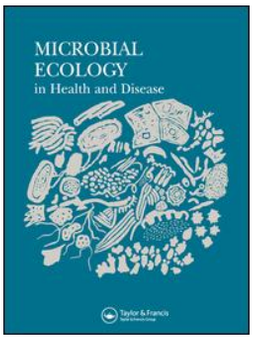

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Isolation, Identification and Prevalence of *Streptococcus anginosus*, *S. intermedius* and *S. constellatus* from the Human Mouth

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This study was carried out to determine the prevalence and proportional distribution of the potentially pathogenic species *Streptococcus anginosus*, *S. intermedius* and *S. constellatus* at sites within the human mouth. The samples from 162 different subjects included supragingival plaque and saliva (18 subjects), subgingival samples (14 subjects) and mouthrinsings (130 subjects). All three species were recovered on nalidixic acid sulphamethazine selective agar at rates comparable with those obtained on a non-selective medium (Columbia blood agar). *S. anginosus* was the most frequently isolated of the three species, being found in 61·0 per cent of subjects examined, while *S. intermedius* and *S. constellatus* were recovered from 53 per cent and 13 per cent of subjects respectively. *S. anginosus* was recovered more frequently and in higher numbers at all oral sites examined than the other two species. *S. constellatus* was isolated least often and in lower numbers, except in the subgingival samples where it occurred as frequently and in similar numbers to *S. intermedius*. The majority of sites harboured *S. anginosus* and *S. intermedius* either singly or together, whilst the combination *S. intermedius* with *S. constellatus* or all three species together, occurred rarely. Sites most frequently harboured only a single phenotype of a species (78 per cent of *S. anginosus*, 83 per cent of *S. intermedius* and 91 per cent of *S. constellatus* yielding sites). Two phenotypes of a species were found together much less frequently and three phenotypes were rarely detected from a single site. Of the 129 isolates of *S. anginosus* recovered during this study, only three (2 per cent) were of the so-called broadly fermentative biotype (which ferment mannitol and raffinose), previously associated with urogenital sources.

KEY WORDS—*Streptococcus anginosus*; *S. intermedius*; *S. constellatus*; Oral cavity.

INTRODUCTION

Streptococcus anginosus, *S. intermedius* and *S. constellatus*, previously classified together as *S. anginosus*[8] and sometimes referred to as the '*Streptococcus milleri*-group' (SMG)[14,15] have recently been shown to form a group of distinct but phylogenetically closely related species.[5,26] Although SMG strains are recognised as members of the normal microbiota of the human mouth, gastrointestinal tract and urogenital tract,[1,16–20,22] these streptococci are of clinical significance due to their association with widespread purulent infections at oral and other sites, that may include the brain, liver, lungs and spleen.[3,9,14,21,23]

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Since publication of these species' descriptions,[25] some light has been shed on the associations of *S. intermedius*, *S. anginosus* and *S. constellatus* with particular types of infections,[27,28] but their prevalence and relative proportions at normal body sites remain to be determined. Recently published studies on the distribution of these streptococci[2,7,13,29,30] have been carried out using identification schemes that identified all strains as '*S. milleri*' (synonymous with *S. anginosus* as classified by Coykendall *et al.*)[8] or which relied mainly on lactose fermentation, haemolytic reaction on blood agar and possession of a Lancefield grouping antigen (groups A, C, F, G or ungroupable) to characterise and assign strains to particular taxa or 'species'.[10–12] These latter characteristics are

unsuitable for differentiating between *S. anginosus*, *S. intermedius* and *S. constellatus*, although they may provide useful additional strain information, for example, in epidemiological studies.[28]

In the light of the recent taxonomic revision of the '*S. milleri*-group' the present study was undertaken in order to investigate the frequency of isolation (prevalence) and proportional distribution of these clinically important species within the human oral cavity.

MATERIALS AND METHODS

Recovery of SMG strains on NAS agar

Laboratory-stored strains of *S. anginosus*, *S. intermedius* and *S. constellatus* together with strains of all other currently recognised species of oral streptococci were seeded onto nalidixic acid sulphamethazine (NAS) agar containing: 40 g/l Sensitivity agar (STA Lab 12; Lab M, Amersham, Bury, UK), 30 μg/ml nalidixic acid (Sigma Chemical Co. Ltd, Poole, Dorset, UK), 1000 μg/ml sulphamethazine, (4-amino-*N*-[4,6-dimethyl-2-pyrimidinyl]-benzenesulphonamide) (Sigma) and 5 per cent vol/vol defibrinated horse blood (Becton-Dickinson UK Ltd, Oxford, UK). The selection and recovery of SMG strains and other streptococci on NAS agar was compared with the growth and recovery obtained on Columbia agar (Gibco BRL, Paisley, Scotland) plus 5 per cent (vol/vol) defibrinated horse blood (Becton-Dickinson UK Ltd).

Oral sites examined

Supragingival plaque and saliva were collected from 18 laboratory personnel of the Department of Medical Microbiology, LHMC. Plaque was collected by sterile toothpick from the buccal surfaces of the upper right first and second molars and homogenised for 1 min in 1 ml thioglycollate broth (Difco Laboratories, Detroit, MI, USA) using a glass grinder. Unstimulated saliva (1–2 ml) from these subjects was collected in sterile plastic tubes. Subgingival samples were collected from periodontal pockets (>5·0 mm in depth) of 14 patients attending the LHMC Dental Institute using a sterile paperpoint and dispersed into 1 ml thioglycollate broth (Difco) by vortexing for 30 s together with 3·5–4·5 mm diameter glass beads (BDH Laboratory Supplies, Merck Ltd, Leicestershire, UK).

In order to obtain a measure of the prevalence of SMG species present in the whole mouth, mouthrinses from 130 healthy students (non-dental) participating in another study were collected using 10 ml distilled water to rinse the mouth for 15 s.

Subjects studied were medically healthy and, with the exception of those patients providing subgingival plaque samples, were also dentally healthy. None of the subjects were receiving or had recently been receiving any antibiotic treatment at the time of sampling.

Isolation and identification

After collection and homogenisation as required, all samples were serially diluted 10-fold in thioglycollate broth. Fifty microlitres of diluted supragingival plaque, subgingival sample or saliva and 100 μl diluted mouthrinse were spread onto: (1) Columbia blood agar for total bacterial colony forming units (c.f.u.), (2) Mitis-Salivarius agar (Difco) with 0·001 per cent potassium tellurite for total streptococcal c.f.u., and (3) NAS agar. All media were incubated at 37°C for 2 d anaerobically in an atmosphere of 10 per cent H_2, 10 per cent CO_2, 80 per cent N_2.

The colonies cultured on the NAS agar were differentially counted and two representative examples of each colony type subcultured onto Columbia blood agar prior to identification using 4-methylumbelliferyl (4-MU)-linked substrates together with tests for the ability to ferment carbohydrates, aesculin hydrolysis and production of ammonia from arginine, carried out in microtitre trays as previously described.[4,28] Isolates recovered on NAS agar were also tested for Gram reaction and catalase production.

RESULTS

Selectivity of NAS medium and recovery of S. anginosus, S. intermedius *and* S. constellatus

The results of testing the growth and recovery of pure cultures on NAS medium are shown in Table 1. All strains of *S. anginosus*, *S. intermedius* and *S. constellatus* grew on this medium with sufficiently high recovery when compared to incubation on Columbia blood agar to demonstrate the suitability of this medium for selecting these species from clinical specimens. Marginally lower recoveries were obtained for *S. constellatus* strains than for the other two species, but with 8/9 of the former strains tested recovered at ≥50 per cent of the counts on Columbia agar, this was not considered to be a serious drawback for detection. Of the other currently

Table 1. Percentage recovery of pure cultures of *S. anginosus*, *S. intermedius*, *S. constellatus* and other oral streptococci on nalidixic acid sulphamethazine selective medium

Species	Recovery (%)*	
	Range	Median
S. intermedius ($n=8$)	78–100	96
S. anginosus ($n=10$)	67–100	82
S. constellatus ($n=9$)	35–100	66
S mutans ($n=8$)	0–97	76
S. sobrinus ($n=5$)	0–72	0

*% recovery: (c.f.u./ml on NAS/c.f.u./ml on Columbia agar) × 100.
Not recoverable: *S. crista*, *S. gordonii*, *S. sanguis*, *S. parasanguis*, *S. oralis*, *S. mitis*, *S. salivarius*, *S. vestibularis* (two strains of each tested).

recognised species of oral streptococci, only *S. mutans* (6/8 strains) and *S. sobrinus* (1/5 strains) grew on NAS agar, indicating that it is reasonably selective for the isolation and enumeration of *S. anginosus*, *S. intermedius* and *S. constellatus*. During the course of examining the 180 oral samples in this study, a total of 129 strains of *S. anginosus*, 99 strains of *S. intermedius* and 22 strains of *S. constellatus* were isolated together with 69 strains of *S. mutans*, eight strains of *S. salivarius* and 54 unidentified gram-positive, catalase-negative cocci or rods. The latter strains were not examined further for the purposes of this investigation.

Isolation of S. intermedius, S. anginosus *and* S. constellatus *from oral sites*

S. anginosus was isolated most frequently at all sites examined, being identified in 109/180 (60 per cent) of the oral samples (Table 2). In contrast, *S. constellatus* was isolated least often, with overall isolation in 23/180 (13 per cent) of the oral samples examined. An exception to this trend was observed in the subgingival samples where *S. constellatus* was identified as frequently as *S. intermedius* (i.e. in 5/14, 36 per cent of cases). Of the total of 162 subjects included in this study, 99 (61·0 per cent) harboured *S. anginosus*, 86 (53 per cent) *S. intermedius* and 27 (13 per cent) *S. constellatus*.

In the majority of cases, subjects harboured *S. anginosus* and *S. intermedius* either singly or together (Table 3). In contrast, *S. intermedius* and *S. constellatus* were rarely found together in the same subject. Overall, of the total of 180 oral samples examined, 91 samples (approximately 50 per cent) harboured only one of these species and 63 samples (35 per cent) contained two species. All three species were not encountered together in supragingival plaque, subgingival samples or saliva and were detected in only 4/130 (3 per cent) of the mouthrinse samples.

The majority of the oral sites and samples examined harboured only one phenotype of a particular species (differentiated on the bases of biochemical test results and colonial morphology): 85/109 (78 per cent) of the samples in which *S. anginosus* was identified harboured only one phenotype of this species, 17/109 (16 per cent) harboured two phenotypes and 4/109 (4 per cent) harboured three phenotypes. Single phenotypes of *S. intermedius* were isolated from 75/90 (83 per cent) of samples in which this species was found to be present, two phenotypes were detected in 10/90 (11 per cent) and three phenotypes in 3/109 (3 per cent) of the samples; 21/23 (91 per cent) of the oral samples in which *S. constellatus* was identified harboured only one phenotype of this species and 2/23 (9 per cent) were found to contain two phenotypes.

In two of the 18 individuals from whom both saliva and plaque were collected, the species of the SMG found to be present at these sites were different. In an additional seven cases no member species of the '*S. milleri*-group' were recovered from the dental plaque sampled, despite being isolated from the saliva of these subjects. These observations might have been due to site-to-site variation in the incidence of these species in dental plaque, although this must remain speculative until further studies involving sampling dental plaque from different sites within an individual have been undertaken.

Of the *S. anginosus* strains isolated during the course of this investigation, only 3/129 (2 per cent) were of the more widely fermentative biotype, characterised by their ability to ferment both mannitol and raffinose.[2] *S. anginosus* strains formed the largest proportion of both the total bacterial and streptococcal microbiota in all sites and sample types compared with *S. intermedius* and *S. constellatus* and comprised up to 14·8 per cent and 43 per cent of these microbiota in supragingival plaque respectively (Table 4), although a longer incubation time than 2 d for the non-selective anaerobic plates might have reduced these estimates to some extent.

Table 2. Frequency of isolation of *S. intermedius*, *S. anginosus* and *S. constellatus* from oral sites

Site/sample	*S. anginosus*	*S. intermedius*	*S. constellatus*
Supragingival plaque ($n=18$)	13 (72)	8 (44)	2 (11)
Supragingival sample ($n=14$)	7 (50)	5 (36)	5 (36)
Saliva ($n=18$)	12 (67)	8 (44)	4 (22)
Mouth-rinses ($n=130$)	77 (59)	69 (53)	12 (9)
Total no. of sites ($n=180$)	109 (60)	90 (50)	23 (13)
Total no. of subjects ($n=162$)	99 (61)	86 (53)	21 (13)

Numbers in parentheses are percentages of samples of particular sites from which the species were detected.

Table 3. Combinations of *S. anginosus*, *S. intermedius* and *S. constellatus* identified at oral sites

Species	Supragingival ($n=18$)	Subgingival ($n=14$)	Saliva ($n=18$)	Mouth-rinses ($n=130$)	Total ($n=180$)
S. intermedius alone	6	7	28	24	21
+ *S. constellatus*	0	0	0	<1*	1
+ *S. anginosus*	39	28	17	28	28
S. constellatus alone	0	21	6	<1	3
+ *S. anginosus*	11	7	17	5	7
S. anginosus alone	28	14	33	25	25
All three species	0	0	0	3	2
Not isolated	17	21	0	14	13

Numbers are the proportion (percentage) of samples of a particular type with the species combination shown.
* <: less than 1 per cent of samples having species combination indicated.

DISCUSSION

The results obtained using NAS semi-selective agar demonstrate that this medium is well suited to facilitate the quantitative isolation of the three species *S. anginosus*, *S. intermedius* and *S. constellatus* from the clinical specimens. The use of a sulphonamide as an agent for the selection of certain species of streptococci has been reported previously for the isolation of *S. mutans*[6] and, together with nalidixic acid, in a broth for the enrichment of '*S. milleri*' prior to seeding on solid media,[10,20] or together with nalidixic acid in an agar medium for the selection of '*S. milleri*' in faeces.[24] However, as *S. anginosus*, *S. intermedius* and *S. constellatus* were not recognised according to their current descriptions at the time of these studies, the present study was needed to demonstrate that all three member species of the SMG can be recovered from mixed bacterial ecosystems using this medium. The slight reduction observed in the recovery of some reference strains of *S. constellatus* using NAS agar indicates that the numbers of this species present in a clinical specimen would not be seriously underestimated. Even assuming only approximately 50 per cent recovery for all strains of *S. constellatus* on this medium (an overly conservative assumption not supported by the actual results obtained using test strains of which 4/9 (44 per cent) were recovered at >70 per cent), the overall picture of the prevalence and numbers of this species at oral sites, relative to *S. anginosus* and *S. intermedius*, would not be markedly altered.

As mentioned above, direct comparison of these results with previous studies is hindered by the differences in the phenotypic criteria employed to characterise strains and the failure previously to recognise the three distinct species. Mejàre and

Table 4. *S. anginosus*, *S. intermedius* and *S. constellatus* as a percentage (mean ± SD) of the total bacterial and streptococcal concentrations (c.f.u./ml) of first dilution obtained at each site

	% of total bacterial count			% of total streptococcal count		
%	*S. anginosus*	*S. intermedius*	*S. constellatus*	*S. anginosus*	*S. intermedius*	*S. constellatus*
Supragingival plaque ($n=18$)						
Mean	2·2 (±3·85)	0·5 (±1·09)	0·12 (±0·52)	6·6 (±10·9)	1·75 (±4·03)	0·23 (±0·77)
Range	0–14·8	0–4·5	0–2·2	0–43·0	0–17·2	0–3·3
Median	0·5	0	0	1·2	0	0
Subgingival plaque ($n=14$)						
Mean	0·3 (±0·45)	0·4 (±1·29)	0·007 (±0·01)	2·2 (±4·7)	1·24 (±2·9)	1·0 (±2·3)
Range	0–2·0	0–4·7	0–0·05	0–16·3	0–10·3	0–8·4
Median	0·003	0	0	0·02	0	0
Saliva ($n=18$)						
Mean	0·4 (±0·54)	0·04 (±0·07)	0·024 (±0·06)	1·4 (±2·42)	0·1 (±0·12)	0·02 (±0·07)
Range	0–1·4	0–0·24	0–0·2	0–10·0	0–0·4	0–0·2
Median	0·14	0	0	0·23	0	0
Mouth-rinses ($n=130$)						
Mean	0·35 (±0·99)	0·06 (±0·35)	0·06 (±0·35)	0·8 (±2·18	0–0·56 (±1·34)	0·105 (±0·6)
Range	0–9·0	0–1·25	0–1·25	0–22·0	0–12·7	0–5·6
Median	0·045	0·028	0	0·13	0·7	0

Edwardsson[17] and Ball and Parker[2] reported few β-haemolytic or serologically groupable strains of '*S. milleri*' at oral sites, with most strains being able to produce acid from lactose. Our previous studies have shown lactose non-fermenting β-haemolytic strains belong to *S. constellatus*. Therefore these published observations would seem to support our findings that *S. constellatus* is relatively rare at oral sites compared to *S. anginosus* and *S. intermedius*. A recent study of '*S. milleri*' in human dental plaque also reports the isolation of mainly non-haemolytic strains of which only approximately half were able to ferment lactose[29] but virtually all of which were able to hydrolyse aesculin. In an earlier study,[2] strains reported to be unable to ferment lactose were also frequently unable to hydrolyse aesculin, an association which we have also recognised recently with some strains of *S. constellatus* (unpublished data). These apparent differences in the reported phenotypes of strains isolated in separate studies may be due to the way in which these tests were carried out in the respective laboratories. Interestingly, Yakushiji *et al.*[29] also reported an average of four to five strains (phenotypes) of '*S. milleri*' from a single dental plaque sample. This relatively high average number of strains in these authors' study can probably be attributed to their recognising only a single species '*S. milleri*' for these strains, instead of the three now known to exist.

The low incidence in this study of *S. anginosus* strains able to ferment mannitol and raffinose is in accord with previous studies where this biotype has been found to be the most frequently isolated from urogenital sources.[27,28]

Frandsen *et al.*[13] reported that 88 per cent of *S. anginosus* strains (defined according to Coykendall *et al.*[8]) from subgingival plaque, produced neuraminidase activity. Of the three species described since this paper was published, only *S. intermedius* has been shown to produce this activity, leading to the conclusion that these authors were isolating high proportions of *S. intermedius* from subgingival pockets. We did not obtain similar results, noting only a slightly higher proportion of *S. constellatus* in subgingival samples compared with the relative numbers found in supragingival plaque. Although Frandsen *et al.*,[13] only examined six periodontal pockets compared to 14 reported here, the data from both studies are sufficiently different to indicate that a more thorough, longitudinal study of the incidence and proportion of the SMG at these sites would be worthwhile.

At present no explanation can be offered for the frequent association detected between *S. intermedius* and *S. anginosus* in contrast to the relatively rare association of *S. intermedius* and *S. constellatus*. These observations clearly warrant further investigation.

In view of the difficulties encountered when trying to compare between studies which have been undertaken at different stages in the understanding of the taxonomy of the '*S. milleri*-group', any detailed and extensive comparison of the data from this study and those reported previously is clearly limited in scope. However, with the recent taxonomic revision of the '*S. milleri*-group', recognition of *S. anginosus*, *S. intermedius* and *S. constellatus* and the availability of a semi-selective agar medium for the isolation and enumeration of these species from mixed bacterial populations, more detailed ecological studies are now possible. Further work is required to reveal the distribution of these clinically important streptococci at other specific oral sites, including a range of mucosal surfaces and at all other normal sites in the human body at which these bacteria are found.

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