Incidence and Characterization of Beta-Hemolytic *Streptococcus milleri* and Differentiation from *S. pyogenes* (Group A), *S. equisimilis* (Group C), and Large-Colony Group G Streptococci

JODY LAWRENCE, DAVID M. YAJKO, AND W. KEITH HADLEY*

Department of Laboratory Medicine, University of California, San Francisco General Hospital Medical Center, San Francisco, California 94110

Received 7 May 1985/Accepted 12 August 1985

The biochemical characteristics of 172 clinical isolates of group A, C, F, or G or "nongroupable" beta-hemolytic streptococci were examined. Among these isolates, 91 were identified as beta-hemolytic strains of *Streptococcus milleri*. The remaining isolates included 20 *Streptococcus pyogenes*, 21 *Streptococcus equisimilis*, 37 large-colony group G streptococci, and 3 unidentified nongroupable isolates. A majority (84%) of the *S. milleri* strains possessed Lancefield group antigen (3 A, 27 C, 41 F, and 5 G), whereas 15 *S. milleri* strains (16%) were nongroupable. Serological tests did not differentiate *S. milleri* isolates with group A, C, or G antigen from *S. pyogenes* (group A), *S. equisimilis* (group C), or large-colony group G streptococci. Biochemical tests which were found useful for differentiation included the Voges-Proskauer test, hydrolysis of pyrog glutamate acid and beta-d-glucuronic acid, bacitracin susceptibility, and acid production from ribose. *S. milleri* represented 56% of the group C, 100% of the group F, and 83% of the nongroupable beta-hemolytic streptococci isolated in our clinical laboratory, whereas the incidence of *S. milleri* among group A and group G streptococci was estimated to be low. The role of beta-hemolytic *S. milleri* as a cause of human infection remains obscure by the failure to routinely differentiate *S. milleri* from other beta-hemolytic streptococci.

*Streptococcus milleri* is considered an important cause of purulent disease in humans (28). In one study, *S. milleri* was the streptococcus most often isolated from abscesses of internal organs (28). Various infections in which *S. milleri* has been implicated include brain abscesses (10, 23, 28, 33), liver abscesses (5, 25, 28), appendicitis (30), peritonitis (25, 28), and endocarditis (25, 28).

Although most strains of *S. milleri* are nonhemolytic, it is estimated that 25% are beta-hemolytic and may possess Lancefield group A, C, F, or G antigen (2). Accurate identification of beta-hemolytic strains of *S. milleri* is hampered by the practice of identifying beta-hemolytic streptococci solely by their Lancefield group antigen. If emphasis is placed on serological criteria for identification, beta-hemolytic *S. milleri* with group A, C, or G antigen may not be distinguished from *Streptococcus pyogenes* (group A), *Streptococcus equisimilis* (group C in humans), and the large-colony group G streptococci. Differentiation of *S. milleri* from these organisms is required before its pathological role can be accurately determined.

The taxonomic status of the species *S. milleri* is somewhat controversial. The name *S. milleri* was first proposed in 1956 by Guthof (16) for a group of streptococci isolated from dental abscesses and other oral lesions. These strains were nonhemolytic and lacked Lancefield group antigen. Related organisms have been variously characterized as *Streptococcus anginosus* (1, 12, 36), minute hemolytic streptococci (4, 22), group F streptococci (21, 22), *Streptococcus MG* (24), *Streptococcus intermedius* (17, 18), *Streptococcus constellatus* (17, 18), *Streptococcus MG-intermedius* (11), *Streptococcus anginosus-constellatus* (11), and *Streptococcus intermedius-MG-anginosus* group (32). In 1972, Colman and Williams (8) presented a classification system which was based on transformation studies, cell wall analysis, physiological characterization, and computer-assisted numerical analysis of the streptococci. In this system, Guthof's strains of *S. milleri*, indifferent streptococci described by Ottens and Winkler (26), *Streptococcus MG*, and minute hemolytic streptococci were placed within a single species which Colman and Williams characterized anew but placed under the old name *S. milleri* (8). Subsequent studies by Ball and Parker (2) and Poole and Wilson (29, 31) contributed to the current description of *S. milleri*; (i) alpha hemolytic, beta hemolytic, or nonhemolytic, (ii) serologically heterogeneous, possessing one of the Lancefield group A, C, F, or G antigens or no group antigen, (iii) often enhanced by and variably requiring increased CO₂ for growth, and (iv) generally associated with the biochemical pattern of acid production from sucrose, salicin, trehalose, and lactose; hydrolysis of arginine and esculin; production of acetoin from glucose (Voges-Proskauer [VP] reaction); and resistance to bacitracin and nitrofurazone.

Although the term *S. milleri* is well established in European nomenclature, a different taxonomy for this group of organisms is used by Facklam at the Centers for Disease Control (12). This latter system uses only species names which are already in the Approved Lists of Bacterial Names (34). Facklam's nomenclature emphasizes hemolysis and acid production from lactose in separating organisms that would be included under *S. milleri* by other investigators (11, 12). In Facklam's system, non-beta-hemolytic *S. milleri* strains are divided into the lactose-negative species *S. intermedius* and the lactose-negative species *S. constellatus*, whereas minute beta-hemolytic *S. milleri* strains are classified separately according to their Lancefield group antigen as subspecies of *S. anginosus* (12). From a genetic standpoint, separation of these organisms into different species may not be justified. In the DNA hybridization studies of Welborn et al. (37), the American Type Culture Collection type strains of *S. intermedius* and *S. constellatus* as well as two clinical

* Corresponding author.
isolates of beta-hemolytic group F streptococci (i.e., *S. anginosus*) were all shown to be closely related genetically. These data support the inclusion of these organisms under a single species. Farrow and Collins (15), using optimum renaturation conditions, confirmed the results of Welborn et al. (37). Kilpper-Bälz and Schleifer (19) and Kilpper-Bälz et al. (20) obtained similar results, except that when more stringent renaturation conditions were used, the Deutsche Sammlung für Mikroorganismen type strains of *S. anginosus* and *S. constellatus* hybridized less than 50% to each other. Thus, although considerable DNA studies have been done, the question of the taxonomy of *S. milleri* strains is still unresolved.

According to conventional identification schemes, the streptococci are initially separated by their ability to hemolyze erythrocytes. Beta-hemolytic streptococci are subsequently identified by serological methods, whereas non-beta-hemolytic streptococci are differentiated to the species level by physiological tests (11, 13). This dichotomous approach can pose a problem with regard to the identification of *S. milleri*. Although non-beta-hemolytic *S. milleri* strains are accurately identified by the physiological methods used to differentiate the viridans streptococci, beta-hemolytic *S. milleri* strains are likely to be differentiated by serological tests only as group A, group C, group F, group G, or nongroupable streptococci. Consequently, beta-hemolytic *S. milleri* strains with group A, C, or G antigen may be mistaken for other species of beta-hemolytic streptococci which have been traditionally associated with these Lancefield group antigens (i.e., *S. pyogenes*, group A; *S. equisimilis*, group C in humans; and the large-colony group G streptococci).

In this study, clinical isolates of beta-hemolytic streptococci were biochemically characterized in an effort to determine practical methods for differentiating beta-hemolytic *S. milleri* from *S. pyogenes*, *S. equisimilis*, and the large-colony group G streptococci. A detailed identification of these isolates provided an estimate of the relative incidence of *S. milleri* among various serological groups of beta-hemolytic streptococci isolated in our clinical laboratory.

**MATERIALS AND METHODS**

**Bacterial strains.** The beta-hemolytic streptococci used in this study were clinical isolates from patients at San Francisco General Hospital. The streak-stab method (13) was used in determining hemolysis on Trypticase soy agar plates (BBL Microbiology Systems, Cockeysville, Md.) containing 5% sheep blood. The cultures were examined after 18 to 24 h of incubation at 35°C in 5% CO2.

Isolates were serologically screened with the Streptex rapid latex testing system (Wellcome Reagents Div., Burroughs Wellcome Co., Research Triangle Park, N.C.) for detecting Lancefield groups A, B, C, D, F, and G. The number of isolates which were further characterized biochemically was limited by including only a sampling of the group A and group G isolates that were morphologically typical of *S. pyogenes* and the large-colony group G streptococci, respectively. However, all minute-colony isolates with group A or group G antigen were tested. In addition, all isolates of group C, group F, and nongroupable (i.e., not group A, B, C, D, F, or G) streptococci obtained during this study were included. Ultimately, 172 clinical isolates of group A, C, F, or G or nongroupable beta-hemolytic streptococci were characterized by using a battery of biochemical tests.

**Biochemical tests.** The following tests were incubated at 35°C in an ambient atmosphere, unless stated otherwise. Acid production from glucose, inulin, lactose, mannitol, melibiose, raffinose, salicin, sorbitol, sucrose, trehalose, and ribose was tested with bromocresol purple pH indicator in heart infusion broth base (Difco Laboratories, Detroit, Mich.) containing 1% (wt/vol; final concentration) carbohydrate (all from Difco, except for ribose, which was from Sigma Chemical Co., St. Louis, Mo.) as described by Facklam (11). Esculin hydrolysis was performed in heart infusion broth medium containing 0.03% esculin (Difco) but not bromocresol purple pH indicator. The inoculum for each test was 1 drop from an 18- to 24-h culture in Todd-Hewitt broth (GIBCO Diagnostics, Madison, Wis.) supplemented with pyridoxal hydrochloride (final concentration, 0.001%; Sigma). The carbohydrate broths were read for acid production over a 7-day incubation period. Esculin hydrolysis was read after 5 days by adding 2 drops of 1% ferric ammonium citrate solution.

The method used for detecting the production of acetoin from glucose was adapted from previously described methods for rapid VP testing (5, 7). A tube containing 0.2 ml of methyl red-VP broth (GIBCO) was inoculated with a loopful of growth from an overnight culture on blood agar and incubated for 4 to 6 h. After incubation, 1 drop each of the following solutions was added: 0.5% creatine (Sigma), alpha-naphthol (Analytab Products, Plainview, N.Y.), and 40% KOH. The tube was shaken and observed for the development of a pink-red color within 30 min.

Four rapid tests were used for the detection of preformed enzymes. These included a chromogenic substrate test for the hydrolysis of pyrogallitic acid (PYR) and fluorogenic substrate tests for the hydrolysis of N-acetyl-beta-D-glucosaminide (NAG), beta-D-glucuronide (beta-GUR), and beta-D-galactoside (beta-GAL). The method used filter paper disks impregnated with substrate. A loop or wooden stick was used to inoculate the disks with colonies taken from an overnight culture on brain heart agar. A pyrogallatynylnaphthylamide derivative (0.5 mg/ml in methanol; EY Laboratories, San Mateo, Calif.) was used in the PYR test. After the disk was inoculated, 1 drop of fast garnet dye (0.1 mg/ml; EY Laboratories) was added. The hydrolysis of PYR was indicated by the development of a pink-red color within 10 min at room temperature. An adaptation of the method described by Sliikin and Gil (35) was used to detect the hydrolysis of fluorogenic 4-methylumbelliferyl conjugates (Sigma) of NAG, beta-GUR, and beta-GAL. The disks were impregnated with 1-mg/ml solutions of the individual substrates in 0.05 M phosphate-buffered saline, pH 6.5. The disks were inoculated and incubated at 35°C for 30 min. One drop of a saturated sodium bicarbonate solution was added, and the disks were observed immediately for fluorescence under a long-wave (366-nm) UV lamp.

Isolates were tested for susceptibility to bacitracin with Taxi A disks (0.04 U of bacitracin; BBL Microbiology Systems) (13).

**RESULTS**

Of the 172 clinical isolates of beta-hemolytic streptococci characterized in this study, 91 proved to be biochemically identical to *S. milleri*. The remaining isolates included 20 *S. pyogenes* (group A), 21 *S. equisimilis* (group C), 37 large-colony group G streptococci, and 3 unidentified isolates. Based on serological agglutination tests, a majority (84%) of the *S. milleri* strains were found to have Lancefield group antigen (3 A, 27 C, 41 F, and 5 G). However, 15 *S. milleri*
strains (16%) did not react with any of the antisera to group A, B, C, D, F, or G. These strains are described here as nongroupable. Table 1 shows the distribution of isolates by general site of isolation. Since not all isolates of S. pyogenes and the large-colony group G streptococci were included in the study, it was not possible to determine the incidence of beta-hemolytic S. milleri in various sites or specimens. However, beta-hemolytic S. milleri strains were frequently recovered from wounds and abscesses and from both the upper and lower respiratory tracts.

The biochemical reactions of the organisms are summarized in Table 2. Isolates identified as S. milleri characterized produced small to pinpoint colonies on 5% sheep blood agar after 24 h of incubation in 5% CO₂. Variations in colony morphology and subtle differences in beta hemolysis were observed among these strains. Microscopic examination of various cultures of S. milleri confirmed their production of beta hemolysis as defined by Facklam and Carey (13).

S. milleri is usually considered a member of the viridans group of streptococci. Although carbohydrate tests are used for identifying species of viridans streptococci (11), these tests were not very useful in differentiating beta-hemolytic S. milleri strains from other beta-hemolytic species of streptococci (Table 2). S. milleri isolates were distinguished from other beta-hemolytic streptococci biochemically by being positive in the VP test, negative for the hydrolysis of PYR and β-GUR, and negative for acid production from ribose (Table 3). Some variation in the other biochemical reactions was found when the S. milleri strains were divided according to their serological reactions (Table 2). Of the S. milleri strains with group C antigen, 15 (56%) were susceptible to bacitracin, whereas all other S. milleri strains were resistant. In addition, an association was observed in the group C strains between bacitracin susceptibility and a lack of acid production from trehalose. Only 4 of 15 (27%) of the bacitracin-resistant strains but 10 of 12 (83%) of the bacitracin-resistant strains of S. milleri with group C antigen produced acid from trehalose. Two isolates of S. milleri with group G antigen were unique among the isolates in this study in that they produced acid from raffinose and melibiose.

The biochemical patterns for S. equisimilis (group C) and the large-colony group G streptococci were virtually identical. With few exceptions, the strains in both groups were characterized by acid production from ribose, hydrolysis of both β-GUR and NAG, and a negative VP test. Thus, S. equisimilis and the large-colony group G streptococci could only be differentiated from one another by their Lancefield group antigen. Approximately one-third each of S. equisimilis and the large-colony group G streptococci (33 and 27%, respectively) was susceptible to bacitracin, but none hydrolyzed PYR. In contrast, all strains of S. pyogenes were susceptible to bacitracin and hydrolyzed PYR. Although S. pyogenes, S. equisimilis, and the large-colony group G streptococci generally produced larger colonies than did S. milleri, colony size was not a definitive characteristic. Several isolates of group C streptococci produced colonies of only moderate size, yet they were identified as S. equisimilis because they produced acid from ribose, hydrolyzed β-GUR, and were negative in the VP test. Three strains of group G streptococci which were biochemically most consistent with the large-colony form appeared morphologically similar to S. milleri.

Three isolates remained unidentified by the methods used in this study. All three were nongroupable in the Streptex system. One strain, isolated from a throat specimen, produced a biochemical pattern similar to that described by Facklam (11) for Streptococcus sanguis II. S. sanguis II, however, is described by Facklam (11) as not being beta hemolytic. The remaining two isolates, one from a hand abscess and the other from a throat specimen, produced unusual but identical biochemical patterns. These two strains do not fit any of the 28 phenons of streptococci described by Bridge and Sneath (6) and may represent a new species of streptococci.

In terms of serological identification, the incidence of beta-hemolytic S. milleri among the clinical isolates of beta-hemolytic streptococci in our study was as follows: S. milleri represented 27 of 48 (56%) isolates of group C streptococci, all isolates of group F streptococci, and 15 of 18 (83%) isolates of nongroupable streptococci. The precise incidence of beta-hemolytic S. milleri among clinical isolates of group A and group G streptococci was not determined in this study because not all isolates with group A or G antigen were included. However, this incidence is estimated to be low, since only three isolates of S. milleri with group A antigen and five isolates of S. milleri with group G antigen were found as a result of examining all clinical isolates of group A and group G streptococci for colony morphology.

### DISCUSSION

The results of this study indicate that Lancefield serological grouping does not provide sufficient information for
<table>
<thead>
<tr>
<th>Group</th>
<th>Characteristics</th>
<th>Other Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>No reaction with auxin B C D F</td>
<td>Acetone, methanol, chloroform, ethyl acetate, and ethyl alcohol.</td>
</tr>
<tr>
<td>B</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Positive</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 2: Biochemical reactions of 'traditional' beta-hemolytic streptococci and beta-hemolytic 'S. milleri' in relation to Lancefield group antigens.
The serological and biochemical heterogeneity observed among the *S. milleri* strains in this and previous investigations (2, 8) suggests the possibility that *S. milleri* may represent more than one species. DNA hybridization studies (15, 37) support the inclusion of *S. intermedius*, *S. constellatus*, and the beta-hemolytic group F streptococci (*S. anginosus*) under a single species (i.e., *S. milleri*). However, genetic evidence is still needed to verify the additional inclusion of minute beta-hemolytic strains with Lancefield group A, C, or G antigen or with no group antigen under *S. milleri*. Hybridization studies may also determine whether there is any taxonomic significance associated with bacitracin susceptibility among minute beta-hemolytic group C streptococci or with acid production from raffinose and melibiose among minute beta-hemolytic group G streptococci and among the strains described by Ball and Parker (2). In the absence of further genetic data, the inclusion of these strains under *S. milleri* is based on their serological and physiological similarities to accepted members of this species.

All of our *S. milleri* isolates were positive in the VP test (4 to 6 h) and were differentiated from *S. pyogenes*, *S. equisimilis*, and the large-colony group G streptococci on this basis. Our data agree with the findings of Bucher and von Graevenitz (7). In studies by Ball and Parker (2) and Poole and Wilson (29), although the vast majority of *S. milleri* strains were positive in the VP test, a few were negative when a method requiring 5 days of incubation was used. In addition, Ball and Parker (2) found that 11% of the *S. pyogenes* strains thus tested produced a positive VP reaction. This suggests that the value of the VP reaction as a differential test may depend on the method used and that additional tests may be necessary to differentiate beta-hemolytic *S. milleri* strains from other species of beta-hemolytic streptococci. Our data indicate that several tests can be used as alternatives or supplements to the VP test for this purpose.

Although only three isolates of beta-hemolytic *S. milleri* with group A antigen were found, all three strains were negative in the PYR test. In contrast, all *S. pyogenes* strains were positive. Thus, the PYR test may serve as a rapid method for differentiating these organisms. Our limited data suggest that the bacitracin test may also be used for this purpose. However, the PYR test has the advantage of differentiating between *S. pyogenes* and the bacitracin-susceptible strains of either *S. equisimilis* or the large-colony group G streptococci, since all strains of the latter are PYR negative (14; J. Lawrence, D. M. Yajko, and W. K. Hadley, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 19, 1984).

We found that serological testing is necessary to differentiate between *S. equisimilis* and the large-colony group G streptococci. As evident from this study and from previous descriptions of these organisms (9, 27), *S. equisimilis* and the large-colony group G streptococci have virtually identical biochemical patterns. Recent DNA-DNA hybridization tests have shown that *S. equisimilis*, large-colony group G streptococci, and group L streptococci actually represent a single species (15).

The clinical significance of *S. milleri* has been examined by several investigators (25, 28–31, 33). However, since in most clinical laboratories beta-hemolytic isolates of *S. milleri* are not distinguished from other species of streptococci, the incidence and pathogenicity of *S. milleri* may be underestimated. In the present study, a high incidence of *S. milleri* was observed among the clinical isolates examined. The
occurrence of streptococcal group A, C, and G antigens among beta-hemolytic strains of \textit{S. milleri} demonstrates that correct identification of beta-hemolytic streptococci to the species level requires a combination of serological and biochemical tests. The rapid biochemical tests described here have been found to be useful for this purpose.

**ADDENDUM IN PROOF**

After this manuscript was submitted for publication, K. L. Ruoff, L. J. Kunz, and M. J. Ferraro published similar findings (J. Clin. Microbiol. 22:149–151, 1985). They found that \textit{S. milleri} accounted for 75% of group C, 15% of group G, 100% of group F, and 75% of nongroupable beta-hemolytic streptococci of clinical origin. No \textit{S. milleri} with group A antigen were found in their study.

**LITERATURE CITED**


