Use of rapid carbohydrate utilisation test for identifying "Streptococcus milleri group"

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Abstract

A short series of biochemical and serological tests were developed for the rapid presumptive identification of "*Streptococcus milleri* group" isolates. One hundred and seventy seven streptococcal isolates were recovered from the mouths of 10 out of 12 healthy adult volunteers by use of a simple sampling procedure and a single selective medium. In all, 127 oral "*S milleri* group" isolates were identified by biochemical and serological tests, confirming the indigenous nature of these streptococci in the mouth. Most (70·1%) of "*S milleri* group" isolates were non-haemolytic, 26% were α-haemolytic, and 3·9% β-haemolytic. Fifty four (42·5%) were serologically typable, of which 46 were Lancefield group F, suggesting that the mouth is an important source of Lancefield group F streptococci. A collection of group F streptococci from a range of sources was indistinguishable from a collection of oral "*S milleri* group" isolates on the basis of the tests used, supporting the general synonymity of group F streptococcus with the broader "*S milleri* group".

The battery of tests was cheap and simple to perform, and was capable of identifying "*S milleri* group" isolates from a range of sources, including variants with wide sugar fermentation patterns.

The viridans streptococci have long been regarded as low grade pathogens contributing to the complex resident flora of a number of mucosal sites. Streptococci belonging to the "*milleri* group" are currently classified with the viridans streptococci, and are known to contribute to the resident flora of the gastrointestinal tract,[1] the urogenital system,[2] the upper respiratory tract,[3] and the mouth, where a particular association with sheltered sites on hard surfaces has been shown.[4 5] They are, however, unusual among the viridans streptococci in their association with deep-seated sepsis in a range of sites, including surgical sepsis,[6] hepatic abscesses,[7] pleural empyema,[8] intracranial sepsis[9] and odontogenic abscesses.[10] The mouth has often been regarded as the source of "*Streptococcus milleri* group" strains involved in abscess formation in many of these sites.

Despite its widespread use in the clinical setting, the title "*S milleri*" has no official status in streptococcal taxonomy[11] and is a title used to describe a collection of strains which have variously been referred to as *Streptococcus anginosus*,[12] minute colony forming β haemolytic streptococci,[13] *Streptococcus MG*,[14] *Streptococcus milleri*,[15 16] *Streptococcus intermedius*,[17 18] *Streptococcus constellatus*,[17 18] *Streptococcus anginosus-constellatus*,[19] and *Streptococcus MG-intermedius*.[19] The "*S milleri* group", therefore, represents a complex aggregate of organisms which may display α, β, or non-haemolysis on blood agar culture media, and may variably possess Lancefield group antigens A, C, G, or F. Heterogeneity in traditional biochemical tests has also been observed.[20] Recognised differences notwithstanding, these strains are currently classified within a single species, under the approved name *S anginosus*.[21] Taxonomic uncertainty persists, however, and recent studies have shown three distinct genotypes within the "*S milleri* group"[22]; one homology group including the type strain of *S constellatus*, one including the type strain of *S intermedius*, and the final group including the type strain *S anginosus*. A very recent report from the same authors has also described a biochemical scheme for the reliable phenotypic differentiation of these three genotypes, suggesting that they are incorrectly classified within a single species.[23]

The aims of the current study were: (i) to design a simple and reliable protocol for the recovery of "*S milleri* group" isolates from defined sites in the oral cavity; (ii) to develop a simple, rapid, and cheap battery of biochemical and serological tests for the presumptive identification of "*S milleri* group" isolates from the mouth and other sources that would be capable of recognising the various haemolytic types, serological groups, and biotypes known to exist within this taxon; and (iii) to establish a culture collection of group F streptococcal strains from a range of sites, and of different haemolytic and biotypes.

Methods

Dental plaque was recovered on sterile McCall's curettes from two non-inflamed sites in the mouths of 12 healthy adult volunteers (five men, seven women), lingual to a lower first permanent molar tooth and distolabial to an upper canine tooth. The plaque was dispersed into 1·0 ml reduced transport fluid (RTF).[24] Samples were transported to the laboratory within one hour and dispersed in RTF by vortexing before serial dilution in RTF. Dilu-

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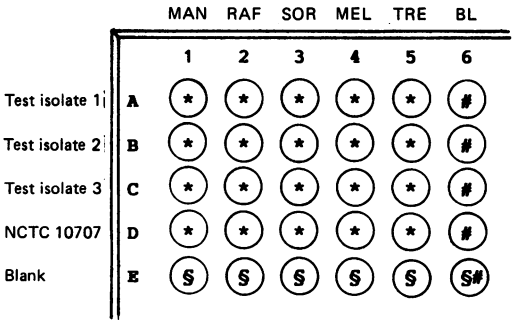
Table 1 List of reference and clinical strains included in this study

Reference strains:		
1 "*S milleri* group" reference strains:		
Number	*Species*	
NCTC 10707	Lancefield group F streptococcus	
NCTC 10714	Lancefield group F streptococcus	
NCTC 5389	Lancefield group F streptococcus	
NCTC 8037	*S anginosus* (Lancefield group F)	
NCTC 11065	"*S milleri*"	
NCTC 11063	"*S milleri*"	
NCTC 11169	"*S milleri*"	
NCTC 10708	"*S milleri*"	
Other streptococcal reference strains:		
NCTC 7864	*S sanguis*	
NCTC 8606	*S salivarius*	
NCTC 10449	*S mutans*	
NCTC 10712	*S mitior*	
Clinical strains:		
	Diagnosis	*Source*
CBB1	"*S milleri*" (Lancefield group F)	High vaginal swab
CBB2	"*S milleri*" (Lancefield group F)	High vaginal swab
CBB3	Group F streptococcus	Pilonidal sinus
CBB5	Group F streptococcus	Infected dental cyst
CBB6	Group F streptococcus	Neonatal skin swab
CBB8	Group F streptococcus	Throat swab
CBB9	"*S milleri*" (Lancefield group F)	Arm abscess
CBB10	Group F streptococcus	Denture swab
H957	Group F streptococcus	Chin abscess

tions (0·2 ml of 10^{-3}, 10^{-4}, 10^{-5}) were spread on to the surface of a single selective medium (MC agar: a sulphonomide-containing medium similar in composition to mitis salivarius agar described by Carlsson in 1967,[25] and modified in the current study by the replacement of sulphadimetine with sulphadimidine) with sterile glass spreaders, and incubated in 10% CO_2, 90% H_2, in anaerobic jars without catalysts for 48 hours at 37°C. Colonies morphologically resembling "*S milleri*"[24] were selected and subcultured on blood agar. One or more further subcultures was sometimes required to obtain pure cultures which were subjected to identification procedures.

Table 1 shows additional clinical "*S milleri* group" isolates, obtained from the Clinical Bacteriology Laboratory, University of Edinburgh, and reference strains included in this study.



Schematic representation of RCUT test prepared for identification of three test isolates. Numbers 1–6 and letters A–E represent row numbers of a microtitre plate.
MAN = Mannitol MEL = Melibiose
RAF = Raffinose TRE = Trehalose
SOR = Sorbitol BL = Blank
*Wells marked * received both indicated organism and sugar.*
Wells marked ♯ received organism, but no sugar.
Wells marked § received sugar, but no organism.
The well marked §♯ received neither sugar nor organism.

BIOCHEMICAL IDENTIFICATION

Carbohydrate fermentation tests

Fermentation of mannitol, raffinose, sorbitol, melibiose and trehalose was by a method modified from the rapid carbohydrate utilisation test (RCUT) of Young.[26] The RCUT was conveniently performed in the wells of microtitre plates as follows:

(1) 100 µl of buffered salt solution containing neutral red indicator (BSS)[26] were added to six wells in a microtitre plate, and 250 µl to a small, sterile glass tube for each organism to be examined. Control wells were also set up as indicated in the figure.

(2) 25 µl of 10% (w/v) aqueous solutions of mannitol, raffinose, sorbitol, melibiose and trehalose were added, respectively, to one of five wells for each test organism, and to appropriate control wells as indicated in the figure.

(3) Growth from a fresh, overnight blood-agar culture was harvested with a cotton-tipped swab, and used to make a dense suspension in the 250 µl BSS contained in the test tube. Volumes (25 µl) of this suspension were added to test wells of the microtitre plate, and to appropriate control wells (figure).

(4) Plates were incubated at 37°C by floating in a waterbath, and a definitive reading (colour change of red to yellow) was made after four hours.

Each day, a fresh culture of Lancefield group F streptococcus (NCTC 10707) was used as a positive "*S milleri*" control.

Voges-Proskauer (VP) reaction

This was achieved by the rapid method of Bucher and von Graevenitz,[27] using fresh culture of Lancefield group F streptococcus (NCTC 10707) as a positive control.

Arginine hydrolysis

This was carried out according to the method of Niven *et al*[28] as described by Cowan and Steel,[29] with a stock strain of *Enterococcus faecalis* as a positive control.

Aesculin hydrolysis

This was carried out using two methods: (i) the method of Cowan and Steel[29]; and (ii) the method of Brown *et al.*[30] A stock strain of *E faecalis* was the positive control in each test.

SEROLOGICAL IDENTIFICATION

Serological identification was performed by gel-diffusion in Ouchterlony gels.[31]

Antigenic extracts

C antigen was extracted from the cells contained in a heavy sweep from a fresh blood-agar culture by the method of El Kholy *et al.*[32 33] Each day fresh cultures of laboratory stock strains of Lancefield group A, C, and G streptococci, and Lancefield group F streptococcus (NCTC 10707) were used as controls.

Antisera

Antiserum was raised in a Dutch rabbit by the method of Poxton.[34] The vaccine was 10^9 bacteria which had been irradiated with a lethal dose of ultraviolet light. Antiserum was raised

Table 2 Biochemical characterisation of some streptococcal reference strains

Strain	*MAN*[1]	*RAF*[1]	*SOR*[1]	*MEL*[1]	*TRE*[1]	*ARG*[2]	*AESC*[2]	*VP*[3]
S sanguis (NCTC 7864)	−	+	−	+	+	−	−	−
S salivarius (NCTC 8606)	−	+	−	+	+	−	+	−
S mutans (NCTC 10449)	+	+	+	+	+	−	+	+
S mitior (NCTC 10712)	−	+	−	+	+	−	−	−
Lancefield group F streptococcus (NCTC 10707)	−	−	−	−	+	+	+	+
Lancefield group F streptococcus (NCTC 10714)	−	−	−	−	+	+	−	+
Lancefield group F streptococcus (NCTC 5389)	−	−	−	−	+	+	+	+
S angiosus (NCTC 8037)	−	−	−	−	+	+	+	+
"*S milleri*" (NCTC 11065)	−	−	−	−	+	+	+	+
"*S milleri*" (NCTC 11063)	−	−	−	−	+	+	−	+
"*S milleri*" (NCTC 10708)	−	−	−	−	+	+	+	+
"*S milleri*" (NCTC 11169)	−	−	−	−	+	+	+	+

1 Rapid carbohydrate utilisation test:
MAN = Mannitol; RAF = Raffinose; SOR = Sorbitol; MEL = Melibiose; TRE = Trehalose.
2 Hydrolysis reactions:
ARG = Arginine; AESC = Aesculin.
3 Production of acetoin from glucose:
VP = Voges-Proskauer reaction.
+ = Positive test reaction.
− = Negative test reaction.

against Lancefield group F streptococcus (NCTC 10707). Commercial antisera (groups A, C, G, and F) were obtained from Wellcome.

Results

A collection of reference strains was first examined by the short series of biochemical tests to establish its ability to differentiate "*S milleri* group" strains from single strains of *S sanguis* (NCTC 7864), *S salivarius* (NCTC 8606), *S mutans* (NCTC 10449), and *S mitior* (NCTC 10712), and to establish the reproducibility of results. Table 2 shows the results of tests, which were repeated on three consecutive days and gave entirely reproducible results. RCUT tests were clearly read after four hours, and Voges-Proskauer tests after five hours. Determination of arginine hydrolysis required incubation for 24 hours, while aesculin hydrolysis often required incubation for 48 hours before a definitive reading could be made.

The eight "*S milleri* group" reference strains tested represented a relatively homogeneous collection of strains, fermenting only trehalose, giving a positive Voges-Proskauer reaction, hydrolysing arginine, and in all but two cases hydrolysing aesculin.

Although differentiating "*S milleri* group" strains from the single representatives of *S sanguis*, *S salivarius*, *S mutans*, and *S mitior*, differentiation among the other strains was not entirely clear. The single *S mutans* strain, however, was clearly identified by its wide carbohydrate utilisation pattern, positive Voges-Proskauer reaction, aesculin hydrolysis, and its failure to hydrolyse arginine.

Serological tests correctly identified Lancefield group F streptococci (NCTC 10707, NCTC 5389, NCTC 10714), and *S anginosus* (NCTC 8037) as group F strains. None of the other strains reacted with the range of antisera used in this study. Serological results were clearly read within five hours.

A simple sampling procedure, in combination with a single selective medium, permitted the isolation of 177 streptococcal isolates which were examined further. Within this collection of isolates 127 "*S milleri* group" isolates were identified by the short series of tests from the mouths of 10 of the 12 subjects sampled.

The results of biochemical and serological tests for 127 "*S milleri* group" isolates, repeated in duplicate, are shown in table 3. Most isolates fermented trehalose, hydrolysed arginine and aesculin, and gave a positive Voges-Proskauer reaction. A few, however, utilised mannitol, raffinose, sorbitol or melibiose. Most (70·1%) isolates were non-haemolytic, and of the five (3·9%) β-haemolytic isolates, three were Lancefield group G and two were ungroupable within the range of antisera used. In total, 54 (42·5%) of the 127 oral "*S milleri* group" isolates were serologically groupable, most (n = 46) of these strains belonging to Lancefield group F. In all instances the results obtained with antiserum raised against Lancefield group F streptococcus (NCTC 10707) agreed with the commercial group F antiserum. In addition to the 46 oral group F strains obtained in the current study, nine clinical group F strains and four NCTC reference strains (table 1) contributed to a collection of 59 Lancefield group F streptococcal strains. The range of biochemical profiles

Table 3 Summary of characteristics of 127 "S milleri group" isolates recovered from mouths of 10 volunteers

		Number (%) of isolates positive
Biochemical tests		
Mannitol		6 (4·7)
Raffinose		6 (4·7)
Sorbitol		6 (4·7)
Melibiose		2 (1·6)
Trehalose		119 (93·7)
Arginine		118 (92·9)
Aesculin		103 (81·1)
VP		119 (93·7)
Serological group	A	0 (0)
	C	1 (0·8)
	F	46 (36·2)
	G	7 (5·5)
	No group	73 (57·5)
Haemolysis	α	33 (26·0)
	β	5 (3·9)
	None	89 (70·1)

VP = Voges-Proskauer reaction.

Table 4 Biochemical characterisation of 59 group F streptococci, including oral, clinical, and NCTC isolates

Number of isolates with given biochemical profile		*MAN*[1]	*RAF*[1]	*SOR*[1]	*MEL*[1]	*TRE*[1]	*ARG*[2]	*AESC*[2]	*VP*[3]
	44	−	−	−	−	+	+	+	+
	4	−	−	−	−	+	+	+	−
	2	−	−	−	−	+	+	−	+
	2	−	+	−	+	−	+	+	+
	2	−	+	−	−	+	−	+	+
	1	−	−	−	−	+	−	−	+
	1	+	−	+	−	+	+	+	+
	1	−	−	−	−	+	+	−	−
	1	−	−	−	−	+	−	+	+
	1	−	−	−	+	−	−	−	+
Total:	59	1	4	1	3	56	54	54	54
(%)	(100)	(1·7)	(6·8)	(1·7)	(5·1)	(94·9)	(91·5)	(91·5)	(91·5)

1 Rapid carbohydrate utilisation test:
MAN = Mannitol; RAF = Raffinose; SOR = Sorbitol; MEL = Melibiose; TRE = Trehalose.
2 Hydrolysis reactions:
ARG = Arginine; AESC = Aesculin.
3 Production of acetoin from glucose:
VP = Voges-Proskauer reaction.
+ = Positive test reaction.
− = Negative test reaction.

encountered in this culture collection is shown in table 4. Thirty eight (64·4%) were non-haemolytic, 16 (27·1%) α-haemolytic, and five (8·5%) β-haemolytic. Most (74·6%) formed a biochemically homogeneous group, but strains were found which displayed differing fermentation profiles. No differentiation could be made between Lancefield group F and non-Lancefield group F "*S milleri* group" isolates on the basis of the biochemical tests used in this study.

Discussion

In the first part of this study a series of simple biochemical and serological tests were developed for the presumptive identification of "*S milleri* group" reference strains. Differentiation of "*milleri* group" streptococci from a small collection of oral streptococcal reference strains was readily achieved, though differentiation among other species tested was not always clear. Although beyond the scope of the current study, it is suggested that a fuller evaluation of this system with a broad range of oral streptococci of known identity would be required to modify the range of tests for the presumptive identification of a greater number of species.

Tests for the production of hyaluronidase, and the ability to degrade synthetic fluorogenic substrates described recently by Whiley *et al*,[23] were not included in this study, and the differentiation of strains within the "*S milleri* group" corresponding to *S anginosus*, *S intermedius*, and *S constellatus* was consequently not possible.

The single selective medium used in this study proved effective for the recovery of "*S milleri* group" isolates from the mouth and confirmed the association of this group with the human gingival crevice.[4 35]

Oral "*S milleri* group" isolates represented a relatively homogeneous group on the basis of the tests undertaken, though a small number of isolates (9·4%) was encountered which were able to ferment mannitol, raffinose, sorbitol, or melibiose. This proportion of oral "*S milleri* group" isolates with wide sugar fermentation profiles was comparable with the findings of Ball and Parker.[20]

In common with previous studies on "*S milleri* group" isolates from the mouth[4 20 23 35 36] most isolates found in our study were non-haemolytic and possessed no recognisable Lancefield grouping antigen, properties typical of the *S intermedius* taxon described by Whiley *et al*.[23] A high proportion of oral "*S milleri* group" isolates, however, were serologically groupable, and of these, Lancefield group F was by far the commonest, which agrees with the findings of other workers.[35 36] All of the oral group F isolates found were either α or non-haemolytic, suggesting that they would most likely belong to the *S anginosus* taxon.[23]

Further tests would be required to confirm these intuitive associations because it is recognised that haemolytic reactions, Lancefield group status, and behaviour in traditional biochemical tests are of little practical value in identifying the three taxa shown within the "*S milleri* group".[23] The results of this study supported this observation because a collection of Lancefield group F streptococci from a range of sources was indistinguishable from a collection of oral "*S milleri* group" isolates on the basis of the tests used.

These observations notwithstanding, the system of tests described in the current report were regarded as satisfactory for the rapid presumptive identification of "*S milleri* group" isolates from a range of sources. Inoculation of tests from a pure plate culture, in addition to short incubation times in non-nutrient medium, reduced to a minimum the potential problems of contamination, while the small volumes of reagents and the use of non-sterile microtitre plates kept the cost of media and consumables low.

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