Recognition of Group D Streptococcal Species of Human Origin by Biochemical and Physiological Tests

RICHARD R. FACKLAM

Center for Disease Control, Atlanta, Georgia 30333

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The speciation of 262 strains of group D streptococci isolated from human sources is described. One hundred forty-two isolates from blood cultures were included; 96 of these were submitted as isolates from clinical cases of subacute bacterial endocarditis. The results show that 98 Streptococcus faecalis, 29 S. faecalis var. zymogenes, 44 S. faecalis var. liquefaciens, 27 S. faecium, 13 S. durans, 44 S. bovis, and 7 unspeciated S. bovis-like group D isolates were identified. No S. faecium var. casseliflavus, S. equinus, or S. avium (group Q streptococci) were identified among the human isolates. The speciation procedures and techniques are detailed. The procedures and limitations of the tests used are discussed. Ninety-eight percent of the 262 strains were speciated by a spectrum of tests that allowed us to recognize atypical as well as typical strains within species.

Shattuck (19) in 1962 and Deibel (2) in 1964 presented classification schemes for group D streptococci that included the two nonenterococcal group D streptococci, Streptococcus bovis and S. equinus. The inclusion of S. bovis and S. equinus into the group D classification is based on the demonstration of the group D antigen in extracts of cells of these two species (17, 20). Shattuck (19), Deibel (2), and Hartman et al. (9) have also included S. faecium in their classification schemes. The results of studies by Barnes (1), Deibel et al. (3), and Shattuck (17) clearly show that S. faecalis and S. faecium are distinct species. The terms "fecal streptococci," "enterococci," and "group D streptococci" are discussed by Hartman et al. (9). It is now evident that these terms are not synonymous. The term fecal streptococci has no definitive meaning and should not be used. Various investigators use the term to describe many different streptococcal species of fecal origin (9). The term enterococci may be defined as including S. faecalis and its varieties (zymogenes and liquefaciens), S. faecium, and S. durans. Group D streptococci may be defined as all those streptococci possessing the group D antigen. This includes all the enterococcal species plus S. bovis and S. equinus. It is probably advisable to retain the use of the term enterococcus because of the difference in antibiotic therapy for patients with enterococcal infections and those with nonenterococcal infections. Clinical studies which have demonstrated multiple antibiotic resistance have been done on enterococci and not on the entire group D streptococci. Even though the term has no real taxonomic meaning, it does have clinical significance.

A review of the literature on speciation of group D streptococci isolated from human sources will not be attempted but the reader is referred to early reviews by Thomson and Thomson (21), Dible (4), Graham and Bartley (8), and an excellent review by Evans and Chinn (6). Earlier classification schemes (before 1962) did not include the species S. faecium, S. bovis, or S. equinus. Recently, during a study of antibiotic resistance of human isolates, Toala et al. (22, 23) speciated the enterococci, and included S. faecium in their identification procedures. Although they did not include the two nonenterococcal group D species (S. bovis and S. equinus) in their identification scheme and although the number of differentiating tests was somewhat limited, their interpretations appear sound. Pleceas (16) advocated the use of a limited number of differential tests to recognize all the group D species. She included phage typing as a means of dif-
ferentiating between species and recommended that atypically reacting strains be recognized as separate entities. Duma et al. (5) did not feel that a limited number of tests would sufficiently speciate the group D streptococci, but they did feel that a few tests would adequately place them into the division set forth by Deibel (2). Isenberg et al. (10) attempted speciation of group D streptococci, but failed to incorporate sufficient tests into their scheme to differentiate the species accurately. Food and water microbiologists have been using the identification procedures summarized by Shattuck (19) and Deibel (2) for several years, and many publications have appeared showing the successful speciation of food, water, and fecal isolates.

It is the purpose of this investigation to demonstrate that speciation of group D streptococci isolated from clinical material can be accomplished if the recommendations of Deibel (2) are heeded. Deibel emphasized that, in all identification procedures, dependence must be placed on a spectrum of characteristics possessed by the strain in question, and its failure to comply in a few specific tests does not constitute sufficient grounds to negate speciation. Deibel also cautioned against the attempt to establish species on the basis of a few differing characteristics and emphasized the need to recognize transitional strains as types. Accurate speciation procedures are necessary to determine the species distribution of group D streptococci in human infection and to determine differences among species in regard to their susceptibility to various antibiotics.

**MATERIALS AND METHODS**

**Cultures.** A total of 262 strains of group D streptococci isolated from the clinical sources listed in Table 1 were tested. These strains were received at the Center for Disease Control (CDC) between July 1968 and September 1971 and constituted all the group D streptococcal isolates from human sources excluding feces. An additional 6 S. faecalis, 39 S. faecalis var. zymogenes, 5 S. faecalis var. liquefaciens, 9 S. faecium, 14 S. faecium var. casseliflavus, 12 S. durans, 16 S. bovis, 18 S. equinus, and 6 S. avium (group Q streptococci) stock strains were specified to determine test proficiency. The reactions of these stock strains are not included in the following tables. The majority of stock strains of the group D streptococci were obtained from R. L. Lancefield, S. D. Elliot, and J. O. Mundt.

**Preparation of media.** The procedures for serological grouping; Gram staining; determining hemolytic and catalase activities; and testing for reactions on bile-esculin medium (BEM), growth in 6.5% NaCl broth, acid reaction in Streptococcus faecalis broth (SF, Difco), and growth in 0.1% methylene blue milk (MBM) have been previously described (17).

All of the following media were sterilized by autoclaving for 15 min at 15 psi (121 C) unless otherwise stated. All pH measurements were conducted with a pH meter. Growth at 10 and 45 C was determined by inoculating 5 ml of Todd-Hewitt broth (THB, Difco); after incubation the tubes were examined for growth by rotating them in front of an incandescent lamp.

Heart infusion agar (HIA, Difco), 40 g per liter, was used as the base medium for preparation of 40% blood-bile, 0.1% tetrazolium, 0.04% tellurite, 2% starch, and 5% sucrose-agar plates. These media were poured into 15 by 75 mm plastic petri dishes (10 ml per dish).

Blood-bile-agar was prepared by adding 40 g of oxgall (Difco) per liter of HIA. A 50-ml amount of defibrinated rabbit blood was added to 1 liter of the sterilized cooled medium just before the plates were poured. Tolerance to 40% bile was determined by examining the plates for growth daily for 3 days.

The tetrazolium plates were prepared by adding 5 g of dextrose per liter of HIA. The mixture was adjusted to pH 6.0 by adding 1 N HCl. Ten milliliters of a 1% solution of 2, 3, 5-triphenyl tetrazolium chloride, sterilized by filtration, was added to the sterilized, cooled medium (50 C) just before the plates were poured. A positive reduction of the tetrazolium was recorded when examination of the plates revealed brick-red colonies at any time interval within 3 days.

The base tellurite medium was prepared by adding 1 N HCl to 1 liter of HIA to pH 6.0. Fifty milliliters of defibrinated rabbit blood was added to the sterilized medium at 90 C. The potassium tellurite solution (0.5 g of K-tellurite per 150 ml of distilled water sterilized by filtration) was added to the cooled medium (less than 50 C) just before the plates were poured. Tolerance to tellurite was recorded as positive when examination of the plates revealed black colonies after any time interval up to 3 days.

The starch agar was prepared by adding 20 g of soluble starch (Merck) per liter of HIA. The medium was sterilized and poured when cooled. Hydrolysis of starch was determined by flooding the surface of the plate with Gram's iodine 48 hr after inoculation and incubation. A zone of hydrolysis appeared colorless, and a dark blue to purple color indicated that the starch had not been hydrolyzed.

The sucrose plates were prepared by adding 50 g of sucrose per liter of HIA. The medium was sterilized and poured when cooled. Plates were examined daily for 3 days for large gummy colonies and colonial adherence to the media characteristic of extracellular polysaccharide production.

The medium for determining pH 9.6 tolerance was prepared by adding NaOH pellets to Heart Infusion Broth (HIB, Difco) until a pH of 9.6 was reached as indicated by a pH meter. The medium was dispensed in 3-ml amounts into screwcap tubes (13 by 100 mm) and sterilized. Tolerance was determined by observing the medium for growth daily for 3 days.
TABLE 1. Number and sources of group D streptococcal species tested

| Source          | Division I | Division II | Division III | Total |
|-----------------|------------|-------------|--------------|-------|
|                 | faecalis   | liquefaciens| zymogenes    |       |
| Blood (SBE)*    | 35         | 20          | 7            | 96    |
| Blood*          | 14         | 6           | 2            | 46    |
| Urine           | 22         | 5           | 11           | 45    |
| Wounds          | 2          | 4           | 2            | 17    |
| Vagina/cervix   | 2          | 5           | 2            | 12    |
| Urethra         | 7          | 0           | 0            | 8     |
| Teeth           | 4          | 0           | 0            | 6     |
| Joint fluid     | 1          | 1           | 0            | 3     |
| Sputum          | 1          | 0           | 0            | 3     |
| Pleural fluid   | 0          | 0           | 0            | 2     |
| Miscellaneous   | 6          | 2           | 3            | 16    |
| Not given       | 2          | 0           | 2            | 5     |
| Total           | 98         | 44          | 29           | 262   |

* Specimens submitted as isolates from patients with subacute bacterial endocarditis (SBE); only one isolate per patient is listed here.

* Includes bacteremias, septicemias, fever of unknown origin, and no diagnosis given.

Cerebral spinal fluid.

by rotating the tube in front of an incandescent lamp.

The medium for determining hydrolysis of gelatin was prepared by adding 120 g of gelatin to 1 liter of HIB (11). The medium was dispensed in 5-ml amounts into 15 by 125 mm screwcap tubes and sterilized. Hydrolysis of the gelatin was determined after incubation for 3 days by refrigerating (10°C) the growth tube and an uninoculated control tube. When the control tube had solidified, the tubes were removed and inverted. Tubes that remained unsolidified were recorded as positive, all others as negative. Litmus milk (Difco) was prepared and dispensed in 10-ml amounts into cotton-stoppered tubes (15 by 150 mm) and sterilized. The reactions of acid production and clotting of litmus milk were noted daily for 3 days.

Heart infusion broth (HIB) was used as the base medium for 1% broths of sucrose, raffinose, man- nitol, inulin, lactose, esculin, sorbitol, glycerol, and arabinose. One hundred milliliters of a 10% solution of each carbohydrate and 1 ml of indicator solution (1.6 g of brom cresol purple in 100 ml of 95% ethanol) were added to 900 ml of HIB. The medium was dispensed in 3-ml amounts into screwcap tubes (13 by 100 mm). The medium was sterilized by autoclaving for 10 min at 15 psi. A positive reaction was recorded when the indicator changed from purple to yellow, which indicated that acid had been produced. Recordings were made daily for 3 days.

The 5% sucrose broth was prepared by adding 28.5 g of dehydrated thio broth (Difco), 10.0 g of K2HPO4, and 12.0 g of sodium acetate to 500 ml of distilled water. This solution was autoclaved separately as was 50 g of sucrose in 500 ml of distilled water. After sterilization, the solutions were mixed and dispensed into screwcap tubes (16 by 125 mm). The medium was checked daily for 3 days for an increase in viscosity.

All media were inoculated with a Pasteur pipette that dispensed one to two drops of a 24-hr THB culture of the specimen being tested. Media were incubated at 35°C aerobically unless otherwise indicated.

RESULTS

Speciation by a spectrum of physiological tests. Table 2 shows the reactions used to specify the group D isolates. This table is compiled from various publications (1, 2, 3, 7, 9, 14, 15, 18, 19) and from our own results with 125 stock strains representing all species of group D and Q streptococci. All strains studied were gram-positive cocci and varied in chain length. All strains studied failed to release O2 from H2O2, with the exception of half of the S. faecium var. casseliflavus strains. Very weak reactions were observed with most of these strains, and at least two of them, upon several laboratory transfers, lost the ability to release O2 from H2O2. Thus this was not considered a stable characteristic of the species.

For a strain to fit the speciation scheme perfectly, all the reactions had to agree with those listed in Table 2. However, there are many variants within a species, and, to best place the organism into a particular species, a spectrum of reactions was used.
| Test                              | Division I (faecalis) | Division II  | Division III |
|----------------------------------|-----------------------|--------------|--------------|
|                                  | faecium | durans | casseli-flavus | avium | bovis | equinus |
| Group D reaction                 | +a      | +      | +              | +     | +     | +       |
| Bile-esculin                     | +       | +      | +              | +     | +     | +       |
| Growth on 40% bile               | +       | +      | +              | +     | +     | +       |
| Growth at 45 C                   | +       | +      | +              | +     | +     | +       |
| Acid in litmus milk              | +       | +      | +              | +     | +     | -       |
| Growth in 6.5% NaCl broth        | +       | +      | +              | +     | +     | -       |
| Growth in S. faecalis broth      | +       | +      | +              | +     | +     | -       |
| Growth in pH 9.6 broth           | +       | +      | +              | +     | +     | -       |
| Growth in methylene blue milk    | +       | +      | +              | -     | -     | V       |
| Growth at 10 C                   | +       | +      | +              | -     | -     | -       |
| Reduction of tetrathionate       | +       | -      | -              | V     | -     | V       |
| Resistance to tellurite           | +       | -      | -              | V     | -     | -       |
| Clostr in litmus milk            | +       | -      | +              | V     | -     | +       |
| Hydrolysis of gelatin            | V       | -      | -              | -     | -     | -       |
| Hydrolysis of starch             | -       | -      | -              | -     | -     | +       |
| Beta-hemolysis                   | V       | -      | V              | -     | -     | -       |
| Acid from:                       |          |        |                |       |       |         |
| Esculin                          | +       | +      | +              | +     | +     | +       |
| Lactose                          | +       | +      | +              | +     | +     | +       |
| Sorbitol                         | +       | -      | -              | +     | -     | -       |
| Sucrose                          | +       | +      | V              | +     | +     | +       |
| Glycerol                         | +       | -      | -              | -     | -     | -       |
| Mannitol                         | +       | +      | -              | +     | V     | -       |
| Arabinose                        | -       | +      | +              | +     | V     | -       |
| Raffinose                        | -       | V      | V              | V     | +     | V       |
| Inulin                           | -       | -      | V              | -     | V     | -       |
| Production of extracellular polysaccharide on: |          |        |                |       |       |         |
| 5% Sucrose agar                  | -       | -      | -              | -     | -     | V       |
| 5% Sucrose broth                 | -       | -      | -              | -     | -     | V       |

* Symbols: +, positive reaction; -, negative reaction; V, reaction is variable; some strains are positive while others are negative.

*S. faecalis* (division I) and its varieties, *zymogenes* and *liquefaciens*, are differentiated by hemolytic activity and gelatinase production. All other characteristics of these taxons are similar in our system. There is very little difference between these three taxons, and it is doubtful that they need to be treated as separate entities. Other investigators have noted that the hemolytic action of *S. faecalis* var. *zymogenes* on blood-agar depends on the kind of blood used in the pour plates (6, 12, 24). We have observed this same phenomenon with some of our isolates. By definition, *S. faecalis* is not beta-hemolytic and does not liquefy gelatin; *S. faecalis* var. *liquefaciens* is not beta-hemolytic but liquefies gelatin; and *S. faecalis* var. *zymogenes* is beta-hemolytic and may or may not liquefy gelatin.

We arbitrarily placed *S. faecium* var. *casseli-flavus* and *S. avium* in division II because of certain similarities to *S. faecium*. The variety *casseli-flavus* is a relatively poorly definable species in our system. The species shares characteristics of both division I and II. The positive reactions on tellurite and tetrathionate media indicate that the organisms belong to division I, but the negative reaction in sorbitol and positive reaction in arabinose broths are characteristics of *S. faecium* (division II). The *casseli-flavus* strains produced a characteristic yellow-pigmented, mucoidal colony on 5% sucrose agar, whereas all other members of division I and II were white (nonpigmented) and mucoidal on the same media. We failed to identify any strains from our collection which fit this particular spectrum of reactions.

We also failed to identify strains resembling *S. avium* in our collection from human sources.
although they are easily recognized in our system. All of our division II species grew in MBM and all but three grew at 10°C (Table 3). None of our stock strains of S. auium was able to grow in MBM or at 10°C.

The failure to initiate growth in litmus milk and to form acid in lactose broth are characteristics unique to S. equinus in the group D streptococci. We feel that these characteristics are distinctive and make the species easily recognizable. We failed to recognize any strains resembling S. equinus in our collection from human sources.

Recognition of streptococci as members of group D. Table 3 shows the percentages of positive reactions of the various species of group D streptococci in our collection. Extracts of four S. durans, two S. bovis, and one S. faecium failed to react with CDC group D antisera. All of these strains showed typical reaction patterns of their respective species described in Table 2. One strain failed to blacken BEM. This strain failed to form acid in any carbohydrate (CH) broth tested, including esculin, but was still classified as a S. faecalis var. liquefaciens by the remaining tests. Growth on 40% bile, growth at 45°C, and acid from esculin are not properties unique to group D streptococci but are characteristics shared by the majority of strains of all group D streptococcal species.

The results of the MBM-tolerance test support our previous contention (7) that MBM should not be used as a differential test for enterococci or group D streptococci. Although nearly all of the enterococcal strains (divisions I and II) reduced MBM, 60% of the nonenterococcal strains (division III) were able to reduce MBM. This variability of division III strains to reduce MBM limits the usefulness of the test to differentiate accurately either enterococci or group D streptococci from other streptococci. It does serve as a useful test in recognizing S. auium strains as previously discussed.

| Test                                      | Positive reactions (%) of streptococci from |
|-------------------------------------------|-------------------------------------------|
|                                           | Division I | Division II | Division III |
|                                           | faecalis  | durans      | bovis        | variant     |
| Group D reaction                          | 100        | 96          | 69           | 96          | 100         |
| Bile-esculin medium                       | 99         | 100         | 100          | 100         | 100         |
| Growth on 40% bile                        | 100        | 100         | 100          | 100         | 100         |
| Growth at 45 C                            | 99         | 100         | 100          | 93          | 100         |
| Acid in litmus milk                       | 100        | 100         | 100          | 100         | 100         |
| Growth in 6.5% NaCl broth                 | 100        | 100         | 100          | 2           | 0           |
| Growth in S. faecalis broth               | 98         | 96          | 100          | 2           | 0           |
| Growth in pH 9.6 broth                    | 98         | 86          | 100          | 39          | 67          |
| Growth in methylene blue milk             | 98         | 100         | 100          | 61          | 43          |
| Growth at 10 C                            | 95         | 89          | 100          | 2           | 0           |
| Reduction of tetrazolium                  | 99         | 0           | 0            | 30          | 14          |
| Resistant to tellurite                    | 90         | 0           | 0            | 0           | 0           |
| Clot in litmus milk                       | 89         | 33          | 62           | 100         | 100         |
| Hydrolysis of gelatin                     | 28         | 0           | 0            | 0           | 0           |
| Hydrolysis of starch                      | 0          | 0           | 0            | 100         | 0           |
| Beta-hemolysis                            | 17         | 0           | 0            | 0           | 0           |
| Acid from:                                |            |             |              |             |             |
| Esculin                                   | 99         | 100         | 100          | 100         | 100         |
| Lactose                                  | 95         | 100         | 100          | 100         | 100         |
| Sorbitol                                 | 97         | 7           | 0            | 2           | 0           |
| Sucrose                                  | 97         | 89          | 77           | 100         | 100         |
| Glycerol                                 | 78         | 4           | 0            | 0           | 0           |
| Mannitol                                 | 98         | 100         | 0            | 91           | 0           |
| Arabinose                                | 4          | 96          | 0            | 2           | 0           |
| Raffinose                                | 6          | 67          | 39           | 98          | 29          |
| Inulin                                   | 4          | 11          | 0            | 50          | 0           |
| Production of extracellular polysaccharide|            |             |              |             |             |
| on MBM                                    |            |             |              |             |             |
| 5% Sucrose agar                          | 0          | 0           | 0            | 84          | 0           |
| 5% Sucrose broth                         | 0          | 0           | 0            | 57          | 0           |
| No. of specimens tested                  | 171        | 27          | 13           | 44          | 7           |
Placement of strains into divisions and species. The ability of enterococci (divisions I and II) to grow in SF broth and in 6.5% NaCl broth and to initiate growth at 10 C differentiates enterococci from nonenterococcal (division III) group D streptococci. Table 3 shows that very few strains of division III give positive reactions in these three tests. Table 3 also shows that pH 9.6 broth did not clearly differentiate between enterococcal and nonenterococcal group D species; however, the pH of this medium was adjusted before rather than after autoclaving, and this may have affected the results. We did not rely on this test to establish placement of the organisms into division categories.

The reduction of tetrazolium, resistance to tellurite, and acid production in sorbitol and glycerol broths are characteristics shared by division I organisms. Division II species (faecium and durans) ordinarily fail to give positive reactions on these tests, but exceptions do occur (Table 3). The same exception occurs with acid from arabinose by S. faecium: S. faecium routinely forms acid in arabinose, whereas most other group D species do not. Table 3 shows that only 78% of division I specimens formed acid in glycerol broth within 3 days. This was not totally unexpected since the test was designed as an anaerobic test and we ran all our tests aerobically. We did not rely on acid from glycerol to place the organisms into division I.

Acid production from mannitol, arabinose, and sucrose and failure to clot litmus milk are tests generally considered to differentiate S. faecium from S. durans. Acid from mannitol and arabinose clearly demonstrated this capacity, but 10 of 13 strains that were mannitol- and arabinose-negative were sucrose-positive. By our system, these strains were closer to S. durans than S. faecium. We do not feel that they should be recognized as distinct entities but rather that they should be considered as a biotype within S. durans. Clot formation in litmus milk was not used to differentiate between S. faecium and other group D species. The test was unreliable because many strains of faecium clot litmus milk and some strains of faecalis and durans do not (Table 3).

The division III organisms are recognized by the failure to grow in SF broth at 10 C and in 6.5% NaCl broth. Table 3 shows that the hydrolysis of starch and slime formation in 5% sucrose broth and agar are unique characteristics of S. bovis among the group D isolates. We recognized a variant in division III organisms that did not hydrolyze starch, form slime in 5% sucrose broth or agar, or form acid in mannitol as did nearly all the S. bovis strains. These seven variant strains were all very similar to one another and were easily distinguished from typical S. bovis. We feel that they may be a separate species or a biotype of S. bovis. These isolates are unlike S. equinus which fails to grow in litmus milk or form acid in lactose broth. All seven strains were positive in both tests.

Table 4 shows the distribution of the 262 strains according to the speciation scheme in Table 2 and the number of exceptional reactions that occurred. The reactions listed as variable were not counted for this data. The formation of a clot in litmus milk, the formation of acid from glycerol, and growth in pH 9.6 broth were considered unreliable tests for speciation under our conditions and were not used for speciation or counted in the exceptional reactions. Table 4 shows that nearly 73% of the specimens fit the spectrum of reactions for the species perfectly, whereas 20% had one exceptional reaction. Therefore, of the specimens tested, almost 93% had one or no exceptional reactions according to our spectrum of reactions in Table 2. Fourteen specimens had two exceptional reactions, one had three, three had five, and one had six. The correct identity of the specimen with six exceptional reactions may be questioned, but we believe that the other 261 strains have been correctly identified. The three strains with five exceptional reactions were similar to one another. Most of the exceptional reactions of these three strains were the result of the failure to form acid in CH broths.

DISCUSSION

Significance of the isolates. Clinical information on some of these isolates is not available, but a significant portion of the clinical

| Table 4. Number of group D streptococcal species yielding exceptional reactions |
|---------------------------------|----------------|----------------|------------------|----------------|
| No. of exceptional reactions | Div. I (faecalis) | Div. II (faecium) | Div. III (durans) | Total | Per cent of total |
|-----------------------------|-----------------|-----------------|-----------------|-------|-----------------|
|                            | faecium | durans | bovis | varient |                |               |
| 0                           | 117     | 20     | 8     | 39     | 7             | 191           | 72.7           |
| 1                           | 31      | 5      | 5     | 0      | 52            | 20.0          |
| 2                           | 12      | 2      | 0     | 0      | 14            | 5.4           |
| 3                           | 1       | 0      | 0     | 0      | 1             | 0.4           |
| 5                           | 3       | 0      | 0     | 0      | 3             | 1.1           |
| 6                           | 1       | 0      | 0     | 0      | 1             | 0.4           |
| Total                       | 165     | 27     | 13    | 44     | 7             | 262           | 100.0          |
information was provided with many of the isolates. Although these specimens are not a random sample of isolates from the United States, we believe that they represent a distribution of species of group D streptococci that is found in human infections. This supposition is supported by the fact that 54% of the specimens were blood isolates; 39% of these were from subacute bacterial endocarditis.

It is also significant that in our series of subacute bacterial endocarditis (SBE) isolates from the Mayo Clinic (J. Washington), 9 of 48 (19%) group D isolates were S. bovis or S. bovis variants. In our series from the Cleveland VA Hospital (P. I. Lerner), 6 of 25 (24%) group D isolates were S. bovis. This high percentage of nonenterococcal group D isolates demonstrates the necessity of correct identification of enterococci for the best management of these infections.

**Recognition of group D and enterococcal streptococci.** Group D streptococci are defined as those streptococci possessing the group D antigen. Other investigators (17, 20) have demonstrated that S. bovis and S. equinus possess the group D antigen and recommended that these two species be included in the group D classification. We have previously shown that the BEM reaction correlates with the group D serological reaction (7). Identification of group D streptococci can be presumptively made by the BEM reaction and definitively made by demonstrating a serological group D reaction.

The inclusion of S. bovis and S. equinus into the group D classification (2, 9, 19) and the occurrence of S. bovis strains in human infections (Table 1) indicates that the clinical laboratories cannot use the serological or BEM reactions to establish enterococcal identification. Twenty percent of the group D isolates in our collection were S. bovis, all were BEM positive, and 96% reacted with group D antisera. A significant number of misidentifications would result if either the BEM or group D reactions were used as indicators of enterococci. Only limited evidence is available but apparently S. bovis strains do not resemble enterococci in their susceptibility to antibiotics (25). S. bovis infections probably do not require the intensive treatment required by enterococcal infections.

Mundt and Graham (14) described a new streptococcal species, *S. faecium* var. *caseiisflavus*. We were able to demonstrate a satisfactory group D precipitin reaction with Lancefield extracts of all 14 of their strains. Although Isenberg et al. (10) felt that several of their isolates resembled *S. faecium* var. *caseiisflavus*, they failed to perform a sufficient number of tests to identify accurately any of their group D isolates. Although *S. faecium* var. *caseiisflavus* physiologically resembles *S. faecalis* in some respects and *S. faecium* in others, we feel that the species can be recognized by the set of physiological characteristics listed in Table 2: pigment production and release of O₃ from H₂O₂ by some of the strains. This spectrum of characteristics was not observed among any of the human isolates reported here.

Nowlan and Deibel (15) described the physiological and serological characteristics of *S. avium* (group Q streptococci). We were able to demonstrate weak group D reactions in Lancefield extracts with five of six strains of *S. avium* (7). This serological reaction was very weak and required more than the usual 30-min time limit to develop. Lancefield extracts of *S. equinus* stock strains reacted in the same manner. We would not have accepted these reactions as bona fide group D reactions in extracts of diagnostic strains of streptococcal isolates. Increased group D precipitin reactions (both tube and gel diffusion) were reported by Nowlan and Deibel (15), who used a method of concentrating Lancefield extracts of *S. avium*. The physiological characteristics of *S. avium* are very similar to those of *S. faecium*. However, failure of *S. avium* to tolerate MMB and the formation of acid in sorbitol broth differentiates the species from *S. faecium*. All of our strains of *S. faecium* grew in MMB and only two fermented sorbitol. The three strains of *S. faecalis* that failed to grow in MMB did not resemble *S. avium*. These three *S. faecalis* isolates tolerated tellurite. *S. avium* does not tolerate tellurite.

Recognizing *S. equinus* is not difficult. The species has two very distinctive physiological characteristics—failure to grow in litmus milk and failure to form acid in lactose broth. Like *S. faecium* var. *caseiisflavus* and *S. avium*, strains resembling *S. equinus* were not encountered among our 262 human group D isolates.

Thus, to differentiate between enterococcal and nonenterococcal streptococci, laboratory personnel need to perform additional tests after they have established presumptive or confirmatory identification of a streptococcal isolate as a group D streptococcus. The results show that growth at 10°C in 6.5% NaCl broth, or production of acid in SF broth will provide an acceptable differentiation between members of division I and II (enterococci) and those of division III (nonenterococci).
Differentiation of species. Other characteristics can be used further to differentiate the enterococcal specimens (divisions I and II) from each other. Tolerance to tellurite, the ability to reduce tetrazolium and form acid from sorbitol and glycerol, and the inability to form acid from arabinose indicate that the organism is *S. faecalis* or one of its varieties; whereas, the opposite reaction on all five tests indicates that the organism does not belong to division I. A clinical laboratory would probably never need to determine the varieties of *S. faecalis*. The differentiation of these two varieties (lance fasciens and zymogenes) is on very questionable grounds. Updyke (24) showed that 88% of 90 enterococcal isolates were alpha-hemolytic in sheep blood and beta-hemolytic in rabbit, horse, and human blood-agar pour plates. Deibel (2) has pointed out that *S. faecalis* strains show frequent loss of hemolytic activity and vary in their proteolytic activity (depending on the type of medium used to test for hydrolysis of gelatin). He thus suggested that the designation of varieties of *S. faecalis* be discontinued. We are in full agreement with Deibel in this respect.

Division II species can be differentiated from species in division I (*faecalis*) and division III (bouis and equinus) by a number of tests. The formation of acid in arabinose, sucrose, and mannitol broths is used to differentiate *S. faecium* from *S. durans*. *S. faecium* should form acid in all three carbohydrates, but *S. durans* should not. However, 10 of 13 strains of *S. durans* formed acid in sucrose broth; thus, it should not be used as a differential characteristic for these organisms. These data also support the contention that *S. durans* should be considered a variety of *S. faecium*.

Like Duma et al. (5) and Toala et al. (22, 23), we encountered very few *S. durans* isolated from human sources. No *S. faecium* or *S. durans* species were identified in the collection of 73 group D SBE isolates received from the Mayo Clinic (Rochester, Minn.) and the Cleveland, Ohio, V.A. Hospital.

The failure of *S. equinus* to form acid in lactose broth or to grow in litmus milk serves sufficiently to differentiate the species from *S. bouis*. The variant species of division III described here does not resemble *S. equinus* and does not have all the characteristics of *S. bouis*. We are hesitant to designate this biotype of group D as a new species because of the limited number of strains reported here and the limitations of our spectrum of tests. Additional isolates and characteristics must be studied before conclusions can be made as to the best classification of this organism.

These data indicate that the spectrum of tests used here will differentiate about 98% of the group D streptococci isolated from human sources. Emphasis must be placed not on a limited number of tests but on a larger spectrum of tests to recognize varieties within the species.

The clinical laboratories probably do not need to worry about species like *S. faecium* var. *casseliflavus*, *S. acidi*, or *S. equinus*; however, they should be aware of the fact that these entities do exist and must be dealt with if correct speciation is attempted. We would like to caution the workers in clinical laboratories against relying on any single test that might be advocated for identifying enterococci. Investigators studying any aspect of species differences should perform at least the number of tests reported here to be of differential value.

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