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Journal of Dentistry 28 (2000) 45–50

Journal of Dentistry

www.elsevier.com/locate/jdent

Composition of the oral streptococcal flora in healthy children

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Received 16 February 1999; received in revised form 19 May 1999; accepted 14 June 1999

Abstract

Objectives: To identify the predominant streptococcal species in the mouths of healthy children and to investigate the composition of the oral streptococcal flora over a period of 4 months.

Patients and methods: The subjects were 33 fit, healthy schoolchildren aged between 5 and 16 years. These children were part of a large study and were the matched controls for a group of subjects undergoing bone marrow transplantation.

The oral flora was sampled using an oral rinse technique on two separate occasions 4 months apart. The outcome measures were the number of each streptococcal species per millilitre of oral rinse; the isolation frequency of each species; the proportion of each species as a percentage of both the total streptococcal count and the total anaerobic count.

Results: The predominant species were *Streptococcus salivarius*, *S. oralis* and *S. mitis*. There was no significant variation in the composition of the oral streptococcal flora over the 4 month period.

Conclusions: The oral rinse technique provides a reliable method of sampling the streptococcal flora of children.

Keywords: Stability; Reproducibility; Oral streptococci; Oral rinse

1. Introduction

This study was part of a large microbiological [1], immunological and oral health survey [2] of children undergoing bone marrow transplantation. Each bone marrow transplant child was matched with a healthy control child for age, gender, social class and ethnicity. Indices were recorded for dental caries, bacterial dental plaque and gingivitis, and saliva was collected using an oral rinse technique for microbiological and immunological analysis. The oral rinse method has been used by other workers and found to be effective for longitudinal screening [3]. For the matched control children these procedures were carried out on two separate occasions 4 months apart. The microbiological data from the control children are presented here.

The oral cavity is usually sterile at birth and although there is the close contact with the resident bacteria of the birth canal, these do not appear to colonise the neonate's mouth [4]. Within a few hours bacteria may be isolated from the oral cavity [5,6], e.g. streptococci, lactobacilli and veillonella [5]. *Streptococcus salivarius* in particular, becomes well established within 2 days of birth [4,5]. The oral streptococcal flora is affected by physiological changes such as tooth eruption that is essential for the establishment of the mutans species [7,8], plaque accumulation [8], deprivation of the normal diet [1,9], sucrose restriction [10,11] and antibiotic therapy [12,13].

Several workers have examined the acquisition and development of the oral streptococcal flora. Streptococci were found to be the main components of the oral flora of 51 neonates and 44, one-month-old infants, comprising 98% of the total cultivatable bacteria [5]. The oral flora of the one-month-old infants was examined over a period of 1 year and at the end of this time streptococci were still predominant comprising 70% of the total bacterial flora. The species most commonly isolated was *S. salivarius*. Subsequent studies also reported the early acquisition of *S. salivarius* [14,15].

In later studies the streptococcal flora of infants has been investigated taking into account the recent developments in the taxonomy of the genus *Streptococcus*. The prevalence

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0300-5712/99/$ - see front matter
PII: S0300-5712(99)00048-2

and proportions of a wider range of streptococcal species were reported from a group of infants before and after tooth eruption [15]. *S. mitis* biovar I was found to be the major component of the early streptococcal flora and although the prevalence of *S. salivarius* was high, 94%, the proportion of this species, as a percentage of the total bacterial count, was low. *S. oralis* and *S. anginosus* were isolated from approximately one-third of both pre-dentate and dentate infants during the first year of life. *S. sanguis* was not isolated from the pre-dentate infants and from only 50% of those with teeth. Tappuni and Challacombe [8] evaluated the salivary streptococcal counts in a group of mothers and their pre-dentate and dentate infants. The mean streptococcal counts were lowest in the pre-dentate group and highest in the adults. *S. mitis*, *S. oralis* and *S. salivarius* were the species most frequently isolated from all three groups. In a later study of neonates during the first month of life, *S. mitis* biovar I and *S. oralis* comprised 55% of the pioneer streptococci isolates, *S. salivarius* 25.3% and *S. anginosus*, *S. mitis* biovar II, *S. sanguis*, *S. gordonii* collectively 11.4% [16].

These studies have been concerned primarily with the acquisition of a normal oral flora and have concentrated on the establishment of the oral streptococci in neonates and pre-school children. None of the studies determined the overall stability of the streptococcal flora in terms of the percentage composition and frequency isolation of individual species. In the work reported here, the composition of the oral streptococcal flora has been investigated in healthy children aged between 5 and 16 years, over a 4 month period.

2. Subjects and methods

2.1. Subjects

Thirty-three children were recruited from schools in the Merton and Sutton Area Health Authority of Greater London. The criteria for selection were firstly, no underlying medical disorder. Any child taking medication (e.g. Ventolin) or who had been treated with antibiotics within the previous 4 weeks was not included. These children was matched for age, gender, social class and ethnicity with a child undergoing bone marrow transplantation. Three-day diet diaries were given to each child to be completed the evening before the baseline saliva sample was taken.

The mean age of the group was 9.5 years ±1.9 range 5–15.9 years. Twenty-four children were boys. Indices were recorded for dental caries at the beginning of the study using the WHO criteria [17]. The proportion of children who were caries free was 57.6%. The dmft was 1.4, the DMFT was 0.14 and the percentage of untreated disease was 21.1% in the deciduous dentition.

2.2. Oral rinse procedure

Each child was asked to rinse the mouth with 5 ml of sterile 0.9% (w/v) saline for 30 s. This was returned to, and collected in, a sterile container. This method of saliva collection was chosen following a pilot study with bone marrow transplant children. It was similar to the mouth-care regimen and all the children were able to manage it.

2.3. Reproducibility

To establish the reproducibility of the oral rinse as a reliable sampling method, two oral rinses were collected from each of 18 subjects 30 min apart and subjected to microbial analysis.

2.4. Composition of the oral microflora

To investigate the composition of the oral flora, an oral rinse was collected from each child on two separate occasions approximately 4 months apart (range 104–134 days). This time interval was selected since if there was to be any significant change in the composition of oral streptococci, it would be apparent within this time scale. The oral rinses were collected between 09:30 and 11:30 hours on each occasion and processed in the laboratory within 3 h of collection.

2.5. Microbiological processing of the oral rinses

Tenfold serial dilutions of the oral rinses were prepared in Fastidious Anaerobe Broth (Lab M, Bury, Lancs) and 100 μl aliquots of the appropriate dilutions were inoculated onto both selective and non-selective media. Mitis salivarius agar (MSA) (Oxoid Unipath Ltd, Basingstoke, Hampshire) supplemented with 0.1% potassium tellurite (Lab M) was prepared for the growth of the oral streptococci. Mutans streptococci (*S. mutans* and *S. sobrinus*) were isolated from MSA with bacitracin 0.2 units/ml (Sigma–Aldrich Co. Ltd, Poole, Dorset) and sucrose 15% w/v (BMSA) after Gold et al. [18]. The plates were incubated anaerobically at 37°C for 3 days. Fastidious Anaerobe Agar supplemented with 5% (v/v) defibrinated horse blood was prepared to determine the total aerobic and anaerobic counts. The plates for the aerobic counts were incubated in air supplemented with 5% CO_2 at 37°C for 3 days. The other plates were incubated anaerobically in a chamber with an atmosphere consisting initially of 90% nitrogen, 5% hydrogen and 5% carbon dioxide, at 37°C for 7 days.

2.6. Identification of the oral streptococci

Streptococcal colonies from both MSA and BMSA plates were characterised initially by gram staining. Between three and 10 different colony types were identified from the rinses of each child on both occasions. Two of each colony type of the gram-positive cocci were subcultured into separate 4 ml volumes of sterile Todd–Hewitt (Oxoid) broth and

Table 1
Total bacterial counts as colony-forming units per millilitre oral rinse (log_{10}) (nd = not detected; % = isolation frequency)

Species	First oral rinse						4 months later					
	%	Mean	Std. dev.	Median	Min	Max	%	Mean	Std. dev.	Median	Min	Max
S. mutans	33.3	1.10	1.67	nd	nd	4.79	24.2	0.74	1.41	nd	nd	4.81
S. mitis	60.6	3.45	2.91	4.70	nd	7.08	72.7	4.36	2.76	5.73	nd	7.78
S. oralis	66.7	4.07	2.97	5.90	nd	7.90	78.8	4.78	2.62	6.04	nd	7.61
S. sanguis	39.4	2.45	2.86	nd	nd	6.95	45.5	2.69	3.02	nd	nd	7.20
S. parasanguis	51.5	2.78	2.77	4.48	nd	6.15	51.5	2.84	2.83	4.30	nd	6.43
S. gordonii	6.1	0.34	1.38	nd	nd	6.30	6.1	0.36	1.45	nd	nd	6.23
S. salivarius	93.9	5.72	1.59	6.04	nd	7.20	93.9	5.60	1.57	5.89	nd	7.49
S. vestibularis	9.1	0.55	1.77	nd	nd	6.42	6.1	0.37	1.48	nd	nd	6.71
S. intermedius	6.1	0.36	1.46	nd	nd	6.30	3.0	0.21	1.20	nd	nd	6.90
Total aerobic count		7.26	0.50	7.26	5.97	8.22		7.26	0.48	7.09	6.53	8.21
Total anaerobic count		7.50	0.50	7.63	6.61	8.37		7.53	0.50	7.61	6.53	8.38

incubated at 37°C for 48 h. These cultures were checked for purity by gram staining the broth and by inoculation of each broth onto Columbia base agar (Oxoid) supplemented with 5% (v/v) defibrinated horse blood. The blood agar plates were incubated anaerobically at 37°C for 24 h. Any mixed or contaminated streptococcal cultures were subcultured on blood agar until pure. The pure cultures were subjected to a range of carbohydrate fermentation tests using the sterile microtitre format and tests to detect pre-formed enzyme activity [19]. *S. mitis* biovar I and biovar II were differentiated on the basis of arginine hydrolysis [20]. Regular quality testing was carried out by culturing type strains in the same manner. The type strains used were: *S. mutans* NCTC 10449, *S. sobrinus* NCTC 12279, *S. mitis* NCTC 10712, *S. oralis* NCTC 11427, *S. sanguis* NCTC 7863, *S. parasanguis* NCTC 5898, *S. salivarius* NCTC 8618, *S. vestibularis* MM1, *S. gordonii* NCTC 7865, *S. cristae* CR 311, *S. anginosus* NCTC 10713, *S. constellatus* NCDO 2226 and *S. intermedius* NCDO 2227. NCTC and NCDO are abbreviations for National Type Culture Collection and National Collection of Dairy Organisms. *S. cristae* CR311and *S. vestibularis* MMI were provided by Dr R.A. Whiley.

2.7. Detection limits

The detection limits for individual streptococcal species was 0.5% of the total streptococcal count, except for *S. mutans* which was 10 colony forming units per millilitre of oral rinse.

2.8. Analysis of data

The data were tested for normality using the Shapiro–Wilks test [21] and neither the raw nor log_{10} transformed data were normally distributed. All analyses were undertaken on the raw data using appropriate non-parametric statistical tests. The mean, standard deviation and median were calculated for each data set. The data are presented as colony forming units (cfus) per millilitre of oral rinse. The proportion of each streptococcal species is expressed as a percentage of the total streptococcal count and as a percentage of the total anaerobic count. The number of species in each rinse was recorded.

3. Results

3.1. Reproducibility of sampling procedure

The oral rinse sampling method was found to be highly reproducible. There were no significant differences in the total aerobic and anaerobic counts, recovery of mutans streptococci and oral streptococci between the two sets of samples ($p > 0.05$). The total bacterial colony counts and streptococcal counts were significantly correlated ($p < 0.0001$).

3.2. Total bacterial counts

There were no significant differences in the mean total aerobic bacterial counts between the first oral rinse and the second rinse 4 months later (7.26 ± 0.50 and 7.26 ± 0.48) or the total anaerobic counts (7.50 ± 0.50 and 7.53 ± 0.50, respectively) (Table 1). The total anaerobic count was significantly greater than the total aerobic count both at baseline ($p < 0.003$) and after 4 months ($p < 0.001$). There was a significant correlation between the total aerobic ($\rho = 0.36$, $p < 0.04$) and anaerobic counts ($\rho = 0.61$, $p < 0.0001$) at the beginning and end of the study time (Table 1).

3.3. Oral streptococci-isolation frequency

A wide variety of species of oral streptococci was isolated from the oral rinses of these children including *S. mutans*, *S. mitis* biotypes I and II, *S.oralis*, *S. gordonii*, *S. sanguis* biotypes I, II and III, *S. parasanguis*, *S salivarius*, *S. vestibularis*, and *S. intermedius* (Table 1). The most frequently isolated species on both sampling occasions were *S. salivarius*, *S. oralis*, *S. mitis* and *S. parasanguis* (Table 1). The streptococcal flora of the children appeared to be stable as

Table 2
Streptococcal species isolated from oral rinses

Species	Number of children	
	Isolated from first sample (but not 4 months later)	Isolated 4 months later (but not from first sample)
S. mutans	5	2
S. mitis	4	8
S. oralis	4	6
S. sanguis	3	5
S. parasanguis	3	3
S. gordonii	1	1
S. salivarius	2	2
S. vestibularis	1	0
S. intermedius	1	2

there was no significant change in the isolation frequency of individual species from the oral rinses or in the concentration of each species (cfu/ml) between the baseline and after 4 months ($p > 0.05$) (Table 1). There was also no significant difference in the mean number of streptococcal species isolated at baseline (3.7 ± 0.9) or at the end of the study (3.9 ± 1.0).

There were some small differences in isolation frequency of streptococcal species in individual children (Table 2). The concentration of each species was not significantly different between baseline and 4 months later except for *S. sanguis* ($p < 0.04$).

3.4. Individual streptococcal species as a percentage of the total anaerobic count

There was no significant difference in the total streptococcal count as a percentage of the total anaerobic count between the first sample $17.9\% \pm 14.9$ and the second sample 4 months later, $19.6\% \pm 22.1$. There were no significant differences in the proportion of individual species as a percentage of the total anaerobic count between baseline and 4 months later ($p > 0.05$) (Table 3). The mean values of the species showed no significant variation over the period of 4 months, e.g. *S. salivarius* $6.39\% \pm 6.8$ at the beginning and $5.7\% \pm 12.8$ after 4 months; *S. oralis* $5.4\% \pm 9.4$ and $6.7\% \pm 14.6$; *S. mitis* $3.05\% \pm 5.2$ and $3.6\% \pm 5.4$ (Table 3). The mean proportion of *S. mutans* was low on both occasions 0.01 ± 0.03 and 0.004 ± 0.02.

3.5. Individual streptococcal species as a percentage of the total streptococcal count

There was a significant decrease in the proportion of *S. salivarius* as a percentage of the total streptococcal count from a baseline mean value of 39.1 to 28.5 after four months ($p < 0.01$) (Table 4). There were no significant differences in the proportions of any other species isolated at the beginning and the end of the study, e.g. *S. oralis* mean value $22.1\% \pm 23.2$ and $28.0\% \pm 26.9$ ($p > 0.05$); *S. mitis* mean value $14.42\% \pm 20.9$ and $21.1\% \pm 23.4$ ($p > 0.05$).

4. Discussion

The streptococcal flora of the oral cavity of children was investigated and was found to be particularly stable. The isolation frequency of individual species did not change significantly over the 4 month study period. The percentage composition of the streptococcal flora, both as a percentage of the total anaerobic colony count and as a percentage of the total streptococcal colony count was not significantly different after 4 months. The oral microflora was sampled by using an oral rinse technique that is a preferred method as all the oral surfaces are sampled. It was demonstrated to be reproducible and easy to use which is particularly important in sick children from whom discrete samples of dental plaque and microflora from the mucosal surfaces cannot be easily collected because of an increased risk of septicaemia. This oral rinse technique is quick and non-invasive which is important for children who are in hospital for long periods of time undergoing intensive treatment regimens.

Microbial counts have been reported to vary from day to day [22]. This is most probably due to differences in sampling technique and for this reason it has been suggested that quantitative analysis of salivary microorganisms should

Table 3
Proportion of each streptococcal species as a percentage of the total anaerobic count (nd = not detected)

Species	First oral rinse					4 months later				
	Mean	Std. dev.	Median	Min	Max	Mean	Std. dev.	Median	Min	Max
S. mutans	0.01	0.03	nd	nd	0.15	0.004	0.02	nd	nd	0.09
S. mitis	3.05	5.20	0.42	nd	22.88	3.61	5.38	1.33	nd	24.90
S. oralis	5.40	9.44	1.54	nd	40.50	6.67	14.57	3.89	nd	83.67
S. sanguis	1.23	2.96	nd	nd	11.84	1.78	3.16	nd	nd	12.59
S. parasanguis	1.53	3.46	0.10	nd	14.44	1.56	3.78	0.07	nd	20.15
S. gordonii	0.16	0.89	nd	nd	5.13	0.22	1.06	nd	nd	6.01
S. salivarius	6.39	6.82	4.03	nd	27.03	5.72	12.79	2.47	nd	73.13
S. vestibularis	1.35	6.88	nd	nd	39.39	0.38	1.68	nd	nd	9.20
S. intermedius	0.11	0.48	nd	nd	2.63	0.10	0.58	nd	nd	3.32

Table 4
Proportion of each streptococcal species as a percentage of the total streptococcal count (nd = not detected)

Species	First oral rinse					4 months later				
	Mean	Std. dev.	Median	Min	Max	Mean	Std. dev.	Median	Min	Max
S. mutans	0.07	0.21	< 0.001	nd	1.14	0.03	0.15	< 0.001	nd	0.86
S. mitis	14.42	20.92	3.09	nd	72.97	21.07	23.42	12.5	nd	94.37
S. oralis	22.10	23.15	15.46	nd	79.21	28.03	26.91	25.58	nd	90.41
S. sanguis	9.78	18.02	< 0.01	nd	81.82	10.64	15.44	< 0.01	nd	57.69
S. parasanguis	9.76	15.92	1.10	nd	57.14	7.14	11.36	2.02	nd	47.72
S. gordonii	0.82	4.52	< 0.01	nd	25.97	1.42	6.96	< 0.01	nd	39.54
S. salivarius	39.06[a]	25.25	34.15	nd	97.75	28.50[a]	24.23	25.13	nd	99.99
S. vestibularis	3.06	11.94	< 0.01	nd	64.67	2.93	11.94	< 0.01	nd	57.30
S. intermedius	0.92	3.76	< 0.01	nd	18.18	0.24	1.38	< 0.01	nd	7.92

[a] Significant decrease from baseline $p < 0.01$.

be based on several samples from the same individuals collected on different days. In the present study, one investigator using a standardised and reproducible method of oral rinsing. No significant variation was found in the microbial counts even though the two oral rinses were taken 4 months apart.

A wide variety of species was isolated from each child but the predominant species were *S. salivarius*, *S. oralis* and *S. mitis* which is in agreement with earlier studies [8,14,16]. The stability of the oral streptococcal flora of children has not been previously investigated. There have been numerous studies that have been limited to reporting the stability of the salivary concentration of mutans streptococci or lactobacilli in the mouths of children of similar ages. In these studies, the salivary levels of mutans streptococci remained relatively stable in the absence of carious lesion formation, as in the current study. Similar observations have been made at the strain level for mutans streptococci where DNA fingerprinting techniques have demonstrated that the predominant strains of *S. mutans* persist over long periods [23]. There is no other information available on the genotype stability of other streptococcal species in the human oral cavity. However, the genotypic diversity of each species from each child in the two samples could be demonstrated using restriction endonuclease analysis [24], but was beyond the remit of this study. *S. mutans* was isolated from the oral rinse samples of only 33% of the children at baseline and from 24.2% of them 4 months later. These isolation frequencies compare with 83% in pre-school children [25], between 70 and 94% depending on caries status in 6–7 year olds [26] and 77% in young adults [27]. In these studies whole stimulated saliva was used as the source of bacteria while in this investigation the oral flora was sampled using an oral rinse technique.

S. anginsosus has been isolated by other workers [15,16]. Although *S anginosus* was not isolated from the oral rinses children in this study, it was isolated from some of the bone marrow transplant children with whom they were matched [1]. Members of the Anginsosus Group, *S. intermedius* were isolated from some children.

Only one child shed deciduous teeth and eruption of the mandibular first permanent molars occurred in three further children during the study period with no significant effect on the streptococcal flora. The response to the three-day diet diaries was poor so that no relationship could be accurately identified between the diet and the streptococcal flora.

5. Conclusions

The oral rinse technique provides a reliable method of sampling the streptococcal flora of children. It was used in this study to demonstrate the overall stability of the oral streptococcal flora of healthy school children. This is particularly important in clinical research projects involving groups of sick children.

Acknowledgements

We would like to thank the Sir Jules Thorne Charitable Trust for supporting this work and the children in Merton and Sutton Area Health Authority for taking part in the study.

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