

DIAGNOSTIC MICROBIOLOGY

Speciation of presumptive viridans streptococci from early onset neonatal sepsis

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Twenty isolates resembling viridans streptococci, 16 from blood and four from gastric aspirates, from 17 cases of early onset neonatal sepsis were identified by the API20 Strep, Rapid ID 32 Strep and conventional tests plus hydrolysis of methylumbelliferyl glycoside substrates. Nineteen of the isolates were identified as species of viridans streptococci and one as a *Leuconostoc* sp. Ten of the isolates were *Streptococcus oralis*, three *S. mitis* biotype 1, two *S. mitis* biotype 2 and one each of *S. sanguis*, *S. vestibularis*, *S. salivarius* and *S. intermedius*. The Rapid ID 32 Strep and conventional plus methylumbelliferyl tests gave the same species identity for 17 of the isolates. *S. intermedius* was identified by the Rapid ID 32 Strep as *S. constellatus* and *S. salivarius* as *S. equinus*, with *S. salivarius* at lower probability. The API20 Strep failed to identify *S. vestibularis* and identified *S. salivarius* as *S. defectivus*. The absence of certain critical tests, including urea hydrolysis, does not allow the API20 Strep to identify all the currently recognised species of viridans streptococci. The species distribution was unexpected and the incidence of *S. oralis* and other viridans streptococci in vaginal swabs from prenatal patients is being investigated further.

Introduction

Infection is a major cause of mortality and morbidity in neonates and *Streptococcus* spp. have frequently been implicated as pathogens [1, 2]. *S. pyogenes* predominated in the first half of the century [3] to be replaced by *S. agalactiae* in the 1960s [4]. In the 1980s several hospitals reported increased isolations of so called 'viridans streptococci', which in some cases exceeded the number of isolations of *S. agalactiae* during the same period [2, 4]. An earlier study of neonatal septicaemia reported on 99 positive blood cultures obtained from 94 neonates. Specimens from 17 babies yielded viridans streptococci, a total which was exceeded only by *Escherichia coli* and *Klebsiella pneumoniae* and was higher than *S. agalactiae*, which was isolated from the blood cultures of six neonates [5]. The latter study and others [2, 4] have been mainly clinical and species identification has either not been included, or details of the identification methods used have not been given. Adams and Faix [6] reported six cases of neonatal infection, all early onset, and stated the identification method used. These workers identified their isolates as

S. mitis by the API Rapid Strep; however, the biochemical characteristics of the isolates were not reported.

Beighton and colleagues [7] showed that *S. oralis* and to a lesser extent *S. mitis* are the species of viridans streptococci most frequently isolated from the blood cultures of adult neutropenic cancer patients. They attribute this to the ability of these organisms to obtain nutrients from host-derived glycoproteins by virtue of their glycolytic and proteolytic abilities [8, 9]. Beighton *et al.* [10] have developed an identification scheme for viridans streptococci based on hydrolysis of methylumbelliferyl-linked glycosidase substrates together with tests for arginine and aesculin and urea hydrolysis and carbohydrate fermentation. Kilian *et al.* [11] also proposed a classification scheme based on hydrolysis of α -naphthyl- and nitrophenol-linked substrates and a methylumbelliferyl substrate for sialidase activity, in addition to conventional tests.

In the report by Beighton *et al.* [7] there was poor agreement between results obtained with the API20 Strep, (bioMérieux, La Balme les Grottes, France) and Beighton's conventional and methylumbelliferyl substrate methods, as *S. oralis* was not included in the version of the API20 Strep profile register used.

However, *S. oralis* is included in the most recent version (V5.1) that has been issued by bioMérieux. BioMérieux have also introduced the Rapid ID 32 Strep which includes more tests for glycosidases and proteases than the API20 Strep, and identifies viridans streptococci according to nomenclature which more closely fits currently accepted taxonomy [10–12].

Until 1995 we identified neonatal streptococci by the API20 Strep system. We have now converted to the Rapid ID 32 Strep, and during the transition period isolates were profiled with both systems. Isolates were also tested by conventional tests based on those of Waitkins *et al.* [13, 14] and with methylumbelliferyl substrates as described by Beighton *et al.* [10].

Materials and methods

Bacterial strains

Twenty isolates from blood cultures or gastric aspirates of 17 neonates aged ≤ 2 days, reported as presumptive 'viridans streptococci' on initial isolation, were investigated. Storage of clinical isolates and control strains was as reported previously [14].

All biochemical tests were performed with cultures grown on Columbia Blood Agar (Oxoid CM331) with sheep blood (Saudi Prepared Media) 5% incubated anaerobically for 18–24 h. All cultures and biochemical tests were incubated at 37°C. Inoculation of conventional tests was as described previously [14].

Biochemical tests

All the organisms were catalase negative, gram-positive cocci or coccobacilli, resistant to optochin.

Arginine hydrolysis was performed by a modification of the method of Waitkins *et al.* [13, 14]. Aesculin hydrolysis was tested both in Blood Agar Base (Oxoid CM271) and Brain Heart Infusion Agar (BBL 11065) supplemented with aesculin 0.1% and ferric chloride 0.05% and dispensed in 2-ml volumes in 5-ml bottles; results were recorded daily for 5 days. Acetoin production (Voges-Proskauer test) was determined by the method of Waitkins *et al.* [13]. Dextran and levan production were tested on Trypticase Soy Agar (Oxoid CM131) with sucrose 5% incubated in air for 3 days, and examined for glossy adherent colonies. Greening on chocolate agar was tested on blood agar base with sheep blood 5% heated for 15 min at 80°C before pouring. Hydrogen peroxide production was detected with 1-cm squares of blotting paper impregnated with diamino-benzidine 0.1% w/v (50 μ l) and glucose 1% (50 μ l). Chocolate agar plates were heavily inoculated with test strains and incubated for 6 h in air. Paper squares with diamino-benzidine and glucose were placed on the growing cultures and on uninoculated control plates. A drop of hydrogen peroxide 3% was dropped on to the

surface of the chocolate agar close to a paper as a positive control. The plates were re-incubated in air and strains that produced a black zone around the paper were recorded as positive for hydrogen peroxide production. Urease activity was tested in Urease Broth (BBL 21098) which was inoculated to give a turbidity of 1.0 McFarland units and incubated for 24 h. Vancomycin susceptibility was performed on Mueller Hinton Agar (Oxoid CM337) with lysed sheep blood 5%. Streptococcal grouping was carried out with the Avipath-Strep kit (Omega Diagnostics). Hydrolysis of methylumbelliferyl substrates was performed according to the method of Beighton *et al.* [10], except that 50- μ l volumes of substrate and bacterial suspension were used. API20 Strep and Rapid ID 32 Strep methods (BioMérieux), were performed according to the manufacturer's instructions. API results were read with APILAB plus software V5.1 for the API20 Strep and V1.1 for the Rapid ID 32 Strep.

Results

All the organisms tested were isolated from blood or gastric aspirate cultures of babies aged ≤ 2 days. The source of the organisms and results obtained with conventional tests and methylumbelliferyl substrates are shown in Table 1, together with species identification. The isolates were numbered sequentially after identification to simplify presentation and discussion of the results. Isolates from the same baby that showed biochemical differences were given the same serial number, and recorded as A and B variants. Weak reactions in the Voges-Proskauer (VP) test and for aesculin hydrolysis in conventional media are shown, but were considered as negative for the purpose of assigning species identity.

API20 Strep and Rapid ID 32 Strep profiles and identities derived from the profile number are shown in Table 2. In generating the profile number weak VP and aesculin reactions were recorded as negative.

Ten of the isolates (1–9A) were identified as *S. oralis* based on their reactions in conventional tests and production of β -galactosidase, sialidase and N-acetyl- β -glucosaminidase. All the isolates showed alkaline phosphatase activity in the API20 Strep. The same species identity was obtained for all the isolates with the Rapid ID 32 Strep at probabilities of $\geq 98.2\%$, and with the API20 Strep for six isolates at probabilities of $\geq 99.0\%$. The four exceptions with the API20 Strep were one isolate that was aesculin positive, identified as *S. oralis* with 89.6% probability, two isolates identified as *S. sanguis* at low probability and one identified as *S. mitis*. The strains identified as *S. sanguis* were recorded as negative for starch fermentation (0270450), but had this reaction been positive (0270451) would have been identified as *S. oralis* at 99.0% probability. The strain identified as *S. mitis* was recorded α -galactosidase negative

Table 1. Sources of and identities of presumptive viridans streptococci by conventional tests and hydrolysis of methylumbelliferyl glycoside substrates

Serial no.	Species identity	Source	Biochemical characteristics													
			arg	aes	VP	β -glu	α -gal	β -gal	sial	β -nag	α -ara	H ₂ O ₂	poly	AIP	urea	
1	<i>S. oralis</i>	Blood	-	-	-	-	-	+	+	+	-	+	+	+	-	-
2	<i>S. oralis</i>	Blood	-	+	-	-	-	+	+	+	-	+	+	+	-	-
3	<i>S. oralis</i>	Blood	-	-	-	-	+	+	+	+	-	+	+	+	-	-
4	<i>S. oralis</i>	Blood	-	-	-	-	+	+	+	+	-	+	+	+	-	-
5A	<i>S. oralis</i>	Blood	-	-	-	-	+	+	+	+	-	+	+	+	-	-
5B	<i>S. oralis</i>	Blood	-	-	-	-	+	+	+	+	-	+	+	+	-	-
6	<i>S. oralis</i>	Blood	-	-	-	-	+	+	+	+	-	+	+	+	-	-
7	<i>S. oralis</i>	Blood	-	-	-	-	+	+	+	+	-	+	+	+	-	-
8	<i>S. oralis</i>	Blood	-	-	-	-	+	+	+	+	-	+	+	+	-	-
9A	<i>S. oralis</i>	Blood	-	±	-	-	+	+	+	+	-	+	+	+	-	-
10A	<i>S. mitis</i> biotype 1	Gast asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	<i>S. mitis</i> biotype 1	Gast asp	-	-	-	-	+	+	+	+	-	+	+	+	-	-
12	<i>S. mitis</i> biotype 1	Gast asp	-	-	-	-	+	+	+	+	-	+	+	+	-	-
13	<i>S. mitis</i> biotype 2	Blood	+	-	-	-	-	-	-	-	-	-	-	-	-	-
14	<i>S. mitis</i> biotype 2	Gast asp	+	-	-	-	-	-	-	-	-	-	-	-	-	-
9B	<i>S. sanguis</i>	Blood	+	●	-	-	+	+	+	+	-	-	-	-	-	-
10B	<i>S. vestibularis</i>	Blood	-	-	±	-	-	+	+	+	-	-	-	-	-	+
15	<i>S. salivarius</i>	Blood	-	+	+	-	-	-	-	-	-	-	-	-	-	-
16	<i>S. intermedius</i>	Blood	+	+	+	-	-	+	+	+	-	-	-	-	-	-
17	<i>Leuconostoc</i>	Blood	-	-	+	+	+	+	+	+	-	-	-	-	-	-

arg, arginine hydrolysis; aes, aesculin hydrolysis; VP, Voges-Proskauer test. Methylumbelliferyl glycoside tests: β -glu, β -glucosidase; α -gal, α -galactosidase; β -gal, β -galactosidase; sial, sialidase; β -nag, N-acetyl- β -glucosaminidase; α -ara, α -arabinosidase; H₂O₂, hydrogen peroxide production; poly, dextran production; AIP, alkaline phosphatase reaction (from API20 Strep); urea, urease activity. +, positive reaction; -, negative reaction; ±, positive reaction weaker than control.

Table 2. Speciation of presumptive isolates of viridans streptococci by the Rapid ID 32 Strep and API20 Strep systems

Serial no.	Rapid ID 32 Strep		API20 Strep	
	Profile no.	Identity (%)	Profile no.	Identity (%)
1	46076643100	<i>S. oralis</i> (99.8)	0270451	<i>S. oralis</i> (99.0)
2	66076643120	<i>S. oralis</i> (99.8)	4270451	<i>S. oralis</i> (89.6)
3	46076643100	<i>S. oralis</i> (99.9)	0270441	<i>S. oralis</i> (99.9)
4	46052643120	<i>S. oralis</i> (99.9)	0270441	<i>S. oralis</i> (99.9)
5A	66072443121	<i>S. oralis</i> (99.4)	0270450	<i>S. sanguis</i> (54.5)
5B	46056643100	<i>S. oralis</i> (99.9)	0270451	<i>S. oralis</i> (99.9)
6	46072443100	<i>S. oralis</i> (99.2)	0270451	<i>S. oralis</i> (99.9)
7	66076641120	<i>S. oralis</i> (99.1)	0270451	<i>S. oralis</i> (99.0)
8	46076643120	<i>S. oralis</i> (99.9)	0270450	<i>S. sanguis</i> (54.5)
9A	02052463120	<i>S. oralis</i> (98.2)	0070441	<i>S. mitis</i> (88.1)
10A	02052043100	<i>S. mitis</i> 1 (50.6)	0240440	<i>S. sanguis</i> (43.4)
11	00012441100	<i>S. mitis</i> 1 (99.1)	0040400	<i>S. mitis</i> 2 (97.0)
12	02052443100	<i>S. mitis</i> (68.7)	0240440	<i>S. sanguis</i> (43.4)
13	50016441100	<i>S. mitis</i> 2 (99.9)	0051401	<i>S. mitis</i> 1 (99.5)
14	50076441100	<i>S. mitis</i> 2 (82.7)	0051401	<i>S. mitis</i> 1 (99.5)
9B	30032441120	<i>S. sanguis</i> (99.6)	4041411	<i>S. sanguis</i> (86.6)
10B	60036401102	<i>S. vestibularis</i> (94.5)	0050410	<i>S. oralis</i> (55.1)
15	20022341110	<i>S. equinus</i> (68.9)	5040211	<i>Listeria</i> (38.8)
16	54023001110	<i>S. constellatus</i> (99.1)	5061010	<i>S. intermedius</i> (88.0)
17	62054003101	<i>Leuconostoc</i> (99.9)	1210450	<i>S. defectivus</i> (52.6)

(0070401), but had α -galactosidase been positive (0270441) would have been identified as *S. oralis* at 99.9% probability. Five isolates (10A–14) were identified as *S. mitis* by the Rapid API 32 Strep. Three of these (10A, 11 and 12) met the criteria for *S. mitis* given by Beighton *et al.* [10] and are recorded as *S. mitis* biotype 1, following the terminology of Kilian *et al.* [11]. Two of these isolates were identified as *S. mitis* 1 by the Rapid ID 32 Strep, but as *S. mitis* 2 by the API20 Strep. Isolate 12 was identified as *S. mitis* by the Rapid ID 32 Strep at 68.7% probability, and as *S. sanguis* (43.4%) by the API20 Strep, which would have identified *S. oralis* at 95.6% had the starch hydrolysis result been positive. Two isolates (13 and 14) were identified as *S. mitis* biotype 2 according to the terminology of Kilian *et al.* [11]. These strains were arginine positive and aesculin negative, but were excluded from *S. sanguis*, *S. parasanguis*, *S. gordonii* and *S. crista* (tufted fibril group) by the absence of N-acetyl- β -glucosaminidase, β -glucosidase, α -galactosidase, α -fucosidase, β -fucosidase or α -arabinosidase activity [10]. They were identified as *S. mitis* 2 by the Rapid ID 32 Strep, but as *S. mitis* 1 by the API20 Strep. Strain 9B was a typical *S. sanguis*, and was identified as this species by all the methods used. Isolate 10B showed a typical biochemical pattern for *S. vestibularis* in conventional tests (weak VP and urease positive) as well as α -arabinosidase activity [15], and was identified as this species by the Rapid ID 32 Strep. However, the API20 Strep software does not include *S. vestibularis*, and the organism was identified as *S. oralis* with low probability. Isolate 15 was identified as *S. salivarius* as it was β -glucosidase and α -arabinosidase positive, arginine negative and produced levan on sucrose 5% agar. The organism was identified as *S. equinus* (68.9%) by the Rapid ID 32 Strep and *Listeria* sp. (38.8%) by the API20 Strep

system. Both identities were rejected as the organism was negative for Lancefield group D antigen and catalase activity. The commercial systems offered *S. salivarius* at lower probability for this isolate (22.1 and 12.5% respectively). Isolate 16 was identified as *S. intermedius* by conventional methods and methylumbelliferyl substrates and by the API20 Strep, but as *S. constellatus* (99.1%) by the Rapid ID 32 Strep. These species plus *S. anginosus* were previously often reported as '*S. milleri*' [16], but were separated by differences in hydrolysis of methylumbelliferyl glycoside substrates [17, 18]. Isolate 17 was identified as a *Leuconostoc* sp. at high probability (99.9%) by the Rapid ID 32 Strep and this identity was accepted because the organism was vancomycin resistant in addition to being pyrrolidonylamidase and leucine aminopeptidase negative in both API systems [19]. The API20 Strep for this isolate gave *S. defectivus* at 52.6% and *Leuconostoc* sp. at 36.3% probability.

Discussion

All the organisms identified in this study were from early onset cases of suspected neonatal sepsis. Therefore, we believe that the organisms were acquired from the mother by vertical transmission. Ten of the 16 blood culture isolates were typical *S. oralis* as defined by Beighton *et al.* [10]. Although *S. oralis* is the species most frequently reported from adult neutropenic patients we have not noted any reports of isolations of this species from neonates, and did not recognise it before 1995, because it was not included in versions of the API20 Strep profile register prior to V5.1., in which *S. oralis* is included. All the isolates of *S. oralis* were alkaline phosphatase positive, a result that concurred with the description by Kilian *et al.* [11].

A further five isolates were identified as *S. mitis* based on the descriptions of Beighton *et al.* [10] and Kilian *et al.* [11], although *S. mitis* biotype 2 isolates in the present study were negative for N-acetyl- β -glucosaminidase and β -glucosidase activity with methylumbelliferyl substrates. Kilian *et al.* [11] proposed *S. mitis* biotype 2 for arginine positive strains from his collection which had *S. mitis*-type cell-wall composition (Lys-direct peptidoglycan and ribitol teichoic but lacking rhamnose). The cell-wall composition of the isolates was not investigated in the present study. Both API systems include two *S. mitis* types, referred to as *S. mitis* 1 and *S. mitis* 2 in the list of species identified. *S. mitis* 1 is given as arginine negative and *S. mitis* 2 99% arginine positive in the Rapid ID 32 Strep literature; however, the API20 Strep profile register includes arginine positive and negative variants in both *S. mitis* 1 and *S. mitis* 2. Hence an isolate may be identified as *S. mitis* 1 by one API kit and as *S. mitis* 2 by the other. Both Spigelblatt *et al.* [2] and Adams and Faix [6] reported *S. mitis* from neonatal blood cultures and all the cases documented by Adams and Faix [6] were early onset. *S. salivarius* and *S. sanguis* isolates were reported by Spigelblatt *et al.* [2].

Members of the '*S. milleri* group' have been reported from neonates and from vaginal specimens of the mothers of infected babies [16]. *S. vestibularis* has been reported from the blood culture of an adult patient [20], but not to our knowledge from neonates. Members of the genus *Leuconostoc* have been reported from cases of sepsis, including a late onset case in a study of neonatal meningitis [21, 22].

The spectrum of organisms found in the present study, with the exception of *S. oralis*, is similar to that recorded by Spigelblatt *et al.* [2] who identified 10 of 19 neonatal blood culture isolates as *S. mitis*, eight as single isolates and two in mixed cultures with another *Streptococcus* sp., and also identified *S. sanguis* and *S. salivarius*. Some of the isolates identified as *S. mitis* by Spigelblatt *et al.* [2] could have been *S. oralis*, as although this species was first proposed by Bridge and Sneath in 1982 [23] and an emended description was given by Kilian *et al.* in 1989 [11], it was rarely reported from clinical specimens before publication of the identification scheme of Beighton *et al.* [10] in 1991 and its introduction into API profile registers.

There was excellent agreement in species identity between the Rapid ID 32 Strep and conventional plus methylumbelliferyl substrate tests, with agreement to species level for 17 of 19 streptococcal isolates, provided that two *S. mitis* biotypes were accepted. *S. intermedius* was identified as *S. constellatus*, a closely related species [17, 18]. The *Leuconostoc* sp. was also correctly identified. The only major discrepancy was in the identification of the isolate that was identified as *S. salivarius* based on conventional and rapid

methods; however, both API systems did offer *S. salivarius* at lower probability. The API20 Strep system performed less satisfactorily than the Rapid ID 32 Strep, as it does not contain certain critical tests such as urea and N-acetyl- β -glucosaminidase which are essential to identify all the currently recognised species of viridans streptococci.

The predominance of *S. oralis* was a novel finding and the occurrence of this and other species of viridans streptococci in vaginal swabs taken from prenatal patients is currently being investigated.

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