et al.* 2002, Pinheiro *et al.* 2003). The bacterial species recovered from root filled teeth may reside in accessory canals or alongside the root filling (Nair *et al.* 1990); they appear to be a subset of those found in untreated teeth although the diversity is much reduced. These studies have collectively found that when the technical quality of root canal treatment was satisfactory, not only was the diversity of residual species reduced but they were dominated by Gram-positive facultative

Correspondence: K. Gulabivala, Unit of Endodontology, Eastman Dental Institute for Oral Health Care Sciences, 256 Gray's Inn Road, London WC1X 8LD, UK (Tel.: 020-7915-1033; fax: 020-7915-1273; e-mail: k.gulabivala@eastman.ucl.ac.uk).

organisms, in contrast to the flora in previously untreated teeth with periapical disease (Wittgow & Sabiston 1975, Sundqvist 1976).

The most frequently identified species has been *Enterococcus faecalis* (Molander *et al.* 1998, Sundqvist *et al.* 1998, Hancock *et al.* 2001, Peciuliene *et al.* 2001, Pinheiro *et al.* 2003), although some studies have failed to identify it (Gomes *et al.* 1996, Cheung & Ho 2001, Rolph *et al.* 2001). Other frequently occurring species include *Propionibacterium* species, *Streptococcus* species, *Lactobacillus* species and Yeasts (Waltimo *et al.* 1997, Molander *et al.* 1998, Sundqvist *et al.* 1998, Cheung & Ho 2001, Hancock *et al.* 2001, Peciuliene *et al.* 2001, Egan *et al.* 2002).

The most important problem facing the investigators was the retrieval of samples from root canal systems obturated with root filling material, without biasing the retrieved microbial flora. Use of either mechanical instruments or organic solvents (chloroform, xylene, halothane) to remove the root filling material may potentially kill remaining microorganisms by generation of frictional heat or direct contact with the solvent (Molander *et al.* 1998). Reporting of chloroform use for root filling removal is inconsistent amongst the studies: some mention its use (Möller 1966, Gomes *et al.* 1996), others define the cases in which it was used (Engström 1964, Molander *et al.* 1998, Egan *et al.* 2002) and yet others deny its use (Sundqvist *et al.* 1998, Cheung & Ho 2001, Hancock *et al.* 2001, Peciuliene *et al.* 2001, Pinheiro *et al.* 2003). Alternative approaches have included adoption of rotary mechanical instrumentation (Rolph *et al.* 2001, Pinheiro *et al.* 2003) and sampling from extracted teeth *in vitro* which does not pose the same problems of removing root filling material (Fukushima *et al.* 1990).

Fukushima *et al.* (1990) recovered a wider spectrum of bacteria including *Eubacterium*, *Lactobacillus*, *Propionibacterium*, *Actinomyces*, *Peptostreptococcus*, *Streptococcus*, *Bacteroides*, *Fusobacterium*, *Selenomonas* and *Veillonella* species. They found mixed cultures in 52% and single culture infections in 10% of cases. It was unclear whether this was a function of sampling protocol or technical quality of previous treatment. Cheung & Ho (2001) noted that teeth with acceptable coronal restorations and technically unsatisfactory root fillings contained a broader range of bacterial species. A trend of single culture infections has also been evident in other studies: Sirén *et al.* (1997) found that a third of *E. faecalis* cases were in pure culture, Waltimo *et al.* (1997) found 13% of yeasts in pure culture, Sundqvist *et al.* (1998) found single species infections in 19 cases, Molander *et al.* (1998) found only one to two strains in 85% of the teeth and Peciuliene *et al.* (2000) often found *E. faecalis* in pure culture.

Treatment failure is due to residual bacteria, either surviving treatment protocols (Byström & Sundqvist 1981, 1983, 1985, Cavalleri *et al.* 1989, Gomes *et al.* 1996, Chávez de Paz *et al.* 2003) or introduced during or after treatment, for instance because of coronal leakage (Engström 1964, Myers *et al.* 1969, Sirén *et al.* 1997, Waltimo *et al.* 1997). It is certainly established that unsatisfactory coronal restorations are associated with lower rates of complete apical healing (Heling *et al.* 2002). It would be of interest to establish the nature of the infecting flora in root filled teeth with persistent apical periodontitis where subtle evidence of coronal microleakage was evident. In particular, this was a preliminary study to identify and locate the microbial flora in such teeth using an *in vitro* sampling protocol that would reduce the potential of killing the residual bacteria in the process.

Materials and methods

Eight freshly extracted, root filled permanent teeth were collected from seven patients who attended the Oral and Maxillo-facial Surgery Department, Eastman Dental Hospital. The sample selection criteria were: (i) root canal treatment completed more than 4 years previously (or frank evidence of treatment failure if 4 years had not lapsed), (ii) clinical or radiographic evidence of apical periodontitis, (iii) absence of periodontal involvement and (iv) permanent coronal restoration with *subtle* clinical evidence of marginal leakage. All patients were examined (by VA) prior to tooth extraction to confirm presence of periapical disease and gather clinical data. Teeth were extracted as atraumatically as possible by the same operator with minimal pumping action during the procedure (Kapalas *et al.* 2002) The teeth were transferred to the anaerobic chamber in the laboratory within 10 min of the extraction. Marginal leakage and integrity were assessed by the presence of staining and the use of a probe (EXS 9; Hu-Friedy®, Chicago, IL, USA) under magnification (×10) (Stemi 2000 Stereomicroscope; Zeiss, Jena, Germany) within this time.

Bacterial sampling procedures

The laboratory processing of the teeth was carried out in an isolated chamber under strict anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) at 37 °C and 80%

relative humidity (MACS-MG-1000 Anaerobic Workstation; Don Whitley Scientific, Shipley, UK).

Attached soft tissue and calculus were removed using a scalpel and scalers (MacFarlane SMF 2/3; Hu-Friedy®), followed by surface decontamination using 30% (v/v) hydrogen peroxide solution (Sigma Chemical Ltd, Poole, UK) and 10% (w/v) povidine–iodine solution (Betadine, Seton Healthcare Group plc, Oldham, UK) according to a validated protocol (Ng *et al.* 2003); the iodine solution was inactivated by 5% (v/v) sodium thiosulphate solution (Sigma). Sterile paper points soaked in reduced transport fluid (RTF) (Syed & Loesche 1972) were used to sample the decontaminated root surface. The samples were treated as described below for the cultivation of bacteria. The tooth was sectioned transversely at the crown/restoration level and then pre-grooved longitudinally around each root with a sterile diamond disc (Prodont-Holliger, Vence, France) driven by a straight hand-piece and electric motor (Schick SM78; Georg Schick GmbH, Schemmerhofen, Germany) running at 10 000 rpm. The pre-grooved root was then split with a sterile flat plastic instrument (6 SH; Claudius ASH, Potters Bar, UK). The gutta-percha (GP) root filling material usually remained fixed to one half of the root. Bacterial samples were taken from each of the relevant surfaces; dentine, restoration or GP at three levels (crown, coronal half of the root and apical half of the root). Each sample was taken with three, white, sterile paper points (ROEKO, Langenau, Germany) soaked in RTF. A total of 46 samples were obtained from eight teeth; a single representative root sample was taken from each root site or surface from molar teeth. Neither chemicals nor heat were used to remove the GP. All samples were immediately placed in 100 μL RTF, serially diluted and cultured on fastidious anaerobic agar (FAA) medium and blood agar (BA) medium (Lab M, Bury, UK) both containing 5% v/v defibrinated horse blood. FAA plates were incubated anaerobically (80% N_2, 10% CO_2, 10% H_2) and BA plates were incubated aerobically, at 37 °C for 7 and 3 days, respectively. All primary isolates from the root canals were subcultured on appropriate media until pure strains were obtained.

Bacterial identification

The isolates were identified by a combination of routine microbiological tests and Rapid ID kits (BioMérieux, Basingstoke, UK). Initial characterization according to colony morphology, Gram stain, growth atmosphere, haemolysis, catalase and oxidase tests were performed. On the basis of the primary tests, presumptive identifications were made to genus level and the isolates were grouped accordingly. Further characterization and identification was carried out using a range of Rapid ID kits (Rapid ID 32A, Rapid ID Strep and Rapid ID Staph). Strains were grown, inoculated on to the test strips, incubated, developed and recorded according to manufacturer's instructions. API Lab software (BioMérieux) was used to ascertain strain identification on the basis of the numeric code generated.

Results

Clinical and radiographic data

Eight teeth were collected from seven adult patients, whose details are presented in Table 1. All the teeth except one (no. 1) had been treated endodontically more than 4 years previously; tooth no. 1 was included as it had obvious clinical signs of persistent periapical disease.

Clinical examination confirmed subtle marginal leakage at the restorative margins as evidenced by staining of the marginal gap and associated tooth tissue and in some areas probe penetration.

The quality of the root fillings was assessed radiographically by the presence of voids between the root filling and canal wall as well as within the body of the root filling. It was judged by each root and was found to be satisfactory for all teeth except teeth 2, 5 and 6, which were rated poor. Some roots contained no root filling because of sclerosis and in fact did not yield any bacteria; these were not considered further. In all cases the quality of the root filling was confirmed on splitting the roots. In the final analysis the microbial data were considered for a single representative root from each multi-rooted tooth.

Microbiological findings

The 'sterility control' samples yielded no bacterial growth from the decontaminated tooth surfaces.

The microbiological findings from the eight samples are presented in Tables 2–5. The number of strains and species recovered from each sampling site per tooth is presented in Table 2. The identity of the Gram-positive isolates and their location by crown (dentine/restoration), root dentine or GP is given in Table 3. The identity of the Gram-negative isolates and yeasts, with their distribution by the same sites, are given in Tables 4 and 5, respectively. All teeth in this study

Table 1 Sample details

Patients' details and tooth type[a]				Oral examination [b]				Radiographic examination			
Tooth no.	Age	Sex	Tooth type	Oral hygiene	Coronal restoration	Symptoms at the time of extraction	Crown/root fracture	No. of roots	Root type	Size of periapical lesion (mm)	Presence of post
1	26	F	UL7	Fair	Ceramo-metal crown	TTP	–	3	Mesio-Buccal[c]	7	–
									Disto-Buccal[d]	7	–
									Palatal[d]	4	–
2	32	M	LL6	Fair	Ceramo-metal crown	NIL	–	3	Mesio-Buccal[e]	7	+
									Mesio-Lingual[e]	8	–
									Distal[e]	–	–
3[f]	69	F	UR1	Fair	Not present	NIL	–	1	Single rooted[d]	2	–
4[g]	51	F	LL7	Good	Composite resin	TTP	–	3	Mesio-Buccal[c]	–	–
									Mesio-Lingual[c]	–	–
									Distal[d]	–	–
5	42	M	LR6	Fair	Amalgam	TTP	–	3	Mesio-Buccal[e]	10	–
									Mesio-Lingual[e]	10	–
									Palatal[e]	10	+
6	31	F	UR6	Fair	Ceramo-metal crown	TTP	–	3	Mesio-Buccal[e]	8	+
									Distal-Buccal[e]	8	–
									Palatal[e]	8	–
7	45	M	UL4	Good	Ceramo-metal crown	TTP	–	1	Single rooted[d]	3	+
8	45	M	UL5	Good	Temporary filling	NIL	+ (root fracture)	2	Buccal[d]	10	–
									Palatal[d]	10	–

[a]All patients had no significant medical history and none had systemic antibiotics in the previous 6 months.
[b]None of the sampled teeth had carious lesions or sinus tracts and the periodontal probing depths were within normal limits.
[c]There was no root filling in the canal because of canal sclerosis and could not be satisfactorily sampled.
[d]The quality of root filling was judged satisfactory.
[e]The quality of root filling was judged poor.
[f]Crown fractured with no restoration over the root filling.
[g]Absence of radiographic evidence of periapical lesion.
TTP, tender to percussion.

Table 2 Number of species and isolates recovered from different sites within each tooth (n = 8)

Site of sampling	Tooth 1	Tooth 2	Tooth 3	Tooth 4	Tooth 5	Tooth 6	Tooth 7	Tooth 8
Total no. of species (isolates) recovered	11 (17)	41 (77)	16 (27)	16 (25)	21 (39)	27 (51)	6 (7)	8 (9)
Crown dentine	4 (5)	6 (6)	–	7 (7)	1 (1)	7 (7)	1 (1)	2 (2)
Restoration	2 (2)	11 (11)	–	5 (5)	4 (4)	2 (2)	1 (1)	0 (0)
Coronal root dentine	3 (3)	16 (17)	6 (6)	2 (2)	8 (8)	9 (10)	1 (1)	3 (3)
Coronal root GP	2 (2)	17 (18)	11 (11)	4 (4)	3 (3)	14 (16)	2 (2)	3 (3)
Apical root dentine	2 (2)	9 (11)	1 (1)	1 (1)	13 (13)	6 (7)	1 (1)	0 (0)
Apical root GP	3 (3)	12 (14)	9 (9)	6 (6)	9 (10)	9 (9)	1 (1)	1 (1)

GP, Gutta-percha; –, absence of coronal dentine and restoration.

contained a poly-microbial flora and recovery ranged from six (tooth no. 7) to 41 species (tooth no. 2) (Table 2).

There was a predominance of facultative anaerobes (189/252, 75%), followed by obligate anaerobes (43/252, 17%), aerobes (14/252, 5.6%) and *Candida* spp. (6/252, 2.4%). Gram-positive strains (209/252, 82.1%) were more frequently found than Gram-negative strains (37/252, 14.7%). Of the facultative anaerobes, Gram-positive facultative cocci (146/252, 57.9%), *Staphylococcus* spp. (48/252, 19%), *Streptococcus* spp. (44/252, 17.5%) and *Enterococcus* spp. (20/252, 7.9%) were the most prevalent. Staphylococci (6/8 teeth) and enterococci (5/8 teeth) could be found in most teeth. Facultative anaerobic rods were the second most commonly recovered, of which *Actinomyces* spp. (20/252, 7.9%) was the most prevalent. The most frequent anaerobes were *Peptostreptococcus* species (17/252, 6.7%) whilst *Pseudomonas* species were the most frequently isolated aerobes (12/252, 4.8%).

The total number of species isolated from coronal samples was 28 whilst 54 species were isolated from root samples (Tables 3–5).

Discussion

The unique feature of this study was the use of an *in vitro* sampling protocol to retrieve bacteria from root filled teeth without risking their death by organic solvents or mechanical instrumentation. The approach, which has been used before only in previously untreated teeth (Ando & Hoshino 1990, Sato *et al.* 1993), also allowed differential sampling to gauge distribution over dentine, root filling material or restoration, in coronal or apical parts. One drawback of this protocol however, is that it can only be applied to extracted teeth, and therefore limits the range of samples that can be selected for investigation.

The validity of the collected data in this approach is dependent upon assurance that root surface contaminants had been eliminated. This was tested by 'sterility control' samples; the initial sterility of the paper points was ascertained and paper points wetted with RTF were used to sample areas of the tooth surface following the decontamination procedures. None were found to be contaminated, confirming the validity of collected data. A more aggressive sampling protocol involving dispersal of the GP itself or dentine filings may possibly have retrieved more bacteria. It was however the intention to mimic the clinical scenario of paper point sampling for comparison. There was a risk that seepage of decontaminating antibacterial agents into the root canal system via the apical foramina may have killed some bacteria but this was minimized by using judicious (but appropriate, as evidenced by sterility controls) amounts of the agent without flooding the root surface.

In this study, microbial isolates were recovered from all the teeth (8/8, 100%). This is in contrast to other reports where bacteria were recovered from only 38 to 68% of the teeth (Engström 1964, Molander *et al.* 1998). The discrepancy could be attributed to the difference in criteria for tooth selection, methods of root filling removal and microbial sampling protocol. All the teeth selected in the present study were associated with *subtle* evidence of coronal leakage (except sample 3) and periapical radiolucent lesions (except sample 4).

It has been suggested previously that oral microorganisms could enter the root canal system during inadequate isolation of the operative site, via leaking coronal restorations (during or after treatment) or during the use of the 'open drainage' technique (Engström 1964, Myers *et al.* 1969, Sirén *et al.* 1997, Waltimo *et al.* 1997). The relationship between coronal leakage and the diversity of microorganisms isolated from root filled canals has not specifically been investigated previously. Only one study had inferred that

Table 3 Number of Gram-positive isolates (209/252) recovered from different sites in all teeth (n = 8)

Bacterial species	Crown dentine	Crown restoration	Root dentine*	Root gutta-percha*	Total
Gram-positive facultative cocci					
Aerococcus viridans	0	0	1	5	6
Enterobacter sp.	0	0	0	1	1
Enterococcus durans	1	0	0	0	1
Enterococcus faecalis	2	0	7	10	19
Gemella haemolysans	0	0	1	0	1
Gemella morbillorum	1	1	3	5	10
Micrococcus luteus	0	0	3	1	4
Staphylococcus aureus	1	0	2	1	4
Staphylococcus auricularis	0	0	1	0	1
Staphylococcus epidermidis	2	2	2	7	13
Staphylococcus kloosii	1	0	1	0	2
Staphylococcus lentus	3	3	4	5	15
Staphylococcus scuiri	0	0	1	2	3
Staphylococcus simulans	0	0	0	1	1
Staphylococcus warneri	0	0	2	0	2
Staphylococcus xylosus	1	1	2	3	7
Stomatococcus mucilaginosus	0	1	2	3	6
Streptococcus acidominimus	0	1	3	4	8
Streptococcus agalactiae	0	0	0	1	1
Streptococcus alactolyticus	0	0	0	3	3
Streptococcus anginosus	0	0	0	1	1
Streptococcus gordonii	0	1	1	1	3
Streptococcus lentus	0	0	1	0	1
Streptococcus mitis	0	0	1	2	3
Streptococcus mutans	0	0	3	2	5
Streptococcus oralis	0	1	2	3	6
Streptococcus parasanguinis	0	0	3	2	5
Streptococcus sanguinis	0	0	3	3	6
Streptococcus simulans	0	0	0	1	1
Streptococcus uberis	0	0	1	0	1
Not identified	4	0	0	2	6
Subtotal	16	11	50	69	146
Gram-positive facultative rods					
Actinomyces myeri	0	2	2	2	6
Actinomyces naselundii	1	0	0	1	2
Actinomyces odontolyticus	0	1	3	1	5
Actinomyces viscosus	0	1	4	2	7
Bacillus spp.	0	0	2	0	2
Lactobacillus spp.	2	1	0	2	5
Propionibacterium acnes	1	1	1	1	4
Propionibacterium propionicus	0	0	0	2	2
Not identified	0	0	0	1	1
Subtotal	4	6	12	12	34
Gram-positive anaerobic cocci					
Leptobacillus	0	1	0	0	1
Peptostreptococcus magnus	1	4	6	5	16
Peptostreptococcus micros	0	0	1	0	1
Subtotal	1	5	7	5	18
Gram-positive anaerobic rods					
Eubacterium lentum	0	0	0	2	2
Eubacterium limosum	0	0	1	0	1
Eubacterium spp.	0	0	2	2	4
Bifidobacterium spp.	0	0	1	1	2
Subtotal	0	0	4	5	9

Table 3 Continued

Bacterial species	Crown dentine	Crown restoration	Root dentine*	Root gutta-percha*	Total
Gram-positive aerobic cocci					
Not identified	1	0	0	0	1
Gram-positive aerobic rods					
Not identified	0	1	0	0	1
Total	22	23	73	91	209

*Apical and coronal root samples pooled.

Table 4 Number of Gram-negative isolates (37/252) recovered from different sites of all teeth (n = 8)

Bacterial species	Crown dentine	Crown restoration	Root dentine*	Root gutta-percha*	Total
Gram-negative facultative cocci					
Not identified	1	1	0	2	4
Gram-negative facultative rods					
Capnocytophaga spp.	0	0	3	2	5
Subtotal	1	1	3	4	9
Gram-negative anaerobic cocci					
Veillonella spp.	1	0	2	3	6
Gram-negative anaerobic rods					
Prevotella spp.	1	0	1	1	3
Bacteriodes distasonis	0	0	1	0	1
Black pigmented anaerobes	0	0	0	1	1
Fusobacterium nucleatum	0	0	3	2	5
Subtotal	2	0	7	7	16
Gram-negative aerobic rods					
Pseudomonas spp.	2	1	2	7	12
Total	5	2	12	18	37

*Apical and coronal root samples pooled.

Table 5 Number of yeast isolates (5/252) from different sites of all teeth (n = 8)

Species	Crown dentine	Crown restoration	*Root dentine	*Root gutta-percha	Total
Candida spp.	2	0	1	3	6

*Apical and coronal root samples pooled.

'enteric bacteria' (*Enterococcus* spp., *Enterobacter* spp., *Klebsiella* spp., *Acinetobacter* spp.) were more frequently recovered from root filled canals associated with an inadequate coronal seal (Sirén *et al.* 1997). The results of the present study (Table 2) are however at variance with the findings of other studies that have found fewer than two species per canal in many teeth (Möller 1966, Molander *et al.* 1998, Sundqvist *et al.* 1998). Far from a restricted flora, all teeth in this study contained a poly-microbial flora and the number of species recovered per tooth ranged from six (tooth no. 7) to 41 species (tooth no. 2). This even exceeded the number of species reported in previously untreated teeth (Wittgow & Sabiston 1975, Sundqvist 1976, 1994). The nature of the flora was different from that obtained from untreated teeth in that it was dominated by Gram-positive facultative organisms, in agreement with others (Engström 1964, Möller 1966, Molander *et al.* 1998, Sundqvist *et al.* 1998). This discrepancy may be attributed to the difference in the criteria for sample selection as well as the method of microbial sampling. The removal of GP by mechanical or solvent aids, in addition to killing bacteria (Molander *et al.* 1998), may also leave residues of root filling material on canal walls, thereby obstructing adequate sampling. The method adopted for collecting microbiological samples in this study is more likely to have avoided false negative outcomes than a conventional *in vivo* approach (Engström 1964, Molander *et al.* 1998, Sundqvist *et al.* 1998).

The profile of species recovered matched the findings of Molander *et al.* (1998) and Sundqvist *et al.* (1998),

with some exceptions, but the mono-infections reported previously were not found. In the present study, the predominant species were staphylococci (48/252 isolates; 6/8 teeth) which were only reported in 6% (7/117) of the isolates recovered by Molander *et al.* (1998) and none were recovered by Sundqvist *et al.* (1998). The recovery of staphylococci has been attributed to contamination (Engström 1964) but given the stringent measures used in this study and the sterility control results, it is unlikely that this was so. This view is also supported by other studies, which consider these species to be indigenous to the site rather than being contaminants (Sulitzeanu *et al.* 1964, Wyman *et al.* 1978, Dahlén & Möller 1992, Johnson *et al.* 1999). There is no reason to suspect these organisms as contaminants any more than other Gram-positive species such as streptococci and enterococci, widely reported in retreatment cases (Molander *et al.* 1998, Sundqvist *et al.* 1998).

Enterococci were the predominant genus recovered by Molander *et al.* (1998) (32% of teeth) and Sundqvist *et al.* (1998) (45% of teeth) and were isolated from most teeth (62.5%) in the present study but they constituted only 7.9% of all the isolates. Their higher prevalence by proportion of sample may be due to coronal leakage. Of the Gram-positive facultative rods, *Actinomyces* were more frequently recovered from the sampled teeth (62.5%) (7.9% of all isolates) than reported by Molander *et al.* (1998) or Sundqvist *et al.* (1998).

The origin of species found in root filled teeth is a matter of some debate. The obvious explanation is that they were likely to be a subset of the original infection that were capable of evading the killing agents used during treatment (Estrela *et al.* 1999, Haapasalo *et al.* 2000, Evans *et al.* 2002, Chávez de Paz *et al.* 2003). This subset would presumably be further modified by the ability of certain members to survive in a nutrition-depleted environment (Sundqvist 1994). Interestingly, previous studies have failed to identify species capable of resisting the antimicrobial agents used during treatment (Byström & Sundqvist 1981, 1983, Gomes *et al.* 1996). A more recent study, in contrast concluded that some bacterial groups (nonmutans streptococci, enterococci, lactobacilli) appeared to survive commonly following root canal treatment of teeth with apical periodontitis (Chávez de Paz *et al.* 2003) but they did not evaluate the preexisting infection.

Under the conditions of this study it was not possible to determine the origin of the microbial groups. It is possible that a combination of relatively unsatisfactory root canal treatment (Cheung & Ho 2001) and coronal leakage allowed the wide spectrum of species to flourish. This is supported by the observation in this study that the three teeth rated with poor root fillings (2, 5, 6) yielded the highest number of isolates and species (Table 1).

Obvious gradations in the diversity of species in the coronal and apical sites may provide some insight into the dynamics of infection progress in future studies. The total number of species in the coronal parts of the teeth was; 29 (crown dentine)/25 (crown restoration), whilst in the root the comparable numbers were 86 (root dentine)/112 (root GP). The diversity of bacteria was highest in the coronal parts of the canals in four teeth (2, 3, 6, 8), more-or-less equal in the coronal and apical parts of the canals in three teeth (1, 4, 7) and heaviest in the apical part of the canal in one tooth (5). The latter tooth was associated with one of the largest periapical lesions, consistent with the findings of Sundqvist (1976).

It was surprising that bacterial recovery from GP was at least equal to that from dentine. The implication is that poorly compacted GP not only acts as a surface for bacterial growth but also acts as a channel for infection spread to the apical parts of the root canal system. The weak antibacterial activity of GP (due to zinc oxide content) reported by Moorer & Genet (1982) did not appear to afford any protection against bacterial colonization according to this study.

The high frequency of Gram-positive facultative anaerobes, especially enterococci in root canals with persistent infection may have significant implications in root canal treatment. The resistance of enterococci to commonly used root canal medication has raised concerns (Estrela *et al.* 1999, Haapasalo *et al.* 2000, Evans *et al.* 2002, Chávez de Paz *et al.* 2003) and new strategies may need to be found to deal with them.

Successful outcome of root canal treatment is dependent not only upon effective treatment of the initial infection (Byström & Sundqvist 1981, 1983, 1985, Chávez de Paz *et al.* 2003) but also prevention of regrowth by resistant strains between visits (Sjögren *et al.* 1991) and prevention of microbial ingress during and after treatment. The coronal restoration must therefore be considered an integral part of the endodontic treatment (Heling *et al.* 2002, Hommez *et al.* 2002).

Conclusions

A laboratory-based sampling protocol revealed a diverse microbial flora in root filled teeth with persistent

apical periodontitis and coronal leakage. The predominant group of bacteria recovered was Gram-positive facultative anaerobes of which staphylococci followed by streptococci and enterococci were the most prevalent. Whilst the sample size was not large enough to reveal statistical associations between sample site/surface and bacterial flora, the method has the potential to reveal such associations if they exist.

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