Gram-positive rods prevailing in teeth with apical periodontitis undergoing root canal treatment

L. E. Chávez de Paz[1,2], A. Molander[2] & G. Dahlén[1]

[1]Departments of Oral Microbiology and [2]Endodontics/Oral Diagnosis, Faculty of Odontology, The Sahlgrenska Academy at Göteborg University, Göteborg, Sweden

Abstract

Chávez de Paz LE, Molander A, Dahlén G. Gram-positive rods prevailing in teeth with apical periodontitis undergoing root canal treatment. *International Endodontic Journal*, **37**, 579–587, 2004.

Aims To identify Gram-positive rods from root canals of teeth with apical periodontitis and to examine their associations with other species.

Methodology Consecutive root canal samples (RCSs) from 139 teeth undergoing root canal treatment were analyzed prospectively for cultivable microbes. Gram-positive rods in the first RCS submitted after chemo-mechanical preparation were categorised to genus level by selective media and gas–liquid chromatography (GLC), and identified to species level by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Associations between organisms were measured by odds ratios (OR).

Results In the first samples submitted a total of 158 Gram-positive rods, 115 Gram-positive cocci, 26 Gram-negative rods and 9 Gram-negative cocci, were identified. At genus levels Gram-positive rods were classified into: *Lactobacillus* spp. (38%), *Olsenella* spp. (18%), *Propionibacterium* spp. (13%), *Actinomyces* spp. (12%), *Bifidobacterium* spp. (13%) and *Eubacterium* spp. (6%). The most frequent species were *Olsenella uli*, *Lactobacillus paracasei* and *Propionibacterium propionicum*. In subsequent samples taken during treatment, Gram-positive rods were also identified, although the number of strains was considerably reduced. Positive associations were observed between members of the genus lactobacilli and Gram-positive cocci (OR>2).

Conclusions *Olsenella uli* and *Lactobacillus* spp. predominated over other Gram-positive rods. A possible association exists between *Lactobacillus* spp. and Gram-positive cocci in root canals of teeth with apical periodontitis receiving treatment.

Keywords: bacterial resistance, endodontic therapy, microbiology, prospective study, pulpal infection.

Received 13 October 2003; accepted 22 April 2004

Introduction

Gram-positive facultative anaerobes predominate the bacterial flora remaining after root canal treatment (Chávez de Paz *et al.* 2003). Gram-positive rods constitute an important segment of this remaining flora even after root canal filling (Molander *et al.* 1998). However, only few species of this group of organisms have been recognised to be directly implicated with apical periodontitis (Happonen 1986, Sjögren *et al.* 1988).

Culture-growing Gram-positive rods are not usually reported in species levels. By contrast, considerable efforts have been undertaken to classify Gram-negative anaerobes (Haapasalo 1993, Sundqvist 1993) and in recent years *Enterococcus* spp. (Dahlén *et al.* 2000, Molander *et al.* 2002). In addition, a consistent methodology to classify cultured Gram-positive rods from root canal samples (RCSs) is lacking. Methods used so far do not have universal approval and are considered unreliable, e.g. commercial kits based on biochemical tests (Debelian *et al.* 1996). Molecular based techniques have proven to be excellent tools to identify

Correspondence: Luis Eduardo Chávez de Paz Villanueva, Department of Oral Microbiology, Faculty of Odontology, The Sahlgrenska Academy at Göteborg University, Box 450, SE-405 30 Göteborg, Sweden (Tel.: +46 31 7733266; fax: +46 31 825733; e-mail: luis.chavez.de.paz@odontologi.gu.se).

specific bacteria from plaque samples (Ashimoto *et al.* 1996), and root canals (Siqueira & Roças 2003). However, since microbial DNA fragments used for PCR amplification may also belong to non-living organisms, the capacity of these methods to only detect species surviving after chemo-mechanical procedures or root filling is uncertain. Identification of culture-growing bacteria may yet be approached by analyses of phenotypic and structural characteristics. By means of gas–liquid chromatography (GLC) and electrophoresis separation of cellular proteins (sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE) small divergences between bacterial strains can be revealed (Moore *et al.* 1980, Eribe & Olsen 2002).

The mechanisms by which Gram-positive rods remain viable in the root canal after instrumentation or filling may be determined by interactions with other bacteria (Dahlén & Möller 1992). Due to the limited availability of nutrients in these cases, associations between genus or species may serve as a mechanism to survive and increase pathogenicity (Sundqvist *et al.* 1998). While in necrotic pulps a broad range of complex correlations have been recognised (Sundqvist 1992), in root canals receiving root canal treatment bacterial interactions have yet to be evaluated.

The objectives of this study were to classify culture-growing Gram-positive rods to species levels, assess their relative occurrence in root canals associated with apical periodontitis undergoing root canal treatment and to observe associations with other surviving bacteria.

Material and methods

The material consisted of consecutive RCS from teeth with clinical and/or radiographic evidence of apical periodontitis. Teeth were sampled while receiving root canal treatment already initiated at one, two or several previous appointments prior to sampling (Fig. 1). A sterility control from the operation field was submitted in conjunction with the RCS. If the operation field control presented bacterial growth, the RCS was excluded from the study. Further selection was based on results from the microbial analyses of the RCS; only cases presenting Gram-positive rods were included.

A total of 139 teeth from the same number of patients were selected (78 females and 61 males with an age range 18–80 years). Patients were treated in the public dental service (66%) and private dental clinics in Göteborg (34%) by experienced general practitioners or endodontists who were regular users of the sampling methods established by Möller (1966). A number of cases included in this study (86) have been also part of a previous report (Chávez de Paz *et al.* 2003).

Procedures prior to root canal treatment

Teeth were isolated using rubber dam; then disinfected with 30% hydrogen peroxide and a 10% iodine tincture in accordance with the protocol advocated by Möller (1966). The sterility of the operation field was checked after inactivation of the iodine with 5% sodium thiosulphate solution. Part of the fluid was aspirated with a charcoal-impregnated cotton pellet, which was transferred to transport medium VMGA III (Dahlén & Möller 1992). Following removal of the interappointment temporary cement, the canal was flushed with sampling fluid (VMG I, Möller 1966) and instrumented to remove $Ca(OH)_2$ if present. Then and in cases where IKI had been used for interappointment medication, VMG I was added and the canal walls were filed. The suspension was absorbed into charcoal impregnated paper points (Möller 1966), which were transferred to VMGA III. This sample was designated Sample 1 (Fig. 1). In cases with bacterial growth evident in a previous sample, irrigation and medicament procedures were repeated, and a subsequent sample was taken using the same sampling method. These samples were designated as appropriate Samples 2, 3 and 4.

Culturing procedures

All RCS arrived at the microbiological laboratory within 24 h of sampling. Transport tubes were placed in 37 °C for 15 min and vigorously mixed for 20–30 s using a vortex mixer. Dilution aliquots of 100 µl were distributed on to two Brucella agar plates (BBL Microbiological Systems, Cockeysville, MD, USA); one plate was used for aerobic incubation for 3–5 days at 37 °C and the other plate was used for anaerobic incubation using the hydrogen combustion method for 5–7 days at 37 °C (Möller 1966). Charcoaled paper points, as well as the remaining fluid, were placed in a semi-liquid medium (HCMG-Sula, Möller 1966) and incubated at 37 °C for up to 14 days. In cases of no growth on the agar plates, tubes were checked daily for turbidity during 14 days.

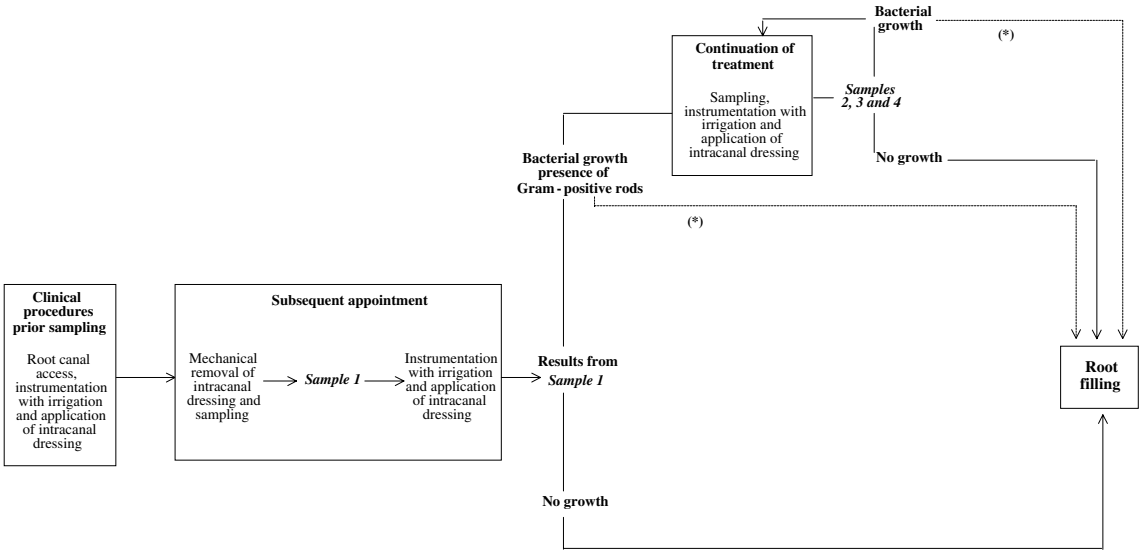

Figure 1 Outline of clinical and laboratory procedures. (*)Despite bacterial growth, treatment was continued or finalised in few cases.

Bacteria growing on agar plates or semi-liquid medium were sub-cultured for identification on the basis of anaerobic or aerobic growth and further identification was performed as outlined by Dahlén & Möller (1992). Colonies were semi-quantified according to the method described in Chávez de Paz *et al.* (2003). Briefly: 1–10 colony forming units (CFU) were considered as very sparse growth, 11–100 as sparse growth, 101–1000 moderate, 1001–10 000 as heavy and more than 10 000 as very heavy growth. Strains isolated were Gram-stained and classified by colony morphology, oxygen tolerance and biochemical tests into species, genus or other main groups.

Microbial identification

Selective media were used for sub-culturing the following strains: Mitis Salivarius agar plates (MS agar, Difco Laboratories, Detroit, MI, USA) on which distinction was made between mutans group and non mutans group streptococci, MSB agar plates (Mitis Salivarius with 3.3 mg ml^{-1} of Bacitracin (Sigma, St Louis, MO, USA) for identification of *Streptococcus mutans*/*Streptococcus sobrinus*, *Staphylococcus* spp. medium agar plates (Difco) on which *Staphylococcus aureus* was distinguished from coagulase-negative *Staphylococcus* spp. by being DNase positive, Enterococcus agar plates (BBL Microbiology Systems, Cockeysville, MD, USA) for identification of *Enterococcus* spp., Rogosa agar plates (Difco) for identification of *Lactobacillus* spp. and *Bifidobacterium* spp., CFAT agar plates (Zylber & Jordan 1982) for identification of *Actinomyces* spp., Drigalski agar plates for identification of lactose positive enterobacteria and Saboraud dextrose agar plates (Difco) for identification of yeasts (*Candida* spp.). Gram-positive rods not growing on selective media, e.g. *Propionibacterium* spp., *Clostridium* spp. and *Eubacterium* spp. were identified after sub-culturing on Brucella blood agar (BBL) enriched with defibrinated horse blood plus 0.5% haemolysed horse blood and 5 mg ml^{-1} of menadione, and were analysed by GLC. Other obligate anaerobic strains, e.g. *Peptostreptococcus* spp., black-pigmented (*Prevotella* spp., *Porphyromonas* spp.) and nonpigmented Gram-negative anaerobes (*Fusobacterium* spp., *Veillonella* spp.) were sub-cultured on Brucella blood agar plates and further identified by GLC. Plates for anaerobic culturing were incubated in anaerobic jars for 7–10 days while other plates were incubated in air with 10% CO_2 for 3–5 days. Gas–liquid chromatography was performed as outlined by Holdeman *et al.* (1977) to classify Gram-positive rods into genus level. Strains were grown in RS-30 medium (Department of Oral Microbiology, Göteborg University, Sweden) for 5 days under anaerobic conditions. The metabolic

Table 1 Gram-positive rods used as positive controls

Actinomyces
A. israelli (OMGS G64)
A. meyeri (CCUG 21024)
A. naeslundii (OMGS 1188)
A. odontolyticus (NCTC 9935)
A. radicidentis (CCUG 36733)
A. viscosus (OMGS 3353)
Bifidobacterium
B. adolescentis (CCUG 18363)
B. bifidum (OMGS 3126)
B. breve (CCUG 18365)
B. dentium (CCUG 17360)
B. longum (CCUG 28903)
Eubacterium
E. alactolyticum (Re-classified) (ATCC 23263)
E. lentum (Re-classified)(OMGS G63)
E. limosum (OMGS G173)
E. nodatum (OMGS 3356)
Lactobacillus
L. acidophilus (OMGS 3185)
L. casei (OMGS 3184)
L. crispatus (ATCC 33820)
L. curvatus (OMGS 3181)
L. delbrueckii (OMGS 3183)
L. fermentum (OMGS 3182)
L. gasserie (OMGS 3178)
L. helveticus (ATCC 15009)
L. paracasei (OMGS 3180)
L. plantarum (ATCC 14917)
L. reuteri (OMGS 3129)
L. rhamnosus (OMGS 3179)
L. salivarius (ATCC 11741)
Olsenella uli (OMGS 3358)*
Propionibacterium
P. acidpropionici (ATCC 25562)
P. acnes (ATCC 11828)
P. propionicum (CCUG 28903)

*Formerly *L. uli*.
OMGS: Collection of the Department of Oral Microbiology, The Sahlgrenska Academy at Göteborg University, Göteborg, Sweden; CCUG: Culture Collection of Göteborg University; ATCC: American Type Culture Collection.

products were analysed with an ionisation detector. The glass column of chromatography was packed with 5% AT 1000 (Altech Associates Inc., Deerfield, MA, USA) on Chromosorb GHP 100/120 mesh (Johns-Manville, Denver, CO, USA). The carrier gas was nitrogen (30 mL min^{-1}) with an injection port temperature of 120 °C. One microlitre portions of the ether-extracted and methylated samples were used, and the results were compared with those obtained with standard solutions of volatile fatty acids. Type strains were used as positive controls (Table 1).

SDS-PAGE identification of Gram-positive rods to species level

One-dimension polyacrylamide gel electrophoresis SDS-PAGE of whole cell protein extracts was used.

Polyacrylamide gels

To screen high molecular proteins, a 10% gel was composed of 3.33 mL acrylamide/BIS, and for low molecular proteins a 12% gel was composed of 4 mL acrylamide/BIS. Gels polymerised after 2 h. A stacking gel solution (4%) added to form grids for sample inoculation was composed of 1.35 mL acryl amide/BIS. Polymerisation of this gel also lasted for 2 h.

Sample preparation and inoculation

Colonies were transferred into 500 μL phosphate buffer solution up to a concentration of 10^8 cells mL^{-1} calculated by a spectrophotometer Novaspec II (Biochrom, Cambridge, UK). The suspension was sonicated for 20 s with a cell disruptor (Sonifier B12- Branson Sonic Power Company, Denbury, CT, USA), in order to lyse the bacterial cell wall. 10 μL of the suspension was then added to 10 μL sample buffer and 2 μL pyromin. This solution was boiled for 5 min and 12 μL was inoculated in the grids formed in the stacking gel.

Electrophoresis and staining

Gels were placed in an electrode chamber and filled with SDS electrophoresis buffer. Electricity was supplied by the Power Pac-300 (BIO RAD, Solna, Sweden), set at 200 v and run for 45 min. After electrophoresis, staining was achieved with a solution of Coomasie blue for 30 min and distained with an acetone-based solution for 3 h.

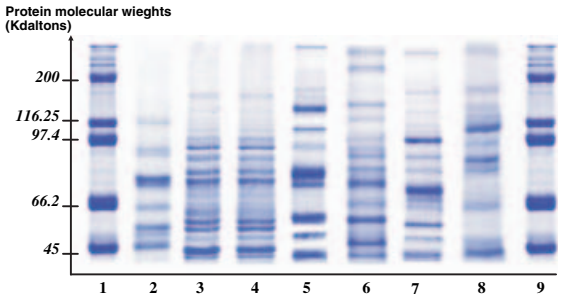

Figure 2 Protein patterns of clinical strains in one-dimensional SDS-PAGE gel 10% (high molecular weight protein standard, Coomasie-blue stained): (1) Standard, (2) *Olsenella uli*, (3) *Lactobacillus paracasei*, (4) *L. casei*, (5) *Propionibacterium propionicum*, (6) *Actinomyces meyerii*, (7) *Bifidobacterium dentium*, (8)*Eubacterium nodatum*, and (9) Standard.

A scanner (Canon N676U; Canon Inc, Tokyo, Japan) was used for digitalisation of the gels. Images were interpreted using the photographic software Adobe Photoshop version 6 (Adobe Systems Inc., Seattle, WA, USA). Banding patterns of clinical Gram-positive rods strains were superimposed and compared with those of the control strains; bands were counted and their positions localised (Fig. 2).

Bacterial associations with Gram-positive rods

The odds ratio (OR) described by Socransky *et al.* (1988) was used to measure the associations of other bacteria with Gram-positive rods. A positive association existed when the OR was above 2 and an OR below 0.5 was considered a negative association. The proportion of the organisms in each sample was not considered in these calculations.

Results

Microbial findings

From the 139 cases included, in the first RCS submitted, a total of 308 strains were isolated. One hundred and fifty eight of these were Gram-positive rods and were distributed into genus levels as: *Lactobacillus* spp. (38%), *Olsenella* spp. (18%), *Propionibacterium* spp. (13%), *Bifidobacterium* spp. (13%), *Actinomyces* spp. (12%) and *Eubacterium* spp. (6%). A total of 115 Gram-positive cocci strains were also identified: *Streptococcus* spp. (56%), *Enterococcus* spp. (25%), coagulase negative *Staphylococcus* spp. (11%) and *Peptostreptococcus* spp. (8%). Twenty-six Gram-negative rods: *Prevotella* spp. (11 strains), *Fusobacterium* spp. (seven strains), enterobacteria (lactose positive) (seven strains) and

Table 2 Distribution based on isolation frequency of Gram-positive rods

	First sample						Second sample				Third sample		
	+	++	+++	++++	+++++	Total	+	++	+++	Total	+	++	Total
Olsenella uli	2	11	7	6	2	28	4	3	1	8	2		2
Lactobacillus paracasei	1	6	5	7	1	20		5	1	6	1		1
L. casei	2	6		2		10		2*		2			0
L. delbrueckii	2	3	3	1		9	1	1		2			0
L. crispatus		6	1	1		8	1			1			0
L. rhamnosus		1	2			3	1			1			0
L. acidophilus		1	2			3	1			1			0
L. salivarius	2	1				3				0			0
L. plantarum		1	1	1		3		1	1	2			0
L. curvata			1			1				0			0
Lactobacillus spp.			1			1				0	1*		1
						61				15			2
Propionibacterium propionicum	3	6	3	5	1	18	2	1	2	5	1		1
P. acnes			1			1							
Propionibacterium spp.			1			1	1*			1	1*		1
						20				6			2
Bifidobacterium dentium	1	2	3	3		9	2	1		3			0
B. longum		4	2			6				0			0
B. breve		1	2			3				0			0
Bifidobacterium spp.		1	1			2				0			0
						20				3			0
Actinomyces meyerii		1	4	2		7		1		1			0
A. odontolyticus		2	2	2		6	2	1*		3	1		1
A. naeslundii	1	1	1			3				0			0
A. israelli	1	1				2	1*			1			0
Actinomyces spp.		1				1		1*	1*	2			0
						19				7			1
Eubacterium nodatum	1	3	1	1		6	1*			1			
E. limosum	1	1	1			3				0			0
Eubacterium spp.	1					1	1*			1			0
						10				2			0

*Strain present not previously identified in the previous sample.
Growth rate: +, very sparse (1–10 CFU); ++, sparse (11–100 CFU); +++, moderate (101–1000 CFU); ++++, heavy (1001–10000 CFU) and +++++, very heavy (>10000 CFU).

Porphyromonas spp. (one strain). Nine Gram-negative cocci strains were identified as *Veillonella* spp.

From 65 cases displaying growth in the second sample submitted, 41 strains were identified as Gram-positive rods, from which eight strains were not previously isolated in the preceding sample (Table 2). Other strains isolated belonged to the groups: Gram-positive cocci (26 strains) and Gram-negative rods (one strain).

In eight cases presenting growth in the third sample, seven Gram-positive rods were isolated. Two of these strains were not isolated in previous samples. One strain of *Enterococcus* spp. was also found.

Table 2 shows the distribution of the total Gram-positive rods isolated in three consecutive RCSs. Most predominating species were *Olsenella uli*, *Lactobacillus paracasei*, and *Propionibacterium propionicum*.

Bacterial associations

Odds ratio demonstrated a positive association between *Lactobacillus* spp. and Gram-positive cocci (Table 3). Among individual species within these two groups, *Lactobacillus casei* and *Enterococcus* spp. showed a positive association (Table 4). *Bifidobacterium* spp. showed a positive association with Gram-negative rods (Table 3) on species level. *Bifidobacterium dentium* was associated with *Peptostreptococcus* spp., *Prevotella* spp. and enterobacteria (lactose positive); while *Bifidobacterium longum* was associated with enterobacteria (lactose positive) (Table 4). *Eubacterium* spp. demonstrated in general a negative association with Gram-positive cocci (Table 3), although among individual species the OR between *Eubacterium nodatum* and *Streptococcus* spp. was positive (Table 4).

Discussion

Lactobacillus spp. and *Olsenella uli* predominated at genus and species level, respectively. *Lactobacillus* spp. and Gram-positive cocci were frequently isolated in conjunction, which may indicate a possible interaction during or after root canal treatment.

Gram-positive rods were identified with a combination of selective media, GLC and SDS-PAGE. By examining patterns given by whole cell proteins, similarities can be observed between bacterial genera and species (Moore *et al.* 1980, Eribe & Olsen 2002). In the present study, SDS-PAGE gels were interpreted with commercial photographic software, e.g. banding patterns of clinical strains were superimposed and compared with those of the control strains; bands were counted and their positions localised. Although this method was useful, 11 Gram-positive rods strains were not identified.

Other methods for identification previously tested showed variable results. Commercial kits based on bio-chemical, enzymatic and fatty acid analysis, were found unstable that may have been due to their susceptibility to different bacterial physiological conditions (Vauterin *et al.* 1993, Debelian *et al.* 1996). Molecular analyses based on DNA probes were not considered in the present study. These methods seem to constitute a valuable tool for identification to species and sub-species levels (Siqueira & Rôças 2003). Yet, since DNA fragments can also be obtained from dead bacterial cells, there is still no consistent evidence that compares molecular-based methods with conventional culture techniques in order only to identify viable bacteria from root canals after instrumentation and/or root filling (Rolph *et al.* 2001, Munson *et al.* 2002).

Lactobacillus spp. was the most frequent genus isolated. *Lactobacillus uli*, recently re-classified as *Olsenella uli* (Dewhirst *et al.* 2001) and *Lactobacillus paracasei*, were the most frequent species. Traditionally, lactobacilli have not been regarded as endodontic pathogens and when found in root canals are considered the result of transient contamination (Sundqvist &

Table 3 Associations among bacterial groups

	Gram-positive coccí (104 cases)	Gram-negative rods (26 cases)	Gram-negative coccí (9 cases)
Olsenella uli (28 cases)	1.00	**0.46**	**0.48**
Lactobacillus spp. (63 cases)	**2.18**	0.96	0.58
Propionibacterium spp. (21 cases)	1.10	**0.42**	–
Bifidobacterium spp. (20 cases)	**0.16**	**2.83**	1.78
Actinomyces spp. (23 cases)	0.94	0.91	1.66
Eubacterium spp. (12 cases)	**0.20**	–	–

Boldface: negative ($x < 0.5$) or positive associations ($x > 2.0$); not present in the samples with the actual species.

Table 4 Odds ratios of associations among bacterial species

	Streptococcus spp. (64 cases)	*Enterococcus* spp. (29 cases)	Coagulase-negative staphylococci (13 cases)	*Peptostreptococcus* spp. (9 cases)	*Prevotella* spp. (11 cases)	Enterobacteria (lactose positive) (7 cases)	*Fusobacterium* spp. (7 cases)	*Veillonella* spp. (9 cases)
Olsenella uli (28 cases)	1.22	0.79	0.83	1.48	**0.37**	0.61	0.65	1.48
Lactobacillus paracasei (20 cases)	1.86	1.38	–	–	1.70	**2.38**	**4.69**	0.60
L. casei (11 cases)	1.75	**2.85**	1.39	–	–	–	–	–
L. delbrueckii (9 cases)	0.54	1.13	1.59	**2.07**	1.79	**2.42**	**2.46**	**4.10**
L. crispatus (8 cases)	1.13	0.55	1.82	–	**5.80**	–	–	1.82
Propionibacterium propionicum (18 cases)	1.15	1.64	–	–	–	–	–	–
Bifidobacterium dentium (9 cases)	**0.12**	**0.46**	**4.10**	**2.07**	**4.83**	**2.46**	–	1.59
B. longum (6 cases)	0.54	–	**2.58**	–	–	**3.98**	–	**2.58**
Actinomyces meyerii (7 cases)	**0.43**	–	–	**2.80**	–	–	–	**2.13**
A. odontolyticus (7 cases)	1.13	–	**2.58**	**3.40**	–	**3.98**	–	–
Eubaterium nodatum (7 cases)	**2.38**	–	–	–	–	–	–	–

Boldface: negative ($x < 0.5$) or positive associations ($x > 2.0$); not present in the samples with the actual species.

Carlsson 1974). The findings of this study do not provide evidence of their origin. However, with the exclusion of cases where contamination of the operation fields was suspected, it is reasonable to assume that lactobacilli isolated in this study were indeed root canal colonisers. *Olsenella uli* has also been found in sub gingival pockets and are strong producers of lactic acid, a feature that may be implicated in inflammatory processes (Olsen *et al.* 1991). *Lactobacillus paracasei* is a resident of the human intestine, it is commonly used as probiotic bacteria for preservation of foods and it is a transient inhabitant of the oral cavity (Holzapfel *et al.* 2001). Therefore, it may be of particular interest to explore the mechanisms by how these species ingress, prevail after root canal treatment and are pathogenically implicated in periapical lesions.

Propionibacterium propionicum and *Actinomyces meyerii* were the most common species within their respective genus. These species have been associated with extra-radicular infections (Happonen 1986, Sjögren *et al.* 1988). *Actinomyces* spp. are primary colonisers of the oral cavity and have a crucial role in initiating plaque development (Yeung 1999). Moreover, they have been reported to be involved in persistent root canal infections (Kalfas *et al.* 2001). Their survival in nutritionally deprived environments may be due to the presence of extracellular enzymes, for example those involved in sucrose or urea metabolism that may provide means for survival (Yeung 1999). *Actinomyces meyeri* has been described as having similar infectious characteristics as *Actinomyces israelii* (Clarridge & Zhang 2002). *Propionibacterium propionicum*, formerly *Arachnia propionica*, is a normal inhabitant of the oral cavity that has been isolated from RCSs in mixed infections and as single isolates. Its pathogenic capacity remains unclear (Sjögren *et al.* 1988).

Bifidobacterium spp. are inhabitants of the human gut and commonly used as probiotic bacteria. Most of the species colonising the oral cavity are considered as transient colonisers (Orrhage & Nord 2000). *Bifidobacterium dentium*, one of the few species of this genus that inhabits the oral cavity, has been found to reside in the periodontal pocket. It is interesting that members of this bacterial group remain in the root canal after antimicrobial therapy since no current knowledge exists as to their pathogenicity in apical lesions.

Eubacterium spp. strains were identified in the lowest numbers. These asaccharolytic microorganisms have been isolated from samples of periodontal pockets, advanced caries and dento alveolar abscesses (Goodacre *et al.* 1996). Due to difficulties with its isolation

and nutritional requirements, these bacteria have been poorly studied. In this study, *Eubacterium nodatum* was the most frequently isolated species, which has been previously identified in human periodontitis (Spratt *et al.* 1999).

Odds ratio, initially applied for dental plaque studies (Socransky *et al.* 1988) and later for RCSs (Sundqvist 1992) was a useful tool to explore associations among resistant organisms in the root canal as they may exist *in vivo*. The most frequent interactions in the present study were given by *Lactobacillus* spp. and Gram-positive cocci, e.g. *Lactobacillus casei* with *Enterococcus* spp. Such associations have not been reported previously. Other non-significant associations were also determined possibly due to the low number of strains recovered, thus, it can be speculated that with a larger number of samples such associations may have been found to be significant. Further clarification of the nature and role of these interactions may be important to elucidate the pathogenic role of those bacteria persisting after root canal treatment.

The potential risk of contamination with 'unrelated bacteria' during sampling is a consistent problem in endodontic microbial studies (Möller 1966). Certain bacterial species as staphylococci, streptocci, lactobacilli, spore formers and fungi have been regarded as contaminants (Möller 1966, Sundqvist & Carlsson 1974). However, as these so-called unrelated organisms are also inhabitants of other sites of the microbial oral flora, they may nevertheless be capable of colonising and establishing in root canals. Thus, the potential risk of obtaining false-positive findings may be reduced by only including cases not presenting growth from sterility control of the operation field (Möller 1966).

Conclusion

The results of this study contribute to the study of bacterial flora remaining after root canal treatment. In addition to *Enterococcus* spp. other bacteria seem to remain after root canal treatment. Further research is required to determine whether these prevalent organisms have any pathogenic implications in root canal treatment failures.

Acknowledgements

This study was supported by research grants from Sigge Perssons och Alice Nybergs Stiftelse för Odontologisk Forskning, Göteborg, Sweden.

The authors would like to thank Professors Emeritus Gunnar Bergenholtz and Åke Möller (Faculty of Odontology, The Sahlgrenska Academy at Göteborg University, Sweden) and Dr. Christine Sedgley (UMICH, USA) for their valuable suggestions in the elaboration of the manuscript. We also express our gratitude to Mr. Ingvar Ågren for the elaboration of the gas–liquid chromatographic analysis and Mrs. Lisbeth Bengtsson for technical assistance with the SDS-PAGE.

References

Ashimoto A, Chen C, Bakker I, Slots J (1996) Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiology and Immunology* **11**, 266–73.

Chávez de Paz LE, Dahlén G, Molander A, Möller ÅJR, Bergenholtz G (2003) Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. *International Endodontic Journal* **36**, 500–8.

Clarridge JE III, Zhang Q (2002) Genotypic diversity of clinical *actinomyces* species: phenotype, source, and disease correlation among genospecies. *Journal of Clinical Microbiology* **40**, 3442–8.

Dahlén G, Möller ÅJR (1992) Microbiology of endodontic infections. In: Slots J, Taubman M, eds. *Contemporary Oral Microbiology and Immunology*, 1st edn. *St Louis, Missouri, USA: Mosby-Year Book, pp. 444–75.*

Dahlén G, Samuelsson W, Molander A, Reit C (2000) Identification and antimicrobial susceptibility of enterococci isolated from the root canal. *Oral Microbiology and Immunology* **15**, 309–12.

Debelian GJ, Olsen I, Tronstad L (1996) Electrophoresis of whole-cell soluble proteins of microorganisms isolated from bacteremias in endodontic therapy. *European Journal of Oral Sciences* **104**, 540–6.

Dewhirst FE, Paster BJ, Tzellas N *et al.* (2001) Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the family *Coriobacteriaceae*: description of *Olsenella* gen. nov., reclassification of *Lactobacillus uli* as *Olsenella uli* comb. nov. and description of *Olsenella profusa* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **51**, 1797–804.

Eribe ER, Olsen I (2002) SDS-PAGE of whole-cell proteins and random amplified polymorphic DNA (RAPD) analices of Leptotrichia isolates. *Microbial Ecology in Health and Disease* **14**, 193–202.

Goodacre R, Hiom SJ, Cheeseman SL, Murdoch D, Weightman AJ, Wade WG (1996) Identification and discrimination of oral asaccharolytic *Eubacterium* spp. by pyrolysis mass spectrometry and artificial neural networks. *Current Microbiology* **32**, 77–84.

Haapasalo M (1993) Black-pigmented Gram-negative anaerobes in endodontic infections. *FEMS Immunology and Medical Microbiology* **6**, 213–7.

Happonen RP (1986) Periapical actinomycosis: a follow-up study of 16 surgically treated cases. *Endodontics and Dental Traumatology* **2**, 205–9.

Holdeman LV, Cato EP, Moore WEC (1977) *Anaerobic laboratory manual*, 4th edn. Blacksburg, VI, USA: *Virginia Polytechnic Institute and State University.*

Holzapfel WH, Haberer P, Geisen R, Bjorkroth J, Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *The American Journal of Clinical Nutrition* **73**, 365S–73S

Kalfas S, Figdor D, Sundqvist G (2001) A new bacterial species associated with failed endodontic treatment: identification and description of *Actinomyces radicidentis*. *Oral Surgery Oral Medicine Oral Pathology* **92**, 208–14.

Molander A, Reit C, Dahlén G, Kvist T (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal* **31**, 1–7.

Molander A, Lundqvist P, Papapanou PN, Dahlén G, Reit C (2002) A protocol for the polymerase chain reaction detection of *Enterococcus faecalis* and *Enterococcus faecium* from the root canal. *International Endodontic Journal* **35**, 1–6.

Möller ÅJR (1966) *Microbiological examination of root canals and periapical tissues of human teeth*. PhD Thesis. Göteborg, Sweden: University of Göteborg.

Moore WEC, Hash DE, Holdeman LV, Cato EP, (1980) Polyacrylamide slab gel electrophoresis of soluble proteins for studies of bacterial flora. *Applied Environmental Microbiology* **39**, 900–7.

Munson MA, Pitt-Ford T, Chong B, Weightman A, Wade WG (2002) Molecular and cultural analysis of the microflora associated with endodontic infections. *Journal of Dental Research* **81**, 761–6.

Olsen I, Johnson J, Moore L, Moore W (1991) *Lactobacillus uli* sp. nov. and *Lactobacillus rimae* sp. nov. from the human gingival crevice and emended descriptions of *Lactobacillus minutus* and *Streptococcus parvulus*. *International Journal of Systematic Bacteriology* **41**, 261–6.

Orrhage K, Nord CE (2000) Bifidobacteria and lactobacilli in human health. *Drugs under Experimental and Clinical Research* **26**, 95–111.

Rolph HJ, Lennon A, Riggio MP *et al.* (2001) Molecular identification of microorganisms from endodontic infections. *Journal of Clinical Microbiology* **39**, 3282–9.

Siqueira JF Jr, Rôças IN (2003) PCR methodology as a valuable tool for identification of endodontic pathogens. *Journal of Dentistry* **31**, 333–9.

Sjögren U, Happonen RP, Kahnberg KE, Sundqvist G (1988) Survival of *Arachnia propionica* in periapical tissue. *International Endodontic Journal* **21**, 277–82.

Socransky SS, Haffajee AD, Dzink JL, Hillman JD (1988) Associations between microbial species in subgingival plaque simples. *Oral Microbiology and Immunology* **3**, 1–7.

Spratt DA, Weightman AJ, Wade WG (1999) Diversity of oral asaccharolytic *Eubacterium* species in periodontitis–identification of novel phylotypes representing uncultivated taxa. *Oral Microbiology and Immunology* **14**, 56–9.

Sundqvist G (1992) Associations between microbial species in dental root canal infections. *Oral Microbiology and Immunology* **7**, 257–62.

Sundqvist G (1993) Pathogenicity and virulence of black-pigmented Gram-negative anaerobes. *FEMS Immunology and Medical Microbiology* **6**, 125–37.

Sundqvist G, Carlsson J (1974) Lactobacilli of infected dental root canals. *Odontologysk Revy* **25**, 233–8.

Sundqvist G, Figdor D, Persson S, Sjögren U (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surgery Oral Medicine Oral Pathology* **85**, 86–93.

Vauterin L, Swings J, Kersters K (1993) Protein electrophoresis and classification. In: Goodfellow M, O'Donnell AG eds. Handbook of New Bacterial Systematics. London: Academic Press, pp. 251–80.

Yeung MK (1999) Molecular and genetic analyses of *Actinomyces* spp.. *Critical Reviews on Oral Biology and Medicine* **10**, 120–38.

Zylber LJ, Jordan HV (1982) Development of a selective medium for detection and enumeration of *Actinomyces viscosus* and *Actinomyces naeslundii* in dental plaque. *Journal of Clinical Microbiology* **15**, 253–259.