ORAL MICROBIOLOGY

Identification of pioneer viridans streptococci in the oral cavity of human neonates

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Summary. Three hundred and sixty-seven strains of pioneer streptococci isolated from the mouths of 40 healthy, full-term infants during the first month of life were examined by two taxonomic schemes that incorporated biochemical and physiological characteristics, IgA1 protease production and glycosidase activities. *Streptococcus mitis* biovar 1 and *S. oralis* comprised 55.0% of the pioneer streptococci isolated from neonates. *S. salivarius* constituted 25.3% of the isolates, while *S. anginosus*, *S. mitis* biovar 2, *S. sanguis* and *S. gordonii* accounted collectively for 11.4%. Difficulties in identifying streptococci were encountered and 8.4% of the 367 isolates could not be assigned to a recognised species.

Introduction

From birth the human oral cavity is a highly selective environment^{1,2} and although during delivery the neonate encounters bacteria from the resident flora of the uterus, vagina and perineum of the mother these do not colonise the infant's mouth.³ The normal oral flora of the adult comprises several hundred species, but few become established in the mouth of the neonate. An oral microbiological study of samples from 50 neonates aged 1–5 days who were hospitalised because of surgical, haematological or infectious complications revealed that *Streptococcus salivarius* was the most prevalent micro-organism, followed by coliforms, staphylococci and enterococci.⁴ The only systematic study of the development of the oral flora in infants has been by McCarthy *et al.*,⁵ who sampled 51 newborn and 44 1-month-old infants. Repeat specimens were collected from the latter group at 8 and 12 months. Streptococci were dominant numerically in the mouth throughout the first year of life, comprising 98% of the cultivable bacteria within 2 days of birth and 70% after 12 months. *S. salivarius* was isolated from half of the neonates and very low numbers of staphylococci, *Lactobacillus*, *Neisseria* and *Veillonella* spp. were isolated occasionally. Carlsson *et al.*^{6,7}

confirmed the early establishment of *S. salivarius* in the mouths of neonates, although this represented < 1% of the total cultivable flora.⁷

As part of a longitudinal study of the relationship between bacterial colonisation and the secretory immune response, 367 isolates of pioneer streptococci isolated from the mouths of 40 full-term, healthy infants during the first month of life were identified by two taxonomic schemes.^{8,9}

Materials and methods

Study population

Forty healthy, full-term infants were enrolled in the study without regard to race or sex. Infant-mother pairs were included in the study after obtaining signed informed consent provided that (1) the infant was full term (38–42 weeks of gestation), (2) the weight of the infant was appropriate for gestational age, and (3) the infant had no underlying illnesses. Conditions which served to exclude an infant-mother pair from the study were: perinatal complications of maternal fever, prolonged rupture of fetal membranes (> 24 h), antibiotic treatment of mother or infant during the first 30 days of the infant's life, and serious metabolic or infectious complications during the first 30 days of life.

Sample collection, processing and culture

Swab samples were obtained 1–3 days, 2 weeks and 4 weeks *post partum*. The mucosal surfaces of the

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cheeks, buccal sulci, edentulous ridges, tongue and hard palate were uniformly sampled with a single swab from a Vacutainer Anaerobic Specimen Collector (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA). The swab was transported anaerobically to the laboratory within 1 h of collection, then placed in 2 ml of reduced transport fluid¹⁰ (RTF). Bacteria were released from the swabs by ultrasound at 80 W for 10 s with a Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a microprobe. The dispersed sample was serially diluted 10⁵-fold in RTF and inoculated on to the following media: trypticase Soy Agar containing sheep blood 5% (TSASB) and Columbia Agar containing sheep blood 5%, cysteine HCl 0.5 g/L, palladium chloride 0.33 g/L, dithiothreitol 0.1 g/L and haemin 5 mg/L (CASB) (both from Remel, Lenexa, KS, USA). Samples were inoculated with a spiral plater (Spiral Systems, Cincinnati, OH, USA). TSASB plates were incubated aerobically at 37°C for 48 h and CASB plates for 3–5 days at 37°C in an anaerobic chamber containing an atmosphere of N₂ 80%, CO₂ 10% and H₂ 10%. Colonies on the TSASB and CASB plates were counted and examined with a dissecting microscope at 20× magnification. A representative of each different colony type was subcultured to purity, examined for haemolysis, stained by Gram's method and tested for catalase production. Colony characteristics after growth on TYC sucrose agar¹¹ were recorded. Streptococci were identified by a combination of two biochemical schemes.^{8,9}

Identification of streptococci

Reference strains. The strains listed in table I were used as internal standards for the biochemical tests and for cluster analysis.

Biochemical tests. Streptococci were subcultured to purity on blood agar plates and incubated for 24 h at 37°C in an atmosphere of air plus CO₂ 5%. Inocula for carbohydrate fermentation tests were prepared by growing streptococci for 24 h in 3 ml of Todd-Hewitt Broth (Difco, Detroit, MI, USA), pH 7.8, in an atmosphere of air plus CO₂ 5% at 37°C. The production of acid from mannitol, sorbitol, raffinose, melibiose, trehalose, amygdalin, inulin, lactose, arbutin, *N*-acetylglucosamine and glucose (all from Sigma, St Louis, MO, USA) was determined in 1-ml volumes of a medium containing thioglycollate broth 24 g/L without dextrose or indicator and purple broth base 16 g/L containing bromocresol purple indicator (both from Difco). The pH was adjusted to 7.2 and the carbohydrates were added at a final concentration of 0.5%. An inoculum of 250 µl from the Todd-Hewitt broth was added and cultures were incubated at 37°C in an atmosphere containing air plus CO₂ 5%; results were read at 2 and 7 days. Acid production was indicated by a change in colour from purple to yellow. Reference strains (table I) were evaluated to establish a pH range for acid production. The pH of weak

Table I. Bacterial strains studied

Species	Strains
<i>S. mitis</i> biovar 1	NCTC 12261 ATCC 15914 SK141 SK137 SK145 J22 HI
<i>S. mitis</i> biovar 2	SK71
<i>S. salivarius</i>	NCTC 8618
<i>S. oralis</i>	NCTC 11427
<i>S. sanguis</i> biovar 1	SK108 SK78
<i>S. sanguis</i> biovar 4	SK112
<i>S. anginosus</i>	SK65
<i>S. gordonii</i> biovar 1	SK6 SK120 SK121 SS38
<i>S. gordonii</i> biovar 2	K1 K4 M5 DL1 D105 G102 SK184
<i>S. gordonii</i> biovar 3	SK8 SK9 SK12 SK33 SK186

ATCC, American Type Culture Collection, Rockville, MD, USA; NCTC, National Collection of Type Cultures, Colindale, London, UK; SK, Dr M. Kilian, Royal Dental College, Aarhus, Denmark; D, G, H, J, K, M, SS, strains from Dr J. Cisar, NIDR-NIH, Rockville, MD, USA.

positive producers was determined and any pH value < 5.9 was considered to be a positive result for acid production. The fermentation of glucose was used as a control of viability of the inoculum and tests were repeated on all isolates that gave a negative result. The production of ammonia from arginine was determined in arginine broth, pH 7.0, containing arginine (Calbiochem, San Diego, CA, USA) 0.3%, tryptose 0.5% and yeast extract 0.5% (both from Difco), dibasic potassium phosphate (Sigma) 0.2% and glucose (Fisher Scientific, Silver Spring, MD, USA) 0.05%. Hydrolysis of aesculin was determined on aesculin agar slants (Remel) and H₂O₂ production with Quantofix® peroxide sticks (Gallard-Schlesinger Industries Inc., Carle Place, NY, USA).

The production of the glycosidases, neuraminidase, β -*N*-acetylglucosaminidase, β -*N*-acetylgalactosaminidase, α -L-fucosidase, β -D-fucosidase, α -glucosidase, β -glucosidase and α -L-arabinosidase was determined with 4-methylumbelliferyl-linked glycosides (Sigma) as described previously.^{8,12}

Analysis of envelope protein profiles. Isolates identified as *S. mitis* biovar 1 and *S. oralis* were grown in an ultrafiltrate (mol. wt cut-off 10 kDa, PM 10 membrane, Amicon Inc., Beverly, MA, USA) of Brain Heart Infusion Broth (Difco). After centrifugation at 7740 g for 20 min, the deposit was washed twice with 10 mM HEPES buffer, pH 7.4, and resuspended in the

same buffer containing 5 mM EDTA. The bacterial suspensions were held on ice and subjected to four 1-min cycles of ultrasound with a Daigger GE 50 micro-ultrasonic processor operated at a setting of 6 and equipped with a 3-mm microprobe (Daigger Corp., Wheeling, IL, USA). After sonication, the bacteria were removed by centrifugation and the supernates were assayed for total protein (BCA Protein Assay, Pierce Chemical Co., Rockford, IL, USA); 10 µg of protein from each isolate were subjected to SDS-PAGE under reducing conditions with 12% gels. As an internal standard, an extract of *S. mitis* biovar 1 (NCTC 12261) was run on each gel along with mol. wt standards. Preliminary experiments were performed to ensure the reproducibility of the extraction procedure. Each lane was scanned with an Ultrosan XL Laser Densitometer (Pharmacia-LKB, Uppsala, Sweden). The scans were normalised by converting the migration distances of individual bands to mol. wt by interpolation into a curve constructed from the mol. wt standards with Lab Calc® densitometry software (Galactic Industries Corporation, Salem, NH, USA). R^2 always exceeded 0.90.

Production of IgA1 protease. Isolates were tested for the production of IgA1 protease as described previously¹³ with an IgA1κ paraprotein (Organon Teknika-Cappel, West Chester, PA, USA). *S. sanguis* biovar 1 (ATCC 10556) incubated with IgA1κ served as the positive control and IgA1κ with buffer served as the negative control.

Cluster analysis. Strains were clustered hierarchically by the average linkage method (SAS/PC 6.04 statistical package, SAS Institute Inc., Cary, NC, USA). The fermentation, physiological and substrate hydrolysis data were coded as 0 (negative reaction) or 1 (positive reaction).

Results

Samples were obtained from 27 subjects at 1–3 days, from 40 subjects at 2 weeks and from 38 subjects 4 weeks *post partum*. The species and proportions of streptococci isolated from the neonates at each visit are presented in table II. The dominant species isolated at all three sampling times were *S. mitis* biovar 1, *S. oralis* and *S. salivarius*. These three species comprised 80.1% of the streptococci obtained from the neonates. Together, *S. anginosus*, *S. mitis* biovar 2, *S. sanguis* and *S. gordonii* accounted for 11.4% of the pioneer streptococci isolated. The diversity of the streptococcal species isolated from the mouths of the neonates increased with time. By visit three, 1 month *post partum*, all neonates were colonised by more than one species of *Streptococcus*. Examples of the diversity of oral streptococci isolated from the infants 1 month *post partum* are shown in table III.

Overall, 367 isolates were examined and 8.5% could not be assigned to any species. Twenty-two isolates failed to ferment glucose, were otherwise bio-

chemically unreactive and were excluded from the data, although 16 produced IgA1 protease. Table IV shows the percentages of isolates that gave positive reactions for each of the determinative tests. Whereas *S. mitis* biovar 2 isolates were separated readily from *S. mitis* biovar 1 isolates on the basis of hydrolysis of arginine and lack of IgA1 protease, the separation of *S. sanguis* and *S. gordonii* isolates into biovars proved unsuccessful. The same problem emerged when control strains of *S. sanguis* and *S. gordonii* were analysed.

A high proportion (61%) of isolates identified by biochemical tests as *S. mitis* biovar 1 produced IgA1 protease. Differentiation between *S. mitis* biovar 1 and *S. oralis* was also based on the production of β-N-acetylglucosaminidase and β-N-acetylgalactosaminidase by *S. oralis*. However, in the present study 23% of isolates that produced β-N-acetylglucosaminidase and 13% of isolates that produced β-N-acetylgalactosaminidase were assigned to *S. mitis* biovar 1 because they failed to produce IgA1 protease.

Cluster analysis placed all isolates identified as *S. salivarius* into a single cluster that included the type strain NCTC 8618. The nine *S. gordonii* isolates fell into two closely related clusters that included all of the type strains, but cluster analysis failed to differentiate the biovars of this species. Isolates identified as *S. mitis* biovar 2 were included in a single cluster together with the type strain. However, isolates identified as *S. oralis*

Table II. Percentage of streptococcal species isolated from neonates 1–3 days and 2 and 4 weeks *post partum*

Identity of streptococci	Time <i>post partum</i>		
	1–3 days (n = 70)*	2 weeks (n = 135)†	1 month (n = 162)‡
<i>S. oralis</i>	41.4	24.4	19.8
<i>S. mitis</i> biovar 1	30.0	28.1	29.6
<i>S. mitis</i> biovar 2	4.3	0.7	1.2
<i>S. salivarius</i>	10.0	30.0	28.4
<i>S. sanguis</i>	4.3	3.0	1.9
<i>S. anginosus</i>	2.9	5.2	4.9
<i>S. gordonii</i>	1.4	1.5	3.7
unidentified	5.7	7.4	10.5

*No bacteria isolated from five of 27 neonates sampled.

†No bacteria isolated from two of the 40 neonates sampled.

‡Bacteria isolated from all 38 neonates sampled.

Table III. Examples of the diversity of oral streptococci in neonates sampled 1 month *post partum*

Species	Number of neonates
<i>S. mitis</i> 1, <i>S. salivarius</i>	6
<i>S. mitis</i> 1, <i>S. oralis</i>	2
<i>S. mitis</i> 1, <i>S. oralis</i> , <i>S. salivarius</i>	4
<i>S. mitis</i> 1, <i>S. salivarius</i> , unidentified species	3
<i>S. mitis</i> 1, <i>S. oralis</i> , <i>S. sanguis</i> , unidentified species	6
<i>S. mitis</i> 1, <i>S. oralis</i> , <i>S. mitis</i> 2, <i>S. gordonii</i> , unidentified species	1
<i>S. gordonii</i> , <i>S. sanguis</i> , <i>S. salivarius</i> , <i>S. anginosus</i> , unidentified species	1

Table IV. Results obtained with streptococcal isolates in the determinative tests

Characteristics	Percentage of isolates that gave positive results						
	<i>S. sanguis</i> (n = 10)	<i>S. mitis</i>		<i>S. oralis</i> (n = 94)	<i>S. gordonii</i> (n = 9)	<i>S. salivarius</i> (n = 93)	<i>S. anginosus</i> (n = 17)
		1 (n = 107)	2 (n = 6)				
Colony characteristics							
α-haemolysis	+	+	v	+	+	—	v
γ-haemolysis	—	—	v	—	—	+	v
colony type on TYC	H/A	S	S	S	v	G	v
Acid production from							
mannitol	10	0	0	2	0	1	65
sorbitol	10	3	50	3	33	26	24
raffinose	50	14	100	15	67	68	65
melibiose	40	14	100	16	67	26	47
trehalose	40	9	0	6	33	38	53
amygdalin	90	8	17	3	78	70	77
inulin	30	2	0	1	0	52	41
lactose	80	86	100	95	100	97	100
arbutin	90	16	17	7	89	96	94
<i>N</i> -acetylglucosamine	100	91	100	95	100	91	94
Biochemical characteristics							
arginine hydrolysis	40	3	100	11	89	3	41
aesculin hydrolysis	50	8	67	5	100	97	88
H ₂ O ₂ production	50	90	100	97	100	36	53
IgA1 protease	100	61	0	100	0	0	0
Enzymes							
Neuraminidase	0	43	0	100	11	0	12
β- <i>N</i> -acetylglucosaminidase	0	23	100	100	100	0	29
β- <i>N</i> -acetylgalactosaminidase	0	13	100	97	100	0	12
α- <i>L</i> -fucosidase	0	1	0	0	11	1	0
β- <i>D</i> -fucosidase	10	0	0	26	0	72	41
α-glucosidase	20	59	100	76	100	81	47
β-glucosidase	10	2	17	0	22	71	41
α- <i>L</i> -arabinosidase	10	0	0	1	0	81	47

n, number of strains tested; v, variable reactions; H/A, hard/adherent; G, gummy, S, soft.

Figure. SDS-PAGE electrophoretogram of the envelope protein profiles of *S. mitis* biovar 1 and *S. oralis* strains. Lane 1, *S. mitis* biovar 1 NCTC 11261; 2, *S. mitis* biovar 1 SK137; 3, *S. mitis* biovar 1 ATCC 15914; 4, *S. oralis* NCTC 11427; 5, *S. oralis* MCS166; 6, *S. oralis* MCS258.

and *S. mitis* biovar 1 formed a single large cluster that included the type strains of both species. Attempts to separate these two species into distinct clusters by

increasing the sensitivity of the analysis and by weighting particular differential tests were unsuccessful.

The envelope protein profiles of isolates identified as *S. mitis* biovar 1 and *S. oralis* were very similar. Representative profiles of these species are shown in the figure.

Discussion

Alpha- and non-haemolytic streptococci are pioneer species in the colonisation of the human mouth and comprise a large part of the commensal microbiota of the oral cavity and pharynx.^{1,2} These bacteria are associated with dental caries and account for about half the cases of bacterial endocarditis.¹⁴

In the present study, a non-selective medium was used to isolate streptococci to avoid, as far as possible, inhibiting the growth of any of the strains in the samples. It is accepted that different oral streptococcal species and biovars can show the same colonial morphology. Therefore, it is possible that the colonies we selected did not include all the species or biovars that were in fact present in the primary cultures. However, the investigation has demonstrated the increasing diversity of the oral streptococcal flora during the first month of life and has characterised the predominant oral *Streptococcus* spp. in neonates.

The identification of viridans streptococci is a difficult undertaking due to lack of uniformity in cultural and biochemical characteristics and the existence of different nomenclature and classification schemes.^{8,9,15,16} Two biochemical schemes have been proposed for the speciation of viridans streptococci^{8,9} based on fermentation and hydrolytic tests, the production of IgA1 protease and a range of glycosidase activities. However, both are based predominately on the reactions of relatively small numbers of well characterised strains with few wild-type isolates. The scheme proposed by Kilian *et al.*⁹ has been used to study the ecology of *Streptococci* in the adult oral cavity¹⁷ and a success rate of 97.9% was claimed for speciation.

The identification of wild-type isolates of viridans streptococci remains difficult, mainly because there is uncertainty concerning the division of biovars in certain species. For example, Kilian *et al.*⁹ described four biovars of *S. sanguis*, three biovars of *S. gordonii* and two biovars of *S. mitis*, whereas Beighton and co-workers⁸ divide *S. sanguis* into only three biovars and do not subdivide *S. gordonii* and *S. mitis*. Furthermore, Beighton *et al.*⁸ include five additional species not described by Kilian *et al.*⁹

Although combining the identification schemes proposed by Beighton *et al.*⁸ and Kilian *et al.*⁹ allowed 92% of the isolates to be identified in the current study, several areas of difficulty were encountered. The major difficulty was in differentiating between *S. oralis* and *S. mitis* biovar 1. On the basis of DNA-DNA hybridisation of six strains, *S. oralis* appears to be genetically homogeneous and well separated from other species, including *S. mitis*.¹⁸ Kilian *et al.*⁹ reported that *S. oralis* (18 strains) invariably produced

IgA1 protease, whereas this characteristic was present in only 20% of *S. mitis* biovar 1 strains and never recorded for *S. mitis* biovar 2 strains. These results suggest that the production of IgA1 protease is a valuable test to differentiate between *S. oralis* and *S. mitis* biovar 1. However, 61% of the isolates in our study that were identified by biochemical tests as *S. mitis* biovar 1 also produced IgA1 protease. IgA1 protease-negative strains of *S. oralis* have not been described and, although the strains of *S. mitis* biovar 1 that produce β -N-acetylglucosaminidase and β -N-acetylgalactosaminidase (table IV) could be placed in *S. oralis*, it was decided that, at present, they should be designated *S. mitis* biovar 1. It is significant that cluster analysis in the current study failed to differentiate between *S. mitis* biovar 1 and *S. oralis* and also that protein profiles of isolates of these species were remarkably similar. The reported ability of *S. mitis* to bind amylase may be a useful test to distinguish this species from *S. oralis*.¹⁹

Our finding that almost half of the streptococci colonising the oral mucosa of the neonates produce IgA1 protease²⁰ is consistent with the results of previous studies of adults.^{17,21,22} However, whether or not the production of IgA1 protease by *S. mitis* biovar 1 and *S. oralis* confers an ecological advantage on these streptococci with regard to colonisation of the oral cavity immediately *post partum* remains to be determined. The production of IgA1 protease by such a high proportion of our *S. mitis* biovar 1 isolates may seem unusual but samples were obtained from the oral mucosa rather than from dental plaque. Interestingly, Nyvad and Kilian²³ have reported that the majority of *S. mitis* biovar 1 strains isolated from buccal mucosa and early dental plaque samples produced IgA1 protease.

Since the recent changes in the taxonomy of oral streptococci,^{9,12,24-26} Smith *et al.*²⁷ have examined the bacterial colonisation of infants with a limited number of the determinative tests proposed by Kilian *et al.*⁹ Although this approach precluded assignment of isolates to all of the currently recognised species of viridans streptococci, *S. mitis* biovar 1 was the predominant species isolated from neonates.²⁷ The results of the current study confirm this, but also indicate that *S. oralis* and *S. salivarius* are prominent in the mouths of neonates. In a comprehensive study of the ecology of oral streptococci in six healthy adult subjects,¹⁷ *S. mitis* biovar 1 was a substantial component of the total streptococci recovered from the buccal and pharyngeal mucosa, and the dorsum of the tongue. Interestingly, while *S. oralis* was commonly present on the oral mucosa of the neonates, it was found almost exclusively on the teeth of the adults by Frandsen *et al.*¹⁷ Also, *S. sanguis* was the predominant streptococcal species on the buccal mucosa of the adults,¹⁷ but was rarely isolated from the neonates. *S. mitis* biovar 2 and *S. salivarius* were prominent on the dorsum of the tongue and pharynx of adults¹⁷ and, although the latter appeared prominent in the mouths of the

neonates, the former was rarely isolated. The differences between the distributions of streptococcal species in adults and neonates may reflect the fact that neonates lack teeth which provide non-shedding surfaces for the colonisation of oral bacteria. The eruption of teeth with the concomitant formation of

the gingival sulcus are known to play important roles in allogenic succession in the oral cavity.²⁸

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