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Presumptive Speciation of *Streptococcus bovis* and other Group D Streptococci from Human Sources by Using Arginine and Pyruvate Tests

KATHARINE C. GROSS,* MARION P. HOUGHTON, AND LAURENCE B. SENTERFIT
Laboratory of Microbiology, The New York Hospital—Cornell Medical Center, New York, New York 10021

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A simplified method for speciation of group D streptococci is described. A total of 4,156 streptococcal isolates from human clinical material was tested for ability to hydrolyze esculin in the presence of 40% bile, ferment pyruvate, hydrolyze arginine, and grow in media containing 40% bile or 6.5% NaCl. Streptococci which hydrolyzed esculin in 40% bile, but which did not hydrolyze arginine, were also tested for their ability to ferment raffinose or sorbose. Sixty percent (2,503) of the isolates hydrolyzed esculin in the presence of 40% bile and were thus presumptively identified as group D. By application of the other criteria, 84% of these were speciated as *Streptococcus faecalis*, 7% were speciated as *S. faecium*, 6% were speciated as *S. bovis*, 2% were speciated as *S. avium*, and 1% were not identified. This scheme was shown to be both reliable and practical for use in the diagnostic laboratory. *S. avium* and *S. bovis* isolates were characterized, and 18 *S. bovis* isolates from patients with bacterial endocarditis were compared physiologically with 151 isolates of this species from other sources.

The need for speciation of the group D streptococci isolated from human clinical material has been emphasized by Deibel (2) and by Facklam (8). This investigation was prompted by the isolation of streptococci with the characteristics of *Streptococcus bovis* from blood cultures of patients with bacterial endocarditis. The physiological identification was confirmed by demonstration of Lancefield group D antigen.

Current biochemical procedures for the differentiation of the group D species, however, are overly complex for the large number of streptococcal isolates encountered in the hospital laboratory. As a consequence many laboratories are unfamiliar with the group D species and rely on inadequate methods for identification of streptococci because of the common, but incorrect, assumption that the terms "enterococci," *S. faecalis* and group D streptococci are synonymous. This may result in an inaccurate report which could lead to improper antibiotic therapy (6, 8, 9, 20, 23).

This communication relates an evaluation of a simplified scheme for the presumptive and confirmed identification of the group D streptococcal species according to physiological characteristics. The scheme is based on the classification of group D streptococci as proposed by Deibel (2, 18). In addition, a survey of the incidence of these organisms in certain clinical specimens is presented.

MATERIALS AND METHODS

Cultures. Of 2,503 group D streptococci isolated from clinical specimens in intervals between March 1972 and July 1973, 546 strains of *S. faecalis*, 75 of *S. faecium*, 8 of *S. faecium* var. *durans*, 48 of *S. avium*, and 169 of *S. bovis* were studied. Reference strains obtained from the American Type Culture Collection were *S. faecalis* ATCC 19433, *S. faecium* ATCC 19434, Lancefield Group Q (*S. avium*) ATCC 14025, *S. bovis* ATCC 15351, and *S. equinus* ATCC 9812. *S. faecium* var. *durans* ATCC 8043 (3), listed as *S. faecalis* by the American Type Culture Collection, was donated by D. J. Hutchison, Sloan-Kettering Institute for Cancer Research. *S. bovis* strains TMC-3 and TMC-101 (14) were obtained from M. T. Pavlova, University of Massachusetts. The human endocarditis *S. bovis* strain, SS-964, was supplied by R. R. Facklam, Center for Disease Control.

Isolates were streaked on sheep blood agar plates (SBAP) to check purity and maintained in Todd-Hewitt broth (THB, Difco). Stocks were frozen at −70 C in THB. An 18-h culture, diluted 1:5 in sterile saline, was used to inoculate all test media. Unless otherwise indicated, all cultures were incubated aerobically at 35 C.

Physiological methods. Our presumptive tests were as follows: (i) esculin hydrolysis in the presence of 40% bile, determined on bile esculin agar (Difco) omitting the horse serum (9); (ii) growth on 40% bile

agar, in which the medium was prepared by adding 4% Oxgall (Bioquest) to tryptose blood agar base (Difco); (iii) growth in THB containing 6.5% NaCl; (iv) pyruvate fermentation, detected in the medium of Deibel and Niven (5). The pyruvate fermentation medium was formulated and modified as follows: tryptone (Difco), 10 g; yeast extract (Difco), 5 g; K_2HPO_4, 5 g; NaCl, 5 g; sodium salt of pyruvic acid (Sigma), 10 g; bromothymol blue, 0.04 g; distilled water, 1 liter; pH 7.2 to 7.4. The broth was dispensed into tubes and autoclaved at 118 C for 15 min. The medium could be stored in the refrigerator for 2 or 3 months without adverse effects. The employment of the bromothymol blue afforded direct readings and thus averted the time-consuming optical density estimations previously reported. Generally, if an organism utilized pyruvate as the energy source, the broth turned bright yellow (acid) after 16 to 18 h of incubation at 35 C. If the strain was unable to utilize pyruvate, the broth remained the blue-green color of uninoculated medium, although slight turbidity was often observed. This reaction was consistent only when the pyruvate was added to the basic medium before autoclaving. (v) Arginine hydrolysis was detected in decarboxylase medium base (Difco, Falkow formulation) with 0.5% L-arginine hydrochloride (Sigma) and 0.25% agar. Four-milliliter volumes of this medium were placed in tubes (13 by 100 mm), sterilized by autoclaving, and then inoculated by stabbing deeply. A purple coloration (alkaline) with good visible growth along the stab area after 18 to 24 h of incubation indicated a positive reaction whereas yellowing (acid) of the medium indicated a negative result.

Confirmative fermentation patterns were determined using basal medium of Deibel and Niven (4). The test carbohydrates were sterilized separately and added aseptically to the basal medium. Starch hydrolysis was detected by the method described by Dunican and Seeley (7), except that soluble starch (Bioquest) was used at a concentration of 0.2% and streak plates were incubated for 1 week at 35 C. Growth at 45 C was detected in THB. The ability to initiate growth in an acidified environment was tested as described by Sims (22) using Rogosa SL agar (Difco) and the GasPak anaerobic system (Bioquest).

Serological methods. Serological grouping was performed only on isolates from patients with bacterial endocarditis which had the physiological characteristics of *S. bovis*. Cultures were grown in THB with glucose added to a final concentration of 1%, as recommended by Medrek and Barnes (15). Extracts were prepared by the Lancefield method, and the group reaction was determined by the capillary precipitin technique (12), using grouping antisera provided by R. C. Lancefield, Rockefeller University.

RESULTS

The majority of the specimens examined in this study were associated with infections. Specimens in which the "viridans" group or group D streptococci often or invariably constitute a portion of the normal flora (i.e., nose-throat, sputum, or feces) were excluded. After isolation from initial SBAP or subsequent plating to SBAP from nonselective enrichment broths, streptococci were identified presumptively by their reaction on the bile-esculin agar, by tolerance tests (40% bile, 6.5% NaCl), and reaction in arginine and pyruvate media. If the bile-esculin reaction was positive, but the arginine test was negative, ability to ferment raffinose or sorbose was also determined. Additional tests, not germane to this discussion, were employed to identify streptococci other than group D. A total of 4,156 streptococcal isolates were tested. Of these, 2,503 were identified presumptively as group D by positive reaction on bile-esculin agar (Table 1). These figures represent individual isolates from different patients. Repeat isolates from the same patient were not included in the tabulation.

The group D isolates were divided into two groups on the basis of their ability to hydrolyze arginine. Those which hydrolyzed arginine were tested for capacity to ferment mannitol, melibiose, sorbitol, and arabinose to confirm their identity. Raffinose and sorbose were included because of their use in the presumptive speciation of the arginine-negative group D streptococci. The physiological characteristics of the 629 arginine-hydrolyzing strains examined were consistent with the reactions described in the literature (2, 3, 8, 18) as well as those of the reference strains (Table 2). The results indicate the usefulness of this scheme for the speciation of the arginine-hydrolyzing group D streptococci. The presumptive identification did not reveal varietal differences among *S. faecalis* and *S. faecium*, but it is doubtful that any clinical significance can be associated with this differentiation.

The group D streptococci that are unable to hydrolyze arginine comprise three species: *S. avium* which often contains the group Q antigen (18), *S. bovis*, and *S. equinus* (2). No *S. equinus* strains were encountered. A total of 217 *S. avium* and *S. bovis* isolates were identified by assaying for ability to grow at 45 C, hydrolyze starch, and ferment lactose, sucrose, melezitose, trehalose, and inulin, in addition to the previously described presumptive and confirmatory tests (Table 3). None of the isolates fermented pyruvate after 18 h of incubation, but *S. avium* usually produced acid after 3 to 4 days of incubation. In other respects, the *S. avium* isolates were compatible with previous descriptions (11, 18).

The isolates of *S. avium* were most frequently recovered from postsurgical infections of the abdominal or rectal area, or abscesses of the

extremities. Very few were isolated from genitourinary areas or urine, and none occurred in blood or cerebrospinal fluid (CSF) specimens. However, the most frequent sources of the *S. bovis* isolates from specimens other than blood of patients with endocarditis were adult female genitourinary areas, urine, or newborn infants either colonized or infected with this organism.

The physiological characteristics of the 151 strains of *S. bovis* isolated from sources other than endocarditis during this study are presented with 18 strains isolated from patients with diagnosed endocarditis (Table 3). This collection of human endocarditis isolates included 10 strains recovered at The New York Hospital prior to this study, some of which were identified by reevaluation of organisms reported as "viridans streptococci" as well as eight strains isolated during this study. All *S. bovis* strains were uniform in their presumptive reactions and, significantly, none grew in pyruvate broth. Isolates from endocarditis were more homogeneous with respect to confirmative characteristics than the isolates from other clinical material.

None of the HCl extracts of *S. bovis* isolates from endocarditis reacted serologically with the group D antiserum when the organisms were grown in THB containing the usual 0.2% glucose. All 18 strains, however, were shown to possess the antigen when grown in THB modified by increasing the glucose concentration to 1% (15).

Sims (22) observed that *S. bovis* grew in an acid medium, which is normally inhibitory for this organism, only if the cultures were incubated in an atmosphere containing 5% CO_2 or if the CO_2 produced by the organism was prevented from escaping from the culture. Since no other streptococci grew under these conditions, this suggested a simple test which would differentiate *S. bovis*. Our results with a limited number of streptococcal strains (57 strains of *S. bovis* and 40 other streptococcal strains) did not entirely confirm this observation (Table 4). Although 42 of the *S. bovis* isolates (including all of the reference strains) grew well under these conditions, the growth of 13 isolates could not be distinguished from growth observed with other group D species and *S. mutans*.

Table 5 summarizes the relative frequency of

TABLE 1. *Streptococcal isolates recovered from various clinical materials*

Streptococci by group	No. of isolates recovered			
	Blood and CSF	Urine	Other[a]	Total
Beta hemolytic, Lancefield groups other than D	14	160	587	761
Viridans, other than group D	40	61	791	892
Group D	37	756	1,710	2,503
Total	91	977	3,088	4,156

[a] Abscesses, lesions, postsurgical infections from various anatomical origins; genitourinary tract, umbilicus, peritoneal, pleural fluids, and postmortem specimens.

TABLE 2. *Arginine-hydrolyzing group D streptococci from human sources showing physiological reactions comparable to reference strains*

Physiological characteristic	*S. faecalis*		*S. faecium*		*S. faecium* var. *durans*	
	ATCC 19433	Clinical isolates (546 strains)	ATCC 19434	Clinical isolates (75 strains)	ATCC 8043	Clinical isolates (8 strains)
Presumptive:						
Bile-esculin	+[a]	545+; 1−	+	74+; 1−	+	+
40% Bile	+	+	+	+	+	+
6.5% NaCl	+	541+; 5−	+	74+; 1−	+	+
Pyruvate	+	+	−	−	−	−
Arginine	+	540+; 6−	+	+	+	+
Confirmative:						
Mannitol	+	+	+	73+; 2−	−	−
Melibiose	−	545−; 1 (+)	+	65+; 10−	+	6+; 2−
Sorbitol	+	+ or (+)	−	64−; 11+, (+)	−	−
Arabinose	−	−	+	+	−	−
Raffinose	−	543−; 3 (+)	−	53−; 22+, (+)	−	4−; 4 (+)
Sorbose	−	−[b]	−	−	−	−

[a] Symbols: +, positive reaction; (+), delayed positive reaction; −, negative reaction.
[b] Only 110 strains were tested.

TABLE 3. *Arginine-negative group D streptococci from human sources showing physiological reactions comparable to reference strains*

Physiological characteristic	*S. avium* (group Q)		*S. bovis*					
	ATCC 14025[a]	Clinical isolates (48 strains)	ATCC 15351[a]	TMC-3[a]	TMC-101[a]	SS-964[a]	Isolates from endocarditis (18 strains)	Isolates from other sources (151 strains)
Presumptive:								
Bile-esculin	+[b]	+	+	+	+	+	+	+
40% Bile	+	+	+	+	+	+	+	+
6.5% NaCl	(+)	47+; 1−	−	−	−	−	−	−
Pyruvate	(+)	41 (+); 7−	−	−	−	−	−	−
Arginine	−	−	−	−	−	−	−	−
Raffinose	−	43−; 5+	+	+	+	+	+	+
Sorbose	+	47+; 1−	−	−	−	−	−	−
Confirmative:								
45 C	+	+	+	+	+	+	+	139+; 12−
Starch	−	−	+	+	+	+	+	149+; 2−
Lactose	+	+	+	+	+	+	+	+
Sucrose	(+)	45+; 3−	+	+	+	+	+	+
Mannitol	+	+	−	−	+	+	16+; 2−	141−; 10+
Melibiose	−	32−; 16 (+)	+	+	+	(+)	(+) or +	(+) or +
Sorbitol	+	+	−	−	−	−	−	−
Arabinose	+	47+; 1−	−	−	−	−	−	150−; 1+
Melezitose	+	43+; 5−	−	−	−	−	−	−
Trehalose	+	+	−	+	+	+	+	110−; 41+
Inulin	−	−	+	+	(+)	−	9−; 9+	139−; 12+

[a] Reference strains.

[b] Symbols: +, positive reaction; (+), delayed positive reaction; −, negative reaction.

isolation of the streptococci and shows there was no seasonal fluctuation in the incidence of the different groups or species. Varietal differentiation was not maintained after the first interval. The group D isolates constituted over 59% of the streptococci recovered in our laboratory. Eighty-four percent of these were easily recognized as *S. faecalis* by the simplified method for speciation. These data indicate that 97 to 99% of the group D streptococci were presumptively speciated by this procedure. With this assurance, speciation by the presumptive scheme is now used routinely in this laboratory.

DISCUSSION

The brief scheme for presumptive speciation of the group D streptococci presented in this paper has been shown to be reliable and clinically feasible. Hopefully, this will facilitate further accumulation of data concerning incidence and distribution of the group D streptococcal species in human infections and their differences in response to antibiotics.

Facklam and Moody (9, 10) established that hydrolysis of esculin in medium containing 40% bile is the test of choice to identify presumptively the group D streptococci. Our findings in preliminary studies were similar. Media containing esculin, azide, and only 10% bile allowed too many false positive reactions by non-group D viridans streptococci to make it reliable as a single presumptive identification test.

The results of this study verify that pyruvate fermentation and arginine hydrolysis are dependable and useful tests for the differentiation of the group D species. The rapid fermentation of pyruvate by *S. faecalis* was consistently demonstrable, but only when the pyruvate was autoclaved with the other constituents during preparation of the medium. The degree of reliability of the arginine test may also depend upon the medium used. Although most *S. faecalis* and *S. faecium* isolates produced a strong positive reaction in the arginine stab medium (Falkow), occasional isolates showed a positive reaction only when tested in the more sensitive decarboxylase base Moeller (Difco) arginine broth overlaid with sterile mineral oil. Routine use of the Falkow arginine stab medium was chosen for economic reasons and ease of preparation.

By using the bile-esculin reaction, salt tolerance, pyruvate and arginine tests, and fermentation of raffinose or sorbose (when necessary), about 98% of the group D streptococci isolated

from human clinical material were speciated presumptively. Speciation was confirmed by additional biochemical reactions (Tables 2 and 3) also selected for their dependability. Some commonly used tests, such as growth at pH 9.6, in *S. faecalis* broth, or methylene blue milk, reduction of tetrazolium, resistance to tellurite, and tyrosine decarboxylation were rejected because of the variability or difficulty of interpretation reported by others (3, 8, 9) and in our own past experience. All media used in this brief scheme fit our criteria for good diagnostic tests, i.e., they are easily prepared, there is a distinct difference between positive and negative reactions after overnight incubation, and there is no special procedure for inoculation.

TABLE 4. *Growth of various streptococci on Rogosa agar*[a]

Streptococcus group or species	No. of strains tested	No. giving each type reaction			
		++[b]	+[c]	±[d]	−[e]
Group A	1				1
Group B	1				1
Group N (*S. lactis*)	1				1
S. mitis	2				2
S. mutans	2		2		
Other viridans	1				1
Group D					
S. faecalis	18			4	14
S. faecium	4		2	2	
S. avium	9		9		
S. bovis	57	42	13	2	
S. equinus	1	1			

[a] Incubated at 35 C in 8 to 10% CO_2 in H_2 for 3 to 4 days.

[b] ++, Full growth to colony size equal to or larger than that on blood agar.

[c] +, Growth, but colony is smaller than normal.

[d] ±, Trace or questionable growth.

[e] −, Complete lack of growth.

S. faecalis was the *Streptococcus* most commonly isolated from human infections (Table 5). It was readily distinguished from other group D streptococci by the singular ability to ferment pyruvate rapidly. Non-group D viridans streptococci never were able to utilize this substrate, but occasional strains of beta hemolytic streptococci not belonging to group D fermented pyruvate weakly.

The proportion (31%) of raffinose-fermenting strains of *S. faecium* observed in this study when compared with that (67%) of Facklam (8), also with human isolates, and that (83%) of Medrek and Barnes (13) with isolates from cattle and sheep indicate considerable variability in the capacity of *S. faecium* to ferment raffinose. These observations point out that *S. bovis* is not the only group D species which can ferment this sugar.

The unique ability of *S. avium* to ferment the polyol sorbose ($C_6H_{12}O_6$), a derivative of sorbitol ($C_6H_{14}O_6$), differentiated it from the other group D streptococci (18). This species represented 2% of the total group D streptococcal isolates from clinical material in this study. The sources of our *S. avium* isolates were similar to those reported by Guthof and also were usually present with other intestinal organisms (11).

TABLE 5. *Streptococci isolated*[a] *at The New York Hospital in different seasons*

Streptococci by group	Spring 1972			Fall 1972			Winter-Spring 1973		
	No. of isolates	% Total	% Group D	No. of isolates	% Total	% Group D	No. of isolates	% Total	% Group D
Beta hemolytic, Lancefield groups other than D	213	18		181	16		367	20	
Viridans, other than group D	335	28		251	23		306	16	
Group D									
S. faecalis	529		84	576		84	989		84
S. faecium	44		7	41[b]		6	75[b]		6
S. faecium var. *durans*	5		1						
S. avium	9		1	16		2	24		2
S. bovis	36		6	35		5	87		7
Unspeciated	9		1	17		3	11		1
Total Group D	632	54		685	61		1,186	64	
Total	1,180			1,117			1,859		

[a] From all cultures (except nose-throat, sputum, or feces) received during these intervals.

[b] Includes both *S. faecium* and *S. faecium* var. *durans* isolates.

Therefore the distribution of *S. avium* in human infections and normal flora appears to be similar to that of the established enterococci, although none was recovered from blood or CSF. The *S. avium* strains characterized in this study conformed to the earlier descriptions (11, 18) with only minor differences. Our data and Guthof's work (11) showed that these organisms did not ferment inulin, whereas Nowland and Deibel (18) reported that all the strains they studied fermented this substrate. The inability to utilize inulin resembled the response seen with other enterococci (8). Nowland and Deibel also observed that pyruvate was not utilized, but failed to recognize that growth might occur with prolonged incubation.

The association of *S. bovis* with bacterial endocarditis has long been known (17). Recent reports (8, 20, 23) indicate renewed awareness of the differences between the enterococci and other group D streptococci and of the need for correct identification of isolates from patients with endocarditis. The presence of *S. bovis* in routine clinical specimens other than blood, however, had not been investigated. Our results show that this species accounted for 6% of the group D streptococci isolated in this laboratory.

The 169 isolates of *S. bovis* examined during this survey were distinctive and unvarying in the presumptive reactions (Table 3). The ability to hydrolyze esculin and ferment raffinose and the inability to hydrolyze arginine or grow in 6.5% NaCl are characteristics previously reported for *S. bovis* isolated from various animals (2, 14, 16, 21). Our results show that this species also lacks the ability to ferment pyruvate and sorbose. Whereas Deibel (2) described *S. bovis* as variable in fermentation of sorbitol, other authors (14, 16, 21) reported uniform inability to ferment sorbitol and our findings with human isolates are in agreement with the latter. The invariable fermentation of melibiose, though frequently delayed, and failure to ferment melezitose are consistent with Deibel's characterization of this species.

It is also apparent from this investigation that certain differences in fermentative abilities within this species may be dependent upon the source of isolation. Our *S. bovis* isolates from patients with endocarditis usually fermented mannitol and trehalose, fermentation of inulin was variable, and arabinose was not fermented. This is similar to other studies of *S. bovis* from humans with endocarditis (8, 14, 16). Isolates from other human sources, however, often did not ferment any of these four sugars. In contrast, *S. bovis* isolates of animal origin almost always fermented inulin and frequently arabinose, whereas mannitol fermentation was relatively uncommon (14, 21).

The ability to hydrolyze starch is an important characteristic in the identification of *S. bovis*. Pavlova et al. (19) suggested standardization to 0.2% starch in agar. Their basic medium, however, would not support the growth of our strains as consistantly as the medium of Dunican and Seeley (7). This study revealed considerable variation in the ability of *S. bovis* isolates of human origin to hydrolyze starch. The use of this low concentration of starch with prolonged incubation made it possible to detect weak as well as strong hydrolyzing abilities. Generally, the endocarditis isolates were strongly positive within 18 to 24 h.

Infrequent strains of non-group D viridans streptococci give equivocal reactions on bile-esculin agar (9, 10). Such observations indicate the occasional need for the full spectrum of confirmatory tests, including serological, in identification of atypical organisms and of streptococcal endocarditis isolates. Much of the biochemical pattern of *S. mutans*, in particular, resembles *S. bovis* (1), and since our data showed that an occasional strain of *S. bovis* failed to hydrolyze starch or grow at 45 C, an additional differential test would be of value. Unfortunately, Sims' test (22) for growth on acid medium in presence of CO_2 did not prove to be sufficiently accurate to reliably distinguish *S. bovis* from other arginine-negative streptococci tested.

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