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# Biochemical differentiation of beta-hemolytic streptococci isolated from humans; incidence and characterization of beta-hemolytic isolates of *Streptococcus milleri*

by

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THESIS

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# DEDICATION

To my parents and my sister

whose love provides constant inspiration

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# INTRODUCTION

In 1874, Billroth used the term "streptococcos" to describe chain-forming cocci that were found in wounds and discharges and associated with erysipelas (18). The genus Streptococcus, however, was not established until 1884 when Rosenbach assigned the name Streptococcus pyogenes to chain-forming cocci that had been isolated from suppurative lesions in man (18). The streptococci are now generally defined as catalase negative, homofermentative gram positive cocci that form pairs and chains. They are facultative anaerobes and usually have complex nutritional requirements (9).

Early classification of the streptococci was dependent on morphological and physiological criteria such as chain length and fermentation of various sugars (1). However in 1933, a serological method for differentiating beta-hemolytic streptococci was introduced by Lancefield (19). Lancefield (19,20) defined several serological groups among the streptococci by using a precipitin test to detect group specific antigen present in the streptococcal cell wall. Beta-hemolytic streptococci isolated from humans were found to possess Lancefield group A, B, C, D, F, or G antigen (19,20).

In 1937, Sherman (32) combined physiological and serological criteria to produce the first systematic classification of the streptococci. In this system, the streptococci were divided into four categories based primarily on the tolerance of these organisms to temperature, salt, alkali, and methylene blue. The four categories were designated pyogenic, viridans, lactic, and enterococcus. According to Sherman (32), enterococci (which included Lancefield group D streptococci) and lactic streptococci grew at 10°C, produced strong reducing action, and were tolerant to methylene blue. Enterococci, however, were unique in being able to grow in 6.5% NaCl and at pH 9.6. In contrast, pyogenic and viridans streptococci did not grow at 10°C, had weak reducing power, and were not tolerant to methylene blue, salt or alkali. Viridans streptococci were differentiated from pyogenic streptococci by not being beta-hemolytic, by not producing ammonia from peptone, and by usually growing at 45°C. All beta-hemolytic streptococci (except beta-hemolytic enterococci) were classified as pyogenic streptococci and were divided into species based primarily on their Lancefield serological group. However, the group C streptococci were further divided into human and animal species based on biochemical tests.

According to current classification, the streptococci

isolated from humans normally fall under the categories of pyogenic or beta-hemolytic streptococci, viridans streptococci, enterococcal and non-enterococcal group D streptococci, and pnuemococci (26). Lancefield serological grouping has become the standard method for identifying beta-hemolytic streptococci. However, this method has not proved useful in differentiating non-beta-hemolytic species. Facklam (11) showed that among the viridans streptococci, Lancefield antigens A-G are found only occasionally and without specificity to species. None of the Lancefield group antigens are present among pneumococci. And although serological tests may be used to identify group D streptococci, these methods do not differentiate enterococcal species from non-enterococcal species. Due to these differences among the streptococci, a dichotomous system for streptococcal identification has been established in which isolates are initially separated on the basis of hemolysis. According to this system, subsequent identification of beta-hemolytic streptococci is accomplished by serological methods, whereas non-beta-hemolytic streptococci are identified to the species level using physiological tests (12).

There are several drawbacks to the current system of streptococcal identification. One concerns the emphasis which is placed on the single characteristic of hemolysis.

Strict separation of the streptococci according to their hemolytic ability can lead to inaccurate identification of non-beta-hemolytic isolates of pyogenic streptococci (e.g., S. pyogenes and S. agalactiae) or beta-hemolytic isolates of viridans streptococci (e.g., S. milleri). Another drawback to the current system is that heavy reliance on serological methods for the identification of beta-hemolytic streptococci discourages biochemical testing of these organisms. As a result, streptococci that are biochemically distinct but have identical group antigen may not be recognized as different species. For example, isolates of S. equisimilis, S. zooepidemicus, S. equi, and some isolates of S. milleri are all identified as beta-hemolytic group C streptococci based on serological tests, although these organisms can be separated biochemically. Over-emphasis on serological characteristics can also lead to excessive formation of species among organisms that differ in their group antigen but are biochemically and genetically homologous. This appears to be the case among isolates of S. milleri.

The original purpose of this study was to find a method for separating Lancefield group A, B, C, F, or G beta-hemolytic streptococci from one another using rapid chromogenic substrates. However, difficulties were encountered when preliminary testing revealed that each

Lancefield group of streptococci yielded more than one biochemical pattern. In particular, it was noticed that minute colony isolates of group C and group G streptococci were biochemically different from large colony isolates within the same Lancefield group (i.e., these were heterogeneous groups of organisms). Subsequent testing showed this heterogeneity to be caused by beta-hemolytic isolates of S. milleri. Additional isolates were therefore studied to determine the incidence of beta-hemolytic S. milleri among various Lancefield groups. Further biochemical testing was performed to evaluate methods for differentiating S. milleri from other species of beta-hemolytic streptococci.

S. milleri is considered an important cause of purulent disease in humans (27). In one study, S. milleri was the streptococcus most often isolated from abscesses of internal organs (27). Various infections in which S. milleri has been implicated include brain abscesses (10,22,27,33), liver abscesses (5,24,27), appendicitis (29), peritonitis (24,27), and endocarditis (24,27).

S. milleri is often considered a member of the viridans streptococci (8,11,24). However, Parker (26) recently placed S. milleri with the pyogenic streptococci. This conflict in classification reflects the serological and

hemolytic diversity among S. milleri and the divergent manner in which S. milleri isolates are identified. Most strains of S. milleri are non-hemolytic, but about 25% are beta-hemolytic and may possess Lancefield group A, C, F, or G antigen (2). According to conventional schemes, non-hemolytic S. milleri isolates are identified by physiological tests used to differentiate the viridans streptococci (8,11). However, beta-hemolytic S. milleri isolates are likely to be identified serologically as group A, group C, group F, group G, or "non-groupable" streptococci. Since the Lancefield groups A, C, and G are frequently equated with other species of streptococci (i.e., S. pyogenes, group A; S. equisimilis, group C in humans; and large colony group G streptococci), over emphasis on serological criteria for the identification of beta-hemolytic streptococci can hinder the recognition of beta-hemolytic S. milleri isolates with these group antigens. Accurate identification of S. milleri is needed in order to determine its role in pathogenesis.

The taxonomic status of the species S. milleri is somewhat controversial. The name S. milleri was first proposed in 1956 by Guthof (15) for a group of streptococci isolated from dental abscesses and other oral lesions. These strains were non-hemolytic and lacked Lancefield group antigen. Related organisms have been variously

characterized as Streptococcus anginosus (1,36), minute hemolytic streptococci (4,21), group F streptococci (20,21), Streptococcus MG (23), Streptococcus intermedius (17), Streptococcus constellatus (17), Streptococcus MG-intermedius (11), Streptococcus anginosus-constellatus (11), and Streptococcus intermedius-MG-anginosus group (31). 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 (25), 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 (28,30) have contributed to the current description of S. milleri: 1) gamma-, beta-, or alpha-hemolytic, 2) serologically heterogeneous, possessing one of the Lancefield group A, C, F, or G antigens or no group antigen, 3) often enhanced by and variously requiring increased $CO_2$ for growth, and 4) generally associated with the biochemical pattern of acid production from lactose, sucrose, trehalose, and salicin; hydrolysis of arginine and esculin; production of acetoin from glucose (Voges Proskauer 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 (13). This latter system includes only species names which are already on the approved list 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 (11,13). Thus in Facklam's system, the non-beta-hemolytic S. milleri strains are divided into the lactose positive species S. intermedius and the lactose negative species S. constellatus, whereas the minute beta-hemolytic strains are classified separately according to their Lancefield group antigen as subspecies of S. anginosus (13). From a genetic standpoint, separation of these organisms into different species does not seem justified. The evidence from DNA hybridization studies by Welborn et al. (37) supports the inclusion of S. intermedius, S. constellatus, and the beta-hemolytic group F streptococci (i.e., S. anginosus) under a single species based on genetic relatedness. Although additional hybridization studies are necessary to confirm the genetic homology among beta-hemolytic isolates of S. milleri with group A, C, or G antigen or with no group antigen, the name

S. milleri as currently defined seems appropriate for describing these physiologically similar organisms.

In this study, biochemical tests were evaluated for their usefulness in identifying the various species of beta-hemolytic streptococci isolated from humans. Emphasis was especially placed on finding methods that would separate S. milleri with group A, C, or G antigen from S. pyogenes (group A), S. equisimilis (group C), and large colony group G streptococci. The biochemical tests that were most useful for this purpose include the Voges Proskauer reaction; hydrolysis of pyroglutamic acid, beta-D-glucuronide, and N-acetyl-beta-D-glucosaminide; bacitracin sensitivity; and acid production from ribose. As a result of this investigation, a combination of biochemical and serological tests is suggested for accurate identification of the beta-hemolytic streptococci.

## MATERIALS AND METHODS

### Bacterial strains

Clinical isolates of beta-hemolytic streptococci were obtained from routine cultures at San Francisco General Hospital. Isolates were identified as streptococci on the basis of Gram staining and catalase testing. The streak-stab method (12) was used to determine hemolysis on blood agar plates with Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) and 5% sheep blood. Cultures were examined after 18-24 hours incubation at 35°C in 5% $CO_2$.

Working cultures were streaked for isolation on trypticase soy blood agar plates and grown 18-24 hours at 35°C in 5% $CO_2$. For prolonged storage, the cultures were either maintained on blood agar slants at 4°C or were frozen at -70°C in Trypticase soy broth supplemented with 15% glycerol.

### Serological testing

Isolates were serologically identified using the Streptex rapid latex testing system (Wellcome Diagnostics, Research Triangle Park, N.C.) for detecting Lancefield groups A, B, C, D, F, and G. Isolates that did not react with any of the antisera to these group antigens are

referred to in this study as "non-groupable".

**Bacitracin sensitivity**

Sensitivity to bacitracin was determined by streaking the cultures for isolation on trypticase soy blood agar plates and placing a Taxo A disc (0.04 U bacitracin; BBL) on the second quadrant of inoculation (i.e., on an area of moderate to heavy growth). The cultures were incubated overnight at 35°C in 5% $CO_2$. Any zone of inhibition was considered indicative of sensitivity.

**Biochemical tests**

The following tests were incubated at 35°C in ambient atmosphere, unless stated otherwise.

**Test Series I:**

The purpose of the first part of this study was to test four rapid chromogenic tests for their usefulness in differentiating beta-hemolytic streptococci of Lancefield groups A, B, C, F, and G. The four substrates (obtained from Sigma Chemical Co., St. Louis, Mo.) included L-pyrrolidonyl-beta-naphthylamide (a naphthyl derivative of pyroglutamic acid, PYR; 1mg/ml), p-nitrophenyl-N-acetyl-beta-D-glucosaminide (pnp-NAG; 2mg/ml), 6-bromo-2-naphthyl-beta-D-glucuronide (bn-β-GUR; 1mg/ml), and p-nitrophenyl-beta-D-galactopyranoside (pnp-β-GAL; 2mg/ml). Substrate

solutions were prepared in methanol with 0.2% Tween 80. Filter paper disks (1/4 inch diameter; Schleicher & Schnell, Inc., Keene, N.H.) were impregnated with 50µl of the appropriate substrate solution and were allowed to dry. The tests were performed as follows:

1. The disks were inoculated in either of two ways.
   a) In the first method, a heavy bacterial suspension was made in a buffered solution of lysozyme (1% lysozyme (Sigma) in 0.05M phosphate buffer, pH 6.4). One drop of this bacterial suspension was pipetted onto each disk.
   b) In the second method, bacterial colonies were taken directly from blood agar and rubbed onto the dry disks using a loop or wooden stick. One drop of a buffered lysozyme solution (same as above) was then pipetted onto each disk.

2. The disks were incubated for 1 hour at 35°C.

3. After incubation, the chromogenic reactions were developed by adding 20µl per disk of either a Fast Garnet dye (0.1mg/ml; EY Laboratories, San Mateo, CA) for the PYR and bn-β-GUR tests or a saturated solution of sodium bicarbonate for the pnp-NAG and pnp-β-GAL tests. The hydrolysis of

PYR and bn-β-GUR was detected by the formation of a red color, whereas the hydrolysis of pnp-NAG and pnp-β-GAL resulted in a yellow color.

**Test Series II:**

In an attempt to explain the biochemical heterogeneity seen among organisms in the first series of tests, many of these isolates as well as additional isolates were characterized using a battery of biochemical tests. One objective was to identify the minute colony isolates of beta-hemolytic streptococci since it appeared that these isolates were partially responsible for the biochemical heterogeneity found among various Lancefield groups. These organisms were suspected of being beta-hemolytic isolates of S. milleri. Since S. milleri does not possess group B or group D antigen and no minute colony isolates had been observed among the group B streptococci, only those isolates which were serologically identified as group A, C, F, or G or as "non-groupable" beta-hemolytic streptococci were characterized using the entire series of tests. In order to limit the study to a practical number of isolates, only a sampling of the group A and group G streptococci were selected for testing. All minute colony isolates with group A or group G antigen were included. However, many isolates that were morphologically typical of S. pyogenes

(group A) or large colony group G streptococci were eliminated. In contrast, all clinical isolates of group C, group F, and "non-groupable" streptococci were included in order to determine the incidence of S. milleri among these Lancefield groups. Ultimately, 172 isolates were characterized using the following battery of tests.

Acid production from glucose, inulin, lactose, mannitol, melibiose, raffinose, salicin, sorbitol, sucrose, trehalose, and ribose was tested using bromocresol purple pH indicator in heart infusion broth base (Difco Laboratories, Detroit, Mich.) containing 1% (wt/vol; final concentration) carbohydrate (Difco; except ribose, Sigma) as described by Facklam (11). Esculin hydrolysis was determined in heart infusion broth medium containing 0.03% esculin (Difco) without bromocresol purple indicator. The inoculum for each test was 1 drop from an 18-24 hour culture in Todd Hewitt broth (Gibco Diagnostics, Madison, Wis.) supplemented with pyridoxal HCl (0.001% final concentration; 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 Voges-Proskauer (VP) testing (3,7). A tube containing 0.2 ml of MR-VP Broth (Gibco) was inoculated with a loopful of growth from an overnight culture on blood agar and incubated 4-6 hours. Subsequently, one drop each of the following solutions was added: 0.5% creatine (Sigma), alpha naphthol (API, Analytab Products, Plainview, NY), and 40% KOH. The tube was shaken and observed for development of a pink-red color within 30 minutes.

Four rapid enzymatic tests which were similar to the tests used in the first part of this study were evaluated. These included a chromogenic test for the hydrolysis of pyroglutamic acid (PYR) and three fluorogenic tests for detecting the hydrolysis of N-acetyl-beta-D-glucosaminide, beta-D-glucuronide, and beta-D-galactoside. Fluorogenic substrates were used instead of the previous chromogenic derivatives in an attempt to reproduce the patterns reported by Slifkin and Gil (35) and to reduce the time required to achieve an identification. The method employed filter paper disks which were impregnated with 50µl of substrate and allowed to dry before testing. A loop or wooden stick was used to inoculate the disks with colonies taken from an overnight culture on blood agar. For the PYR test, disks were impregnated with a pyroglutamyl-naphthylamide derivative (0.5 mg/ml in methanol) obtained from EY Laboratories. The PYR test was performed by

inoculating the disk and then adding a drop of Fast Garnet dye (0.1 mg/ml; EY Laboratories). The hydrolysis of PYR was indicated by the development of a pink-red color within 10 minutes at room temperature. Positive reactions were usually apparent within 30 seconds. The three fluorogenic tests were performed using an adaptation of the method described by Slifkin and Gil (35). The substrates included 4-methylumbelliferyl-conjugates (Sigma) of N-acetyl-beta-D-glucosaminide (4MEU-NAG), beta-D-glucuronide (4MEU-β-GUR), and beta-D-galactoside (4MEU-β-GAL). Disks were impregnated with 1 mg/ml solutions of the individual substrates in 0.05M phosphate-buffered saline, pH 6.5. The tests were performed as follows: 1) The disks were moistened with water, inoculated, and incubated at 35°C for 30 minutes; 2) one drop of a saturated sodium bicarbonate solution was then added; and 3) the disks were observed immediately for fluorescence under a long wave (366 nm) ultraviolet lamp.

After completing the identification to species level of 172 isolates of group A, C, F, and G and "non-groupable" streptococci using the entire series of biochemical tests, efforts were focused on determining whether five of these tests (including VP, PYR, 4MEU-NAG, 4MEU-β-GUR, and 4MEU-β-GAL) might be used to differentiate all species of beta-hemolytic streptococci isolated from humans. Since

the previous isolates did not include any group B or group D streptococci, 20 additional isolates including 15 strains of group B streptococci and 5 beta-hemolytic strains of group D streptococci were studied using these five tests. The beta-hemolytic group D streptococci were obtained from a frozen stock collection of isolates maintained in Dr. W. K. Hadley's research laboratory at San Francisco General Hospital. These strains were identified as enterococci using the bile-esculin and 6.5% NaCl tests (12). They were further identified as S. faecalis 2 (2 strains), S. faecalis 3 (1 strain), and S. faecium durans 1 (2 strains) using the DMS Rapid Strep system (DMS Laboratories, Inc., Flemington, NJ). According to this system, all five strains gave a positive Voges Proskauer reaction; hydrolyzed esculin, pyrrolidonyl-2-naphthylamide, and arginine; and produced acid from ribose and starch. They did not produce acid from L-arabinose, inulin, raffinose, or glycogen, nor did they hydrolyze 2-naphthyl-phosphate (alkaline), or naphthol-AS-BI-beta-D-glucuronate. The strains of S. faecalis 2 and S. faecalis 3 produced acid from mannitol, sorbitol and trehalose. Acid production from lactose was variable among these three strains. S. faecalis 3 was differentiated from S. faecalis 2 by being positive for hydrolysis of hippurate and 2-naphthyl-beta-D-galactopyranoside. The two strains of S. faecium durans 1 were negative for acid production from

mannitol and sorbitol. They were positive for acid production from lactose and for hydrolysis of beta-D-galactopyranoside. In the DMS differential table, beta hemolysis is listed as being found among 96% of S. faecium durans 1 but among only 2% of S. faecalis 2 or 3.

## RESULTS

**Test Series I:**

In the first part of this study, 102 clinical isolates of beta-hemolytic streptococci which were serologically identified as group A, B, C, F, or G were tested for rapid hydrolysis of PYR, pnp-NAG, bn-β-GUR, and pnp-β-GAL. The purpose was to see whether these tests could be used instead of serological tests for differentiating the various groups of beta-hemolytic streptococci. The reaction patterns are summarized in Table 1. All 13 strains of group A streptococci were distinguished from the streptococci of groups B, C, F, and G by being positive for the hydrolysis of PYR. All except one (96%) of the group B streptococci were differentiated from the other Lancefield groups by being negative for the hydrolysis of pnp-NAG but positive for the hydrolysis of bn-β-GUR. Most (90%) of the group F strains were distinguished by being negative for all tests except the hydrolysis of pnp-β-GAL. All group G strains and all except one (95%) of the group C strains were biochemically differentiated from groups A, B, and F, but they were not differentiated from one another. Two distinct patterns were associated with both group C and group G streptococci. One of these patterns was produced by 57% of the group C strains and all except one (95%) of the group G strains. These strains were positive for all

tests except the hydrolysis of PYR. The other pattern was produced by 38% of the group C strains and one (5%) of the group G strains. These latter strains differed from the other group C and group G strains biochemically by being negative for the hydrolysis of bn- -GUR and morphologically by forming much smaller colonies on blood agar. The formation of minute colonies was otherwise more typical of group F streptococci than of group C and group G streptococci. One isolate each from groups B and C and two isolates from group F were negative on all four tests. Therefore out of 102 isolates, 4 isolates (4%) could not be biochemically distinguished as belonging to a specific Lancefield group based on these four tests. The other 98 isolates (96%) were biochemically differentiated as belonging to group A, group B, or group F, or as belonging to either group C or group G. Since a high degree of biochemical heterogeneity was observed among these isolates (particularly among the group C and group G streptococci), futher testing was indicated.

**Test Series II:**

In the second part of this study, a battery of biochemical tests was used to characterize many of the isolates from the first part of this study as well as additional isolates of group A, C, F, or G or "non-groupable" streptococci. One of the purposes was to

identify these organisms to the species level. Among the 172 isolates tested, 91 proved 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 reactions, a majority (84%) of the S. milleri strains were found to have Lancefield group antigen (3A, 27C, 41F, and 5G). However, 15 S. milleri strains (16%) did not agglutinate with any of the antisera to groups A, B, C, D, F, or G. These strains are referred to here as "non-groupable". Table 2 shows the distribution of isolates by general source of isolation. Approximately half of the isolates are known to have come from wounds or abscesses. No information regarding source was found for 28 (16%) of the isolates. The biochemical reactions of these organisms are summarized in Table 3.

The isolates identified as S. milleri characteristically produced small to pinpoint colonies on 5% sheep blood agar after 24 hours incubation in 5% $CO_2$. Variations in colonial morphology and subtle differences in beta hemolysis were observed among these strains. However, microscopic examination of various cultures of S. milleri confirmed their production of beta hemolysis as defined by Facklam (12). The carbohydrate tests used for the identification of species of viridans streptococci (11)

were not very useful in differentiating beta-hemolytic S. milleri strains from S. pyogenes (group A), S. equisimilis (group C), and large colony group G streptococci (Table 3). S. milleri isolates were distinguished from these organisms biochemically by being positive for the VP test, negative for the hydrolysis of both PYR and 4MEU-β-GUR, and negative for acid production from ribose. Some variance among the other biochemical reactions was found when the S. milleri strains were divided according to their serological reaction (Table 3). Among the S. milleri with group C antigen, 15 strains (56%) were sensitive to bacitracin, whereas all other S. milleri strains were resistant. In addition, there appeared to be some correlation between this sensitivity to bacitracin and a lack of acid production from trehalose. Among the S. milleri with group C antigen, only 4 of 15 (27%) of the bacitracin-sensitive strains but 10 of 12 (83%) of the bacitracin-resistant strains produced acid from trehalose. Two minute-colony group G isolates were unique among the isolates of this study in producing acid from raffinose and melibiose. These strains were otherwise typical of S. milleri and resembled certain isolates described by Ball and Parker (2).

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 4MEU-β-GUR and 4MEU-NAG, and a negative VP test. Thus, S. equisimilis and large colony group G streptococci could only be differentiated from one another by their Lancefield group antigen. Approximately one third of the S. equisimilis and the large colony group G streptococci (33% and 27%, respectively) were sensitive to bacitracin, but none hydrolyzed PYR. In contrast, all strains of S. pyogenes were sensitive 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 4MEU-β-GUR, and were negative for 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. One of these group G strains was additionally discrepant in giving negative results on trehalose and 4MEU-β-GUR, but it was placed with the large colony group G streptococci because it produced acid from ribose, was

sensitive to bacitracin, and was negative for the VP test.

Three isolates remained unidentified by the methods of this study. All three were non-groupable by the Streptex system. One strain, isolated from a throat, produced a biochemical pattern similar to that described by Facklam (11) for S. sanguis II. S. sanguis II, however, is described by Facklam (11) as not being beta-hemolytic. This particular isolate formed large colonies which appeared alpha-hemolytic on surface growth but produced beta-hemolysis in deeply stabbed areas of agar when incubated in 5% $CO_2$. Under anaerobic conditions this isolate was completely beta-hemolytic. The other two unidentified isolates, one from a hand abscess and the other from a throat, produced unusual but identical biochemical patterns. Both isolates formed minute beta-hemolytic colonies on sheep blood agar when incubated either in 5% $CO_2$ or anaerobically. They were resistant to bacitracin and negative for hydrolysis of PYR, 4MEU-NAG, 4MEU-β-GUR, and 4MEU-β-GAL. No reaction was obtained from either strain on the VP test or in the heart infusion carbohydrate broths used in this study. However, when tested anaerobically in pre-reduced, anaerobically sterilized peptone yeast carbohydrate broths (16), both strains produced acid from glucose, mannitol, and mannose. Neither strain produced acid from arabinose, cellobiose,

esculin, lactose, maltose, raffinose, rhamnose, salicin, sucrose, trehalose or starch. 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 streptococcus.

The distribution of species among the 172 beta-hemolytic isolates in this study and the overall incidence of S. milleri is not representative of that normally found in the clinical laboratory since many group A and group G isolates were excluded. Nevertheless, since all clinical isolates of group C, group F, and "non-groupable" streptococci were included in this study, the relative incidence of beta-hemolytic S. milleri among these particular groups does represent that found in the clinical laboratory at San Francisco General Hospital. S. milleri represented 27 (56%) of the group C isolates, all 41 (100%) of the group F isolates, and 15 (83%) of the "non-groupable" isolates. Although a precise determination of the incidence of S. milleri among group A and group G streptococci was not obtained, this incidence is estimated to be low since only 3 isolates of S. milleri with group A antigen and 5 isolates of S. milleri with group G antigen were found in the course of this study.

Toward the end of this study, another attempt was made

to determine whether rapid biochemical tests could be used to identify beta-hemolytic streptococci isolated from humans. Five of the tests (including VP, PYR, 4MEU-NAG, 4MEU-β-GUR, and 4MEU-β-GAL) used previously in this study were evaluated for this purpose. The results from testing 172 clinical isolates of groups A, C, F, and G and "non-groupable" streptococci were given earlier as percent positive strains (Table 3). Table 4 lists the distinct reaction patterns found among these isolates and includes the additional results from testing 15 strains of group B streptococci and 5 strains of beta-hemolytic group D streptococci. All strains of S. pyogenes were distinguished by being positive for the PYR test but negative for the VP test. The five beta-hemolytic group D strains were positive for both the PYR and the VP tests. All S. milleri isolates were positive for the VP test, negative for the PYR test, and negative for the 4MEU-β-GUR test. All S. equisimilis strains and all except one strain of large colony group G streptococci were characterized by being negative for both the PYR and the VP tests but positive for the 4MEU-NAG, 4MEU-β-GUR, and 4MEU-β-GAL tests. Most (87%) of the group B streptococci were differentiated by being positive either for only the 4MEU-β-GUR test or for both the 4MEU-β-GUR and the 4MEU-β-GAL tests. Two strains of group B streptococci and two unidentified "non-groupable" strains were negative on

all 5 tests. A third unidentified "non-groupable" isolate produced a reaction pattern identical to an aberrant strain of large colony group G streptococci. A summary of the reactions which were found to differentiate the various species of beta-hemolytic streptococci is presented in Table 5.

Eighty-one isolates (including strains from groups A, B, C, F, and G) were tested on both the chromogenic and the fluorogenic derivatives of N-acetyl-beta-D-glucosaminide, beta-D-glucuronide, and beta-D-galactoside (Tables 1 & 4). Five discrepancies between fluorogenic and chromogenic substrates were found. All of these involved strains that were negative on one of the chromogenic assays using p-nitrophenyl-derivatives (pnp-NAG or pnp-β-GAL) but were positive on the fluorogenic assays using 4-methyl-umbelliferyl-derivatives (4MEU-NAG or 4MEU-β-GAL). One strain of S. milleri with group C antigen and two strains of group F streptococci were negative on pnp-β-GAL but were positive on 4MEU-β-GAL. Two group F isolates that were positive on pnp-β-GAL but negative on pnp-NAG were positive on both 4MEU-β-GAL and 4MEU-NAG. For the remaining 76 (94%) isolates, the reaction patterns on the chromogenic and on the fluorogenic substrates were identical.

## DISCUSSION

The results of this study indicate that Lancefield serological grouping does not provide sufficient information for species identification among beta-hemolytic streptococci of Lancefield groups A, C, and G. Among the beta-hemolytic streptococci isolated from humans, these group antigens are not exclusively associated with *S. pyogenes* (group A), *S. equisimilis* (group C), and large colony group G streptococci; rather they can also be found among beta-hemolytic isolates of *S. milleri*. The incidence of beta-hemolytic *S. milleri* among group A and group G streptococci is estimated to be low. However, 56% of the group C isolates, all of the group F isolates, and 83% of the "non-groupable" isolates of beta-hemolytic streptococci in this study proved to be *S. milleri*. The overall incidence of *S. milleri* among clinical isolates of beta-hemolytic streptococci was not determined. *S. milleri*, however, is undoubtedly one of the most frequently isolated streptococci in the clinical laboratory. This study also shows that the conventional biochemical tests used for identifying species of viridans streptococci are unable to distinguish beta-hemolytic *S. milleri* strains from other beta-hemolytic species of streptococci.

Slifkin and Gil (35) recently presented a non-serological method for identifying beta-hemolytic streptococci of groups A, B, C, F, and G isolated from throat cultures. Their method uses three fluorogenic substrates (4MEU-NAG, 4MEU-β-GUR, and 4MEU-β-GAL) for the detection of bacterial enzymes and the lectin of Dolichos biflorus for the specific agglutination of group C streptococci. In the present study, the three fluorogenic substrates used by Slifkin and Gil (35) were examined in addition to chromogenic derivatives of these three sugars (pnp-NAG, bn-β-GUR, and pnp-β-GAL). The results suggest that p-nitrophenyl-derivatives are not as sensitive as the corresponding 4-methylumbelliferyl-derivatives for detecting bacterial enzymes. In the five cases where a discrepancy between the two sets of substrates was found, the chromogenic test was negative whereas the fluorogenic test was positive. Using fluorogenic substrates, Slifkin and Gil (35) found distinct reaction patterns among their isolates of groups A, B, and F. However, a common pattern was produced by group C and some group G streptococci. These investigators therefore suggested a lectin agglutination test be used to identify the group C streptococci. Most of the reaction patterns obtained by Slifkin and Gil (35) were also seen in this study (Table 4). However, several additional patterns were found among the isolates in this study which made differentiation

of these organisms based on the three fluorogenic tests impossible. In the present study, all isolates of S. equisimilis (group C), all except one strain of large colony group G streptococci, and 35% of the S. pyogenes (group A) isolates produced an identical pattern using the fluorogenic substrates employed by Slifkin and Gil (35). These isolates were positive on all three tests. Two other patterns were also found among more than one serological group. However, these patterns were attributed to isolates of S. milleri. The S. milleri varied in their ability to hydrolyze 4MEU-NAG. However, all S. milleri isolates were negative for hydrolysis of 4MEU-β-GUR. Therefore, the results of this study suggest that additional tests to those proposed by Slifkin and Gil (35) are required for accurate separation of beta-hemolytic streptococci. These results also indicate that hydrolysis of 4MEU-β-GUR may be used as a rapid test for differentiating S. milleri from S. equisimilis and large colony group G streptococci.

Another rapid enzymatic test, the PYR test, was examined in this study for the identification of S. pyogenes (group A). Facklam et al. (14) used an agar-based PYR test and found that the hydrolysis of PYR was more specific than the bacitracin test for group A streptococci and was at least as specific as the 6.5% NaCl tolerance test for enterococci. In the present study a

rapid disk method was developed for the PYR test. The results of this study indicate the PYR test is specific for S. pyogenes rather than for group A streptococci in general since all three isolates of S. milleri with group A antigen were negative for the PYR test. The PYR test is therefore suggested as a rapid method for differentiating these organisms. Facklam et al. (14) also found one isolate of group A streptococci that was PYR-negative and bacitracin resistant, but their isolate was not identified to the species level. Although the bacitracin test can be used to separate S. pyogenes from S. milleri with group A antigen, the PYR test has the additional advantage of differentiating between S. pyogenes and the bacitracin-sensitive strains of either S. equisimilis or the large colony group G streptococci since all strains of the latter two species are PYR negative (J. Lawrence, D. M. Yajko, and W. K. Hadley, Abstr. Annu. Meet. ICAAC 1984, 19, p. 93). In the present study, five isolates of beta-hemolytic enterococci were also positive for the PYR test. These isolates were differentiated biochemically from S. pyogenes isolates by giving a positive reaction in the VP test.

The VP test was added to the battery of biochemical tests primarily for the identification of S. milleri. All of the S. milleri isolates in this study were positive for the VP reaction using a 4-6 hour test and were differen-

tiated from S. pyogenes, S. equisimilis, and large colony group G streptococci on this basis. These results confirm the findings of Bucher and von Graevenitz (7). In their study, the rapid VP test was used to separate the beta-hemolytic S. milleri with group C or G antigen from what they referred to as "true" group C or G streptococci (i.e., S. equisimilis and large colony group G streptococci) which were isolated from throat cultures. In the studies by Ball and Parker (2) and Poole and Wilson (28), although the vast majority of S. milleri strains were positive for the VP reaction, a few were negative using a method requiring 5 days incubation. In addition, Ball and Parker (2) found 11% of the S. pyogenes strains thus tested gave a positive VP reaction. This suggests that the value of the VP reaction as a differential test may depend on the method employed and that additional tests may be necessary to differentiate the beta-hemolytic S. milleri from other species of beta-hemolytic streptococci.

The results from testing 192 isolates of beta-hemolytic streptococci (Table 4) suggest that four biochemical tests (including PYR, VP, 4MEU-β-GUR, and 4MEU-NAG) can be used in combination with serological grouping to differentiate beta-hemolytic isolates of the following streptococcal species: S. pyogenes, S. agalactiae, S. equisimilis, large colony group G streptococci, S. milleri, S. faecalis, and

S. faecium-durans. The expected reactions based on the results of this study are listed in Table 5. Among the streptococci tested in this study, two strains (13%) of group B streptococci and two unidentified "non-groupable" strains were negative on all four tests. Therefore, serological testing is recommended when a negative pattern is obtained. In addition, one aberrant strain of large colony group G streptococci was found that did not hydrolize 4MEU-β-GUR. In such cases, acid production from ribose can be used as a supplementary test to differentiate the large colony group G streptococci (ribose positive) from S. milleri (ribose negative). Serological testing is necessary to distinguish between S. equisimilis and the large colony group G streptococci. As evident from this study and from previous descriptions of these organisms (9,26), S. equisimilis and the large colony group G streptococci have virtually identical biochemical patterns. Tests of genetic relatedness need to be performed to determine whether these organisms actually represent a single species.

The isolates identified in this study as S. milleri conform in most aspects to the description of this species given by Colman and Williams (8) and by Ball and Parker (2). These investigators noted that esculin hydrolysis and acid production from lactose, raffinose, and

melibiose were less common among beta-hemolytic strains than non-beta-hemolytic strains of S. milleri. However, the results of this study and that of Poole and Wilson (28) suggest that these reactions may vary among the beta-hemolytic S. milleri in relation to their Lancefield group antigen (Table 3). In this study a majority of the beta-hemolytic S. milleri with group C or G antigen were positive for esculin hydrolysis and acid production from lactose. In addition, most of these strains hydrolyzed 4MEU-NAG. The opposite was true among the beta-hemolytic S. milleri with group A or F antigen or with no group antigen. Poole and Wilson (28) found a similar pattern for esculin hydrolysis and acid production from lactose among the beta-hemolytic streptococci which they identified as S. milleri. In both studies acid production from trehalose was less common among the beta-hemolytic S. milleri with group C antigen than among the majority of beta-hemolytic S. milleri. In this study, over half (56%) of the S. milleri isolates with group C antigen were sensitive to bacitracin, whereas all other S. milleri isolates were resistant. In addition, among the S. milleri isolates with group C antigen there appeared to be a correlation between sensitivity to bacitracin and a lack of ability to produce acid from trehalose. Only 27% of the bacitracin-sensitive strains but 83% of the resistant strains of S. milleri with group C antigen produced acid from trehalose. Acid

production from raffinose was found only among S. milleri with group G antigen. Two of the five (40%) minute-colony group G strains in this study and 46% of those examined by Poole and Wilson (28) produced acid from raffinose. These strains are biochemically similar to a subset of S. milleri described by Ball and Parker (2) as producing acid from raffinose, melibiose, and less often mannitol.

The serological and biochemical heterogeneity observed among the S. milleri in this and previous investigations (2,8) suggests the possibility that S. milleri may represent more than one species. DNA hybridization studies (37) have confirmed the proper 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. Hybridization studies may also determine whether there is any taxonomic significance associated with 1) bacitracin sensitivity among minute beta-hemolytic group C streptococci and 2) 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.

The clinical significance of S. milleri has been examined by several investigators (24,27-30,33). However, further studies are required to determine the virulence of S. milleri and to define its role in suppurative disease. As discussed by Murray et al. (24), specific identification of S. milleri can provide important information to the physician. All three of their patients with subacute endocarditis caused by S. milleri developed "unexpected and unusual septic complications" (24). In addition, Murray et al. (24) suggest the isolation of S. milleri from a patient's blood may signal the presence of a suppurative process involving the gastrointestinal tract. The need for specific identification of the beta-hemolytic S. milleri strains is also suggested by the high incidence of this species observed among the clinical isolates of beta-hemolytic streptococci examined in the present study. The occurrence of streptococcal group A, C, and G antigens among beta-hemolytic strains of S. milleri demonstrates that correct identification of beta-hemolytic streptococci to the species level requires a combination of serological and biochemical tests. The biochemical tests described here have been found to be useful for this purpose.

## CONCLUSION

One of the purposes of this study was to evaluate rapid biochemical tests for differentiating the various species of beta-hemolytic streptococci isolated from humans. Clinical isolates of beta-hemolytic streptococci were initially tested using chromogenic substrates. The results of these tests indicated isolates within several of the Lancefield groups were biochemically heterogeneous. In addition, the biochemical patterns produced by these isolates differed from previously reported patterns found among beta-hemolytic streptococci using fluorogenic substrates. Subsequent testing of the isolates in this study using the same fluorogenic substrates as employed previously confirmed these differences. Further characterization of these isolates revealed that beta-hemolytic isolates of S. milleri with group A, C, or G antigen were largely responsible for the biochemical heterogeneity found among these Lancefield groups. Lancefield serological grouping of these isolates failed to distinguish S. milleri isolates from other beta-hemolytic streptococci with the same group antigen (e.g. S. pyogenes, group A; S. equisimilis, group C; and large colony group G streptococci). However, several biochemical tests were found to be useful in differentiating beta-hemolytic S. milleri from other beta-hemolytic streptococci. These

tests include the Voges Proskauer reaction, hydrolysis of pyroglutamic acid and beta-D-glucuronide, sensitivity to bacitracin, and acid production from ribose. The incidence of S. milleri among the clinical isolates of beta-hemolytic streptococci obtained at San Francisco General Hospital was found to be 56% among group C streptococci, 100% among group F streptococci, and 83% among "nongroupable" streptococci, whereas the incidence of S. milleri among group A and group G streptococci was estimated to be low. Since none of the biochemical tests examined in this study were able to differentiate S. equisimilis (group C) from large colony group G streptococci, serological tests were required for identification of these species. Based on the results of this study, a combination of serological and biochemical tests is recommended for accurate identification of beta-hemolytic streptococci isolated from humans.

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TABLE 1. Reaction patterns among 102 strains of beta-hemolytic streptococci tested on four chromogenic substrates

| Lancefield Group | No. of strains tested | Reaction Pattern | | | | No. (%) of strains per pattern | |
|---|---|---|---|---|---|---|---|
| | | PYR[1] | pnp-NAG[2] | bn-β-GUR[3] | pnp-β-GAL[4] | | |
| A | 13 | + | + | - | + | 11 | (85) |
| | | + | + | + | + | 2 | (15) |
| B | 26 | - | - | + | - | 24 | (92) |
| | | - | - | + | + | 1 | ( 4) |
| | | - | - | - | - | 1 | ( 4) |
| C | 21 | - | + | + | + | 12 | (57) |
| | | - | + | - | + | 8 | (38) |
| | | - | - | - | - | 1 | ( 5) |
| F | 20 | - | - | - | + | 18 | (90) |
| | | - | - | - | - | 2 | (10) |
| G | 22 | - | + | + | + | 21 | (95) |
| | | - | + | - | + | 1 | ( 5) |

1 L-pyrrolidonyl-beta-naphthylamide
2 p-nitrophenyl-N-acetyl-beta-D-glucosaminide
3 6-bromo-2-naphthyl-beta-D-glucuronide
4 p-nitrophenyl-beta-D-galactopyranoside

TABLE 2: Origin of 172 clinical isolates of beta-hemolytic streptococci from human sources.

| Sources/sites | Total isolates | Total S. milleri | Organisms: S. milleri | S. milleri | S. milleri | S. milleri | S. milleri | S. pyogenes | S. equisimilis | Large colony G | Unidentified |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | A[1] | C | F | G | - | A | C | G | - |
| | No. of isolates | | | | | | | | | | |
| All sources (total) | 172 | 91 | 3 | 27 | 41 | 5 | 15 | 20 | 21 | 37 | 3 |
| Blood | 13 | 3 | | 1 | 2 | | | 4 | 1 | 5 | |
| Peritoneal fluid | 6 | 5 | | 2 | 3 | | | | 1 | | |
| Synovial fluid | 3 | | | | | | | 1 | | 2 | |
| Pleural fluid | 1 | | | | | | | | | 1 | |
| Respiratory: | | | | | | | | | | | |
| mouth, throat, | 10 | 5 | 1 | 1 | 1 | 1 | 1 | | 2 | 1 | 2 |
| nose, sputum | 10 | 8 | | 3 | 2 | 2 | 1 | 1 | 1 | | |
| trachial, bronchial | 10 | 9 | 1 | 4 | 3 | | 1 | | | 1 | |
| Wound, lesion: | | | | | | | | | | | |
| limb | 15 | 5 | | | 5 | | | 3 | 2 | 5 | |
| abdomen | 2 | 2 | | 1 | 1 | | | | | | |
| oral | 5 | 5 | | 1 | 3 | | 1 | | | | |
| other, unspecified | 12 | 5 | | 2 | 1 | | 2 | 1 | 3 | 3 | |
| Abscesses: | | | | | | | | | | | |
| limb, head, neck | 23 | 12 | | 2 | 5 | 1 | 4 | 6 | 1 | 3 | 1 |
| oral, peritonsillar | 4 | 4 | | 1 | 3 | | | | | | |
| abdominal | 2 | 2 | | | 2 | | | | | | |
| perirectal | 6 | 4 | | 1 | 1 | | 2 | 1 | | 1 | |
| pilonidal cyst | 2 | 2 | | 1 | 1 | | | | | | |
| genital | 1 | | | | | | | | 1 | | |
| Barthalian cyst | 1 | | | | | | | | | 1 | |
| other, unspecified | 8 | 5 | | | 2 | | 3 | 2 | | 1 | |
| Genital tract | 5 | 3 | | 1 | 2 | | | | | 2 | |
| Urine | 2 | 1 | | | 1 | | | | 1 | | |
| Miscellaneous[2] | 3 | | | | | | | | | 3 | |
| Unspecified | 28 | 11 | 1 | 6 | 3 | 1 | | 1 | 8 | 8 | |

[1] Serological reaction using Streptex; - "non-groupable"
[2] Includes breast colostrum, gastric aspirate, and eye.

TABLE 3: Biochemical reactions of "traditional" beta-hemolytic streptococci and beta-hemolytic S. milleri in relation to Lancefield group antigen.

(% of strains tested)

| | Group A | | Group C | | Group F | Group G | | Non - groupable[1] | All B-hemolytic |
|---|---|---|---|---|---|---|---|---|---|
| | S. pyogenes | S. milleri | S equisimilis | S. milleri | S. milleri | large colony | S. milleri | S. milleri | S. milleri |
| | (20)[2] | (3) | (21) | (27) | (41) | (37) | (5) | (15) | (91) |
| Acid from | | | | | | | | | |
| Glucose | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Sucrose | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Salicin | 100 | 100 | 90 | 96 | 98 | 76 | 100 | 100 | 98 |
| Trehalose | 100 | 100 | 100 | 52 | 85 | 97 | 100 | 73 | 75 |
| Lactose | 100 | 0 | 75 | 93 | 20 | 70 | 100 | 40 | 48 |
| Raffinose | 0 | 0 | 0 | 0 | 0 | 0 | 40 | 0 | 2 |
| Melibiose | 0 | 0 | 0 | 0 | 0 | 0 | 40 | 0 | 2 |
| Mannitol | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sorbitol | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Inulin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ribose | 0 | 0 | 86 | 0 | 0 | 100 | 0 | 0 | 0 |
| Esculin hydrolysis | 60 | 33 | 10 | 67 | 17 | 38 | 80 | 33 | 38 |
| Voges Proskauer reaction | 0 | 100 | 0 | 100 | 100 | 0 | 100 | 100 | 100 |
| Bacitracin sensitivity | 100 | 0 | 33 | 56 | 0 | 27 | 0 | 0 | 16 |
| Hydrolysis of PYR[3] | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4MEU-NAG[4] | 100 | 0 | 100 | 89 | 10 | 100 | 100 | 0 | 36 |
| 4MEU-β-GUR[5] | 35 | 0 | 100 | 0 | 0 | 97 | 0 | 0 | 0 |
| 4MEU-β-GAL[6] | 100 | 100 | 100 | 100 | 100 | 100 | 80 | 100 | 99 |

1 No reaction with antisera against groups A,B,C,D,F and G.
2 Number of strains tested ( )
3 Pyroglutamyl-naphthylamide
4 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide
5 4-methylumbelliferyl-β-D-glucuronide
6 4-methylumbelliferyl-β-D-galactoside

TABLE 4. Reaction patterns among 192 strains of beta-hemolytic streptococci using five biochemical tests.

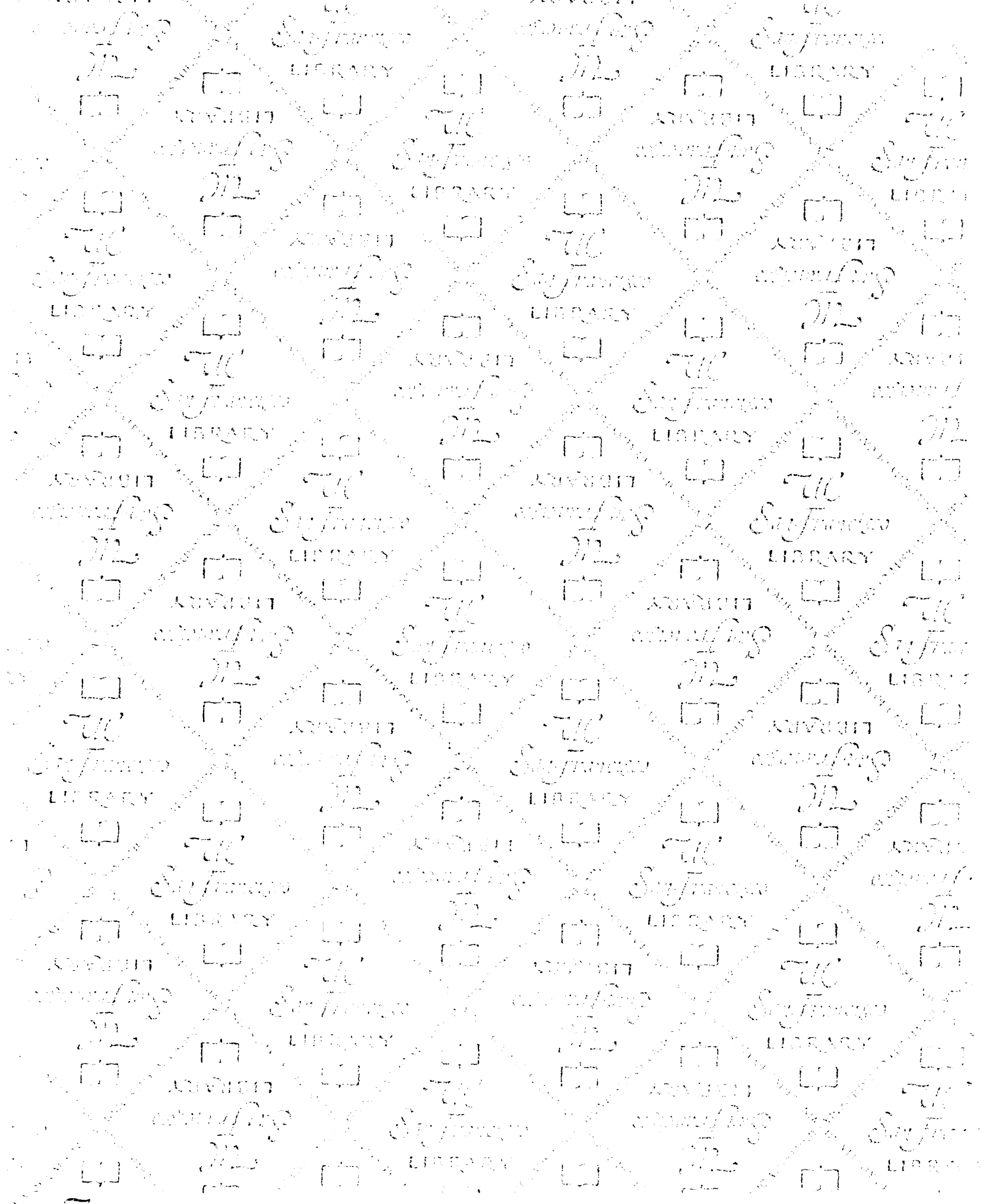
| Lancefield group | Species identification | No. of strains | Reaction Pattern PYR[1] | 4MEU-NAG[2] | 4MEU-β-GUR[3] | 4MEU-β-GAL[4] | VP | No. (%) of strains per pattern |
|---|---|---|---|---|---|---|---|---|
| A | S.pyogenes | 20 | + | + | - | + | - | 13 (65) |
| | | | + | + | + | + | - | 7 (35) |
| | S.milleri | 3 | - | - | - | + | + | 3 (100) |
| B | S.agalactiae | 15 | - | - | + | - | - | 11 (74) |
| | | | - | - | + | + | - | 2 (13) |
| | | | - | - | - | - | - | 2 (13) |
| C | S.equisimillis | 21 | - | + | + | + | - | 21 (100) |
| | S.milleri | 27 | - | + | - | + | + | 24 (89) |
| | | | - | - | - | + | + | 3 (11) |
| D | S.faecalis | 3 | + | + | - | + | + | 3 (100) |
| | S.faecium-durans | 2 | + | + | - | + | + | 2 (100) |
| F | S.milleri | 41 | - | - | - | + | + | 37 (90) |
| | | | - | + | - | + | + | 4 (10) |
| G | large colony group G | 37 | - | + | + | + | - | 36 (97) |
| | | | - | + | - | + | - | 1 (3) |
| | S.milleri | 5 | - | + | - | + | + | 4 (80) |
| | | | - | + | - | - | + | 1 (20) |
| "non-groupable" | S.milleri | 15 | - | - | - | + | + | 15 (100) |
| | unidentified | 3 | - | - | - | - | - | 2 (67) |
| | | | - | + | - | + | - | 1 (33) |

[1] Pyroglutamyl-naphthylamide
[2] 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide
[3] 4-methylumbelliferyl-β-D-glucuronide
[4] 4-methylumbelliferyl-β-D-galactoside
[5] Voges Proskauer reaction

TABLE 5. Differentiation of beta-hemolytic streptococci isolated from humans.

| | Lancefield Group | PYR[1] | VP[2] | GUR[3] | NAG[4] |
|---|---|---|---|---|---|
| S. pyogenes | A | + | - | +/- | + |
| S. agalactiae | B | - | - | +[6] | - |
| S. equisimilis or Large colony group G streptococci[5] | C or G | - | - | +[6] | + |
| S. milleri | A,C,F,G,- | - | + | - | +/- |
| B-hemolytic enterococci | D | + | + | - | + |

1 hydrolysis of pyroglutamic acid
2 Voges Proskauer reaction
3 hydrolysis of β-D-glucuronide
4 hydrolysis of N-acetyl-β-D-glucosaminide
5 Serological grouping is required to separate S. equisimilis from large colony group G streptococci.
6 Negative reactions occasionally occur; in which case identification should be confirmed by serological grouping.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