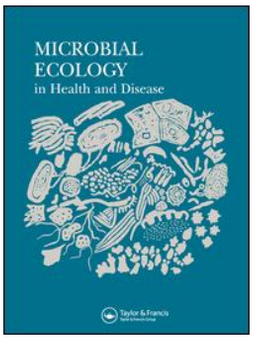

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Predominant Obligate Anaerobes in Necrotic Pulps of Human Deciduous Teeth

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The purpose of this study was to investigate the bacterial composition of necrotic pulps of human deciduous teeth by sampling the split surfaces of freshly extracted teeth and culturing the bacteria present with good anaerobic isolation techniques. Significantly more bacteria were recovered after the incubation in an anaerobic chamber than after aerobic incubation in air with 30 per cent CO_2. Of 276 bacterial isolates, 251 (91 per cent) were obligate anaerobes. These findings suggest that the environment of necrotic pulps in human deciduous teeth is anaerobic and thus favours the growth of anaerobes. Among the 251 obligate anaerobes isolated, strains belonging to the genera *Peptostreptococcus* (25 per cent), *Propionibacterium* (19 per cent), *Eubacterium* (17 per cent) and *Fusobacterium* (13 per cent) were major parts of the bacterial flora of the lesions of human deciduous teeth. *Bifidobacterium* (2 per cent), *Lactobacillus* (1 per cent), *Actinomyces* (1 per cent) and *Veillonella* (0·7 per cent) were minor parts of the flora. The microflora of necrotic pulps of human deciduous teeth is in some respects similar to that reported for the deep layers of dentinal lesions of adults.

KEY WORDS—Children; Deciduous teeth; *Eubacterium*; Necrotic pulps; Obligate anaerobes.

INTRODUCTION

It has been reported that the microflora associated with endodontic lesions of human teeth consists of aerobic and facultative anaerobic bacteria, when conventional bacteriological procedures were used.[4,8,9,25,40] However, by adopting more exacting anaerobic techniques, the flora associated with lesions in human permanent teeth has been shown to consist mainly of obligate anaerobes,[1,5,38,39] suggesting that the environment of the lesions is anaerobic and favours the growth of obligate anaerobes.[1] It is possible that the microflora associated with human deciduous teeth may also consist mainly of obligate anaerobes, but, to date, few attempts have been made to isolate obligate anaerobes from endodontic lesions of deciduous teeth, apart from studies reported by Toyoshima *et al.*[41] and Brook *et al.*[3] Toyoshima *et al.*[41] reported that more than 60 per cent of the isolates in 11 cases were obligate anaerobes, using samples collected from the open root canals after removal of the coronal carious dentine. Unfortunately, this method may give rise to bacterial contamination from carious dentine and dental plaque. Brook *et al.*[3] reported the isolation of obligate anaerobes from periapical abscess in children, although in their study conventional rather than anaerobic cabinet methods were employed.

The purpose of the present study was to investigate the predominant bacteria in necrotic pulps of freshly extracted human deciduous teeth, using an anaerobic cabinet for maximum recovery of obligately anaerobic bacteria.

MATERIALS AND METHODS

Collection of samples

Samples were collected from six human deciduous teeth with endodontic lesions. Immediately after extraction of the tooth, the root apex was sealed with blue inlay wax under a gentle stream of CO_2. After the tooth was notched with a sterile diamond disc to facilitate splitting, it was immediately transferred to an anaerobic cabinet (Model AZ-Hard, Hirasawa, Tokyo, Japan) containing 80 per cent N_2, 10 per cent H_2 and 10 per cent CO_2. While in the cabinet, the tooth was split axially with sterile

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forceps, and infected pulpal tissue exposed on the split surfaces was collected with a sterile excavator.

Isolation of microorganisms

The samples were suspended in 1 ml of sterilised 40 mM potassium phosphate buffer (pH 7·0) and dispersed with a glass homogeniser. Serial 10-fold dilutions (0·1 ml each) were spread onto the surface of brain–heart infusion–blood (sheep) agar (BHI-blood agar) plates[11] and incubated in the anaerobic cabinet and also in air with 30 per cent CO_2 at 37°C for 7 d. All plates, media, buffer solution and experimental instruments were kept in the anaerobic cabinet at least 24 h prior to use. To ensure strictly anaerobic conditions in the cabinet, the reduction of methylviologen (−446 mV) was carefully checked whenever the experimental procedures were carried out.

Because the total number of colony-forming units was higher after anaerobic than after aerobic incubation for every tooth studied, colonies for further inspections were selected only from plates incubated anaerobically. After 7 d of incubation, all colonies from plates with less than 100 colonies were subcultured. Additional colonies did not appear on the plates after a further 7 d incubation period.

Identification

Isolates were identified according to the VPI manual,[11,26] supplemented with information about *Actinomyces*,[35] *Eubacterium*,[10,12,22,30,43,48] *Fusobacterium*,[29] *Peptostreptococcus*,[27] *Porphyromonas*,[13,36] *Prevotella*[13,37] and *Propionibacterium*.[7] Volatile fatty acids (C_2 to C_6), acetoin, diacetyl, alcohols (C_2 to C_6), and free acids and/or methyl derivatives of lactic, succinic, phenyl acetic, phenyl propionic and formic acids, produced in peptone-yeast extract-glucose broth (PYG),[11] were assayed by gas chromatography, as described previously.[14,16,19] In this study, obligate anaerobes were defined as bacteria which grew only in an anaerobic cabinet, and facultative bacteria as those which also grew in air containing 30 per cent CO_2.[1,14,42] It was confirmed at least three times that the obligate anaerobes did not grow in air with 30 per cent CO_2. Bacterial genera and species were identified tentatively by 'key characteristics' described in the VPI manual[11,26] and more conclusively by combining all the morphological and biochemical data.

RESULTS

Out of six human deciduous teeth with endodontic lesions, five teeth had been filled with composite resins. None of the teeth were associated with periodontal pockets. Two teeth had abscesses at the time of sampling (samples 3 and 6), the remaining three teeth did not although they may have previously been abscessed (samples 1, 2 and 4). All of the teeth were beyond restoration and extraction was indicated. The patients were aged between 6 years and 9 years (mean 8 years).

In each case, more bacteria were recovered after anaerobic than aerobic incubation (Table 1), and obligate anaerobes were predominant (mean 91 per cent, range 74–100 per cent, Table 1). In samples 3, 4 and 6, in particular, all the isolates were obligate anaerobes (Table 1).

Of 276 strains isolated as predominant bacteria for identification, 251 (91 per cent) were obligate anaerobes, while 22 (8 per cent) were facultative bacteria and three (1 per cent) were lost before their atmospheric requirements could be tested. The predominant isolates were gram-positive rods (51 per cent) and gram-positive cocci (25 per cent).

The predominant genera of obligate anaerobes isolated were *Peptostreptococcus* (70 isolates, 25 per cent), *Propionibacterium* (52 isolates, 19 per cent), *Eubacterium* (46 isolates, 17 per cent) and *Fusobacterium* (37 isolates, 13 per cent). Other obligate anaerobes were *Bifidobacterium* (six isolates, 2 per cent), *Lactobacillus* (four isolates, 1 per cent), *Actinomyces* (three isolates, 1 per cent), *Veillonella* (two isolates, 0·7 per cent), *Porphyromonas* (one isolate, 0·4 per cent) and *Prevotella* (one isolate, 0·4 per cent), as shown in Table 2. Further identification to species level was also carried out, as shown in Table 2.

The species of *Eubacterium* isolated were *E. alactolyticum*, *E. brachy*, *E. nodatum*, *E. timidum* and *E. lentum* (Table 2). Other isolates assigned to *Eubacterium* (21 isolates, Table 2) could not be classified to any of the established species. Among them, one of the group was assigned to the *Eubacterium* S-group. The morphological and biochemical characteristics of *Eubacterium* S-group are, briefly, as follows: tiny, circular, convex and translucent colonies on BHI–blood agar plates; short-gram-positive rods; poor growth in broth culture with or without carbohydrates; non-reactivity in most of the biochemical tests; no production as end-products from PYG; moderate growth enhancement by arginine. Although their characteristics

Table 1. Number of bacteria recovered from necrotic pulps of deciduous teeth

	Sample number						
	1	2	3	4	5	6	Total
Anaerobic incubation (c.f.u./ml)	$4{\cdot}0 \times 10^1$	$2{\cdot}0 \times 10^6$	$2{\cdot}0 \times 10^6$	$8{\cdot}0 \times 10^2$	$1{\cdot}8 \times 10^4$	$3{\cdot}7 \times 10^6$	
Aerobic incubation	0	$1{\cdot}2 \times 10^5$	$1{\cdot}0 \times 10^1$	NT*	NT*	NT*	
Total of isolation	4	90	74	19	55	34	276
Obligate anaerobes							
No.	3	67	74	19	54	34	251
Per cent	75	74	100	100	98	100	91
Facultative anaerobes							
No.	0	21	0	0	1	0	22
Per cent	0	22	0	0	2	0	8
Asaccharolytic bacteria†							
No.	2	4	74	12	54	12	158
Per cent	50	4	100	63	98	35	57
Saccharolytic bacteria							
No.	1	84	0	7	1	22	115
Per cent	25	93	0	37	2	65	42
Lost‡							
No.	1	2	0	0	0	0	3
Per cent	25	2	0	0	0	0	1

*Aerobic incubation was not tested in these samples.
†Asaccharolytic bacteria were designated as those which were inert in glucose media (PYG).[23,44]
‡Isolates lost before examination of their atmospheric requirements.

resembled those of *E. lentum*, the S-group isolates did not reduce nitrate, unlike known strains of this species. Almost all the isolates belonging to 'asaccharolytic *Eubacterium*', i.e. *E. brachy*, *E. nodatum*, *E. timidum* and *Eubacterium* S-group, appeared as tiny colonies (<0·5 mm diameter) on BHI–blood agar plates.

Of 276 isolates from necrotic pulps of human deciduous teeth, 158 (57 per cent) were asaccharolytic (Table 1). Asaccharolytic bacteria were designated[23,44] as those which were inert in glucose media (PYG).

DISCUSSION

The overwhelming majority (91 per cent) of the isolates from necrotic pulps of deciduous teeth were found to be obligate anaerobes (Tables 1 and 2), even though the definition of obligate anaerobes in this study, described in Methods, is stringent. The result indicates that the environment of necrotic pulps of deciduous teeth is anaerobic and favours the growth of anaerobes. The finding, however, is not unique because obligate anaerobes have been shown to predominate in other studies on samples taken from dentinal and periodontal lesions, when similar isolation procedures were employed.[1,2,14,15,20,21,24,42]

The obligate anaerobic strains, belonging to *Peptostreptococcus*, *Propionibacterium*, *Eubacterium* and *Fusobacterium*, accounted for the majority of isolates from necrotic pulps of deciduous teeth in this study (Table 2), in agreement with the study by Ando and Hoshino[1] on bacteria of endodontic lesions of permanent teeth in adults using similar procedures. In their study, *Lactobacillus*, *Streptococcus* and *Ruminococcus* were also found amongst the predominant genera of isolates. The differences may be due to the fact that samples were taken from the deep layers of infected root dentine of adults' permanent teeth. The present result also agrees with the studies by Sundqvist,[38,39] who isolated *Peptostreptococcus*, *Eubacterium* and *Fusobacterium*, as well as *Lactobacillus*, *Porphyromonas*, *Prevotella* and *Wolinella*, although, in this case, samples were obtained from periapical lesions of intact teeth without carious and traumatic pulpal exposures. These observations indicate that certain specific obligate

Table 2. Bacterial isolates from necrotic pulps of deciduous teeth

	Sample						Total	
	1	2	3	4	5	6	Species	Genus
No. of isolates	4	90	74	19	55	34	276	276 (100%)
Obligate anaerobes	3	67	74	19	54	34	251	251 (91%)
Propionibacterium								52 (19%)
P. propionicum		52					52	
Eubacterium								46 (17%)
E. alactolyticum						17	17	
E. brachy				4			4	
E. nodatum	1				1		2	
E. timidum					1		1	
E. lentum			1				1	
E. S-group			15				15	
E. sp.			6				6	
Bifidobacterium								6 (2%)
B. breve-like				2		1	3	
B. sp.		3					3	
Lactobacillus								4 (1%)
L. plantarum-like	1	3					4	
Actinomyces								3 (1%)
A. odontolyticus				3			3	
Peptostreptococcus								70 (25%)
P. micros	1		3		44	1	49	
P. productus						12	12	
P. sp.		1		3	2	3	9	
Fusobacterium								37 (13%)
F. nucleatum			18		2		20	
F. naviforme			8	3			11	
F. russii					4		4	
F. sp.			2				2	
Porphyromonas								1 (0·4%)
P. levii-like				1			1	
Prevotella								1 (0·4%)
P. bivia-like				1			1	
Veillonella								2 (0·7%)
V. sp.				2			2	
Gram-positive short rods		8						8 (3%)
Gram-negative rods			2					2 (0·7%)
Gram-negative cocci			15					15 (5%)
Unidentified			4					4 (1%)
Facultative anaerobes	0	21	0	0	1	0		22 (8%)
Enterococcus								1 (0·4%)
E. faecalis					1		1	
Lactobacillus								21 (8%)
L. sp.		21					21	
Lost	1	2	0	0	0	0		3 (1%)

anaerobes are common in endodontic lesions of both deciduous and permanent teeth, although their aetiological roles require further investigation.

It has been reported that obligate anaerobes belonging to *Peptostreptococcus*, *Eubacterium* and *Fusobacterium* comprise a significant proportion of the bacterial flora of periodontal pockets[21,28,31,32,42] and are associated with adult periodontal disease.[28,31,42] It appears from the present study that such periodontal disease-associated obligate anaerobes may be present in necrotic pulps of deciduous teeth from early childhood. Thus, it is possible that the teeth with necrotic pulps may act as a reservoir of such obligate anaerobes in children. However, to verify this proposition, a comparative study between children and adults is required. Further speculation that such bacteria in endodontic lesions may sensitize patients during childhood, whilst theoretically possible, lacks convincing proof.

Asaccharolytic obligate anaerobes occupied 57 per cent of the isolates in the present study (Table 1). This indicates that asaccharolytic bacteria, such as peptide- and/or amino acid-degrading bacteria, may have advantages over saccharolytic bacteria in necrotic pulps of deciduous teeth, possibly because of a limited availability of fermentable carbohydrate in this situation. Thus, the environment of the necrotic pulps of deciduous teeth may favour the growth of asaccharolytic bacteria, as suggested previously by Sundqvist.[38,39] Asaccharolytic bacteria are also predominant in periodontal pockets (73 per cent),[23,44] suggesting that the nutritional conditions in necrotic pulps may be in some ways similar to those in periodontal pockets. In contrast, saccharolytic bacteria have been reported to compose the majority (75–95 per cent) in the flora of dental plaque and in dentinal lesions.[23,44]

In this study, asaccharolytic obligate anaerobes were mostly assigned to *Eubacterium*, *Fusobacterium* and *Peptostreptococcus*. Among them, strains assigned to *Eubacterium* S-group, which could not be classified to any of the established species of *Eubacterium*, were predominant among asaccharolytic *Eubacterium* species. Phenotypical characteristics of *Eubacterium* S-group resembled those of *E. lentum*, though some characteristics were atypical. They also resemble *Eubacterium* W2 (Cluster 2) reported by Wade *et al.*[45,46,48] These authors reported that *Eubacterium* Cluster 2 were frequently isolated from acute dentoalveolar abscesses, together with *Prevotella*, α-haemolytic *Streptococcus*, *Peptostreptococcus* and other *Eubacterium* species,[46] although the characteristics of *Eubacterium* Cluster 2 were not given in detail. Recently, various asaccharolytic strains of *Eubacterium*, which cannot be assigned to any established species, have been reported to be isolated as some of the predominant bacterial groups from human periodontal pockets,[10,28,31,42,47,48] and may be associated with periodontal diseases.[10,26,31,42,45,47,48] Thus, the unestablished asaccharolytic species of *Eubacterium*, including *Eubacterium* S-group or *Eubacterium* Cluster 2, may play a considerable role in oral diseases. Further studies on the distribution and the pathogenicity of oral asaccharolytic *Eubacterium* species are required.

It is important to disinfect the bacteria in endodontic lesions for successful treatment,[5,6] especially during periods of root formation or root resorption of deciduous teeth. Several combinations of antibacterial agents have been reported to successfully disinfect carious and endodontic lesions of deciduous teeth *in vitro* and *in situ*,[33,34] as well as carious lesions of permanent teeth.[17,18] In fact, all the bacteria taken from necrotic pulps of deciduous teeth were found to be sensitive to a mixture of antimicrobial agents.[34] Studies are now in progress to evaluate the clinical use of such mixtures in the endodontic treatment of deciduous teeth.

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