JOURNAL OF CLINICAL MICROBIOLOGY, June 1995, p. 1592–1595 Vol. 33, No. 6
0095-1137/95/$04.00+0

Species Identification of Members of the *Streptococcus milleri* Group Isolated from the Vagina by ID 32 Strep System and Differential Phenotypic Characteristics

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Received 14 November 1994/Returned for modification 5 January 1995/Accepted 2 March 1995

The importance of bacterial vaginosis as a risk factor in obstetric and gynecological infections has recently been recognized. The bacterial vaginosis group of organisms includes members of the *Streptococcus milleri* group, the identification of which has caused much confusion. We prospectively surveyed the rates of carriage of *S. milleri* group organisms in 397 high vaginal swabs received in our laboratory. For the identification of 99 clinical isolates and 23 control strains, we compared the results obtained by the rapid ID 32 Strep system (Analytab Products) and by a scheme utilizing six differential phenotypic characteristics (presence of β-*N*-acetylglucosaminidase, α-glucosidase, β-D-fucosidase, β-galactosidase, β-*N*-acetylgalactosaminidase, and β-glucosidase) as described by Whiley et al. (R. A. Whiley, H. Fraser, J. M. Hardie, and D. Beighton, J. Clin. Microbiol. 28:1497–1501, 1990). We identified *Streptococcus anginosus* in 18% and *Streptococcus constellatus* in 0.05% of the specimens examined. Of the isolates of *S. anginosus* that reacted with grouping antisera, 20 of 25 belonged to Lancefield group F. The incubation conditions for bacterial cultures and for reaction mixtures affected the results of phenotypic characterization in the production of α-glucosidase, β-galactosidase, and β-glucosidase. However, by using bacterial cultures grown under hypercapnic conditions and incubating the reaction mixtures aerobically, consistent phenotypic characteristics were obtained, allowing identification similar to that obtained by the ID 32 Strep system. We therefore recommend the phenotypic scheme as an inexpensive, reliable, and convenient method for the initial identification of species of the *S. milleri* group.

Microaerophilic streptococci formerly classified as *Streptococcus anginosus* or as *Streptococcus milleri* group organisms (SMG) by British workers have recently been classified as three distinct species, *S. anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* (13, 15). They occur as commensals in the mouth, nasopharynx, bowel, and vagina and may be associated with purulent infections of the mouth and internal organs (7). Although their clinical significance has long been recognized, there is a paucity of information on the pathogenesis of infections. The identification of these organisms has been the source of much confusion (1, 4). Recently, Freney et al. (6) evaluated the ID 32 Strep system (Analytab Products [API]) for use as a convenient identification method and reported agreement in identification with conventional methods for 95.3% of 413 streptococci and related genera, including 32 strains of SMG.

Genital colonization with the vaginosis group of microorganisms, including viridans group streptococci, may be important etiologically in premature labor and obstetric and gynecological infections (3). Because of the confusion in the taxonomy and nomenclature of SMG, it is difficult to evaluate reports from different centers published during the past decade. As part of a study of the pathogenic role of SMG, we investigated the vaginal carriage of SMG from women attending various clinics in our hospital. We report our findings and problems encountered with the ID 32 Strep system (API) and the identification scheme proposed by Whiley et al. (13, 15).

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MATERIALS AND METHODS

Clinical specimens. Between March and July 1993, high vaginal swabs (HVS) received from the antenatal clinics and the gynecology outpatient department of our hospital were examined for the study. During July and August 1993, antepartum specimens taken because of clinical indications (e.g., premature rupture of membrane) received from the delivery suites were also examined. The HVS were taken under the vision of the Cusco speculum, placed in Stuart's transport medium (Transwab, Sterilin, United Kingdom) and sent to the bacteriology laboratory for examination within 3 h.

Bacteriological methods. (i) Clinical isolates. In addition to routine microbiological examination of HVS, the presence of SMG was investigated by inoculating two extra Columbia blood agar plates (Becton-Dickinson). Both plates were supplemented with 5% (vol/vol) defibrinated horse blood, gentamicin (Roussel) (4 mg/liter), and amphotericin (Squibb) (2 mg/liter); additional metronidazole (May & Baker) (50 mg/liter) was used in one plate only. All plates were incubated at 37°C, one in an aerobic atmosphere plus 5 to 7% CO_2 (hypercapnically) and the other (containing metronidazole) under anaerobic conditions with N_2 (85%), H_2 (10%), and CO_2 (5%). Anaerobic jars were used throughout the study.

All plates were examined after 2 days of incubation and again after 4 days. Small convex streptococcal colonies (≤2 mm in diameter) with an entire edge were sought. Three such colonies from each plate were subcultured onto a MacConkey agar plate and a Columbia blood agar plate, each divided into thirds. After overnight hypercapnic incubation, isolates that grew on Columbia agar plates with poor or no growth on MacConkey plates were examined further. An initial screening to exclude isolates that possessed group antigen B or D was carried out with a Streptex latex agglutination kit (Unipath). This was followed by tests for arginine hydrolysis and acetoin production (5). Isolates that showed positive reactions in both tests were saved for identification and further determination of Lancefield group antigens. In addition, 24 laboratory stock cultures which were obtained in a similar manner previously were revived and included for further work.

The Streptex latex agglutination kit (Unipath) was used to determine Lancefield group antigens A, B, C, D, F, and G. Any strain exhibiting multiple group reactions was retested with diluted extraction enzyme (1:5).

(ii) Control strains. Control strains included nine isolates of *S. anginosus*, four isolates of *S. intermedius*, three isolates of *S. constellatus*, NCTC 10707 (*S. anginosus*), NCDO 2227 (*S. intermedius*), and NCTC 10709 and NCDO 2226 (*S. constellatus*). They had been identified and reported by Whiley et al. (13). In addition, NCTC 10713 (*S. anginosus*), NCTC 11324 (*S. intermedius*), and NCTC 11325 (*S. constellatus*) were obtained from the National Collection of Type

TABLE 1. Differential phenotypic characteristics of *S. constellatus*, *S. intermedius*, and *S. anginosus*[a]

Reaction	Result for[b]:		
	S. constellatus	*S. intermedius*	*S. anginosus*
β-D-Fucosidase	−	+	−
β-*N*-Acetylglucosaminidase	−	+	−
β-*N*-Acetylgalactosaminidase	−	+	−
β-Galactosidase	−	+	−
β-Glucosidase	−	V	+
α-Glucosidase	+	+	V

[a] Modified from reference 15 with permission of the American Society for Microbiology.
[b] −, negative (<15% positive reactions); +, positive (>85% positive reactions); V, variable (16 to 84% positive reactions).

Cultures, Public Health Laboratory Service, Colindale, London, United Kingdom.

Species identification. (i) API system. The rapid ID 32 Strep system (API-bio-Merieux) was used according to the manufacturer's instructions to identify isolates to the species level. Bacterial cultures from Columbia blood agar plates were incubated aerobically for 18 to 24 h. Typically, growth from one entire plate was necessary to prepare a 2-ml suspension in sterile distilled water to be comparable in turbidity to the no. 4 McFarland standard. The individual cupules of the API strip were inoculated with 55 μl of the culture suspension. The color change for a positive reaction was noted visually according to the manufacturer's instructions after 4 h of incubation. The API profiles were interpreted from the computer database for identification. Only one isolate from each plate was identified.

(ii) Identification by differential phenotypic characteristics. Of the eight tests recommended by Whiley et al. (13, 15), six were chosen on the basis of their discriminatory power and convenience for use in a routine laboratory. The patterns of identification were constructed as follows: positive reaction, >85% positive; negative reaction, <15% positive; and variable reaction, 16 to 84% positive (Table 1). Reagents (4-methylumbelliferyl-linked substrates) from Sigma (Poole, United Kingdom) were used to detect the presence of β-*N*-acetylglucosaminidase, α-glucosidase, β-D-fucosidase, β-galactosidase, β-*N*-acetylgalactosaminidase, and β-glucosidase. They were dissolved in a minimum volume of dimethyl sulfoxide, diluted in 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.5) (TES buffer; Sigma) to a final concentration of 100 mg/liter as described by Whiley et al. (15), and stored in the dark at −20°C. For use, 100 μl of each substrate solution was dispensed into individual round-bottomed plastic tubes (50 by 6 mm). With use of a sterile loop and the vortex centrifuge, if necessary, the bacterial suspension for biochemical tests was prepared by emulsifying colonies from Columbia blood agar plates after 48 h of hypercapnic or anaerobic incubation. The turbidity of the suspension was adjusted to be comparable to that of a McFarland no. 4 standard (approximately 10^9 CFU/ml). For the biochemical reaction, 40 μl of the culture suspension was added to the plastic tubes containing the substrate. The reaction mixtures were incubated for 3 h aerobically or anaerobically according to the design of the experiment (see below). Substrate degradation (release of 4-methylumbelliferone) was visualized by viewing the plastic tubes under a hand-held, long-wavelength UV lamp in a dark room. Positive strains produced a blue fluorescence.

The effects of different incubation conditions on the production of the enzymes tested were investigated. Parallel cultures obtained under anaerobic and hypercapnic conditions were used for the reaction mixture which was incubated aerobically. Additional investigations were carried out for α-glucosidase and β-galactosidase reactions. Parallel tests for each strain were carried out as follows: anaerobic culture with the reaction mixture incubated in air, anaerobic culture with the reaction mixture incubated anaerobically, and hypercapnic culture with the reaction mixture incubated in air. All reaction mixtures were incubated for 3 h.

RESULTS

From mid-March to July 1993, a total of 442 HVS (154 from the antenatal clinics and 288 from the gynecology outpatient department) were received in the laboratory. Of these, 306 (115 from the antenatal clinics and 191 from the gynecology outpatient department) were examined for the presence of SMG. From August to October 1993, 91 of 191 HVS received from the delivery suites were also examined. In all, 75 streptococcal isolates were saved for further work. Thus, identification to the species level was carried out for 99 clinical isolates, including 24 isolates revived from laboratory stock cultures.

Identification to species level by API system. For the 23 control strains, identification was carried out once with bacterial cultures incubated anaerobically and once with cultures incubated under a hypercapnic atmosphere. Both modes of testing gave correct identifications.

For the 99 clinical isolates, satisfactory aerobic growth was obtained after 18 to 24 h of incubation, and this incubation time was used for identification. For 30 randomly chosen clinical isolates, the process was repeated with isolates incubated under hypercapnic and anaerobic atmospheres. Similar identifications were obtained with cultures incubated under these three conditions. Two isolates were identified as *S. constellatus* and 97 were identified as *S. anginosus*; 95 of the latter isolates had an API identification percentage of ≥98.4%, and 2 had an API identification percentage of 78.6%.

Identification by differential phenotypic characteristics. The results of characterizations of 23 control strains and 99 clinical isolates by using bacterial cultures obtained under different incubation conditions are shown in Table 2. Most of the positive reactions when hypercapnic culture conditions and aerobic reaction conditions were used became so after 20 min, and all became so after 1 h. The rapid ID 32 API system contains

TABLE 2. Results of differential phenotypic characterization for control strains and clinical isolates under different incubation conditions[a]

Strain category and species (*n*)	Incubation condition for growth	No. of positive reactions					
		β-*N*-Acetylglucosaminidase	α-Glucosidase	β-D-Fucosidase	β-Galactosidase	β-*N*-Acetylgalactosaminidase	β-Glucosidase
Controls							
S. constellatus (6)	Anaerobic	0	6	0	5	0	0
	Hypercapnic	0	6	0	3	0	0
S. intermedius (6)	Anaerobic	6	2	5	6	6	0
	Hypercapnic	6	6	6	6	6	0
S. anginosus (11)	Anaerobic	0	5	0	7	0	8
	Hypercapnic	0	5	0	9	0	10
Clinical							
S. anginosus (97)[b]	Anaerobic	2	83	3	23	0	79
	Hypercapnic	1	93	0	86	0	97
S. constellatus (2)[b]	Anaerobic	0	1	0	2	0	2
	Hypercapnic	0	2	0	1	0	2

[a] In all cases, the reaction was in air.
[b] Based on results of identification by the API rapid ID 32 Strep system.

TABLE 3. Serogrouping and hemolysis of *S. anginosus* isolated from HVS

Hemolysis	No. of isolates in serogroup:				Total no. of isolates
	F	C	G	NG[a]	
Beta-hemolytic	7	2	0	4	13
Alpha-hemolytic	6	0	1	15	22
Nonhemolytic	7	0	0	21	28
Total	20	2	1	40	63

[a] NG, not groupable.
[b] Excluding 10 isolates with unrecorded hemolysis.

three of the six enzymatic reactions used: of the 99 clinical isolates, 80 were positive for the β-galactosidase reaction, 96 were positive for the β-glucosidase reaction, and none were positive for the for β-*N*-acetylglucosaminidase reaction.

With cultures of control strains incubated under hypercapnic condition, all 6 *S. intermedius* isolates, 3 of 6 *S. constellatus* isolates, and 9 of 11 *S. anginosus* isolates were correctly identified. With cultures grown under anaerobic or hypercapnic conditions, 26 and 87% of 99 clinical isolates were identifiable, respectively. The cause of the discrepancy lies with the β-galactosidase reaction. If this test were to be excluded and the results for hypercapnically incubated cultures were to be used, 96 of 99 clinical isolates would be able to be identified and 22 of 23 control strains would be correctly identified. The three clinical isolates not identified included the two identified by the API system as *S. constellatus*. Both had a positive β-glucosidase reaction and were therefore not able to be identified by using Table 1. Only one control strain of *S. anginosus* was not able to be identified because of its negative β-glucosidase reaction (Table 2). With bacterial cultures incubated anaerobically, three control strains of *S. anginosus* did not produce β-glucosidase and four control strains of *S. intermedius* did not produce α-glucosidase. Therefore, identifications of these isolates were not possible (Table 2).

The possible effects of different incubation conditions used for the bacterial cultures and reaction mixtures were investigated further with 10 randomly chosen clinical isolates of *S. anginosus* for the β-galactosidase reaction and with 8 such isolates for the α-glucosidase reaction. All isolates were tested on three separate occasions under the different combinations of incubation conditions described in Materials and Methods. Hypercapnically incubated cultures with reactions carried out aerobically gave the most consistently positive results for both tests: 29 of 30 β-galactosidase reactions and 21 of 24 α-glucosidase reactions were positive under these conditions.

The overall rate of isolation of *S. anginosus* from our specimens was 18%. No significant difference was found between specimens from the three clinical areas. Improved growth was observed for seven isolates of *S. anginosus* on plates incubated hypercapnically compared with those incubated anaerobically. The reverse observation was made for four other isolates. Three strains of *S. anginosus* were isolated anaerobically only, and one strain was isolated hypercapnically only. Prolonged incubation for up to 4 days did not yield further isolation. The results of streptococcal grouping and the types of hemolysis observed in the clinical *S. anginosus* isolates are shown in Table 3. Only one-third of the isolates were grouped successfully, with group F being the most common. Both *S. constellatus* clinical isolates (identified by the API system) were beta-hemolytic and gave a group F reaction.

DISCUSSION

Although the name *S. anginosus* has been used for that particular species in the United Kingdom, it has been used as the synonym of *S. milleri*, *S. constellatus*, and *S. intermedius* in other parts of the world (1). In addition, there have been major differences in the American and British *Streptococcus* taxonomic schemes with reference to *S. milleri* (4). Recently, after using phenotypic differentiation, DNA-DNA hybridization data, and cluster analysis, Whiley et al. (13, 15) have reported that SMG consist of three genetic groups, i.e., *S. constellatus*, *S. intermedius*, and *S. anginosus*. *S. intermedius* was associated with abscesses of the brain and liver, while both *S. anginosus* and *S. constellatus* were isolated from a wider range of sites and infections. *S. anginosus* strains predominated in both genitourinary and gastrointestinal sources and exhibited a wider range of phenotypes (14).

Viridans group streptococci, together with *Gardnerella vaginalis*, anaerobes, and mycoplasmas, have been found to be significantly associated with bacterial vaginosis (8, 9). Bacterial vaginosis is now recognized as an important risk factor in the development of obstetric and gynecological infections (3). Because of the different taxonomic schemes used in these earlier reports, it is difficult to evaluate the exact role of these streptococci. Using the Facklam method, Rabe et al. (9) studied viridans group streptococci isolated from the female genital tract and identified *S. intermedius* as the most common species (13%) among the viridans group streptococci, followed by *Streptococcus acidominimus* (6%), *S. constellatus* (5%), and others. In contrast, using the rapid ID 32 Strep system or differential phenotypic characteristics, we found *S. anginosus* to be the most common minute-colony streptococcal species among vaginal flora. Since only one isolate which showed arginine hydrolysis and acetoin production and lacked group antigen B or D on the initial screening was identified, the presence of other species of SMG cannot be excluded. Of the 97 isolates identified by the API system as *S. anginosus*, 89% fermented either mannitol or raffinose or both. Whiley et al. (14) also noted a similarly high proportion of fermentative *S. anginosus* isolates from genitourinary sources.

Freney et al. (6), using bacterial cultures incubated anaerobically, evaluated the ID 32 Strep system for use as a convenient identification method and reported that of the 32 strains of SMG studied, 7 were not clearly distinguished: 4 were identified as *S. anginosus*/*S. intermedius*, and 3 were identified as *S. anginosus*/*S. constellatus*. These strains required further tests for identification. In our study, the rapid ID 32 Strep system correctly identified all 23 control strains. The incubation condition did not affect the identification of isolates.

On the basis of Table 1, discordant results for the control strains arose mainly from the β-galactosidase reaction. Whiley et al. (15) reported a positive reaction in 2 and 0% of *S. constellatus* and *S. anginosus* strains, respectively, and thus considered these species to give a negative reaction (i.e., <15% positive). Over half of our control isolates gave a positive result, and they accordingly were unable to be identified. This reaction was also present in the API system and was positive for 80 of the 97 *S. anginosus* isolates. If the reaction is changed from negative to variable (i.e., 16 to 84% positive) in Table 1, correct identification of the control strains and identification comparable to that obtained by the rapid ID 32 Strep system for clinical isolates can be achieved. It may be argued that this test need not be included in the classification scheme as shown in Table 1. For the α-glucosidase reaction, we also noted positive reactions in a higher proportion of control strains than reported previously (15). Whiley and colleagues

used an anaerobic chamber for bacterial cultures and aerobic incubation for reaction mixtures (12). The results of our limited investigations illustrate further that different incubation conditions used for the bacterial culture and reaction mixture may affect the enzymatic reaction. The effect of oxygen on the activity of β-galactosidase is well recognized and has been utilized in studies of bacterial genetics (11). Nevertheless, the possible effect of inoculum size cannot be excluded despite our visual standardization of bacterial suspensions to a McFarland no. 4 standard.

The cost of identification by differential phenotypic characteristics is only a fraction of that with the rapid ID 32 Strep method, although the labor and time required are similar. We therefore suggest the following. First, for the identification pattern of *S. anginosus*, the reaction of β-galactosidase should be changed from negative to variable. Second, hypercapnic culture conditions and aerobic incubation of the reaction mixture give more consistent results and a larger number of positive reactions. For *S. intermedius* and *S. constellatus*, our results for the limited number of control strains tested suggest that the same observation may also be applicable. As an initial differential scheme, we recommend the six phenotypic characteristics on the basis of their convenience to use in a routine laboratory. The use of the other two recommended tests, namely, the production of sialidase and hyaluronidase, may be reserved for clarification if required (for example, for the three unidentifiable clinical isolates in our study) (15).

We found that 44% (28 of 63) of our isolates of *S. anginosus* were nonhemolytic and that only 38% (23 of 63) belonged to identifiable serogroups, with group F being the most common. Ruoff (10) reviewed the literature and concluded that there seemed to be no well-established relationship between hemolytic, serological, and physiological characteristics and the site of isolation among SMG. Efstartiou et al. (2), using a combination of the API 20 Strep and API ZYM systems, noted that three of five strains of *S. milleri* produced α-glucosidase and belonged to group G, whereas the remaining two *S. milleri* strains belonged to group C and did not produce the enzyme. We failed to find any significant difference in the results of the API ID 32 Strep system or the six differential phenotypic characteristics between isolates that belonged to group F and those that were not able to be assigned to a serogroup.

ACKNOWLEDGMENT

We thank R. A. Whiley and colleagues at the Department of Oral Microbiology, The Royal London Medical College, London, United Kingdom, for their generous supply of control strains used in the study.

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