

Associations of endodontic symptoms and signs with particular combinations of specific bacteria

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Summary

Significant associations have been reported between (a) specific bacterial species isolated from root canals and (b) between individual bacterial species and endodontic symptoms and signs. The prime objective of this study was to determine whether particular combinations of specific bacteria are associated with individual endodontic symptoms and signs. Seventy root canals were investigated microbiologically taking care to maintain the viability of obligate anaerobes, which accounted for 64% of the total species isolated, including *Peptostreptococcus micros*, *Prevotella melaninogenica*, *Prevotella oralis*, *Eubacterium aerofaciens*, *Eubacterium lentum*, *Fusobacterium nucleatum*, *Prevotella buccae* and *Prevotella intermedia*. Significant associations were found between individual clinical features and the following pairs of species: (a) pain (37 cases) and *Peptostreptococcus* spp./*Prevotella* spp., *Peptostreptococcus* spp./*Prevotella melaninogenica*, *Pstr. micros*/*Prev. melaninogenica* (all $P < 0.01$); (b) swelling (23 cases) and *Pstr. micros*/*Prevotella* spp. ($P < 0.01$); (c) 'Wet' canal (57 cases) and *Prevotella* spp./*Eubacterium* spp. ($P < 0.01$), *Peptostreptococcus* spp./*Eubacterium* spp. ($P < 0.05$). Thus data from this investigation suggests that statistically significant associations exist between individual endodontic symptoms and signs and particular combinations of specific bacteria.

Keywords: bacterial association, endodontics

Introduction

The microflora of infected root canals and especially of the periodontal tissues consists of a complex polymicro-

bial population. In such heterogenous flora, interactions between microbial species may play a significant role in the growth of organisms. Furthermore these interactions may also occur between bacterial species and host (Baumgartner *et al.* 1992, Wikström *et al.* 1993).

Bacterial associations may be positive or negative depending upon whether the growth of one organism enhances the growth of another (positive association) or whether the relationship is inhibitory to either or to both (negative association) (Gomes *et al.* 1994b). Few *in vivo* studies have shown these associations although microbial interactions in periodontal disease have been demonstrated (Socransky *et al.* 1988, Ohta *et al.* 1991, Wikström *et al.* 1993). In infected dental root canals, associations between microbial species have been reported by Sundqvist (1992b) and Drucker *et al.* (1992), and more recently by Gomes *et al.* (1994b). It seems that different bacterial populations result from these associations as well as from the special environment and selective pressures in the dental root canal (Grenier & Mayrand 1986, Sundqvist 1992a).

Another kind of association is that which occurs between bacteria and clinical features. While many different bacterial species are able to colonize the dental root canals, it has been shown that there is a correlation between the presence of specific bacteria and some endodontic symptoms and signs. Consequently the microflora recovered from asymptomatic teeth are different from those isolated from clinically symptomatic ones. The presence of certain organisms, particularly *Peptostreptococcus*, *Eubacterium*, *Fusobacterium* and the former 'black-pigmented *Bacteroides*', is associated with an increased incidence of pain, swelling and purulent exudate, among other endodontic features. On the other hand, facultative isolates such oral streptococci and enteric bacteria have been commonly found in asymptomatic cases (Yoshida *et al.* 1987).

Thus previous studies of the endodontic microflora have found significant associations between: (a) indivi-

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dual bacterial species and endodontic symptoms and signs (Gomes *et al.* 1994a, Gomes *et al.* in press) and between bacterial species (Drucker *et al.* 1992, Gomes *et al.* 1994b). In addition, a more complex type of association may occur between individual endodontic signs and symptoms and two or more associated species acting in concert. Hence the purpose of the present investigation was to test the hypothesis that there is an association of endodontic symptoms and signs with particular combinations of specific bacteria.

Materials and methods

The method used in this study has been previously reported in detail by the authors (Gomes *et al.* 1994a).

Patient selection and sampling

Seventy teeth, from 60 consecutive patients attending the University Dental Hospital of Manchester for endodontic therapy, were included in the study. This mode of selection was adopted in order to reflect, as far as possible, the range of symptoms and clinical circumstances encountered in general dental practice. Patients' gender and age were noted (patients' ages ranged from 12 to 77 years), with the mean ages of the participating group of men and women being 35.8 and 35.3 years,

respectively. The teeth examined were comprised of 49 single-rooted and only one canal of each of the 21 multi-rooted teeth, in order to preserve the identity of a single endodontic/microbiological ecosystem. Necrotic pulp tissue was observed in 47 root canals, as determined by thermal and electrical testing and during the biomechanical preparation. There was vital pulp in two root canals out of 70 sampled. Previous endodontic treatment with a gutta-percha filling had been carried out in the remaining 21 root canals (Table 1). Most of the teeth used in this study were 'non-intact', i.e. they had suffered breaches of the enamel layer e.g. by restoration, infraction or previous endodontic interventions. However, teeth which could not be well isolated with a rubber dam were rejected.

The disinfection of the sampling area was done according to the procedures described by Gomes *et al.* (1994a). Patency of the root canals was established, with minimal instrumentation if necessary for its confirmation, and without the use of any irrigant. For microbial sampling, a sterile paper point was introduced to the full length of the canal (as determined by a pre-operative radiograph), and retained in position, for 60 s. In cases where a dry canal was identified a further sterile paper point, moistened in sterile saline, was used to ensure viable sample acquisition. Pre-existing root fillings were removed by mechanical means as far as

Table 1 Clinical features

	SR	MR	M	F	U	O	NP	V	P.	Pain	T	S	W	P.	Si	P.
					35	34		P	R		P	W		Ex		R
									C							
									T							
P.	11	10	5	16	13	8	/	/	/	13	13	6	17	0	1	12
RCT																
n=21																
Pain	24	13	9	28	18	19	22	2	13	/	34	17	31	3	5	22
n=37																
TTP	32	17	17	32	28	21	34	2	13	34	/	21	42	5	6	31
n=49																
SW	19	4	6	17	15	8	17	0	6	17	21	/	21	4	5	15
n=33																
Wet	36	21	20	37	33	24	38	2	17	31	42	21	/	6	4	36
n=57																
P.	4	2	3	3	4	2	6	0	0	3	5	4	6	/	1	5
Ex.																
n=6																
Si	4	2	1	5	2	4	5	0	1	5	6	5	4	1	/	4
n=6																
P.R.	26	18	15	29	23	21	32	0	12	22	31	15	36	5	4	/
n=44																

SR, single-rooted; MR, multi-rooted; M, male; F, female; U 35, under 35 years old; O 34, over 34 years old; NP, necrotic pulp; VP, vital pulp; P.RCT, previous root canal treatment; TTP, tenderness to percussion; SW, swelling; W, wet canal; P.Ex, purulent exudate; Si, sinus; P.R., periapical radiolucency.

possible, and in these cases, and in all others where root canal therapy had already been undertaken, the canal was copiously irrigated with sterile water to remove treatment residues prior to microbial investigation. In no case was the sampling preceded by irrigation with any chemically active agent (Gomes *et al.* 1994a).

The infectious material on the paper point was inoculated onto four different, pre-reduced, agar plates (see below). The same paper point was also used to inoculate a semi-liquid, non-selective anaerobic medium (Brewer's thioglycollate medium), which was employed to ensure detection of anaerobes should they fail to grow on the plates. The anaerobic plates were immediately placed in an anaerobic transport bag (Generbag anaer, Bio Merieux SA, Marcy-1'Etoile, France) and these and all the other samples were transported without delay to the microbiology laboratory.

Microbial isolation

Upon reaching the laboratory, plates for anaerobic incubation were transferred from the Generbag into an anaerobic work station (Don Whitley Scientific, Bradford, UK) where they were incubated at 37°C in an atmosphere of 5–10% H₂, 10% CO₂ and 80–85% N₂ for up to 14 days to permit detection of very slowly growing strains. Obligate anaerobes and facultative anaerobes were cultured non-selectively, using 5% blood-FAA (Fastidious Anaerobic agar) agar plates incubated at 37°C for 2, 5 and 14 days. Selection for Gram-positive anaerobes and actinomycetes, involved using 5% horse blood-FAA + NAL (0.001% w/v nalidixic acid) agar plates incubated at 37°C, anaerobically, for 2, 5 and 14 days. Selection for clostridia and other anaerobes, included the use of 5% horse blood-FAA + NEO (0.0075% w/v neomycin) agar plates incubated at 37°C, anaerobically, for 2, 5 and 14 days.

To detect aerobic organisms, the plated samples were inoculated onto 5% horse blood Columbia agar plate and incubated aerobically for 2 days.

All the isolation media were purchased from Lab-M (Bury, UK).

Microbial speciation

Isolates were purified by subculture, then Gram-stained, tested for catalase-production, and their gaseous requirements established by incubation aerobically and anaerobically. This permitted selection of appropriate procedures for speciation as follows: (i) API Staph (Bio Mérieux SA, Marcy-1'Etoile, France) for staphylo-

cocci and micrococci (Gram-positive cocci, catalase-positive), (ii) Rapid ID 32 Strep (Bio Mérieux SA, Marcy-1'Etoile, France) for streptococci (Gram-positive cocci, catalase-negative), (iii) Minitek (Becton Dickinson Co., Cockeysville, MD, USA) for non-sporing Gram-positive rods, (iv) RapID NH system (Innovative Diagnostic Systems Inc., Atlanta, GA, USA) for *Eikenella*, *Haemophilus*, *Neisseria* and *Actinobacillus*, (v) Rapid ID 32A (Bio Mérieux SA, Marcy-1'Etoile, France) for obligately anaerobic Gram-negative rods, (vi) RapID ANA II System (Innovative Diagnostic Systems Inc., Atlanta, GA, USA) for obligately anaerobic Gram-positive cocci.

Because the above kits could not identify all possible isolates, additional tests and/or prolonged culture were used to search for additional species such as *Mitsuokella dentalis* (Haapasalo *et al.* 1986) and *Bacteroides forsythus* (Tanner *et al.* 1986).

Statistical analysis

The data collected were typed onto a spreadsheet (QUATTRO PRO, Borland International Inc., Scotts Valley, CA, USA) and statistically analysed using SPSS/PC+ (SPSS Inc., Chicago, IL, USA). A Pearson chi-square test or a one-sided Fisher's exact test, as appropriate, was chosen to test the null hypothesis that there is no relationship between: (a) endodontic symptoms and signs, and (b) the presence of any particular combination of bacterial species in the root canals sampled (Gomes *et al.* 1994a). Thirteen pairs of specific bacterial species were found to be significantly associated (Gomes *et al.* 1994b). For each pair, three comparisons were performed, looking at the presence of both bacteria in (a) patients with a specific endodontic symptom or sign, (b) in patients without, and (c) in all patients.

Results

From the 70 root canals examined, 242 cultivable isolates were recovered, which belonged to 65 different microbial species.

Individual root canals yielded a maximum of 11 bacterial species while 10 root canals had no cultivable bacteria. Some clinical features were presented in the 10 canals without detectable bacteria, as follows: pain (3/10), tenderness to percussion (3/10), swelling (1/10), sinus (1/10), wet canal (7/10) and periapical radiolucency (5/10). Necrotic pulps were found in 9/10 canals and one canal had been previously root-filled with gutta-percha.

Obligate anaerobes accounted for 64% of total species isolations whereas streptococci constituted 23.1%, with members of the 'milleri- group' streptococci predominating. The anaerobes most commonly isolated were: *Peptostreptococcus micros* (14.2% of species isolations), *Prevotella melaninogenica* (6.4%), *Prevotella oralis* (5.2%), *Eubacterium aerofaciens* (4.5%), *Fusobacterium nucleatum* (3.9%), *Prevotella buccae* (3.2%) and *Prevotella intermedia* (2.6%). Gram-negative bacteria accounted for 45.7% of total species isolations.

Pain was associated with 37 out of 70 root canals; or more correctly 37 teeth, because it was impossible to localize pain to an individual canal within a multi-rooted tooth. Of these 37 root canals, two presented with a vital pulp, 22 had non-vital pulps and 13 previous root canal treatment. Bacteria were recovered from all painful canals. Anaerobes were isolated from 70.3% of painful canals and from 29.7% of pain-free canals, confirming their association with pain. *Prevotella* spp. ($P < 0.01$), (predominantly *P. melaninogenica*, $P < 0.01$) and *Peptostreptococcus* spp. ($P < 0.01$), (especially *Pstr. micros*, $P < 0.05$) were the most common isolates. The combination *Peptostreptococcus* spp./*Prevotella* spp. ($P = 0.00015$) was found in 15 canals with history of pain (83.3%) and only in three canals out of 33 with no history of pain. Also *Peptostreptococcus micros*/*Prevotella* spp. occurred in 14 canals with pain (82.3%) and only in three canals without pain. On the other hand *Prev. melaninogenica* and either *Peptostreptococcus* spp. or *Pstr. micros* (all $P < 0.01$) were found present only in canals with a history of pain (Table 2).

Swelling of the periodontal tissues around the tooth sampled was observed in 23 out of 70 cases and was particularly associated with isolation of *Eubacterium* spp. ($P < 0.01$), *Peptostreptococcus micros*. ($P < 0.05$) and *Prevotella* spp ($P < 0.05$). The combination *Pstr. micros*/*Prevotella* spp. ($P < 0.001$) was found in 9/23 patients with swelling and only in 8/47 with no swelling (Table 3).

Table 2 Pain and significant combination of bacteria (P value)

Species	Patients group	
	RC with pain (n=37)	RC without pain (n=33)
PSS/PS	15 (0.00015) ^a	3 (0.02309)
PSS/PM	9 (0.00074)	0 (0.78788)
PSM/PS	14 (0.00004) ^a	3 (0.01356)
PSM/PM	8 (0.00226)	0 (0.63214)

^aDetermined by Pearson chi-square test, the other P values were determined by Fisher's test because tables were larger than 2×2 and Pearson chi-square significance test was not valid in these situations. RC, root canal; PSS, *Peptostreptococcus* spp.; PS, *Prevotella* spp.; PSM, *Peptostreptococcus micros*; PM, *Prevotella melaninogenica*.

Table 3 Swelling and significant combination of bacteria (P value)

Species	Patients group	
	RC with swelling (n=23)	RC without swelling (n=47)
PSM/PS	9 (0.00178) ^a	8 (0.00007)

^aDetermined by Pearson chi-square test.

RC, root canal; PSM, *Peptostreptococcus micros*; PS, *Prevotella* spp.

'Wet canals' refers to the presence of a clear, haemorrhagic or purulent exudate, detected as a distinct dampening or stain on the sampling paper points (Gomes *et al.* 1994a). Canals classified as 'wet', presented in 57 out of 70 cases, were found to be significantly associated with *Prevotella* spp., *Eubacterium* spp. or *Propionibacterium* spp. (each $P < 0.05$), while facultative bacteria ($P < 0.01$) predominated. 'Wet' canals were significantly associated with the following combinations: *Prevotella* spp./*Eubacterium* spp. ($P < 0.01$), which occurred in 10/57 wet canals but in none of the dry canals (0/13), and with *Peptostreptococcus* spp. and *Eubacterium* spp. ($P < 0.05$) occurring in 9/57 wet canals but in none of the dry canals (Table 4).

Discussion

Basic microbiological methods were adapted to recover as many anaerobic species as possible, such as: inoculation of samples immediately, at the chairside, onto pre-reduced nutritious media, which were promptly incubated under suitable gaseous conditions; prolonged incubation to avoid overlooking slow-growing organisms among others. Nevertheless, 14% of root canals had no cultivable bacteria. However, in several studies (Yoshida *et al.* 1987; Hirai *et al.* 1991; Wasfy *et al.* 1992) some root canals have proved free from microorganisms (Gomes *et al.* 1994b). In the present investigation, a previous endodontic therapy had been performed in only one out of 10 root canals with no bacterial growth. It is possible that the sampling with a paper point in re-treatment cases may result in the detection of a lower

Table 4 Wet canal and significant combination of bacteria

Species	Patients group	
	'Wet' canals (n=57)	'Dry' canals (n=13)
PS/ES	10 (0.00021) ^a	- ^b
PSS/ES	9 (0.03558) ^a	- ^b

^aDetermined by Pearson chi-square test.

^bStatistics cannot be computed when the number of non-empty rows or columns is one.

RC, root canal; PSS, *Peptostreptococcus* spp.; PS, *Prevotella* spp.; ES, *Eubacterium* spp.

number of bacteria, because some organisms may also be removed together with the debris. However, negative results are not associated with re-treatment and can, therefore, indicate either absence of bacteria or failure in the microbiological technique (Gomes *et al.* 1994a).

Whereas it is true that commonly occurring species will statistically tend to occur in combination, it is, of course, equally true that the presence of one species may encourage the survival and growth of a second, particular species, thus giving rise to occurrence of species in combination.

In our previous studies (Gomes *et al.* 1994a,b) significant associations were found between specific organisms and pain, tenderness to percussion, swelling, wet canal, purulent exudate, periapical radiolucency and previous root canal therapy. In some of these cases, only one bacterial species was found to be significantly associated with the clinical feature (e.g. periapical radiolucency and *Peptostreptococcus micros*; tenderness to percussion and *Prevotella melaninogenica*; previous root canal therapy and *Propionibacterium acnes*), although in others, such as purulent exudate, five organisms were involved: *Fusobacterium necrophorum*, *Prevotella loescheii*, *Streptococcus constellatus*, *Fusobacterium* and *Bacteroides* spp. In the present investigation, significant bacterial combinations were found to be associated with three out of seven features investigated, because cases like the ones mentioned, with only one organism significantly associated with the clinical feature, were not examined. In respect to purulent exudate, certain bacterial combinations were present, e.g. *Prevotella loescheii* with *Streptococcus constellatus* or *Fusobacterium necrophorum*, *Fusobacterium necrophorum* with *Streptococcus constellatus* or *Bacteroides* spp. However, these combinations were not found to be significantly associated with purulent exudate.

Microbial interactions have been linked with the development of periodontal disease (Socransky *et al.* 1988, Dzink *et al.* 1988, Ohta *et al.* 1991, Wikström *et al.* 1993). According to Ohta *et al.* (1991) the human periodontal disease process is characterized by a changeover of the subgingival microflora from facultatively anaerobic, Gram-positive bacteria to strictly anaerobic, Gram-negative species. Although the exact causes of this succession are not currently known, several reports have indicated that environmental factors such as oxygen tension, the availability of nutrients and pH all have a strong influence on the composition and activity of the subgingival microflora. In dental root canals although the environment is more protective and the microflora relatively simple compared with

the periodontal flora, the factors mentioned above together with the bacterial interactions are among the most important ecological determinants of the endodontic microflora (Farber & Seltzer 1988, Sundqvist 1992a).

When Socransky *et al.* described the role of microbial associations in periodontally healthy and diseased-tissues (1988), they hypothesized that 'if the individual was fortunate enough to encounter pathogens to which his or her microbiota was antagonistic, then long-term health might occur'. On the other hand, they suggested that, if the subjects had pathogens which could readily colonize subgingival sites due to the absence of competitive species, then periodontal disease would develop. The same principle is valid for the success of therapy. They concluded that 'treatment failures could occur if the pathogens were suppressed by the active phase of therapy, but were permitted to recolonize due to the absence of species incompatible with their re-establishment'.

It cannot be confirmed in this study if it was the presence of those associations that initiated the clinical features or vice versa. Nevertheless such combinations between bacteria may well exert an important role in the development (or on the progress) of symptoms of endodontic pathology. For instance the association *Prevotella* spp/ *Eubacterium* spp. was not found in cases of dry canals. Also *Pstr. micros*/*Prev. melaninogenica* was encountered only in canals with pain. On the other hand the combination of *Pstr. micros*/*Prevotella* spp. was reported in cases of both presence or absence of swelling, although the combination was more readily isolated in cases of swelling. Furthermore it can be postulated that if the endodontic therapy is able to eradicate at least one of the organisms jointly associated with the development of a specific clinical feature, then improvement in health should result.

Fabricius *et al.* (1982) who described the influence of combinations of oral bacteria on periapical tissues of monkeys, concluded that some indigenous oral bacteria are capable of inducing apical periodontitis. Additionally, certain combinations of bacteria are more potent in inducing apical periodontitis than are single strains.

According to Rams *et al.* (1992), the Gram-positive anaerobic coccus, *Peptostreptococcus micros*, has been associated with both endodontic (Brook *et al.* 1981, Williams *et al.* 1983, Yoshida *et al.* 1987) and periodontal abscesses (Newman & Sims 1979). This species is able to enhance the virulence of *P. gingivalis*, *P. melaninogenica*, enteric rods, *Pseudomonas* and other bacteria in mixed experimental infections in animals

(Sundqvist *et al.* 1979, Brook & Walker 1984, Brook 1988). Virulence factors of *Pstr. micros* include marked proteolytic enzyme activity, elaboration of hyaluronidase, production of volatile sulphur compounds from L-cysteine, and inhibition of human gingival fibroblast and epithelial cell growth.

The virulence of black-pigmented '*Bacteroides*' strains has also been investigated. These strains have often been isolated from different types of periodontal and also endodontic disease, and thus identified as key organisms in the aetiology of chronic periodontitis (van Steenberg *et al.* 1982). According to the latter authors, who have investigated the virulence of these strains in experimentally-induced skin lesions in mice, the asaccharolytic '*B. gingivalis*' strains (now *Porphyromonas gingivalis*), which were mostly isolated from subgingival plaque associated with adult destructive periodontitis, were characterized by inducing a spreading type of inflammation, mostly resulting in a gravity abscess or a phlegmonous abscess. Strains of saccharolytic '*B. intermedius*' (now *Prevotella intermedia*) isolated from gingivitis or periodontitis, always induced a localized abscess at the site at which they were injected and '*B. melaninogenicus*' strains caused only minimal inflammation. Similarly '*B. gingivalis*' was shown to produce severe rapidly spreading abscesses, while '*B. intermedius*' and '*B. endodontalis*' (now *Porphyromonas endodontalis*) caused localized abscesses of endodontic origin (van Winkelhoff *et al.* 1985).

The former '*B. melaninogenicus* subspecies *melaninogenicus*' (now *Prevotella melaninogenica*) was found to be associated with pain in the present study. Although it has been described by van Steenberg *et al.* (1982) as non-pathogenic, the association with *Pstr. micros* may have been the reason for the development of pain.

Nevertheless the present study suggests that some microbial combinations might be involved with the development of specific endodontic clinical features. Where large numbers of possible effects are tested statistically, false positive results may be expected. For the less significant findings, confirmation (or refutation) will require study of a greater number of cases.

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