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Reidentification of *Streptococcus bovis* Isolates Causing Bacteremia According to the New Taxonomy Criteria: Still an Issue?[▽]

Beatriz Romero,[1,2] María-Isabel Morosini,[1,2] Elena Loza,[1,2] Mercedes Rodríguez-Baños,[1] Enrique Navas,[3] Rafael Cantón,[1,2] and Rosa del Campo[1,2]*

Servicio de Microbiología, Hospital Universitario Ramón y Cajal, and IRYCIS, Unidad de Resistencia a Antibióticos y Virulencia Bacteriana Asociada al Consejo Superior de Investigaciones Científicas (CSIC),[1] CIBER en Epidemiología y Salud Pública (CIBERESP),[2] and Servicio de Enfermedades Infecciosas, Hospital Universitario Ramón y Cajal,[3] Madrid, Spain

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All *Streptococcus bovis* blood culture isolates recovered from January 2003 to January 2010 ($n = 52$) at the Hospital Universitario Ramón y Cajal were reidentified on the basis of their genetic traits using new taxonomic criteria. Initial identification was performed by the semiautomatic Wider system (Fco. Soria-Melguizo, Spain) and the API 20 Strep system (bioMérieux, France). All isolates were reidentified by PCR amplification and sequencing of both the 16S rRNA and *sodA* genes and by mass spectrometry using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; Bruker, Germany). Results of 16S rRNA/*sodA* gene sequencing were as follows: *Streptococcus gallolyticus* subsp. *gallolyticus*, 14/14 (number of isolates identified by 16S rRNA/number of isolates identified by *sodA* gene sequencing); *Streptococcus gallolyticus* subsp. *pasteurianus*, 24/24; *Streptococcus* spp., 7/0; *Streptococcus infantarius* subsp. *infantarius*, 0/2; *Streptococcus lutetiensis*, 0/5; *Leuconostoc mesenteroides*, 4/0; and *Lactococcus lactis*, 3/3. MALDI-TOF MS identified 27 *S. gallolyticus* isolates but not at the subspecies level, 4 *L. mesenteroides* isolates, 3 *L. lactis* isolates, and 6 *S. lutetiensis* isolates, whereas 12 isolates rendered a nonreliable identification result. Pulsed-field gel electrophoresis grouped all *S. gallolyticus* subsp. *gallolyticus* isolates into 3 major clusters clearly different from those of the *S. gallolyticus* subsp. *pasteurianus* isolates, which, in turn, exhibited no clonal relationship. The percentages of resistance to the tested antimicrobials were 38% for erythromycin, 23% for fosfomycin, 10% for levofloxacin, 6% for tetracycline, and 4% for co-trimoxazole. The most frequent underlying diseases were hepatobiliary disorders (53%), endocarditis (17%), and malignancies (12%). We conclude that sequencing of the *sodA* gene was the most discriminatory method and that *S. gallolyticus* subsp. *pasteurianus* appears to have a higher genetic diversity than *S. gallolyticus* subsp. *gallolyticus*.

Streptococcus bovis, a nonenterococcal group D *Streptococcus*, can be found as part of the human gastrointestinal microbiota in 5 to 16% of individuals (17). However, it causes bacteremia and endocarditis, particularly in men and in the elderly (7, 8). The association of *S. bovis* bacteremia and colon tumors was established in the late 1970s (14). Moreover, recent studies have described a frequent association between its isolation and chronic liver and biliary tract disorders (10).

Streptococcal taxonomy has progressively changed according to the description of new species originally grouped as *S. bovis*. During the late 1990s and at the beginning of the first decade of the 2000s, several authors renamed *S. bovis* biotype I as *Streptococcus gallolyticus* subsp. *gallolyticus* (21), biotype II/1 as *Streptococcus lutetiensis* (18), and finally, biotype II/2 as *S. gallolyticus* subsp. *pasteurianus* (20).

Clinicians still remain unfamiliar with the new taxonomy of *S. bovis* species, mostly due to the complexity of the current nomenclature and specific identification requirements based on molecular microbiology techniques not available in routine clinical laboratories. Nevertheless, due to their specific disease association and their microbiology features, a proper identification of the *S. bovis* isolates is needed. The aim of this study was to review all *S. bovis* bacteremic episodes documented over the last 7 years at the Hospital Universitario Ramón y Cajal, focusing on the new taxonomy and the probable association of different subspecies and pathologies.

MATERIALS AND METHODS

Bacterial identification. All *S. bovis* isolates ($n = 52$, from 51 patients) causing bacteremia recovered from blood cultures between January 2003 and January 2010 were studied. Initially, *S. bovis* identification was routinely performed using the commercial API 20 Strep gallery (bioMérieux, Marcy l'Etoile, France) and the semiautomated Wider system (Fco. Soria Melguizo, Madrid, Spain) (5). The ability to grow on bile esculin agar (BD, NY) was determined after 24 h of incubation at 37°C. Isolates were tested for the presence of Lancefield streptococcal antigen D by agglutination using a Slidex Strepto Plus kit (bioMérieux, Marcy l'Etoile, France).

Subsequently, both 16S rRNA gene PCR with universal primers (primer 16-F [5′-AGGATTAGATACCCTGGTAGTCCA-3′] and primer 16-R [5′-AGGCCC GGGAACGTATTCAC-3′]) and *sodA* PCR with degenerate primers (primer *dl* [5′-CCITAYICITAYGAYGCIYTIGARCC-3′] and primer *d2* [5′-ARRTART AIGCRTGYTCCCAIACRTC-3′]) were performed (18). Amplicons (500 bp for 16S rRNA and 609 bp for *sodA*) were sequenced using an ABI Prism 377 automated sequencer (Perkin Elmer, Norwalk, CT) after purification with ExoSAP-IT (Amersham, Bucks, United Kingdom). Sequences were aligned using the ClustalW tool from the website www.ebi.ac.uk, and phylogenetic trees were constructed with TreeView software (Michael Eisen, Stanford University, Stanford, CA).

Mass spectrometry using a Bruker Biotyper MALDI78 TOF MS matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI TOF MS) system (Bruker Daltonics, Germany) was also performed as part of the bacterial reidentification scheme (3).

* Corresponding author. Mailing address: Servicio de Microbiología, Hospital Universitario Ramón y Cajal, Ctra. Colmenar, Km 9,1, 28034 Madrid, Spain. Phone: 34 91 336 8542. Fax: 34 91 336 8809. E-mail: rosacampo@yahoo.com.
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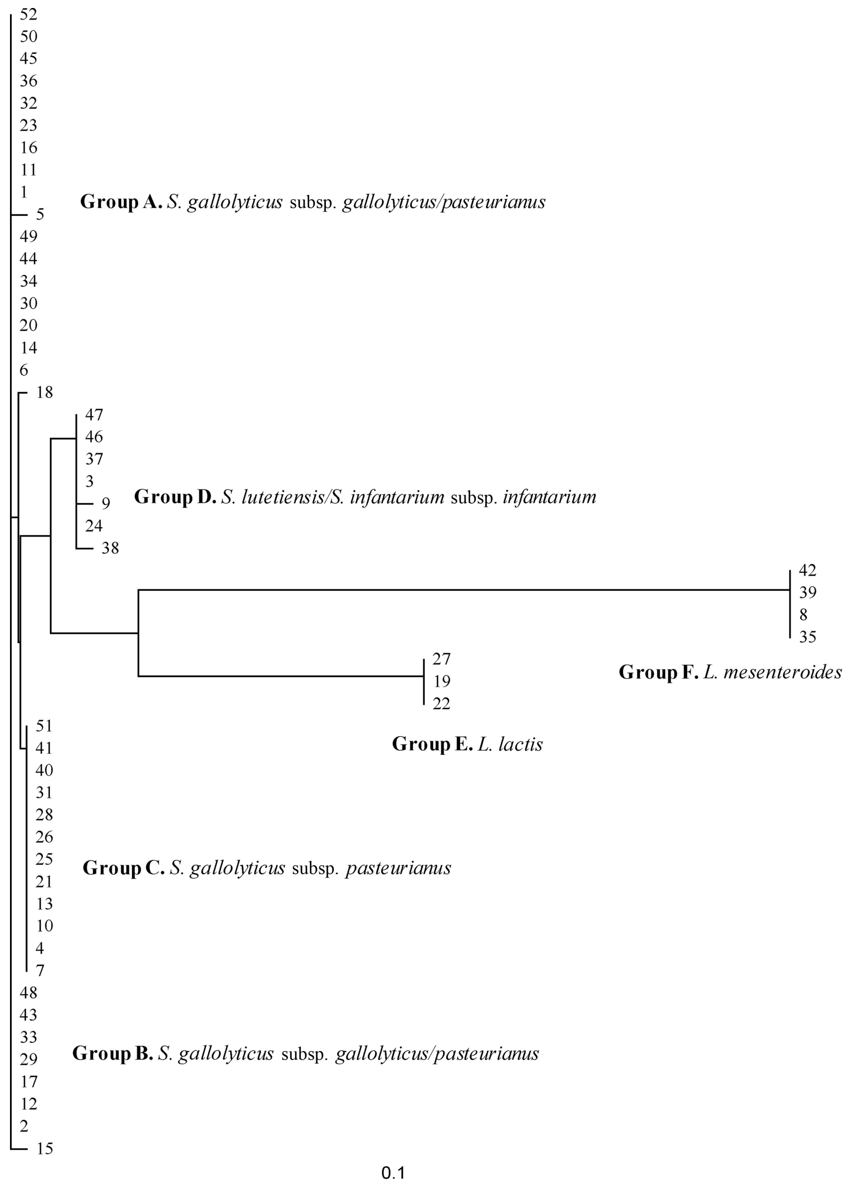

FIG. 1. Clustering of 16S rRNA nucleotide sequences obtained from all 52 isolates and constructed by TreeView software.

Antimicrobial susceptibility testing. Susceptibility testing (microdilution method) was performed using Wider panels for Gram-positive organisms, and the results were interpreted according to Clinical and Laboratory Standards Institute criteria (5, 6).

Genetic diversity. Clonal relatedness was determined by pulsed-field gel electrophoresis (PFGE) using a protocol initially described for *Streptococcus suis* serotype 2 (15). A dendrogram was constructed using the Phoretix (version 5.0) software (Nonlinear Dynamics Ltd., United Kingdom) on the basis of the Dice coefficient.

Patients. Clinical charts of all patients were reviewed with the approval of the Ethics Committee of the Hospital Universitario Ramón y Cajal to assess both demographic and clinical data as well as diagnostics and treatments. All patients in whom colonic pathologies were suspected were submitted to examination by colonoscopy. A possible biliary source of bacteremia was assigned if there was a clinical or surgical diagnosis of acute cholecystitis or cholangitis, after the exclusion of other possible foci of infection.

RESULTS

Isolates previously identified as *Streptococcus bovis* were reidentified using the current nomenclature. All 52 isolates, including the nonstreptococcal ones, had a clear positive reaction with the latex agglutination test for group D *Streptococcus*. Initial data from the API 20 Strep system were not available, and contemporary profiles corresponded to 29 *S. bovis* II/2 isolates (which included 24 *S. gallolyticus* subsp. *pasteurianus* and 5 *S. lutetiensis* isolates, according to the *sodA* identification), 14 *S. bovis* I isolates, 2 *S. bovis* II/4 isolates, 3 *Lactococcus lactis* isolates, and 4 *Leuconostoc* sp. isolates.

Nucleotide sequences of 16S rRNA amplicons (500 bp) allowed the classification of the isolates as *Streptococcus gallolyticus* subsp. *gallolyticus* ($n = 14$), *Streptococcus gallolyticus* subsp. *pasteurianus* ($n = 24$), *Streptococcus* spp. ($n = 7$), *Lactococcus lactis* ($n = 3$), and *Leuconostoc mesenteroides* ($n = 4$) (Fig. 1).

Isolates were also reclassified on the basis of the nucleotide sequence of an internal fragment of the *sodA* gene as *S. gal-*

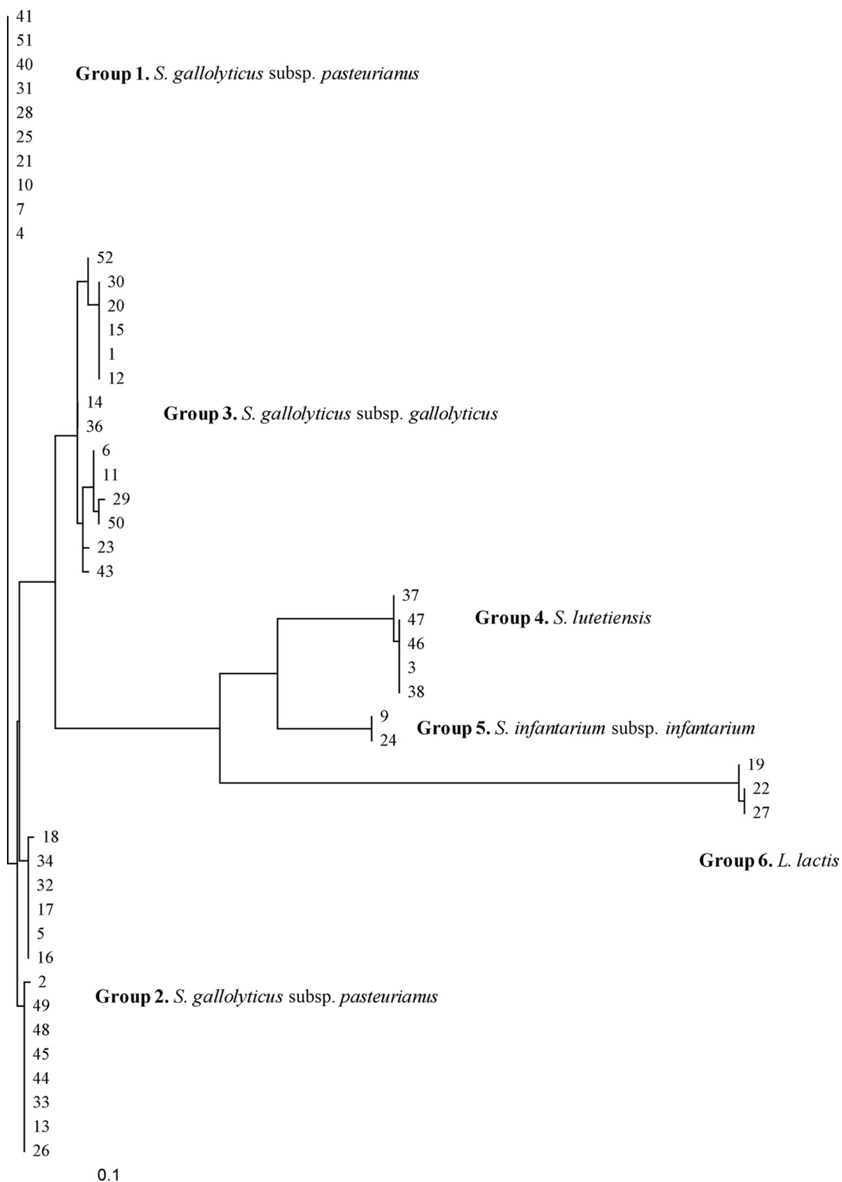

FIG. 2. Clustering of *sodA* nucleotide sequences obtained from non-*Leuconostoc* isolates ($n = 48$) and constructed by TreeView software.

lolyticus subsp. *gallolyticus* ($n = 14$), *S. gallolyticus* subsp. *pasteurianus* ($n = 24$), *Streptococcus infantarius* subsp. *infantarius* ($n = 2$), *Streptococcus lutetiensis* ($n = 5$), and *L. lactis* ($n = 3$). The seven *Streptococcus* sp. isolates not identified at the species level by the 16S rRNA sequence analysis were characterized as *S. infantarius* subsp. *infantarius* and *S. lutetiensis* using *sodA* gene amplification and sequencing. Negative results for *sodA* amplification were consistently obtained in the 4 isolates previously identified as *L. mesenteroides* by 16S rRNA sequencing. The *sodA* nucleotide phylogenetic analysis discriminated the *S. gallolyticus* subsp. *pasteurianus* isolates into two clusters (Fig. 2), although the amino acid sequences remained identical (Fig. 3). However, several amino acid differences were detected in the *S. gallolyticus* subsp. *gallolyticus* group.

Finally, MALDI-TOF MS identified 27 *S. gallolyticus* isolates but not subspecies, 4 *L. mesenteroides* isolates, 3 *L. lactis* isolates, and 6 *S. lutetiensis* isolates, whereas in 12 isolates a nonreliable identification result was obtained.

PFGE analysis with SmaI digestion grouped all *S. gallolyticus* subsp. *gallolyticus* isolates into 3 major clusters clearly different from those of the *S. gallolyticus* subsp. *pasteurianus* isolates, which, in turn, exhibited no related PFGE patterns among them (Fig. 4).

Antimicrobial susceptibility results are shown in Table 1. All isolates remained susceptible to penicillin, ampicillin, amoxicillin-clavulanate, oxacillin, quinupristin-dalfopristin, linezolid, and rifampin. Resistance to the glycopeptides vancomycin and teicoplanin was observed only in *L. mesenteroides* isolates. As expected, all streptococcal isolates ($n = 45$) showed low-level resistance to aminoglycosides. Additionally, 16 isolates exhibited high-level resistance (MIC_{50}, >1,000 mg/liter) to streptomycin, whereas high-level resistance to gentamicin was not

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41 NVNAALEKHPEIGDDLEALLADVDSIPTDIRQAVINNGGGHLNHALFWELLSPEKQEPTTQVLAAIEEAFGSFDEFKAAFTQAATTRFGSGWAWLVVNENGKLEVLSTANQDTPISQGKAP
18
34
2
49
52              E             A                               A        D
30      F       E             A                T              A        D
14              E             A                               A
6               E             A                               A
29              E             A                               A                  V
23              EA         N  A                               A
43              E             A                               V
37  A          L     VI   L K  A            A   S    F         ADV                V  K                KDD    I       N   E  K
46  A          L     VI   L K  A            A   S    F         ADV                V  K                KDD    I       N   E  K
9   A          L     VI  EL K  A            A   S              AD           ED  T                    KD     T       T L E  K
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FIG. 3. Comparison of the amino acid sequences for the different *sodA* alleles of the *sodA* protein detected.

observed. Resistance percentages for the other antimicrobials tested varied according to the bacterial species (Table 1).

When clinical data from all 51 patients with documented *S. bovis* bacteremia were considered, the patients' median age was 73.5 ± 15 years (range, 27 to 98 years; mode, 82 years). The gender distribution was 28 males (54.9%) and 23 females (45.1%). A mean of 6.5 episodes per year was documented, with the maximum (n = 10 episodes) occurring in 2005. An accumulation of cases between April and June (n = 21) was observed, with the incidence from October to December being lower (n = 7). Blood samples for culture were obtained in different wards, with the emergency unit being the most frequent (51%), followed by the general medicine unit (16%), intensive care unit (10%), gastroenterology unit (8%), and others (15%). The most frequent symptoms at hospital admission were fever and abdominal pain.

The patients' underlying diseases are shown in Table 2. The most common feature detected was hepatobiliary disorder

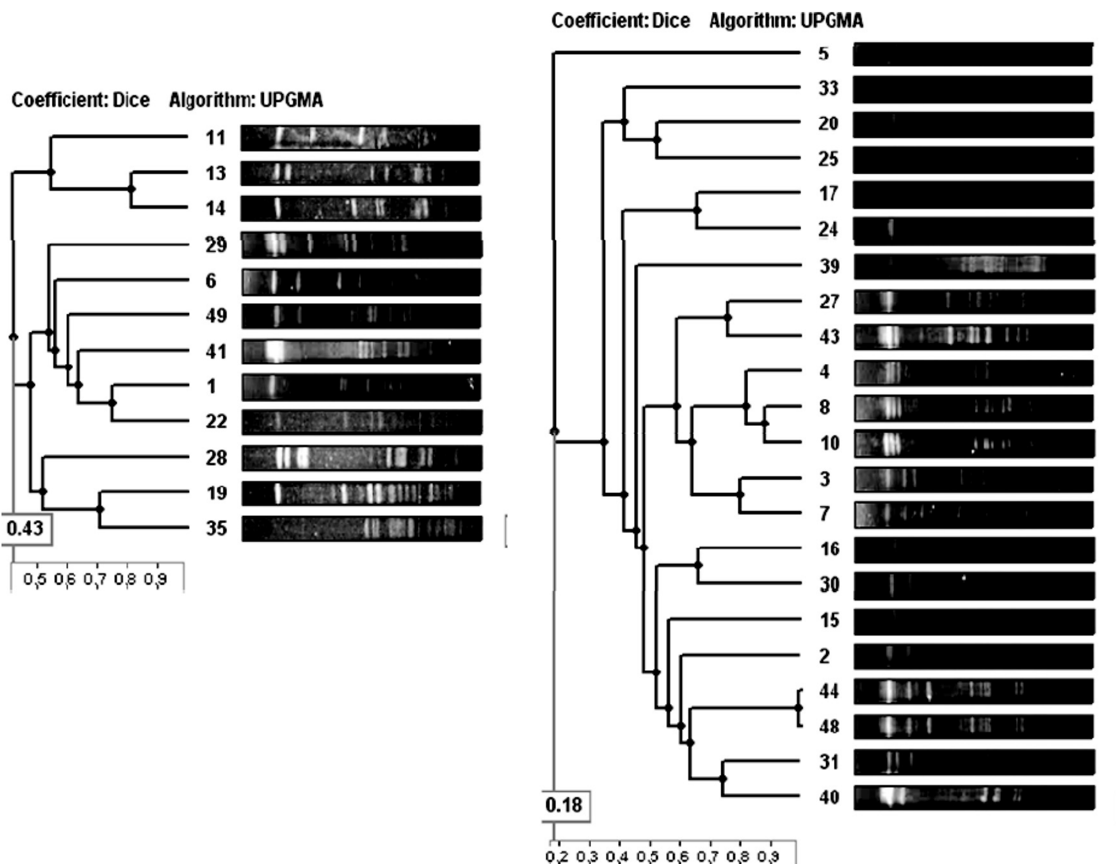

FIG. 4. Dendrogram obtained for the 24 *S. gallolyticus* subsp. *pasteurianus* and 14 *S. gallolyticus* subsp. *gallolyticus* isolates using the unweighted-pair group method using average linkages method and Phoretix (version 5.0) software.

TABLE 1. Antibiotic susceptibility for the different species studied

Species	% isolates antibiotic resistant (MIC_{50})[a]					
	Str	Lev	Ery	Cln	Fos	SxT
S. gallolyticus subsp. *gallolyticus* (*n* = 14)	42.8 (≤1,000)	14.2 (2)	35.7 (≤0.5)	35.7 (≤0.5)	35.7 (≤32)	35.7 (≤1/19)
S. gallolyticus subsp. *pasteurianus* (*n* = 24)	20.8 (≤1,000)	8.3 (≤1)	37.5 (≤0.5)	25.0 (≤0.5)	29.1 (≤32)	25.0 (≤1/19)
S. infantarius subsp. *infantarius* (*n* = 2)	0 (≤1,000)	0 (≤1)	0 (≤0.5)	0 (≤0.5)	0 (≤32)	0 (≤1/19)
S. lutetiensis (*n* = 5)	60.0 (>1,000)	40.0 (2)	60.0 (>2)	60.0 (>2)	20.0 (≤32)	20.0 (≤1/19)
L. lactis (*n* = 3)	66.6 (>1,000)	0 (2)	33.3 (≤0.5)	33.3 (≤0.5)	66.6 (>64)	66.6 (>2/38)
L. mesenteroides (*n* = 4)	0 (≤0.25)	0 (2)	50.0 (≤0.5)	25.0 (≤0.5)	25.0 (≤0.32)	25.0 (≤1/19)

[a] High-level resistance, MIC_{50} > 1,000 mg/liter. Str, streptomycin; Lev, levofloxacin; Ery, erythromycin; Cln, clindamycin; Fos, fosfomycin; SxT, trimethoprim-sulfamethoxazole. The MIC_{50}s, presented in parentheses, are in mg/liter.

(53%), followed by endocarditis (17.6%), cardiovascular disease (11.7%), diverticulitis and/or colon polyps (11.7%), digestive tract carcinomas (8%), ferropenic anemia (6%), and other malignancies (4%). Only one patient died during the bacteremic episode. Interestingly, a female had two different bacteremic episodes separated by 3 years, and these were caused by genetically different *S. gallolyticus* subsp. *pasteurianus* isolates.

DISCUSSION

The former *S. bovis* group is, at present, divided into four major subspecies (12), with two of them (*S. gallolyticus* subsp. *gallolyticus*, formerly *S. bovis* biotype I, and *S. gallolyticus* subsp. *pasteurianus*, formerly *S. bovis* biotype II/2) being genetically closely related and the other two (*S. infantarius* subsp. *infantarius* and *S. lutetiensis*, named *S. infantarius* subsp. *coli* by some authors [2] and formerly *S. bovis* biotype II/1) being more distantly related. *Streptococcus gallolyticus* subsp. *macedonicus* is also a member of the group, but it is generally considered nonpathogenic for humans. However, mainly due to discrepancies still remaining, a deeply genetics-based classification is awaited.

A clear association between *S. gallolyticus* subsp. *gallolyticus* causing bacteremia and/or endocarditis and the presence of colon cancer has been reported (1, 4, 11). Nevertheless, the basis of this association is still not completely understood. In this study, half of the patients had hepatobiliary disorders, whereas endocarditis or cardiovascular diseases were observed in only 17.6 and 11.7% of the patients, respectively. Colonic polyps and carcinomas of the gastrointestinal tract were diagnosed in 11.7 and 8% of the patients, respectively. When only those patients with *S. gallolyticus* subsp. *gallolyticus* bacteremia (*n* = 14) were considered, malignances had previously been diagnosed in 7 of them (50%), although some of them were not related to the gastrointestinal tract (lymphoma, leukemia, or prostate carcinoma), even though colonoscopy exploration was not performed in all patients.

Different authors suggest a positive association between *S. gallolyticus* subsp. *gallolyticus* isolation and male and elderly patients and endocarditis (7, 18). In our case, the gender distribution was balanced (55% of male individuals), the age of presentation was about 70 years, and endocarditis was detected in only 17% of patients. Our results are in concordance with those recently published by another Spanish group (9).

During the study period, 76,089 blood samples from patients with suspected bacteremia were processed in our laboratory. From these, 6,935 (9.1%) were positive, and 52 of them ren-

TABLE 2. Patients' clinical features

Clinical condition or finding	No. of patients					
	S. gallolyticus subsp. *gallolyticus*	*S. gallolyticus* subsp. *pasteurianus*	*S. lutetiensis*	*S. infantarius* subsp. *infantarius*	*L. lactis*	*L. mesenteroides*
Cholecystitis/cholangitis	3	5	1			
Cirrhosis	2	10	1		1	1
HCV[a]		5	1		1	
Acute appendicitis		1				
Colonic adenoma	1	4				1
Colonic polyps	2	7		1		
Rectal carcinoma	1					
Diabetes		2				
Ischemic cardiopathy		3	1	1		1
Endocarditis	1	6				
Mitral stenosis		1				
Renal insufficiency		1				
Urinary tract infection	1				1	
Bladder cancer			1			
Prostate cancer	1					
Pulmonary cancer			1			
Mucosa-associated lymphoid tissue lymphoma	1					
Leukemia	1					

[a] HCV, hepatitis C virus.

dered an *S. bovis* identification (0.74%), although considering the data from our present work, only 45 isolates in fact corresponded to this species (0.64%). During the same period, 270 cases of infective endocarditis were diagnosed at the Hospital Universitario Ramón y Cajal, and these were mostly due to *Staphylococcus aureus* (30.7%), coagulase-negative staphylococci (17.4%), and *Enterococcus* (13.3%). *S. bovis* was identified in 7 cases (6 *S. gallolyticus* subsp. *pasteurianus* isolates and 1 *S. gallolyticus* subsp. *gallolyticus* isolate), representing 2.6% of all cases of endocarditis (E. Navas et al., unpublished results).

Surprisingly, 4 *Leuconostoc mesenteroides* and 3 *Lactococcus lactis* isolates causing bacteremia were initially identified as *S. bovis* by the semiautomated system used in our laboratory. When reidentification was obtained by 16S rRNA gene sequencing, the bacteria were reidentified using the same system, and it was observed that the misleading identification was due to their slow biochemical reactivity; at 24 h of incubation the identification was again *S. bovis*, whereas at 48 h, the identification was correct. Moreover, vancomycin resistance in *Leuconostoc* isolates was observed only at 48 h. Another important factor to be considered is the positive agglutination of our *Leuconostoc* and *Lactococcus* isolates for Lancefield antigen D. We were unable to find similar references to this finding, and this fact has been demonstrated only for *Pediococcus* sp. isolates (16).

As it has been described by other authors (13, 19), we detected differences in isolate identification between the 16S rRNA and the *sodA* gene sequencing methods, with partial sequencing of the *sodA* gene being considered the most accurate method of identification. Recently, a high concordance between MALDI-TOF MS and *sodA* nucleotide sequencing has been described (12), although in our experience, the Bruker technology is not yet able to identify *Streptococcus* isolates, especially *S. gallolyticus* subsp. *pasteurianus*. The results obtained with the contemporary API 20 Strep system agree with the *sodA* sequencing classifications, although 5 *S. lutetiensis* isolates showed the same profile as the S. *gallolyticus* subsp. *pasteurianus* isolates. Nevertheless, the API 20 Strep system can be considered a useful method to discriminate the two main *S. gallolyticus* subspecies, but from clinical and epidemiological points of view, it could also be of interest to further discriminate *S. infantarius* subsp. *infantarius* and *S. lutetiensis* using the *sodA* analysis.

In spite of many efforts that have been made to improve *S. bovis* group identification, data from genetic studies remain unavailable. The multilocus sequence typing tool has not yet been developed, and there has been only one reference describing high genetic diversity among Italian *S. bovis* isolates, which was determined by PFGE (22). In our experience, high genetic diversity was also observed among the 45 streptococcal isolates.

Our study demonstrated that in a routine clinical microbiology laboratory, incorporation of a MALDI-TOF MS system will partially solve the problems with identification of members of the *S. bovis* group. Nevertheless, this system will indicate the presence of *S. gallolyticus* and further molecular testing can be performed if needed. The presence of data entries in the database for different *S. gallolyticus* subspecies should be a goal for future MALDI-TOF MS systems for testing of other species.

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