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16S Ribosomal DNA Sequence Analysis Distinguishes Biotypes of *Streptococcus bovis*: *Streptococcus bovis* Biotype II/2 Is a Separate Genospecies and the Predominant Clinical Isolate in Adult Males

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We characterized 22 human clinical strains of *Streptococcus bovis* by genotypic (16S rRNA gene sequence analysis [MicroSeq]; Applied Biosystems, Foster City, Calif.) and phenotypic (API 20 Strep and Rapid ID32 Strep systems (bioMerieux Vitek, Hazelton, Mo.) methods. The strains, isolated from blood, cerebrospinal fluid (CSF), and urine, formed two distinct 16S ribosomal DNA sequence clusters. Three strains which were associated with endocarditis urinary tract infection (UTI), and sepsis clustered with the *S. bovis* type strain ATCC 33317 (cluster 1); other closely related type strains were *S. equinus* and *S. infantarius*. Nineteen strains clustered at a distance of about 2.5% dissimilarity to the *S. bovis* type strain (cluster 2) and were associated with central nervous system (CNS) disease in addition to endocarditis, UTI, and sepsis. All strains were distinct from *S. gallolyticus*. Within cluster 2, a single strain grouped with ATCC strain 43143 (cluster 2a) and may be phenotypically distinct. All the other strains formed a second subgroup (cluster 2b) that was biochemically similar to *S. bovis* biotype II/2 (mannitol negative and beta galactosidase, alpha galactosidase, beta glucuronidase, and trehalose positive). The API 20 Strep system identified isolates of cluster 2b as *S. bovis* biotype II/2, those of cluster 1 as *S. bovis* biotype II/1, and that of cluster 2a as *S. bovis* biotype I. There was an excellent correlation of biotype and genotype: *S. bovis* biotype II/2 isolates form a separate genospecies distinct from the *S. bovis, S. gallolyticus*, and *S. infantarius* type strains and are the most common isolates in adult males.

S. bovis is a human pathogen associated with endocarditis, sepsis, and meningitis (2, 3, 4, 6, 7, 8, 10). Since the early 1980s genetic and biochemical diversity among *S. bovis* has been noted (4, 7, 8). Several groups studied this diversity and devised schemes to distinguish strains by biotype. *S. bovis* strains from humans are said to be biotype I (or typical) if, among other traits, they ferment mannitol and produce glucan and biotype II (or variant) if they cannot ferment mannitol or produce glucan. The *S. bovis* biotype II strains are further divided into type II/1 and type II/2 by the ability of the later group to produce beta-galactosidase and beta-glucuronidase and ferment trehalose but not glycogen (4, 8).

Recently the *S. bovis* group has been further defined based on 16S rRNA gene sequence, ribotyping, and whole-cell protein electrophoresis patterns. *S. infantarius*, with a 1.8% difference by 16S rRNA gene sequence from the *S. bovis* type strain, is phenotypically like *S. bovis* biotype II/1 (1, 9). Ribotyping patterns were used to differentiate all related species from *S. infantarius* and distinguish two subspecifics within the group (9). *S. gallolyticus*, originally isolated from Koala dung, is phenotypically that of biotype I (5, 9). Devriese et al. examined strains previously identified as *S. bovis* by both phenotypic methods and whole-cell protein electrophoresis and reported that many of the human strains associated with endocarditis and animal (goat, pigeon, and cow) strains were *S. gallolyticus* (5). By comparing our strains with the more recently described species, we wished to determine the distribution of the various *S. bovis* groups within our population. Although there have been some previous reports of frequency of occurrence of the *S. bovis* groups (7), none have been based on 16S rRNA gene sequence analysis of strains from a single population. We characterized by genotypic and phenotypic methods 21 human strains of *S. bovis* that were isolated in a single hospital from adult males and one referred strain from a child. The correlation of phenotype with genotypic cluster is presented here.

MATERIALS AND METHODS

The clinically significant strains of *S. bovis* were collected over a period of 10 years, with no two isolates collected in the same month. From 1998 to 2000, the isolates were sequential. They were not a single genetically related cluster. After initial isolation, strains were frozen at −70°C until further study. Presumptive phenotypic identification was performed by Gram stain, colony morphology, sheep blood hemolysis, and catalase reaction. For biochemical testing, streptococcal strains were grown at 37°C on Columbia agar plates (Remel, Lenexa, Kans., and BBL, Becton Dickinson, Cockeysville, Md.) in anaerobic conditions. Biochemical testing was performed using the API 20 Strep system (version 6) and the Rapid ID32 Strep system (bioMerieux Vitek). The interpretation of all these tests was done according to manufacturers' instructions. 16S rRNA gene sequence identification was performed at MIDI Labs (Newark, Del.) and Houston VA laboratories using the MicroSeq 500 Gene kit (Applied Biosystems) according to the manufacturer's specifications. Test strain sequences were compared against the MicroSeq 16S rRNA gene sequence database. The database contains sequences from 1,297 different species (1,187 type strains), including 36 type strains from the genus *Streptococcus*, 18 type strains from the genus *Enterococcus*, and 2 type strains from the genus *Aerococcus*. Within the *S. bovis* group, *S. bovis* type strain ATCC 33317 was sequenced. Sequence data obtained from

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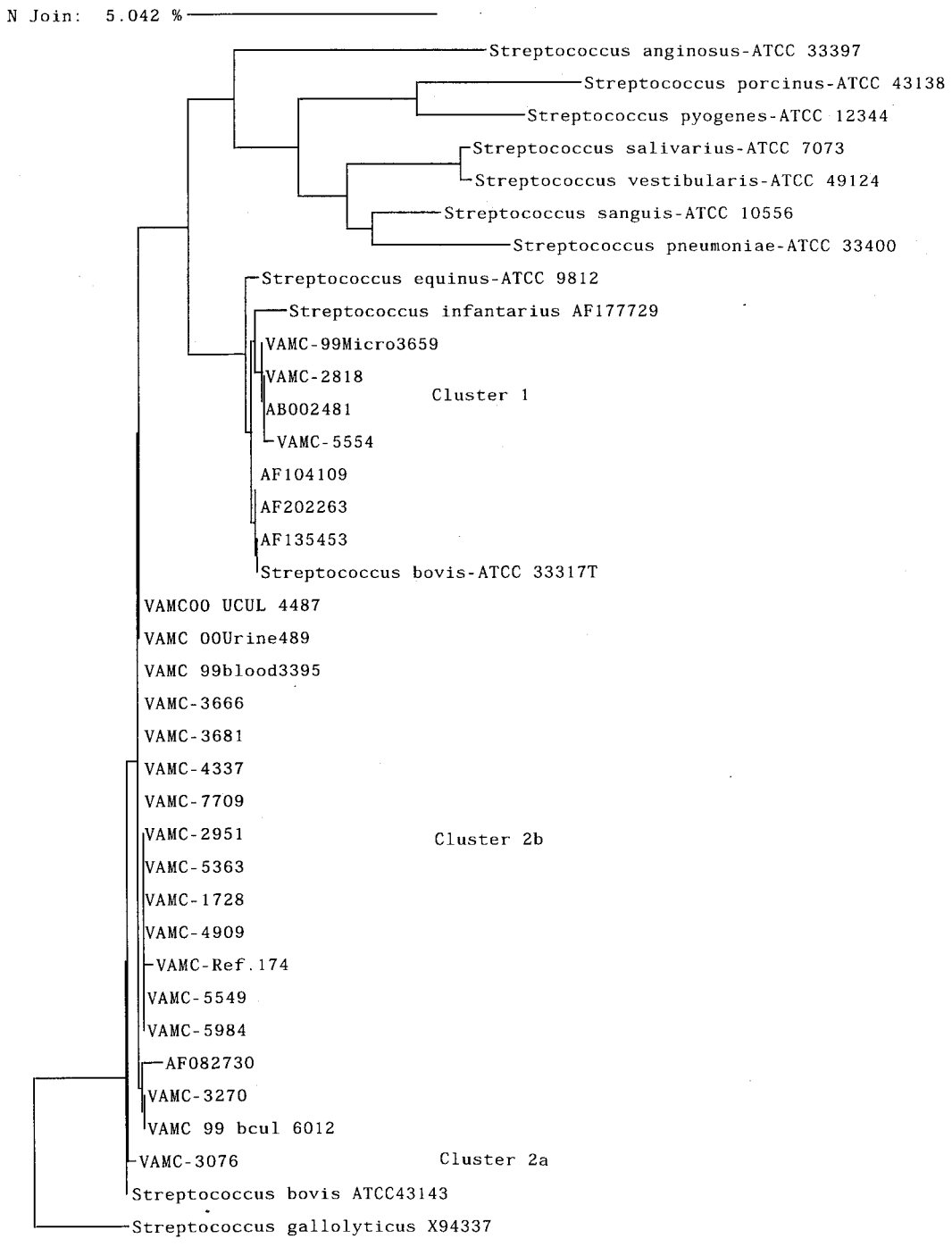

FIG. 1. Dendrogram of phylogenetic relationships among *Streptococcus bovis* isolates and ATCC type strains. Distances were calculated by the neighbor-joining method. The bar at the top indicates a 5% difference measured horizontally.

GenBank on non-type strains was also included in the analysis. These strains and their accession numbers are as follows: *S. bovis*, ATCC 43143; *S. bovis*, AF104114.1; *S. infantarius*, AF177729; and five *S. bovis* sequences—AF082730.1, AF202263.1, AF135453.1, AF104109.1, and AB002481.1.

RESULTS

The relatedness of strains as determined by 16S rRNA gene sequence analysis is shown in a dendrogram (Fig. 1). Although sequence comparisons were performed on all 22 of our isolates, all strains in the MicroSeq database (including 36 streptococcal type strains), and all sequences in GenBank, for convenience, only the closely related or better-known type strains, close sequences from GenBank, and 20 of our strains are shown on the dendrogram. The strains from our study are prefaced with "VAMC", and others are as listed as in Materials and Methods. Details of all clinical strains are given in Table 1. There are two major genogroups into which the

TABLE 1. Biochemical characteristics by strain and genotypic cluster of strains isolated in this study[a]

Cluster	Strain designation	Source	API biotype no.	API 32S biotype no.	Clinical association
1	99B3659	Blood	5240443	22013001110	Sepsis, colon cancer
1	2818	Cardiac tissue	5240441	22013001110	Endocarditis
1	5554	Urine	5240440	22013001110	UTI
2b	00U4487	Urine	ND[b]		UTI
2b	00U489	Urine	5450451		UTI
2b	99B3395	Blood	5650450	63077203150	Sepsis
2b	96B3666	Blood	5650450	63077001150	Sepsis, GI bleed
2b	96B3681	Blood and CSF	5650450	63077201150	Meningitis
2b	4337	Brain tissue	5650450		Subdural empyema
2b	99U7709	Urine	5640450		UTI
2b	2951	Blood	5650450		Sepsis, GI bleed
2b	5363	Blood	5650450	63077001150	Endocarditis
2b	97B1728	Blood	5650450	63077201150	Spontaneous peritonitis
2b	98B4909	Blood	5650450	63077001150	Sepsis
2b	5538	Blood	5650450	23077201150	Sepsis, GI bleed
2b	ref174	CSF	5650450	63077001150	Encephalitis and meningitis
2b	5549	Urine	5650450	23077201150	UTI
2b	5984	Urine	5650450	23077201150	UTI
2b	7057	Urine	5650450	63077003150	Unknown
2b	3270	Blood	5650450	63077001150	Endocarditis
2b	99B6012	Blood	5650450	63077201150	Endocarditis
2a	3076	Blood	5250553	20237061110	Endocarditis, meningitis

[a] The right-hand portion of the strain designation is the same as in Fig. 1. As stated in text, there were isolates associated with UTI endocarditis and sepsis in both larger groups.
[b] ND, not determined.

strains cluster. Cluster 1 includes three clinical strains, three type strains (*S. bovis* ATCC 33317, *S. equinus* ATCC 9812, and *S. infantarius* AF177729), and four sequences from GenBank. Cluster 2 includes 19 clinical strains, *S. bovis* ATCC 43143, and a sequence from GenBank. These two clusters differ by about 2.5%. Within cluster 2, a single strain grouped with ATCC 43143 (cluster 2a), while all the other strains (18) formed a second subgroup (cluster 2b), for which there was only one similar sequence in the databases, belonging to an undescribed organism isolated from a human. Strain 4337 and all strains in the identical group show 2.43% difference in sequence from *S. bovis* type strain ATCC 33317. Cluster 2a differs from 2b by about 0.8%. The *S. gallolyticus* sequence (Fig. 1) did not cluster within the *S. bovis*-*S. infantarius*-*S. equinus* node.

Table 1 shows the source, API20 Strep system biotype, and Rapid ID32 Strep biotype of all the tested clinical strains. The three strains that clustered with the *S. bovis* type strain ATCC 33317 were isolated from blood, cardiac tissue, and urine, while strains in cluster 2 were isolated from the central nervous system (CNS) in addition to blood and urine. All organisms were positive for hydrolysis of esculin, production of acetoin and leucine aminopeptidase, and production of acid from lactose, maltose, raffinose, and sucrose. The significant tests that were negative were hydrolysis of hippurate and urea and production of acid from ribose, L-arabinose, and sorbitol. Our data for tests that were variable or important in distinguishing related strains are shown in Table 2 in the first three data columns. The other seven columns show comparative data from sources as indicated. We found that strains that are different genetically also show consistent biochemical differences. For example, all the *S. bovis* strains in cluster 1 are trehalose negative and those in cluster 2 are trehalose and beta-galactosidase positive. Within this cluster, one strain, 3076 (cluster 2a), is biochemically distinct and genetically closer to *S. bovis* strain ATCC 43143. The rest of the strains are in cluster 2b. The API 20 Strep system identified isolates of cluster 2b as *S. bovis* biotype II/2, those of cluster 2a as *S. bovis* biotype I (although the test results are not as described by others [7] for *S. bovis* biotype I), and those of cluster 1 as *S. bovis* biotype II/1. Both the API 20 Strep system and the Rapid ID32 Strep system identified all strains as *S. bovis*.

DISCUSSION

Recently two new species closely related to the *S. bovis*-*S. equinus* group have been described. *S. infantarius*, so named because it had been isolated from infant's stools, was distinguished based on 16S rRNA gene sequence and ribotyping. *S. gallolyticus* was reported as isolated from both human and animal specimens. We began this study because the genetic and biochemical diversity of the *S. bovis* group had not clearly distinguished whether there would be separate biotype or genotype groups for isolates from different clinical settings, e.g., adults and infants, humans and animals, urine and CNS, or isolates associated with colon cancer and isolates not associated with colon cancer. A previous excellent study indicated that biotype I was more commonly associated with underlying cancer or gastrointestinal (GI) disease and that biotype II/2 occurred less frequently (7). Confirming this and clarifying the occurrence of genetically defined strains could lead to a better understanding of the pathogenic role of *S. bovis*.

In fact, the 22 human strains of *S. bovis* that we characterized by both 16S rRNA gene sequence analysis and biochemical methods formed two distinct 16S rRNA sequence clusters.

TABLE 2. Summary of differential characteristics of the strains[a]

Characteristic	Our data (% positive) for cluster:			Data from references 5 and 9				Data from API 20 Strep chart (% positive)		
	1 (n = 3)	2a (n = 1)	2b (n = 17)	*S. Infantarius*	*S. bovis* biotype II/1	*S. bovis* biotype II/2	*S. gallolyticus*	Typical biotype I	*S. bovis* biotype II/1	*S. bovis* biotype II/2
Production of:										
Beta-glucosidase	100	100	100[b]	v/+	+	+	+	ND[e]	ND	ND
Beta-glucuronidase	0	0	100	−	−	+	−	4	1	88
Alpha-galactosidase	100	0	100	+	+	v	+	71	86	85
Beta-galactosidase	0	100	100	−	−	+	−	14	17	94
Beta-mannosidase	0	0	100[b]	−	−	+	v	ND	ND	ND
Acid from:										
Trehalose	0	100	100	−	−	+	+	90	30	99
Raffinose	100[d]	100[d]	100	v	+	v	+	90	99	72
Inulin	0	0	0	−	+	−	+	63	61	13
Starch[c]	fast	fast	slow	+/v	+	−	+	100	73	40
Glycogen	33	100	0	+/−	+	−	+	90	65	18
Pullulans	33	100	0[b]	v/−	−	−	+	ND	ND	ND
Melibiose	0	0	20[b]	v/−	+	−	−	ND	ND	ND
Mannitol	0	0	0[b]	−	−	−	+	86	0	0

[a] Our data, which were obtained by testing our strains on both the API 20 Strep and API 32 Strep systems, is shown in the first group of columns. It is contrasted with data in subsequent columns from references 5 and 9 combined and the API 20 Strep chart.
[b] Only 11 strains tested.
[c] With extended incubation (1 week), all strips became positive: fast, positive in 24 to 48 h; slow, positive in >48 h.
[d] Positive on API 20 Strep but negative on the rapid ID 32 Strep.
[e] ND, not in API 20 Strep database.

However, the genotypic clusters did not clearly segregate the isolates according to source, age of patient, or type of infection. Both clusters 1 and 2 contained isolates that were obtained from blood and urine and associated with sepsis, endocarditis, and urinary tract disease. The single strain from a child that we examined clustered with the majority of our strains from adults, not with the *S. infantarius* strain as we thought it might. One difference that might be explored further is that the three isolates that were associated with CNS disease were in cluster 2. Although we did not purposefully compare human and animal strains, it is interesting that our analysis of a serendipitous strain isolated from a septic dog placed it in cluster 1 (data not shown) with the *S. bovis* type strain ATCC 33317, also isolated from an animal.

The genotypic clusters did, however, segregate the isolates into biochemically homogeneous biotypes. The three strains that clustered with *S. bovis* type strain ATCC 33317 (originally isolated from cow dung) were trehalose negative and compatible with biotype II/1, as is the type strain (4), and also similar to the published description of *S. infantarius* (see Table 2). All the other strains were similar to *S. bovis* biotype II/2 in that they produced beta-galactosidase and beta-glucuronidase and fermented trehalose but not glycogen. A single strain that was closest to ATCC strain 43143 (originally isolated from human blood) was also most similar biochemically to *S. bovis* biotype II/2; the slightly different biochemical pattern may not be significant. In contrast to the results of Devriese (5), we found no strains phenotypically similar to *S. bovis* biotype I by most descriptions (i.e., none were mannitol positive, even though the Strep API 20 code book called one isolate *S. bovis* biotype I), and none of our *S. bovis* strains clustered with the *S. gallolyticus* sequence.

Thus, in contrast to studies on other populations, *S. bovis* biotype II/2 is the most frequently occurring of the various *S. bovis* group biotypes within our adult, predominantly male population. The phenotypic and genotypic characteristics demonstrate that they are clearly distinct from the *S. bovis, S. gallolyticus*, and *S. infantarius* type strains and represent a hitherto unnamed species.

Nucleotide sequence accession numbers. Sequences for strains VAMC blood3395 and VAMC 3076 have been deposited in GenBank under accession numbers AF313406 and AF313408, respectively.

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