Identification of *Streptococcus bovis* and *Streptococcus salivarius* in Clinical Laboratories

KATHRYN L. RUOFF,[1,2]* MARY JANE FERRARO,[1,3] JUDITH HOLDEN,[1] AND LAWRENCE J. KUNZ[1,2]

Francis Blake Bacteriology Laboratories, Massachusetts General Hospital, Boston, Massachusetts 02114,[1] and Departments of Microbiology and Molecular Genetics[2] and Medicine,[3] Harvard Medical School, Boston, Massachusetts 02115

Streptococci identified as *Streptococcus bovis*, *S. bovis* variant, and *Streptococcus salivarius* were examined with respect to physiological and serological characteristics and cellular fatty acid content. Similarities in physiological reactions and problems encountered in serological analysis were noted, suggesting that an expanded battery of physiological tests is needed to definitively identify these streptococci. Cellular fatty acid analysis provided an accurate method for distinguishing *S. salivarius* from *S. bovis* and *S. bovis* variant.

The impact of Lancefield's serological studies of the streptococci has fostered reliance on antigenic characteristics as a major criterion of streptococcal taxonomy. The establishment of *Streptococcus bovis* as a member of Lancefield's group D (22) led to the taxonomic separation of this species from other nonhemolytic and alpha-reacting streptococci of the viridans group which are devoid of the group D antigen. If serological reactions are disregarded, it becomes apparent that streptococci identified as *S. bovis* are similar in terms of physiological characteristics and antibiotic susceptibilities to certain viridans streptococcal species (5, 8, 23, 26). Although similarities between *S. bovis* and *Streptococcus salivarius* (23), *S. bovis* and *Streptococcus mutans* (8), and *S. bovis* variant and *Streptococcus* MG-*intermedius* (5) have been noted, our experience indicates that among routine clinical isolates, *S. bovis* is most often confused with *S. salivarius* when a small number of physiological tests are employed for presumptive identification.

Since *S. bovis* is an agent of endocarditis (9, 16, 19, 28) and its presence in the bloodstream is correlated with underlying gastrointestinal disease (11, 12, 18, 20), its accurate identification in clinical laboratories is imperative. We therefore examined a collection of *S. bovis*, *S. bovis* variant, and *S. salivarius* organisms with respect to their physiological and serological characteristics and cellular fatty acid profiles, hoping to resolve some of the confusion surrounding the identification of these streptococci.

MATERIALS AND METHODS

Bacteria. Cultures were identified as *S. bovis*, *S. bovis* variant, or *S. salivarius* on the basis of physiological and serological reactions. Of the 20 strains identified as *S. salivarius*, 14 were clinical isolates obtained from the frozen culture collection of the Massachusetts General Hospital Bacteriology Laboratory. The culture collection also yielded *S. salivarius* SS262 and *S. mutans* SS909 and SS980, strains originally obtained from the Centers for Disease Control, Atlanta, Ga. *S. salivarius* TH1, TH2, 4, and 9GS2, originally supplied by R. Gibbons, Forsythe Dental Infirmary, Boston, Mass., were also obtained from the frozen collection, as was ATCC strain 9759. Clinical isolates from the frozen collection accounted for 10 of the 20 *S. bovis* and 5 of the 6 *S. bovis* variant cultures examined. Other *S. bovis* strains (ATCC

9809 and ATCC 15351) were obtained from the American Type Culture Collection, Rockville, Md., and from R. Facklam, Centers for Disease Control (SS964). Seven *S. bovis* strains (78-89186, 78-89187, and 78-89190 through 78-89194) and one *S. bovis* variant strain (78-89188), originally obtained from the Centers for Disease Control, were supplied by C. Wennersten, New England Deaconess Hospital, Boston, Mass. CDC strains of *Streptococcus faecium* (SS961), *Streptococcus durans* (SS497), and *Streptococcus avium* (SS861) were obtained from our frozen collection, as was Lancefield's *Streptococcus faecalis* D76. Suspensions of cells that had been stored frozen in horse blood at −70°C were thawed and streaked onto brucella agar plates containing 5% horse blood (GIBCO Diagnostics, Madison, Wis.). All organisms were propagated on horse blood agar plates incubated at 35°C in the presence of 3 to 5% CO_2.

Physiological tests. Fermentation of carbohydrates (sucrose, lactose, mannitol, sorbitol, arabinose, sorbose, inulin, and raffinose) and hydrolysis of starch were determined with media described by Facklam (3). The method of Facklam and co-workers (7) was used for the determination of hippurate hydrolysis. Fermentation of pyruvate was assayed with the medium of Gross et al. (8), and esculin hydrolysis was determined by the method of Vera and Power (27). Hydrolysis of esculin in the presence of 40% bile and growth in Todd-Hewitt broth containing 6.5% NaCl were determined on media obtained from GIBCO Diagnostics. Moeller semisolid decarboxylase medium containing arginine (Micro-Media Systems, Quincy, Mass.) was used to assay arginine hydrolysis. Urease activity was determined with the medium of Stuart et al. (25). Mitis-salivarius agar (GIBCO Diagnostics) was employed for testing the ability of the streptococci to produce polysaccharides from sucrose after incubation for 48 h at 35°C in the presence of 3 to 5% CO_2. Unless otherwise noted, media and other ingredients were purchased from Difco Laboratories, Detroit, Mich., or Sigma Chemical Co., St. Louis, Mo.

Serological tests. Streptococci were grown overnight in 40 ml of Todd-Hewitt broth containing 1.0% glucose. After the supernatant fluid was centrifuged and decanted, the cell pellet was extracted with the hot hydrochloric acid method of Lancefield as described by Facklam (5). Extracts were tested with group D antiserum obtained from Wellcome Research Laboratories, Beckenham, England, using the capillary precipitin test (5). Extracts yielding negative precipitin reactions were subjected to concentration by alcohol

* Corresponding author.

TABLE 1. Distinguishing physiological characteristics of
S. bovis, S. bovis variant, and S. salivarius[a]

Property	% Positive in each test		
	S. bovis (n = 20)	S. bovis variant (n = 6)	S. salivarius (n = 20)
Bile esculin	100	100	20
Mannitol fermentation	95	0	0
Lactose fermentation	100	100	95
Raffinose fermentation	100	100	80
Inulin fermentation	70	0	90
Starch hydrolysis	100	0	0
Urea hydrolysis	0	0	70
Polysaccharide produced from sucrose	100	0	90

[a] All organisms hydrolyzed esculin and fermented sucrose. None of the streptococci grew in Todd-Hewitt broth containing 6.5% NaCl, hydrolyzed arginine or hippurate, or fermented pyruvate, sorbitol, arabinose, or sorbose.

precipitation (22). The redissolved precipitates were then retested with the capillary precipitin technique. Extracts positive in the precipitin test were subjected to double-diffusion testing with the modified Ouchterlony agar described by Rosan (21), except that 1.2% agarose (FMC Corp., Rockland, Maine) was substituted for agar. Stock strains of enterococci were used as controls in the serological tests.

Cellular fatty acid analysis. Cells used for fatty acid analysis were grown overnight at 35°C in the presence of 3 to 5% CO_2 in 40 ml of Todd-Hewitt broth containing 3.0% glucose (13). The methods of Moss and co-workers (17) were used for extraction and methylation of fatty acids. Samples containing 2 μl were analyzed with a Hewlett-Packard model 5880A gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Avondale, Pa.). Samples were analyzed on a coiled glass column (length, 6 ft [ca. 1.8 m]; outside diameter, ¼ in. [ca. 0.64 cm]; inside diameter, 2 mm) packed with 3% OV-101 coated on 80-100 mesh Chromosorb W-HP (Hewlett-Packard). The injection temperature was 200°C, and the detector temperature was 250°C. After sample injection, the column was temperature programmed from 155 to 260°C at 6°C/min. The carrier gas was nitrogen at a flow rate of 40 ml/min. Fatty acid methyl esters were identified by comparing the retention times of their peaks with those of known standards obtained from Supelco, Inc., Bellafonte, Pa., and Alltech Associates, Deerfield, Ill.

RESULTS

Physiological characteristics of the streptococci. The physiological properties of the 46 cultures examined are summarized in Table 1. All three groups of organisms gave identical

reactions in 9 of the 17 tests employed. Mannitol and inulin fermentation as well as starch hydrolysis and polysaccharide production were useful tests for distinguishing between S. bovis and S. bovis variant. Esculin hydrolysis in the presence of 40% bile and urease production were traits that helped to distinguish S. salivarius from the group D streptococci. The polysaccharide-producing S. salivarius strains formed dome-shaped colonies on mitis-salivarius agar, whereas S. bovis formed mucoid colonies on this medium.

Serological reactions. Hot HCl extracts of all organisms were examined with the capillary precipitin technique, with positive reactions being indicated by the formation of strong precipitin bands within 15 min. All S. bovis extracts produced positive reactions with group D antiserum and formed reactions of identity with extracts of group D stock strains in gel diffusion tests. Three of the six S. bovis variant HCl extracts gave positive precipitin reactions with group D antiserum. The three nonreactive extracts were subjected to alcohol precipitation, and the resulting precipitate was dissolved in a small amount of saline to form the "fraction A" described by Shattock (22) as being rich in the group D antigen. Alcohol-precipitable material from the three extracts produced positive precipitin reactions. Gel difusion tests on either HCl extracts or fraction A of all S. bovis variant cultures revealed reactions of identity with HCl extracts of group D control strains. Of the 20 S. salivarius cultures examined, only 1 yielded an acid extract which produced a precipitin reaction with group D antiserum interpreted as being positive. Alcohol-precipitable material from five S. salivarius extracts produced positive reactions with group D antiserum in the capillary precipitin test. Gel diffusion testing showed that all of these positive precipitin reactions were due to cross-reacting antigens which were not identical to the antigen of group D control strains.

Fatty acid composition of the streptococci. Table 2 summarizes the relative amounts of the major fatty acids common to the S. bovis, S. bovis variant, and S. salivarius strains examined. The most striking difference between the S. bovis and S. salivarius patterns was the relatively high content of eicosenoic acid (20:1) in S. salivarius. Although this fatty acid was detected in all streptococci identified as S. bovis or S. bovis variant, it was present in significantly lower amounts. In addition to the fatty acids in Table 2, small amounts of eicosanoic acid were found in most of the streptococci examined. S. bovis and S. bovis variant cultures also contained small amounts of heptadecanoic acid (17:0), 14-methylhexadecanoic acid (a 17:0), and one or two unidentified peaks with retention times in between those of tetradecanoic acid (14:0) and hexadecenoic acid (16:1). Our results with S. salivarius and 2 S. mutans stock strains (data not shown) agree with those of Lambert and Moss (13), who found eicosenoic acid to be a distinctive feature of the fatty

TABLE 2. Fatty acids common to S. bovis, S. bovis variant, and S. salivarius

Fatty acid	% of total extractable fatty acids[a]					
	S. bovis (n = 20)		S. bovis variant (n = 6)		S. salivarius (n = 20)	
	Mean	Range	Mean	Range	Mean	Range
14:0	4.8	1.9–6.7	8.5	7.4–12.5	2.7	1.3–4.5
16:1	7.8	3.5–10.7	9.5	8.0–10.5	4.2	2.4–5.5
16:0	27.9	23.0–34.7	34.2	30.1–37.8	32.0	25.6–37.2
18:1	30.3	18.5–36.1	18.3	12.5–21.6	28.6	22.2–35.1
18:0	14.8	11.4–18.5	15.7	8.7–21.7	14.4	8.6–18.1
20:1	3.4	1.2–5.5	2.1	1.5–2.9	16.1	11.8–21.1

[a] Extracts of each organism were analyzed in duplicate, and average values for the duplicate determinations were used to calculate the data.

acid profiles of 18 *S. mutans* strains and 5 strains of *S. salivarius*.

We also examined other group D streptococci for fatty acid content. These consisted of *S. faecalis* D76, *S. faecium* SS961, *S. durans* SS497, and *S. avium* SS861. The fatty acid profiles of these organisms were characterized by significant amounts of the 19-carbon cyclopropane fatty acid methyl-*dl-cis*-9,10-methyleneoctadecanoic acid (mean, 20%; range, 13 to 31%). The patterns of these salt-tolerant group D streptococci were similar to those described in the study by Amstein and Hartman (1) on fatty acid profiles of enterococci.

DISCUSSION

The characteristics of the *S. bovis* and *S. salivarius* cultures examined illustrate the difficulty of identifying these streptococci on the basis of presumptive physiological tests alone or in combination with serological tests. These organisms can display similar nonhemolytic colony morphology, and 3 of the 20 streptococci identified as *S. salivarius* produced presumptive physiological reactions (bile esculin positive, growth in the presence of 40% bile, no growth in the presence of 6.5% NaCl, arginine hydrolysis negative, raffinose fermentation positive, pyruvate and sorbose fermentation negative) typical of *S. bovis* by the identification scheme of Gross et al. (8). Gross and co-workers noted that presumptive reactions alone may not be sufficient for the accurate identification of *S. bovis* and that occasionally further testing, including serological examination, may be warranted. Although presumptive physiological tests readily separate *S. bovis* from typical (bile esculin-negative) viridans streptococci, atypical (bile esculin-positive or weakly bile esculin-positive) *S. salivarius* isolates could easily be misidentified as *S. bovis*. Facklam (4) noted that 1% of 81 *S. salivarius* isolates examined by him were bile esculin positive. We noted a higher percentage of bile esculin-positive *S. salivarius* isolates, which may reflect true differences in the organisms examined or only a difference in sample size.

The use of serological criteria for *S. bovis* identification is also problematic. Medrek and Barnes (15) found that *S. bovis* must be grown in the presence of elevated glucose concentrations to ensure detection of the group D antigen. Although we grew our streptococci in the recommended glucose concentration (1.0%), it was necessary to concentrate the group D antigen by alcohol precipitation to detect it in some of the *S. bovis* variant isolates. Facklam (5) noted that not all *S. bovis* isolates produce enough group D antigen to be detected by microprecipitin testing. The deficiencies of latex agglutination and coagglutination techniques for demonstrating the group D antigen in *S. bovis* as well as the cross-reactivity of viridans streptococci tested by these techniques have also been previously reported (2, 6, 14). Data from the study reported here indicate that cross-reacting antigens were released after acid extraction of a number of the *S. salivarius* cultures tested. Kaplan and co-workers (10) have also found that sera from patients with viridans streptococcal endocarditis contain an antibody which cross-reacts with an *S. bovis* antigen released by the acid extraction technique of Lancefield. In addition, we have observed that extracts formed by treatment of *S. salivarius* cells with a mixture of lysozyme and *Streptomyces albus* filtrate (29) show a very high frequency of cross-reaction with group D antiserum, suggesting that the cross-reactive antigens are more effectively released, or less effectively destroyed, by enzymatic extraction. Among 20 consecutive isolates of *S. salivarius* gathered in our laboratory over a 20-month period, enzymatic extracts of 5 isolates produced positive microprecipitin reactions with group D antiserum. Four of these five isolates were scored as bile esculin positive, salt tolerance negative, and arginine hydrolysis negative upon routine examination by our technologists. Three of the four bile esculin-positive isolates were urease positive, suggesting that routine assay of urease activity in *S. bovis* and *S. salivarius* could be useful for identification. Without considering the urease reaction, 20% of these 20 *S. salivarius* isolates could conceivably have been misidentified as *S. bovis* on the basis of the results of three presumptive tests and serological examination with the lysozyme-albus method. Thus, difficulty in detection of the group D antigen in *S. bovis* and *S. bovis* variant isolates, along with the cross-reactions of viridans strains, adds to the problems associated with distinguishing *S. bovis* from *S. salivarius*.

Cellular fatty acid profiles allowed for a clear distinction between *S. bovis* and *S. salivarius*. Although the fatty acid profiles were generally similar, *S. salivarius* contained significantly greater amounts of eicosenoic acid. This elevated eicosenoic acid content is also found in members of the species *S. mutans* (13). Although *S. bovis* and *S. bovis* variant produced fatty acid profiles which lacked any distinguishing features, eicosenoic acid content could be used to distinguish between *S. bovis* and the physiologically similar viridans species *S. salivarius* and *S. mutans*. It is interesting to note that the fatty acid profiles of salt-tolerant group D streptococci (1) are strikingly different from those of *S. bovis* strains. Perhaps this qualitative difference in fatty acid content reflects the basic physiological dissimilarities of these two groups of streptococci.

In surveying previous literature on *S. bovis* endocarditis or *S. bovis* as an indicator of gastrointestinal disease, it appears that although some studies (9, 11, 20, 28) used adequate methods for *S. bovis* identification, others (12, 16, 19) employed only presumptive tests or failed to test all organisms reported on with a complete battery of physiological and serological tests. In the study of Murray and Roberts (18), it appears that full characterization of the isolates was performed, but these authors also noted previous dental manipulation in some patients with bacteremia due to organisms identified as *S. bovis* even though *S. bovis* is not a common inhabitant of the oral cavity. Considering the data presented here, it may be reasonable to question the true identity of some of the isolates in previous reports. Instances of misidentification of isolates of viridans streptococci as *S. bovis* and vice versa have been mentioned by Kaplan et al. (10) and Gross and co-workers (8) and have been observed in our own laboratory.

Although *S. salivarius* has traditionally been considered an inhabitant of the oral cavity, it is also readily isolated from stool cultures (24). In view of the physiological similarities of *S. salivarius* and *S. bovis* and the fact that they both inhabit the gut, it might be hypothesized that *S. salivarius*, like *S. bovis*, could function as a bacteremic indicator of gastrointestinal disease. This possibility is currently under investigation in our laboratory.

The study reported here illustrates the problems associated with distinguishing between *S. bovis* and *S. salivarius* isolates. Our data suggest that a minimal number of presumptive physiological tests alone can lead to misidentification of these streptococci. Due to the problems associated with the serological analysis of these organisms, we recommend that an expanded battery of physiological tests be performed for accurate identification. Commercially available systems might be used for physiological testing (K. L.

Ruoff, M. J. Ferraro, and L. J. Kunz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, C128, p. 258). Cellular fatty acid analysis could also be employed to distinguish between *S. bovis* and *S. salivarius* isolates which produce identical reactions in presumptive physiological tests.

LITERATURE CITED

1. **Amstein, C. F., and P. A. Hartman.** 1973. Differentiation of some enterococci by gas chromatography. J. Bacteriol. **113:**38–41.
2. **Chang, G. T., and P. D. Ellner.** 1983. Evaluation of slide agglutination methods for identifying group D streptococci. J. Clin. Microbiol. **17:**804–806.
3. **Facklam, R. R.** 1972. Recognition of group D streptococcal species of human origin by biochemical and physiological tests. Appl. Microbiol. **23:**1131–1139.
4. **Facklam, R. R.** 1977. Physiological differentiation of viridans streptococci. J. Clin. Microbiol. **5:**184–201.
5. **Facklam, R. R.** 1980. Streptococci and aerococci, p. 88–110. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
6. **Facklam, R. R., R. C. Cooksey, and E. C. Wortham.** 1979. Evaluation of commercial latex agglutination reagents for grouping streptococci. J. Clin. Microbiol. **10:**641–646.
7. **Facklam, R. R., J. F. Padula, L. G. Thacker, E. C. Wortham, and B. J. Sconyers.** 1974. Presumptive identification of group A, B, and D streptococci. Appl. Microbiol. **27:**107–113.
8. **Gross, K. C., M. P. Houghton, and L. B. Senterfit.** 1975. Presumptive speciation of *Streptococcus bovis* and other group D streptococci from human sources by using arginine and pyruvate tests. J. Clin. Microbiol. **1:**54–60.
9. **Hoppes, W. L., and P. I. Lerner.** 1974. Nonenterococcal group-D streptococcal endocarditis caused by *Streptococcus bovis*. Ann. Intern. Med. **81:**588–593.
10. **Kaplan, M. H., H. Chemel, A. Stephens, H.-C. Hsieh, M. J. Tennenbaum, I. R. Rothenberg, and G. R. Joachim.** 1983. Humoral reactions in human endocarditis due to *Streptococcus bovis*: evidence for a common *S. bovis* antigen. J. Infect. Dis. **148:**266–274.
11. **Klein, R. S., M. T. Catalano, S. C. Edberg, J. I. Casey, and N. H. Steigbigel.** 1979. *Streptococcus bovis* septicemia and carcinoma of the colon. Ann. Intern. Med. **91:**560–562.
12. **Klein, R. S., R. A. Recco, M. T. Catalano, S. C. Edberg, J. I. Casey, and N. H. Steigbigel.** 1977. Association of *Streptococcus bovis* with carcinoma of the colon. N. Engl. J. Med. **297:**800–802.
13. **Lambert, M. A., and C. W. Moss.** 1976. Cellular fatty acid composition of *Streptococcus mutans* and related streptococci. J. Dent. Res. **55**(Spec. issue A):96–102.
14. **Levchak, M. E., and P. D. Ellner.** 1982. Identification of group D streptococci by SeroSTAT. J. Clin. Microbiol. **15:**58–60.
15. **Medrek, T. F., and E. M. Barnes.** 1962. The influence of the growth medium on the demonstration of a group D antigen in faecal streptococci. J. Gen. Microbiol. **28:**701–709.
16. **Moellering, R. C., Jr., B. K. Watson, and L. J. Kunz.** 1974. Endocarditis due to group D streptococci. Am. J. Med. **57:**239–250.
17. **Moss, C. W., M. A. Lambert, and W. H. Merwin.** 1974. Comparison of rapid methods for analysis of bacterial fatty acids. Appl. Microbiol. **28:**80–85.
18. **Murray, H. W., and R. B. Roberts.** 1978. *Streptococcus bovis* bacteremia and underlying gastrointestinal disease. Arch. Intern. Med. **138:**1097–1099.
19. **Raverby, W. D., E. J. Bottone, and G. T. Keusch.** 1973. Group streptococcal bacteremia, with emphasis on the incidence and presentation of infections due to *Streptococcus bovis*. N. Engl. J. Med. **289:**1400–1403.
20. **Reynolds, J. G., E. Silva, and W. M. McCormack.** 1983. Association of *Streptococcus bovis* bacteremia with bowel disease. J. Clin. Microbiol. **17:**696–697.
21. **Rosan, B.** 1973. Antigens of *Streptococcus sanguis*. Infect. Immun. **7:**205–211.
22. **Shattock, P. M. F.** 1949. The streptococci of group D; the serological grouping of *Streptococcus bovis* and observations on serologically refractory group D strains. J. Gen. Microbiol. **3:**80–92.
23. **Sherman, J. M.** 1937. The streptococci. Bacteriol. Rev. **1:**3–97.
24. **Sherman, J. M., C. F. Niven, Jr., and K. L. Smiley.** 1943. Streptococcus salivarius and other non-hemolytic streptococci of the human throat. J. Bacteriol. **45:**249–263.
25. **Stuart, C. A., E. Van Stratum, and R. Rustigian.** 1945. Further studies on urease production by Proteus and related organisms. J. Bacteriol. **49:**437–444.
26. **Thornsberry, C., C. N. Baker, and R. R. Facklam.** 1974. Antibiotic susceptibility of *Streptococcus bovis* and other group D streptococci causing endocarditis. Antimicrob. Agents Chemother. **5:**228–233.
27. **Vera, H. D., and D. A. Power.** 1980. Culture media, p. 981. *In* E. H. Lennette, H. Balows, W. H. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
28. **Watanakunakorn, C.** 1974. *Streptococcus bovis* endocarditis. Am. J. Med. **56:**256–260.
29. **Watson, B. K., R. C. Moellering, Jr., and L. J. Kunz.** 1975. Identification of streptococci: use of lysozyme and *Streptomyces albus* filtrate in the preparation of extracts for Lancefield grouping. J. Clin. Microbiol. **1:**274–278.