

# Lancefield grouping and smell of caramel for presumptive identification and assessment of pathogenicity in the *Streptococcus milleri* group

O Brogan, J Malone, C Fox, A S Whyte

## Abstract

**Aim**—To evaluate Lancefield grouping and caramel smell for presumptive identification of the *Streptococcus milleri* group, and to find whether Lancefield group, species, or protein profile correlated with virulence or infection site.

**Methods**—Prospective studies were made of 100 consecutive streptococcal isolates in blood cultures or pus from 100 patients in whom the severity of infection was categorised as serious, moderate, or not significant. The usefulness of Lancefield group and the caramel smell for presumptive identification was examined, and the relation of the *S milleri* species, Lancefield group, and SDS-PAGE protein analysis to severity of infection and infection site was investigated. Lower respiratory tract and genital tract specimens, strict anaerobes, group D streptococci, and strains identified as *Streptococcus pneumoniae*, *Streptococcus pyogenes*, or *Streptococcus agalactiae* were excluded.

**Results**—Most streptococci occurring in pure or significant growth density were *S milleri* group (87/100; 87%, 95% confidence interval 0.81–0.93). Of these, 89.7% (78/87; 0.84–0.96) were associated with infection. Lancefield group F antigen predominated (41/87; 47.1%, 0.38–0.56). Lancefield group F alone or accompanied by the caramel smell had a specificity of 100%, but a sensitivity of only 47.3% for group F alone, and 19.5% for group F accompanied by the caramel smell. There was no significant association between species, Lancefield group, and severity of infection, site of infection, or pathogenicity. SDS-PAGE analysis failed to discriminate between strains.

**Conclusions**—Neither species nor Lancefield antigen was related to the site of infection. The presence of Lancefield group F antigen alone or accompanied by a caramel smell was a useful indicator for the *S milleri* group when present, but was too insensitive to use as a screening test. Most streptococci occurring in pure culture or in significant growth density were of clinical importance. Such organisms should be identified to species level to detect the *S milleri* group.

(J Clin Pathol 1997;50:332–335)

The *Streptococcus milleri* group comprises three species, *Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus constellatus*. They are commensals of the mouth, gastrointestinal tract, and vagina which may cause serious infections, especially abscesses of the brain, liver, and soft tissues.<sup>1,2</sup> Little is known about the properties which confer strain virulence. The problem is that the group is so culturally and biochemically variable<sup>2</sup> that strains are hard to recognise. A caramel smell is produced in perceptible quantities by some strains,<sup>2</sup> but has never been fully evaluated for screening identification of the *S milleri* group. Most strains do not have Lancefield group specific carbohydrate, and those that do usually belong to Lancefield group A, C, F, or G,<sup>3</sup> so the Lancefield group is of little help in recognising possible *S milleri* group cultures. Despite this, both Lancefield grouping and speciation of the *S milleri* group may help to distinguish pathogenic strains, as some organs are said to be the target of *S milleri* group strains of particular Lancefield groups or species.<sup>3–5</sup> Another possibility is the use of protein antigen analysis, a method used to subdivide group A streptococci into the M and T types used as virulence and epidemiological markers.<sup>6</sup> Protein analysis might further subdivide the *S milleri* group and help in the assessment of pathogenicity.

Study objectives were to assess Lancefield grouping and the caramel smell for the presumptive identification of the *S milleri* group, to investigate the relation between *S milleri* group and the severity of infection, and to evaluate *S milleri* species, Lancefield grouping, and protein analysis as indicators of pathogenicity.

## Methods

Studies were made of the first 100 consecutive streptococcal strains cultured in significant growth from blood, pus, or tissue.

## STREPTOCOCCI EXCLUDED FROM THE STUDY

Strains of *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, Lancefield group D streptococci, and anaerobic streptococci were excluded from the study. For this purpose, *S pyogenes* strains were deemed to be  $\beta$  haemolytic streptococci which gave a zone in the diagnostic bacitracin sensitivity test,<sup>7</sup> and anaerobic streptococci to be streptococci which failed to grow in an atmosphere of 7.5% carbon dioxide at first subculture.

Department of  
Microbiology, Fife  
Area Laboratory,  
Hayfield Road,  
Kirkcaldy, Fife KY2  
5AG

O Brogan  
J Malone  
C Fox  
A S Whyte

Correspondence to:  
Dr Oonagh Brogan.

Accepted for publication  
22 January 1996

Keywords: *Streptococcus milleri*; caramel smell; Lancefield group

## CULTURE CONDITIONS

Pus and tissue specimens were cultured on 5–7% horse blood agar with Columbia agar base (Oxoid, Basingstoke) supplemented with 20 µg of Factor V (nicotinamide adenine dinucleotide, Boehringer, London). The plates were 90 mm in diameter, and the volume of medium in each was 20 ml. Blood cultures were in Bactec 460 media (Becton Dickinson, Oxford) subcultured to the blood agar medium described.

## PLATING PROCEDURE

A primary inoculum on the first quadrant of the plate was made from a swab or from one drop (1/200 ml) of liquid specimens. The primary inoculum was then seeded to each remaining quadrant with a sterile loop.<sup>8</sup>

## CONDITIONS OF INCUBATION

One plate was incubated in an atmosphere of 7.5% carbon dioxide in air. A second plate was incubated anaerobically in an atmosphere of 10% carbon dioxide with 5% hydrogen and 85% nitrogen. Plates were incubated at 37°C overnight and then examined.

## ASSESSMENT OF GROWTH AS SIGNIFICANT

Growth was deemed significant if primary culture was pure, regardless of growth density. Mixed cultures were considered significant if the growth density was moderate or heavy. Any isolate from a blood culture was considered significant growth, regardless of density. Moderate growth was defined as growth of at least 30 colonies in the second inoculated quadrant, heavy growth as growth of at least 30 colonies in the third inoculated quadrant.

## CAMEL SMELL

The presence of a caramel smell was recorded on a single occasion by a single observer, at the time when a pure subculture was first obtained. Observations were recorded blind before preliminary identification of the streptococcal species. A 10% vol/vol aqueous solution of diacetyl ISLR (Fisons Laboratory Reagents, London) was used as a positive control.

## SPECIES IDENTIFICATION AND LANCEFIELD GROUPING

Morphology was examined by Gram stain and microscopy. The catalase reaction was tested by a standard method.<sup>9</sup> Catalase negative colonies of Gram positive cocci were subcultured to anaerobic blood agar for further tests.

Preliminary identification was made by the API 20Strep kit system (API bio-Merieux (UK), Hampshire). Lancefield grouping was by Streptex kits (Wellcome Reagents, London), using reagents for groups A–H. Identification of *S milleri* group strains to species as *S anginosus*, *S constellatus* or *S intermedius* was by a published method.<sup>5</sup>

SDS-PAGE METHOD<sup>10</sup>

Overnight blood agar growth was suspended in 2 ml of sterile distilled water, washed three times by centrifugation at 2500 ×g for 10 minutes at 25°C and resuspended in 150 µl sterile

distilled water. PAGE extraction buffer 150 µl (sodium dodecyl sulphate (SDS) 6.0% wt/vol, glycerol 30% wt/vol, 2-mercaptoethanol 15% wt/vol, bromophenol blue 0.001% wt/vol) was added to each suspension. The mixture was boiled for five minutes, cooled, centrifuged at 2500 ×g for 15 minutes at 25°C, and 30 µl supernatant subjected to PAGE.

PAGE was performed in a discontinuous buffer system at a constant current of 20 A until the dye marker reached the bottom of the gel. Gels were stained with Coomassie brilliant blue (0.25 g in 100 ml methano:acetic acid:water, 45:10:45 vol/vol/vol) and destained in the same solvent mix without Coomassie blue. Gels were examined by eye using a light box illuminator, and the bands for each strain compared against each other. Reproducibility was assured by repeat testing of several strains on different gels and by use of molecular weight markers. The similarity of banding patterns was assessed by established criteria.<sup>10 11</sup>

## CLINICAL ASSESSMENT

The clinical significance of strains was made by an experienced general surgeon with the clinician involved. Each patient was assigned to one of three infection categories, designated serious, moderate, or no infection. Serious infection denoted septicaemia, purulent discharge accompanied by a partial or complete dehiscence of the fascial layers of the wound, deep tissue destruction, or spreading cellulitis and lymphangitis which required antibiotic treatment. Moderate infection was discharge of pus without lymphangitis or deep tissue destruction.

## STATISTICAL METHODS

The methods used were confidence intervals, a Yates corrected  $\chi^2$ ,  $\chi^2$  test of trend, and a test of sensitivity and specificity with calculation of positive and negative predictive values.

## Results

The study period was 18 months, and involved 34 210 specimens, of which 100 (0.3%: 95% confidence interval (CI) 0.002–0.003) yielded streptococci which conformed to the study definition. Of the 100 specimens, five were blood cultures and 95 pus or tissue. There were 100 patients.

Of the 100 streptococcal strains, 87 were identified as *S milleri* group (87.0%: 95% CI 0.81–0.93). Of the 87, 78 were associated with infection (89.7%: 95% CI 0.84–0.96). Of the remaining 13 strains not identified as *S milleri* group, 10 were associated with infection (76.9%: 95% CI 0.50–1.02), and eight of the 10 (80.0%: 95% CI 0.50–1.10) were identified as *Streptococcus* spp (Lancefield group G).

PATHOGENICITY OF *S MILLERI* INFECTION

Table 1 shows the identity of the streptococcal strains studied, with the clinical diagnosis and infection site. Table 2 shows the distribution of the 87 *S milleri* group strains and the 13 other streptococcal strains among the three clinical groups, and the proportion of mixed and pure

Table 1 Site of infection and identity of streptococcal strains in 88 patients with infection

Site of infection	Group	Total	Identity of strain				Not S milleri group	
			S milleri group					
			S anginosus	S constellatus	S intermedius	Not speciated		
Blood	Severe	1					<i>Streptococcus sanguis</i>	1
Head and neck	Moderate	0						
	Severe	1				1		
Thorax	Moderate	10	2	3	2	2	<i>Streptococcus</i> spp group G	1
	Severe	1				1	<i>Streptococcus</i> spp	2
Hand	Moderate	4	2					
	Severe	0						
Abdominal	Moderate	12	3	5	1	2	<i>Streptococcus</i> spp group G	1
	Severe	16	8	6	1	1		
Genito-urinary	Moderate	17	5	6	3	3		
	Severe	1		1				
Thigh/lower limb	Moderate	4	2	2				
	Severe	4		3			<i>Streptococcus</i> spp group G	1
Total	Moderate	17	3	3	2	5	<i>Streptococcus</i> spp group G	4
	Severe	16	8	6	1	1		
Total severe + moderate	Moderate	64	25	29	9	15		10
	Severe	24	8	10	1	3		2
Total	Moderate	64	25	29	9	15		8
	Total	88						10

Table 2 Distribution of pure and mixed cultures of streptococci among the three clinical groups into which 100 strains of streptococci were placed, showing the number of *Streptococcus milleri* strains

Clinical group	Result of culture				Total
	S milleri		Not S milleri		
	Mixed	Pure	Mixed	Pure	
Serious	16	6	1	1	24
Moderate	39	17	3	5	64
No infection	7	2	0	3	12
Total	62	25	4	9	100

cultures. There was a statistical trend towards more severe infection if the *S milleri* group was isolated ( $\chi^2$  test of trend,  $p < 0.005$ ).

#### ASSOCIATION OF THE *S MILLERI* GROUP SPECIES WITH SITE OF INFECTION

No statistically significant association was found ( $p > 0.05$ ).

#### LANCEFIELD GROUPING

Of the 87 *S milleri* strains, 41 (47.1%: 95% CI 0.38–0.56) were group F, 30 failed to group (34.5%: 95% CI 0.25–0.43), 11 were group C (12.6%: 95% CI 0.06–0.18), three were group G (3.4%: 95% CI 0.004–0.06), one (1.2%) was group A, and one (1.2%) group B (95% CI 0.01–0.03).

The Lancefield group had no statistically significant association with the site of infection ( $p > 0.05$ ).

#### EVALUATION OF THE USE OF LANCEFIELD GROUPING AND THE CARAMEL SMELL FOR PRESUMPTIVE IDENTIFICATION OF *S MILLERI* GROUP

Table 3 shows the number of caramel smelling strains identified as *S milleri* group. Table 4 shows the sensitivity and specificity of Lancefield group F antigen and caramel smell alone and in combination as screening tests for *S milleri* group, in the 87 *S milleri* group strains.

Table 3 Detection of a caramel smell in 100 strains of nondescript streptococci, showing the number of strains identified as *Streptococcus milleri*

Caramel smell detected	Strain identified as <i>S milleri</i>		
	Yes	No	Total
Yes	49	3	52
No	38	10	48
Total	87	13	100

#### PAGE ANALYSIS

The similarity between banding patterns for any two strains was always equal to or greater than 85%. This suggests that either the strains were indistinguishable, or that the method used was not discriminating enough.<sup>11 12</sup>

#### Discussion

Most accounts of the *S milleri* group are reports of life threatening infection. We found the *S milleri* group in moderate and severe infections, often in mixed culture, as in other studies.<sup>13 14</sup> There were no cases of either liver or brain abscess among our patients, so the suggestion that *S intermedius* has a predilection for these infections<sup>5</sup> could not be evaluated. We found no relation between severity of infection, site of infection, or clinical diagnosis with species, Lancefield group, or SDS-PAGE protein analysis. Apart from one recent report<sup>15</sup> which found statistically significant links between abdominal and urogenital infection and *S anginosus*, studies which suggest such associations<sup>5 16 17</sup> are neither prospective nor analysed statistically. It is possible that they may be biased towards very severe or unusual infections by the use of selected strains, rather than consecutive strains from a prospective study. This may explain the difference between our findings and those of others. Another explanation for the predominance of *S anginosus* is that it is the most numerous *S milleri* group species at abdominal and urogenital sites.<sup>18</sup> Our study did not substantiate this. Most studies do not specify the precise site of

Table 4 Evaluation of the presence of Lancefield group F antigen with and without the perception of a smell of caramel for the screening identification of 87 strains of *Streptococcus milleri*

Test	Sensitivity (%)	Specificity (%)	False negative rate (%)	Predictive value	
				Positive (%)	Negative (%)
Group F antigen present	47.3	100.0	52.8	100.0	27.1
Caramel smell	56.3	76.9	43.6	94.2	20.8
Group F antigen present and caramel smell	19.5	100.0	80.4	100.0	15.6

infection, but use generalised terms. Thus it is not clear whether the term "abdominal" includes both superficial infections of the skin and soft tissue and deep infections originating in the gastrointestinal tract.

The presence of Lancefield group F antigen with or without the smell of caramel had a good specificity and positive predictive value for the *S milleri* group, but occurred too seldom to be a reliable screening test. The caramel smell alone had an even lower specificity and positive predictive value for *S milleri* identification. The caramel smell is due to the formation of the metabolite diacetyl, and it has been suggested that quantitation by gas chromatography may be useful as a rapid presumptive test.<sup>19</sup> Such a test may have potential advantages in increased sensitivity and greater consistency, but is impractical for an ordinary medical microbiology department because of the equipment and expertise needed to screen a relatively small number of isolates.

We found that 88 of 100 streptococci which conformed to the study definition, and which occurred in pure, moderate, or heavy growth on primary culture, were clinically significant. Of these clinically significant streptococci, 78 (89.7%) were *S milleri*, and eight (9.1%) *Streptococcus* spp (group G). Thus streptococci isolated in pure or significantly heavy growth density may often be clinically relevant, and belong to the *S milleri* group or other potentially pathogenic species. It is important to identify *S milleri* group infections because they tend to persist, despite the use of standard antibiotics and surgical drainage,<sup>19, 20</sup> may need extended antibiotic treatment,<sup>21</sup> and show signs of emerging penicillin resistance.<sup>15</sup> Speciation of streptococci to detect the *S milleri* group has practical implications for clinical management. As a minimum, biochemical speciation should be part of the routine laboratory investigation of streptococci cultured in pure, moderate, or heavy growth from tissue or pus from all serious surgical infections, and from all blood cultures.

A Fife Health Board research grant in support of this project, and the advice of Mr James Boyd, Statistician, Fife Health Board, are gratefully acknowledged.

- Gossling J. Occurrence and pathogenicity of the *Streptococcus milleri* group. *Rev Infect Dis* 1988;10:257-85.
- Ruoff KL. *Streptococcus anginosus* ("Streptococcus milleri"): the unrecognised pathogen. *Clin Microbiol Rev* 1988;1:102-8.
- Poole PM, Wilson G. Infection with minute-colony-forming  $\beta$ -haemolytic streptococci. *J Clin Pathol* 1976;29:740-5.
- Ball LC, Parker MT. The cultural and biochemical characteristics of *Streptococcus milleri* isolated from human sources. *J Hyg (Camb)* 1979;82:63-78.
- Whiley RA, Fraser H, Hardie JM, Beighton D. Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus* and *Streptococcus anginosus* strains within the "Streptococcus milleri group". *J Clin Microbiol* 1990;28:1497-501.
- Ross PW. *Streptococcus*. In: Collee JG, Duguid JP, Fraser AG, Marmion BP, eds. *Mackie and McCartney practical microbiology*, 13th ed. Edinburgh: Churchill Livingstone, 1989:317-26.
- Oxoid Ltd. Bacitracin discs. In: *The Oxoid manual*, 5th ed. Basingstoke: Oxoid Ltd, 1982:55.
- Collee JG, Marr W. Cultivation of bacteria. In: Collee JG, Duguid JP, Fraser AG, Marmion BP, eds. *Mackie and McCartney practical microbiology*, 13th ed. Edinburgh: Churchill Livingstone, 1989:121-40.
- Collee JG, Marr W. Tests for identification of bacteria. In: Collee JG, Duguid JP, Fraser AG, Marmion BP, eds. *Mackie and McCartney practical microbiology*, 13th ed. Edinburgh: Churchill Livingstone, 1989:152.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
- Stephenson JR, Millership SE, Tabaqchali S. Typing of *Aeromonas* species by polyacrylamide-gel electrophoresis of radiolabelled cell proteins. *J Med Microbiol* 1987;24:113-8.
- Millership SE, Want SV. Typing of *Aeromonas* species by protein fingerprinting: comparison of radiolabelling and silver staining for visualising proteins. *J Med Microbiol* 1989;29:29-32.
- Singh KP, Morris A, Lang SDR, MacCulloch DM, Bremner DA. Clinically significant *Streptococcus anginosus* (*Streptococcus milleri*) infections: a review of 186 cases. *NZ Med J* 1988;101:575-8.
- Edmiston CE, Walker AP, Krepel CJ, Seabrook GR, Frantzides CT. *Streptococcus milleri* group (*Streptococcus anginosus*): recovery from intra-abdominal and soft tissue sites. *Ann Clin Lab Sci* 1991;21:56-61.
- Bantar C, Fernandez Canigia L, Rellosio S, Lanza A, Bianchini H, Smayevsky J. Species belonging to the "Streptococcus milleri" group: antimicrobial susceptibility and comparative prevalence in significant clinical specimens. *J Clin Microbiol* 1996;34:2020-2.
- Whiley RA, Beighton D, Winstanley TG, Fraser HY, Hardie JM. *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* (the *Streptococcus milleri* group): association with different body sites and clinical infections. *J Clin Microbiol* 1992;30:243-4.
- Gomes-Garces J-L, Alos J-I, Collogos R. Bacteriologic characteristics and antimicrobial susceptibility of 70 clinically significant isolates of *Streptococcus milleri* group. *Diagn Microbiol Infect Dis* 1994;19:69-73.
- Flynn CE, Ruoff KL. Identification of "Streptococcus milleri" group isolates to the species level with a commercially available rapid test system. *J Clin Microbiol* 1995;33:2704-6.
- Chew TA, Smith JMB. Detection of diacetyl (caramel odor) in presumptive identification of the "Streptococcus milleri" group. *J Clin Microbiol* 1992;30:3028-9.
- Tresadern JC, Farrand RJ, Irving MH. *Streptococcus milleri* and surgical sepsis. *Ann R Coll Surg Engl* 1983;65:78-9.
- Murray JW, Gross KC, Masur H, Roberts RB. Serious infections caused by *Streptococcus milleri*. *Am J Med* 1978;64:759-64.