Elsevier Editorial System(tm) for Diagnostic Microbiology and Infectious Disease
Manuscript Draft

Manuscript Number: DMID-16-552R1

Title: Identification, antimicrobial resistance and molecular characterization of the human emerging pathogen Streptococcus gallolyticus subsp. pasteurianus

Article Type: Original Article

Keywords: Streptococcus gallolyticus subsp. pasteurianus, infections, identification, MALDI-TOF, antibiotic resistance, PFGE.

Corresponding Author: Prof. giovanni gherardi,

Corresponding Author's Institution: università campus biomedico

First Author: giovanni gherardi

Order of Authors: giovanni gherardi; claudio palmieri; emanuela marini; arianna pompilio; valentina crocetta; giovanni di bonaventura; roberta creti; bruna facinelli

Abstract: This study aimed to retrospectively identify 22 Streptococcus bovis clinical strains based on the new taxonomy, as well as to investigate their antibiotic-resistance and clonality.
Strains were identified by Phoenix100 system, 16S rRNA sequencing, and two MALDI-TOF MS platforms (Bruker Biotyper, Vitek MS). Antibiotic resistance was determined both phenotypically and genotypically, and clonality was assessed by PFGE.
Most of strains (63.6%) were isolated from urine, and diabetes was the most common underlying disease (31.8%). Phoenix100 system revealed all strains belonged to biotype II, and 16S rRNA sequencing identified all strains as S. gallolyticus subsp pasteurianus (SGSP). Although both MALDI-TOF MS systems correctly identified isolates to the species level, only Bruker Biotyper accurately identified to the subspecies level. Erythromycin-resistant strains (31.8%) were also clindamycin-resistant and positive for erm(B). Strains resistant to tetracycline (68.2%) were also resistant to erythromycin. PFGE showed high genetic variability identifying 17 different pulsotypes, most of which single.

Rome, 18 September 2016

Dear Prof Mendes,

please find enclosed the revised version of the manuscript Ref. No.: DMID-16-552 entitled "**Identification, antimicrobial resistance and molecular characterization of the human emerging pathogen *Streptococcus gallolyticus subsp. pasteurianus***". The authors want to thank the reviewers for their comments and suggestions. We have addressed all the issues raised and we feel that the manuscript has greatly benefited from the modifications made.

Along with the revised marked-up version of the manuscript and Figure 1, with all changes highlighted in yallow, please find submitted a point-by-point replies to the Reviewers' comments highlighted in blue italics.

Thanking you for your consideration, we look forward to hearing from you.

Sincerely,

Giovanni Gherardi

Giovanni Gherardi, PhD, Department of Medicine, Campus Biomedico University, Via Alvaro del Portillo 200, 00128 Rome, Italy. Phone: +39 06 22541 1136. Fax: +39 06 22541 456. E-mail: g.gherardi@unicampus.it.

*Response to Reviewers

Rome, 18 September 2016

Dear Prof Mendes,

please find enclosed the revised version of the manuscript Ref. No.: DMID-16-552 entitled "**Identification, antimicrobial resistance and molecular characterization of the human emerging pathogen *Streptococcus gallolyticus subsp. pasteurianus***". The authors want to thank the reviewers for their comments and suggestions. We have addressed all the issues raised and we feel that the manuscript has greatly benefited from the modifications made.

Along with the revised marked-up version of the manuscript and Figure 1, with all changes highlighted in yallow, please find submitted a point-by-point replies to the Reviewers' comments highlighted in blue italics.

Thanking you for your consideration, we look forward to hearing from you.

Sincerely,

Giovanni Gherardi

Giovanni Gherardi, PhD, Department of Medicine, Campus Biomedico University, Via Alvaro del Portillo 200, 00128 Rome, Italy. Phone: +39 06 22541 1136. Fax: +39 06 22541 456. E-mail: g.gherardi@unicampus.it.

Point-by-point responses to Reviewers' comments:

Reviewer #1:

Specific points are listed below

Major point

1. As the authors stated, the isolate numbers are small. Were all S. bovis isolates recovered during the study period included? Also, the starting time of the isolates differed between L95 and L101 (May 2010 to Jan 2012) and Table 1 (Dec 2009 to Jan 2012). Maybe the authors can expand their study period.

As we stated in the manuscript, the number of isolates is small. However, considering the rarity of isolation of S. bovis from clinical specimens, we believe that the number of isolates we analyzed is congruent enough to obtain some important observations, as we did. We thank the Reviewer for the discrepancy due to an error on the typing in the Table 1 and, therefore, we modified the Table accordingly (page 20, Table 1).

2. L110-L136, the long detailed description on MALDI-TOF data generating procedure is not needed and identification criteria can be shortened considerably. Just highlight parts that are pertinent to ID results.

According to the Reviewer's suggestion we shortened and modified the text relative to MALDI TOF identification, also following the recommendation of Reviewer #2 (page 5, paragraph 2.1, lines 103-115).

3. Since MLST is relatively new for this species and since 17 distinct PFGE types were seen in the 22 isolates studied, the author should consider performing MLST to correlate with PFGE results. The information may provide additional data on the clonality of SGSP.

The Reviewer's observation could be correct since MLST analysis could provide additional data on the clonality of SGSP analyzed in this study. As stated on page 11, lines 263-265 ("Discrepant results might be related to the different typing methods used; different PFGE patterns might depend by the presence of genomic islands probably acquired from other streptococci") we cannot exclude that different PFGE types could share identical, or closely related, ST by MLST. However, this study was first set up to gain information on identification and antimicrobial resistance features of S. bovis strains available, and then we chose PFGE analysis to assess their genetic relatedness. We believe that PFGE analysis is a reliable tool to give a picture on genetic variability of SGSP strains. Moreover, we decided to perform PFGE due to the long-lasting technical experience in this molecular typing method of the group involved in this study and due to the high costs of MLST. All of these are the reasons led us to choose PFGE instead of MLST.

4. L181 - 182 in Results. The authors reported that "The most common underlying condition was diabetes (40%), followed by ischemic cardiopathy (20%) (Table 1)". However, according to Table 1, 6 patients (30%) had cancer, 2 others had myeloproliferative (not mieloproliferaive) disorder, and another had lymphoma. In light of the recent reports linking S. bovis, including SGSP, to different malignancies, the authors should look into their cases more carefully on this association. The authors should also consider careful statistical analysis for clinical data.

In some cases, SGSP isolation was obtained by patients having different malignancies as underlying diseases. Although cancer is more frequently associated with S. bovis group - mainly S. gallolyticus subsp gallolyticus - it has been reported that also SGSP is linked to different malignancies. We looked into these cases when necessary. In fact, we found that the cancer most frequently associated with SGSP isolation was the biliary tract cancer and, for this reason, we decided to discuss this finding as you can see on pages 9-10 , lines 222-225, where we state that "A significant prevalence was found also for biliary tract cancer, thus suggesting the previously described association between SGSP and biliary tract disease (Corredoira et al., 2014; Lee et al., 2003; Sheng et al., 2014), since all strains from bile were associated with biliary tract malignancies.". Moreover, lymphoma was found in a patient with SGSP bacteremia, but in this patient endocarditis was also diagnosed and we think that this latter condition was more important (see page 10, lines 228-229: "…both SGSP bacteraemia were found in elderly patients with endocarditis, and patients died during the bacteremic episodes.").

Statistical analysis was performed to evaluate differences in prevalence of the underlying diseases by calculating the Clopper-Pearson Exact confidence interval (page 7 paragraph 2.5, lines 151-154; page 8, paragraph 3.1, lines 172-174; pages 9-10, Discussion, lines 222-224).

5. L 226-L229 in Discussion, the authors stated that "In this study, most of patients with SGSP bacteriuria were female, thus confirming an association between gender and SGSP isolation in the urinary tract (Matesanz et al., 2015). We found diabetes as the most common underlying condition, thus in agreement with previous studies (Matesanz et al., 2015; Sheng et al., 2014)". First, it is Matsubara, not Matesanz. Second, the report by Matsubara et al was on a case of bacteremia in a boy. Third, the other pediatric SGSP cases summarized by the Matsubara et al study were all from blood and CSF, not urine. Fourth, Matesanz's report did not mention underlying conditions. Finally, the study by Sheng et al focused on association of SGSP bacteremia and malignancies. These inaccuracies bring doubts on other potential problems in the manuscript.

We think that the statement is accurate. In fact the study of Matesanz et al on SGSP isolated from urine reports that "…in the final analysis, 153 adult patients were included, who had a mean age of 67 years [standard deviation (SD) ±20.4; range 14–100 years) and most of them were women (80 %). Most of our patients (65 %) had some underlying disease and 40 % had more than one; the most common (37 %) was urologic disease, followed by diabetes mellitus (27 %),..". Thus, this reference is correct/adequate: an association of gender (women) and SGSP isolation in the urinary tract was found and diabetes was a common underlying condition in patients with SGSP isolation. The paper of Sheng et al. on bacteraemic SGSP reports that: "...those with bacteraemia due to S. gallolyticus subspecies gallolyticus were younger (median age, 59 years vs. 70 years, P Z 0.04) and fewer patients had diabetes mellitus (10% vs. 34%, P Z

0.009) than patients with bacteraemia due to S. gallolyticus subspecies pasteurianus....". Therefore, also this reference is correct/adequate: SGSP was frequently found in patients with bacteraemia that had diabetes mellitus. The Reviewer mentions the paper of Matsubara et al: we believe that this study, a case report on the first isolation of a SGSP that can cause invasive infection in a child older than 3 months, has nothing to do with the statement indicated by the Reviewer.

6. L303-305 in Discussion, what do the authors mean by "the most common methods used in clinical laboratory correctly identified all strains to the species and subspecies level"?
It means that the methods we used, that are also currently the most common methods used in clinical microbiology laboratories (e.g., MALDI TOF), were able to identify S. bovis isolates to the species and subspecies level accurately and consistently. We modified this part according to the reviewer's observation to better clarify it (page 12, Discussion, lines 296-298).

Careful English editing is needed.
We thank Reviewer for this suggestion. We have sent the manuscript to a native English speaker to improve the language.

Below are some (but not all) examples with revisions suggested or problems underlined
1. L47, Resistance to erythromycin and tetracycline was also studied by PCR, looking for …", change to " .. by PCR to look for"
Done, as suggested (page 6, paragraph 2.3, line 135).

2. L68," …bacteremia, highly associated with colorectal cancer"
Done, as suggested (page 3, Introduction, line 68).

3. L80-82, ".. in many clinical microbiology laboratories until becoming the primary method for…"
Done, as suggested (page 4, Introduction, lines 83-84).

4. L88, All 22 isolates resulted positive for group D Streptococcus by the latex agglutination test". Change "resulted" to "were".
Done, as suggested (page 8, paragraph 3.2, line 181).

5. L217, Accordingly to the finding
Done, as suggested (page 9, Discussion, line 210).

6. L234-235, "both cases of SGSP bacteremia regarded elderly patients with endocarditis and fatal outcome"
Done, as suggested (page 10, Discussion, lines 228-229).

7. L267 " prevalently classified into "
Done, as suggested (page 11, Discussion, line 260).

8. L303, Nevertheless, some interesting findings arose from our findings
Done, as suggested (page 12, Discussion, line 296).

9. L206-207, almost one third of the isolates resulted to be both erythromycin and clindamycin -resistant .."
Done, as suggested (page 13, Discussion, line 301).

Reviewer #2:

The authors should clarify some details:

1. Why was the 16S rRNA gene chosen when previous studies have shown that other gene targets (eg. sodA) may be more accurate?

We have chosen 16S rRNA as target to identify S. bovis group isolates because the partial sequencing of the 16S rRNA gene is generally considered adequate for most routine clinical bacterial identification. The use of partial or complete gene sequencing of 16S rRNA has been largely the most prevalent single-target gene used among S. bovis, and specifically among SGSP (Li et al, Vet Microbiol 2013, 162:930-936; Hede et al, J Clin Microbiol 2015: 53:1419-1422; Corredoira et al, Clin Microbiol Infect 2014, 20:405-409; Su et al, J Clin Microbiol 2013, 51:4249-4251; Sheng et al, J Infect 2014, 69:145-153; Matsubara et al, Jpn J Infect Dis 2015, 68:251-253; Beck et al, J Clin Microbiol 2008, 46:2966-2972). All these papers reported that 16S rRNA gene sequencing was able to identify S. bovis isolates at the species- and subspecies-level.

Nevertheless, 16S rRNA gene sequencing is not always able to discriminate among S. bovis subspecies and, for this reason, other target genes have been proposed such as groEL, sodA, and rnpB. In some cases, the gene sequences of these markers have allowed an even more detailed phylogenetic relationships than 16S rRNA gene. Accordingly, Romero et al (Romero et al, J Clin Microbiol 2011, 49:3228-3233) found that both 16S RNA and sodA genes showed excellent and identical performances for the identification of 14 SGSG and 24 SGSP.

In our case, the partial sequencing of the 16S rRNA genes of our strains displayed 100% sequence identity with the reference SGSP strain 906 (accession n. EU163502.1) and with several other SGSP strains available in the GenBank database. Conversely, the sequence homology was 99.7% or even less with other SGSG strains on a shorter sequence length. Indeed, by using the database suggested by Reviewer #3 (https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi) our strains felt into the SGSP cluster (see point 3, Reviewer #3).

Noteworthy, it has been reported that, there are four nucleotide positions in the 16S RNA gene that are distinctive for SGSG and SGSP (Beck et al. "Comprehensive study of strains previously designated Streptococcus bovis consecutively isolated from human blood cultures and emended description of Streptococcus gallolyticus and Streptococcus infantarius subsp. coli". J Clin Microbiol. 2008 Sep;46:2966-72.). Our sequenced portions of the 16S rRNA gene sequences comprised two out four of these signatures (pos. 182 and pos. 238) and they are coherent with the SGSP affiliation.

We suggested that the combined use of a single-gene-based molecular testing (in our case, the 16S rRNA gene) and MALDI-TOF MS can represent a reliable choice for the species- and subspecies- level discrimination.

Other gene targets (single or multiple) can be sequenced to have more chances to increase the confidence level of the identification accuracy, when possible.

Nevertheless, our findings demonstrated that:

1. all strains were identified as belonging to S. bovis biotype II by using Phoenix 100;

2. all strains were identified as SGSP by using MALDI Biotyper;

3. by 16S rRNA partial gene sequencing, all strains showed complete identity with SGSP strains compared to a less identity percentage on a shorter length with SGSG strains. This finding convinced us that the combination of multiple test methods we used were sufficiently accurate and consistent to identify our S. bovis group isolates at the species- and subspecies-level. As we stated at the end of the manuscript, "the combination of proteomic and molecular methods allowed the classification of the S. bovis isolates as SGSP". The main message for clinical microbiologists that can face with this circumstance is that "the application of multiple identification methods along with the clinical presentation of the patient are, therefore, critical factors that need to be carefully considered" (page 13, lines 305-307).

2. The authors should provide details on BioTyper and Vitek MS database versions as this will affect the level of accuracy achieved.

We thank the precious reviewer's recommendation and, therefore, we added in the text the details on Biotyper Bruker and Vitek MS databases used in this study (page, paragraph 2.1, lines 103-115).

3. Lines 116-8: Did the authors run into instances where multiple different species or subspecies scored >2.0? How did the authors discern BioTyper identification?

By Bruker Biotyper, only subspecies SGSP was found to have an identification score $\geq$ 2. In particular, all isolates showed a score of $\geq$ 2.2 for SGSP, with a score < 1.8 for SGSG; thus, according to the identification criteria recommended by the manufacturer and used in our study (identifications with scores $\geq$ 2 were

considered to be reliable at the species- and subspecies-level , identifications with scores between 1.7 and 1.9 were considered to be reliable only at genus levels) we concluded that all isolates could be considered as belonging to SGSP subspecies.

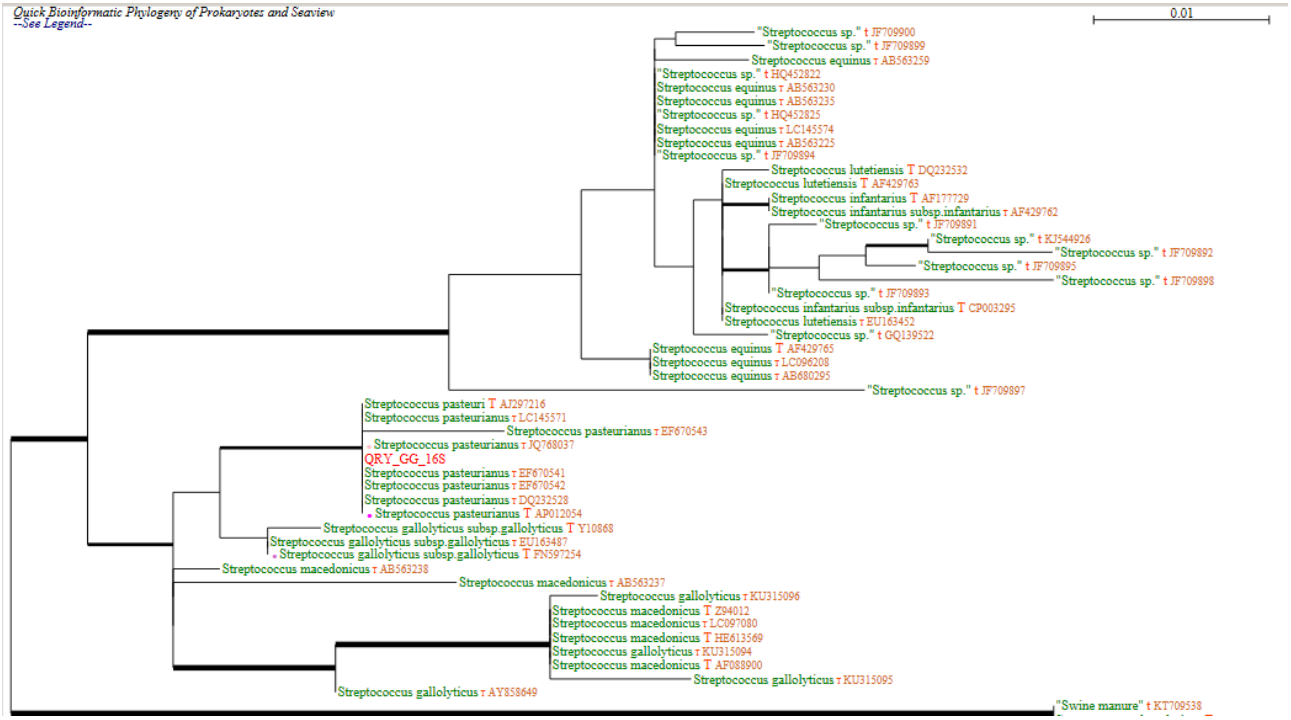
4. Lines 243-6 and 303-5: The authors should rephrase because 16S rRNA is not always considered the gold standard for accurate speciation and suspeciation of the S. bovis group, and other members of the S. bovis group were not analyzed in this study.
According to the Reviewer's suggestion, we modified the text (page 10, Discussion, lines 237-240; page 12, Discussion, lines 296-298).

Reviewer #3:

1. Lines 85-93, if this paragraph was meant to address resistance in several drug classes, it seems very superficial. Authors mentioned "…while variable resistance rates have been observed for clindamycin, erythromycin, tetracycline and levofloxacin". However, after this sentence, authors address only erythromycin and MLSb resistance phenotype, so it seems incomplete. I would suggest authors to either expand or suppress lines 88-93, since these mechanisms are well known.
We decided to omit this part, accordingly.

2. As authors described in line 78-79, "PCR and sequencing of 16S rRNA, rnpB, groEL, and sodA, with different and, in some cases, contradictory results", why at least one target sequence in addition to 16S rRNA was not utilized. There only 22 isolates it should not be much and can provide confirmatory results. Also, is 16S the most appropriate target?
Please, see response to point 1 of the reviewer #2, and response to point 3 below. We believe that the combined use of 16S rRNA gene sequencing and MALDI TOF allowed the identification of all S. bovis isolates as SGSP.

3. Did the authors make use of a curated database (e.g. bibi; https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi) rather than NCBI for bacterial identification?
We thank the Reviewer for the precious suggestion of this curated database. The phylogenetic tree constructed by this database using the stringency option, where SGSG and SGSP clustered into two separated and well-defined groups, revealed that our strains felt into the SGSP cluster (in particular, the closest sequence based on patristic distances is Streptococcus_pasteurianus T AP012054 and belongs to the proximal cluster, see the attached figure below). This finding confirmed the validity of 16S rRNA gene sequencing we used to identify our S. bovis isolates to the species and subspecies-level. (page 5, paragraph 2.1, lines 101-103; page 8, paragraph 3.2, lines 185-188).



4. Lines 110-136, are all those details necessary for the reader. I would tend to suppress whatever is associated with regular use following the manufacturer's instructions.
We eliminated all this part, as suggested (please, see response to major point 2 of reviewer #1) (page 5, paragraph 2.1, lines 103-115).

5. Lines 146-149, any particular for screening of those selected genes. Authors may be aware of additional resistance determinants (e.g. http://faculty.washington.edu/marilynr/).
We agree with the reviewer's observation and we modified the text accordingly (page 6, paragraph 2.3, line 136).

6. Item 2.4, common knowledge. Please, condense. Just explain how types and subtypes were assigned.
We thank the reviewer for this suggestion and, therefore, we shortened this part (page 6, paragraph 2.4, lines 140-141).

7. Lines 194-195, I would suggest to refrain for interpreting the identification results i.e. delete the words "correct".
We deleted the words, as suggested.

8. Line 218, if cMLSb it should be ery and clinda-resistant?
We corrected the sentence, accordingly (page 9, paragraph 3.4, lines 200-201).

9. Item 3.3, this reviewer is having a hard time following, with the exception of the initial first sentence. Authors may want to simplify and take the reader to Table wwhere results are presented. Again just explain how types and subtypes were assigned.
We shortened this part accordingly (page 8, paragraph 3.3, lines 195-196).

10. Line 218, sentence "…most of SGSP isolates were from urine, 3 of which consecutively isolated from the same patient" may refer to a patient having persistent/recurrent infection, as demonstrated by the PFGE results. Therefore, it should not be considered.
The total of isolates were 22, and we found that 3 isolates from the same patients showed indistinguishable PFGE profile. For these reason, we considered only one of these 3 genetically indistinguishable strains

when we considered the underlying conditions, sources, and all other clinical characteristics of patients (see Table 1).

11. Line 220, the polymicrobial information is buried in the manuscript (line 181). As this comes back in the discussion, authors should mention other organisms recovered. Please add additional information.
We added this information, as suggested (page 7, paragraph 3.1, lines 169-171).

12. Line 243, "better performance" may not be the best word (this was not evaluated). I would suggest perhaps "further indentified to subspecies level".
We modified this part, according to the reviewer's suggestion (page 10, Discussion, line 237).

13. Line 263, again having trouble understanding PFGE results ie "and 3 multiple-strain PFGE groups".
We thank the reviewer for this observation and we modified the text accordingly (page 11, Discussion, lines 255-257).

Identification, antimicrobial resistance and molecular characterization of the human emerging pathogen *Streptococcus gallolyticus* subsp. *pasteurianus*

Running title: *S. gallolyticus* subsp. *pasteurianus* in humans

Word counts. Abstract: 150; Text: 3234

Giovanni Gherardi[a*], Claudio Palmieri[b], Emanuela Marini[b], Arianna Pompilio[c], Valentina Crocetta[c], Giovanni Di Bonaventura[c], Roberta Creti[d], Bruna Facinelli[b]

[a] Department of Medicine, Campus Biomedico University, Via Alvaro del Portillo 200, 00128 Rome, Italy.

[b] Department of Biomedical Sciences and Public Health, Unit of Microbiology, Polytechnic University of Marche, Via Tronto 10/A, 60123 Ancona, Italy.

[c] Department of Medical, Oral and Biotechnological Sciences; and Center of Excellence on Aging and Translational Medicine (CeSI-MeT); "G. d'Annunzio" University of Chieti, Via Vestini 31, 66100 Chieti, Italy

[d] Department of Infectious, Parasitic, and Immunomediated Diseases, Istituto Superiore di Sanità, Viale Regina Margherita 299, 00161 Rome, Italy

[*]Corresponding author: Prof Giovanni Gherardi, Centro Integrato di Ricerca (CIR), Università Campus Biomedico, Via Alvaro del Portillo 200, 00128 Rome, Italy, e-mail: g.gherardi@unicampus.it, Phone number: +39 06 225411136, Fax number: +39 06 22541456

ABSTRACT

This study aimed to retrospectively identify 22 *Streptococcus bovis* clinical strains based on the new taxonomy, as well as to investigate their antibiotic-resistance and clonality.

Strains were identified by Phoenix100 system, 16S rRNA sequencing, and two MALDI-TOF MS platforms (Bruker Biotyper, Vitek MS). Antibiotic resistance was determined both phenotypically and genotypically, and clonality was assessed by PFGE.

Most of strains (63.6%) were isolated from urine, and diabetes was the most common underlying disease (31.8%). Phoenix100 system revealed all strains belonged to biotype II, and 16S rRNA sequencing identified all strains as *S. gallolyticus subsp pasteurianus* (SGSP). Although both MALDI-TOF MS systems correctly identified isolates to the species level, only Bruker Biotyper accurately identified to the subspecies level. Erythromycin-resistant strains (31.8%) were also clindamycin-resistant and positive for *erm*(B). Strains resistant to tetracycline (68.2%) were also resistant to erythromycin. PFGE showed high genetic variability identifying 17 different pulsotypes, most of which single.

Keywords: *Streptococcus gallolyticus* subsp. *pasteurianus*, infections, identification, MALDI-TOF, antibiotic resistance, PFGE.

1. Introduction

Streptococcus bovis, a nonenterococcal group D *Streptococcus*, is a commensal inhabitant of the human gastrointestinal tract in 5% to 16% of individuals (Noble, 1978). The association of *S. bovis* bacteremia and colon tumours was established in the late 1970s (Klein et al, 1977) and it has been extensively reported in the literature (Burnett-Hartman et al., 2008; Gupta et al., 2010). *S. bovis* is also responsible for infective endocarditis worldwide, particularly in southern Europe, with the prevalence rising in elderly patients (Durante-Mangoni et al., 2008). An association between isolation of *S. bovis* and chronic liver and biliary tract disorders has been also described (Gonzlez-Quintela et al., 2001).

Traditionally, *S. bovis* has been grouped into three biotypes: I (mannitol-positive), II/1 (mannitol- and glucuronidase-negative), and II/2 (mannitol-negative, glucuronidase-positive) (Dekker and Lau, 2016; Facklam, 2002; Ruoff et al., 1989). Streptococcal taxonomy has progressively changed and using the scheme proposed by Schlegel et al. (2003), that is based on DNA studies, currently comprises 7 (sub)species grouped into four branches, with two *Streptococcus* species of principal interest in human pathogenesis: *S. gallolyticus* - with the subspecies *S. gallolyticus* subsp. *gallolyticus* (SGSG, formerly biotype I), and *S. gallolyticus* subsp. *pasteurianus* (SGSP, formerly biotype II/2) - and *S. infantarius* (formerly biotype II/1), with the subspecies *coli* and *infantarius* (Dekker and Lau, 2016; Poyart et al., 2002).

The identity of *S. bovis* strains in human diseases has not been systematically investigated using modern taxonomy. Moreover, clinicians still remain unfamiliar with the new taxonomy of *S. bovis* species. Considering the specific disease association and microbiology features, an accurate identification of the *S. bovis* isolates is mandatory. In fact, after the introduction of the new nomenclature of *S. bovis* strains, it became clear that SGSG represent the major cause of infective endocarditis and bacteremia, the latter that is often associated with colorectal cancer (Boleij et al, 2011a; Boleij et al., 2011b; Vaska and Faoagali, 2009). SGSP seem instead related to

immunosuppressive comorbidities, polymicrobial bacteraemia and concomitant biliary-pancreatic diseases, urinary tract infection (UTI), osteoarticular infections, and meningitis, mostly in elderly patients (Corredoira et al., 2014; Dekker and Lau, 2016; Fernandez-Ruiz et al., 2010; García-País et al., 2016; Marmolin et al., 2016; Matesanz et al., 2015; Romero et al., 2011; van Samkar et al., 2015).

Phenotypic biochemical methods have been largely used for streptococci identification in routine diagnostic laboratories, though they are time-consuming and have limited differentiation capacity due to phenotypic trait variability (Isaksson et al., 2015; Teles et al., 2011). Several molecular methods have been therefore developed to improve species identification of streptococci, such as PCR and sequencing of 16S rRNA, *rnpB*, *groEL*, and *sodA*, with different and, in some cases, contradictory results (Dekker and Lau, 2016; Glazunova et al., 2009; Hoshino et al., 2005; Isaksson et al., 2015; Teles et al., 2011). In recent years, Matrix-Assisted Laser system Desorption Ionization–Time Of Flight Mass Spectrometry (MALDI-TOF MS) technique has gained considerable interest in many clinical microbiology laboratories, becoming the primary method for bacterial species identification, with a performance comparable or even higher than molecular methods (Seng et al., 2010; Wieser et al., 2012).

Susceptibility to penicillin and vancomycin in *S. bovis* group has remained relatively stable over the years, while variable resistance rates have been observed for clindamycin, erythromycin, tetracycline and levofloxacin (Beck et al., 2008; Romero et al., 2011).

The aim of this study was to retrospectively identify by new taxonomy criteria 22 *S. bovis* isolates recovered at a University Hospital in Rome from May 2010 to January 2012, and to investigate their antibiotic resistance traits and genetic diversity.

2. Materials and Methods

2.1. Bacterial identification

A total of 22 *S. bovis* isolates, collected from 20 patients between May 2010 and January 2012, were studied. Identification of *S. bovis* species was routinely performed using the automated Phoenix100 system (Becton Dickinson [BD], Sparks, MD, USA), and isolates were tested for the presence of the Lancefield streptococcal antigen D (bioMérieux Slidex Strepto Plus kits). All *S. bovis* strains were retrospectively identified by 16S rRNA gene sequencing using universal primers (Edwards et al., 1989) and BlastN research of homologies. Moreover, the website https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi was also used to compare the 16S rRNA gene sequences of our strains to these present into the database. Identification was also performed by MALDI-TOF MS using Bruker Biotyper software package 3.1 with BDAL-5989 database (Bruker Daltonics, Bremen, Germany) and Vitek MS v2.3.3 system (bioMérieux, Marcy l'Etoile, France), according the manufacturers' recommendation, using the full extraction procedure as previously described (Bizzini et al., 2010; Davies et al., 2012; Rychert et al., 2013). The mass spectra generated were compared with the reference spectra BDAL-5989 database, that includes a total of 5989 entries, 5291 of bacterial species, and consists of 294 different spectral profiles within the Streptococcus genus, and specifically 3, 2, and 6 profiles for *S. gallolyticus*, SGSG, and SGSP, respectively. The identification criteria used in our analysis, as outlined by the manufacturer, were as follows: a score of ≥ 2 indicated identification to species level, a score between 1.7 and 1.9 indicated identification to genus level, and a score <1.7 was interpreted as unreliable identification.

The Vitek MS database V2.3.3, allows 35 identifications of subspecies, species, or species group within the Streptococcus genus and it can distinguish the two subspecies SGSG and SGSP. The overall correct and incorrect identification was defined as follows: (i) correct identification to the subspecies level, when the system proposed the reference species identification as a single choice to the subspecies level, with confidence value between 60% and 99.9%, (ii) correct identification to the species level, when the system proposed the reference species identification of the same species with low discrimination to the subspecies level (between 25% and 50%), (iii) correct identification to the genus level, when the system proposed the reference species identification among a set of low

discrimination results including species of the same genera, and (iv) incorrect identification to both species and genus level, when the system proposed the reference species identification among a set of low-discrimination results including species of different genera.

2.2. Susceptibility testing

Susceptibility to penicillin, cefotaxime, vancomycin, meropenem, erythromycin, clindamycin, and tetracycline was performed using the automated Phoenix system for Gram-positive organisms, and the results were interpreted according to EUCAST criteria (www.eucast.org). Resistance to erythromycin and clindamycin was also phenotypically assessed by the Kirby-Bauer double disk diffusion method to assign the $cMLS_B$, $iMLS_B$ and M macrolide resistance phenotypes (Imperi et al., 2011).

2.3. Antibiotic resistance genes

Resistance to erythromycin and tetracycline was also studied by PCR to look for the presence of the antibiotic resistance genes commonly found so far among *S. bovis* group isolates *erm*(A), *erm*(B), *mef*(A), *tet*(M) and *tet*(O), as previously described (Imperi et al., 2011).

2.4. Genetic relatedness

Clonality was determined by Pulsed-Field Gel Electrophoresis (PFGE) essentially as previously described by Tripodi et al.(2005). PFGE patterns were assigned designations following the type/subtype definition according to the previously described criteria (Tenover et al., 1995): isolates with identical profiles were assigned to the same PFGE type and subtype; isolates with similar profiles (i.e., differing by 1 to 5 bands) were assigned to different subtypes within the same PFGE type. PFGE types were also analyzed with Bionumerics software for Windows (version 2.5; Applied Maths, Ghent, Belgium). Comparison was performed by the unweighted pair group method with arithmetic averages (UPGMA) and with the Dice similarity coefficient applying a 1.5%

tolerance in band position. Isolates with a percentage of similarity ≥ 80% resulted to be genetically related thus belonging to the same PFGE cluster.

2.5. Statistical analysis

Differences in prevalence of underlying conditions observed in SGSP-positive patients were statistically evaluated by calculating the Clopper-Pearson Exact confidence interval for the observed proportions. Statistical significance was set at p value < 0.01.

3. Results

3.1. Clinical data

Clinical charts of 20 patients with documented isolation of *S. bovis* were reviewed to assess both demographic and clinical data (Table 1). The majority of *S. bovis* strains were isolated from urine (14 isolates), followed by 3 isolates from bile, 2 isolates from blood, and one isolate from a diabetic leg ulcer. The patients' average age was 72.25 years (range 38 to 91 years). The gender distribution was 7 males (35%) and 13 females (65%) (Table 1). Among 14 patients with bacteriuria, 11 were inpatients and 3 were outpatients. One patient showed recurrent urinary tract infection in three different episodes, seven months apart. Ten out of 14 cases of bacteriuria were UTIs, as demonstrated by clinical symptoms (dysuria, urgency, and/or frequency, and/or fever, and/or back pain) and by the analysis of urinary sediment, with bacteriuria, urinary esterase, and leucocyturia. The remaining 4 cases were asymptomatic bacteriuria. Overall, 5 episodes of bacteriuria were polymicrobial, where *S. bovis* was simultaneously detected with other 2 or more microorganisms, such as *Escherichia coli* (4 cases), *Enterococcus faecalis* (2 cases), *Proteus mirabilis* (1 case), *Staphylococcus epidermidis* (1 case).

The underlying condition with the highest prevalence was diabetes (40%; CI95%: 19.1-63.9, $p<0.01$), followed by ischemic cardiopathy (20%; CI95%: 5.7-43.6, $p<0.01$), biliary tract cancer and cirrhosis (both at 15%; CI95%: 3.2-37.9, $p<0.01$) (Table 1).

Overall, five out of 14 patients (35.7%) where a SGSP was isolated from urine specimens presented with diabetes. The two cases found with SGSP bacteremia were associated with endocarditis, and both patients died during the bacteremic episodes. Moreover, all three cases of SGSP isolated from bile were associated with biliary tract cancer (Table 1).

3.2. Identification

All 22 isolates were positive for group D *Streptococcus* by the latex agglutination test. Initial identification by the automated Phoenix system revealed that all isolates belonged to *S. bovis* biotype II (Table 2). Nucleotide sequences of 16S rRNA amplicons (900 bp) classified all *S. bovis* biotype II isolates as SGSP, showing 100% nucleotide identity to accession number EU163502.1, corresponding to 16S rRNA gene of S. pasteurianus reference strain 906 (Table 2). Moreover, the phylogenetic tree provided by using the curated website (https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi) with the stringency option, where SGSG and SGSP clustered into two separated and well-defined groups, revealed that our strains felt into the SGSP cluster.

A score of > 2 was obtained for all isolates by MALDI Biotyper, properly identifying the isolates as SGSP (Table 2). By Vitek MS, a total of 6 isolates showed identification to the subspecies level as SGSP, with level of confidence of 99.99%; the remaining 16 isolates showed identification to the species level, as *S. gallolyticus*, with low discrimination to the subspecies level (Table 2).

3.3. PFGE genotyping

Overall, 17 different PFGE types were identified among 22 strains, with 3 PFGE groups, that comprised ≥ 2 genetically related strains (Figure 1, Table 3).

3.4. Antibiotic-resistant phenotypes and genotypes

Antimicrobial susceptibility results are shown in Table 3. All isolates were susceptible to penicillin, cefotaxime, vancomycin, meropenem, and chloramphenicol. Seven strains (31.8%) were both erythromycin- and clindamycin-resistant, belonged to the cMLS$_B$ phenotype, and carried *erm*(B) gene. Fifteen strains (68.2%) were tetracycline-resistant: of these, 11 strains (73.3%) carried *tet*(O) and 4 strains carried *tet*(M). All erythromycin-resistant isolates were also resistant to tetracycline.

4. Discussion

In this study, we retrospectively analysed 22 *S. bovis* isolates with the aim to properly identify strains to the species level by the methods currently used in microbiology laboratory, and to investigate their phenotypic and genotypic antibiotic resistance traits and genetic variability.

The revised taxonomy allowed a more precise association between human infections and specific *S. bovis* species (Fernandez-Ruiz et al., 2010). According to the finding that SGSP can be responsible for UTIs (Matesanz et al., 2015), most of SGSP isolates were from urine, 3 of which consecutively isolated from the same patient. A difficult issue in our retrospectively study was to determine the clinical significance of SGSP bacteriuria. Moreover, the co-isolation of SGSP with other microorganisms made questionable their etiological role. Nevertheless, 10 out of 14 patients presenting with SGSP isolation from urine showed clinical symptoms and urinary sediment suggestive for an ongoing infection, and 5 out of 10 patients had monomicrobial bacteriuria. Therefore, although uncommon, SGSP bacteriuria should not always be considered as a contaminant, especially in adult patients.

In this study, most of patients with SGSP bacteriuria were female, thus confirming an association between gender and SGSP isolation in the urinary tract (Matesanz et al., 2015).

We found diabetes as the most common underlying condition, thus in agreement with previous studies (Matesanz et al., 2015; Sheng et al., 2014). A significant prevalence was found also for

biliary tract cancer, thus suggesting the previously described association between SGSP and biliary tract disease (Corredoira et al., 2014; Lee et al., 2003; Sheng et al., 2014), since all strains from bile were associated with biliary tract malignancies. Invasive infection due to SGSP causes, mainly in the elderly, a variety of clinical manifestations, including bacteremia, endocarditis, osteomyelitis, peritonitis, solid organ abscess, and meningitis (Jans et al., 2015; Sheng et al., 2014). In agreement with these observations, both cases of SGSP bacteraemia were found in elderly patients with endocarditis, and patients died during the bacteraemic episodes.

The association between *S. bovis* subspecies and specific pathogenesis strongly suggests the importance of a precise diagnosis and species/subspecies identification. MALDI-TOF MS has recently become the primary technique for microbial species identification, mainly because it is very fast and cheap (Seng et al., 2010; Wieser et al., 2012). In this study, we therefore tested the performance of two MALDI-TOF platforms - namely Biotyper Bruker, and Vitek MS - in identifying *S. bovis* isolates to the subspecies level, comparatively with phenotypic and genotypic methods. Our findings indicated that both systems correctly identified *S. bovis* group isolates, although Biotyper Bruker further identified strains to the subspecies level. Partial sequencing of 16S rRNA gene and Biotyper Bruker allowed the identification of all strains of our collection to the subspecies level as belonging to SGSP, while Vitek MS identified strains to the species level, *S. gallolyitcus*, but only 6 isolates showed identification to the subspecies level.

Our results confirmed that accuracy of MALDI-TOF MS in species identification is highly dependent on the system used and on the spectral databases and algorithms employed (Dekker and Lau, 2016). In some cases, the accuracy is limited to identify a specific bacterial complex or group, as observed for the *S. bovis* group. Romero et al. (2011) found a limitation of MALDI Bruker Biotyper in discriminating the two main *S. gallolyticus* subspecies. Conversely, Hinse et al. (2011) published a reliable method for identifying isolates of the *S. bovis* group to the subspecies level by MALDI-TOF and *sodA* DNA sequencing. Similarly, another study confirmed the usefulness of MALDI Biotyper technology to properly discriminate between SGSG and SGSP (López Roa et al.,

2013). A recent study that used the 16S RNA gene sequencing as gold standard found that both MALDI Biotyper and Vitek MS systems were reliable and accurate in clinical diagnostics for streptococcal identification, including *S. bovis* species (Kärpänoja et al., 2014). In another study, *rnpB* gene sequencing and both MALDI Bruker Biotyper and Vitek MS showed excellent resolving power with full agreement in the identification of bacteraemic streptococcal species, comprising *S. bovis* isolates (Isaksson et al., 2015).

Only few studies have been reported on the molecular epidemiology of the *S. bovis* group. PFGE analysis revealed a high genetic variability, showing a total of 17 different PFGE groups among SGSP strains studied. Our results are consistent with previous studies (Romero et al., 2011; Tripodi et al., 2005). Particularly, an Italian study on bacteraemic *S. bovis* isolates reported 4 unrelated and unique PFGE types among 4 *S. bovis* biotype II/2, presumably SGSP (Tripodi et al., 2005). On the contrary, in another study SGSP isolates from several origins and sources mainly classified into the major single cluster ST14 by MLST (Shibata et al., 2014). A variant of ST14 was also found in a SGSP isolated from a transient bacteraemia in a 3-year old child, thus suggesting the diffusion of a successful clone causing human infections (Matsubara et al., 2015). Discrepant results might be related to the different typing methods used; different PFGE patterns might depend by the presence of genomic islands probably acquired from other streptococci (Jans et al., 2015).

Antimicrobial susceptibility testing indicated that all SGSP isolates of our study were susceptible to penicillin, cefotaxime, vancomycin, meropenem, and chloramphenicol. On the contrary, 31.8% of SGSP strains were simultaneously resistant to erythromycin and clindamycin. Similar rates were found among 24 bacteraemic SGSP isolates recovered over a 7-years period in a Spanish university hospital (Romero et al., 2011). Lower erythromycin resistance rate (approximately 9%) was found among 45 independent and not typed *S. bovis* isolates from Israel (Peretz et al., 2014). Higher erythromycin (ranging from 55 to 78%) and clindamycin (ranging from 51% to 72%) resistance rates were observed in *S. bovis* group, regardless of the species or subspecies involved (Leclercq et al., 2005; Lee et al., 2003; Matesanz et al., 2015; Rodriguez-Avial et al., 2005; Sheng et al., 2014).

We found that all erythromycin-resistant isolates displayed also resistance to clindamycin, and showed the $cMLS_B$ resistance phenotype, carried by the *erm*(B) gene. Similarly, in France the erythromycin resistance was found to be mainly based on the presence of *erm*(B) and rarely on *mef*(A) (Leclercq et al., 2005). Another study from Taiwan found that 21 out of 38 erythromycin-resistant *S. bovis* blood isolates displayed the $iMLS_B$ resistance encoded either by *erm*(B) or *erm*(T), although the majority of isolates belonged to SGSG (Teng et al., 2001). Additionally, 15 SGSP strains in our study were tetracycline-resistant, accounting for 68.2% of all strains, with approximately 50% of tetracycline-resistant isolates that were resistant to 3 different classes of antibiotics (erythromycin, clindamycin, and tetracycline). The majority of tetracycline-resistant strains carried *tet*(O), while a minority harboured *tet*(M).

Taken together, these findings indicated that antibiotic resistance was widespread among the *S. bovis* clinical isolates thus representing a serious problem also considering the emerging infection rates. *S. bovis* reside in the gastrointestinal tract where they can interact with many multi-resistant microbes (Sommer et al., 2009); consequently, the horizontal transfer of antibiotic resistance genes might occur, thus rendering the continuous monitoring of resistance profiles of the outmost importance.

This study showed some limitations: i) its retrospective observational nature, where patients did not receive the same diagnostic procedures or tests; ii) most of patients did not undergo to echocardiography as well as colonoscopy, and management and investigation were mainly based on the clinical assessment; therefore, incomplete diagnoses cannot be excluded; iii) the small sampling size of isolates and the finding of only SGSP subspecies.

Nevertheless, some interesting findings arose from this study: i) the methods in this study (MALDI TOF and 16S rRNA sequencing), that represent the most common methods used in clinical laboratory, allowed the identification of all strains to the species and subspecies level, although with some differences; ii) SGSP isolation is higher in adult females, mostly from the urinary tract, and diabetes was the most common patients' underlying disease; iii) almost one third of the isolates

were both erythromycin- and clindamycin-resistant, all carrying *erm*(B), and almost 70% of isolates were tetracycline-resistant, most of which harboring *tet*(O); and iv) isolates genotyped by PFGE showed a high genetic variability.

In conclusion, the combination of proteomic and molecular methods allowed the classification of the *S. bovis* isolates as SGSP. The application of multiple identification methods along with the clinical presentation of the patient are, therefore, critical factors that need to be carefully considered. The specific disease associations of SGSP described in this study underscore the paramount importance of accurate species- and subspecies-level identification of clinical *S. bovis* isolates.

Funding sources

This work was partly supported by the Italian Ministry of Education, University and Research [PRIN CBSB9Y, 2008].

References

Beck M, Frodl R, Funke G. Comprehensive study of strains previously designated *Streptococcus bovis* consecutively isolated from human blood cultures and emended description of *Streptococcus gallolyticus* and *Streptococcus infantarius subsp. coli*. J Clin Microbiol 2008;46:2966-72.

Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G. Performance of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J Clin Microbiol 2010;48:1549-54.

Boleij A, Muytjens CM, Bukhari SI, Cayet N, Glaser P, Hermans PW, et al. Novel clues on the specific association of *Streptococcus gallolyticus subsp gallolyticus* with colorectal cancer. J Infect Dis 2011;203(8):1101-9.

Boleij A, van Gelder MM, Swinkels DW, Tjalsma H. Clinical Importance of *Streptococcus gallolyticus* infection among colorectal cancer patients: systematic review and meta-analysis. Clin Infect Dis 2011;53(9):870-8.

Burnett-Hartman AN, Newcomb PA, Potter JD. Infectious agents and colorectal cancer: a review of *Helicobacter pylori*, *Streptococcus bovis*, JC virus and human papillomavirus. Cancer Epidemiol Biomarkers Prev 2008;17:2970e9.

Corredoira J, Alonso MP, García-Garrote F, García-Pais MJ, Coira A, Rabuñal R, et al. *Streptococcus bovis* group and biliary tract infections: an analysis of 51 cases. Clin Microbiol Infect 2014;20(5):405-9.

Davies AP, Reid M, Hadfield SJ, Johnston S, Mikhail J, Harris LG, et al. Identification of clinical isolates of α-hemolytic streptococci by 16S rRNA gene sequencing, matrix-assisted laser desorption ionization-time of flight mass spectrometry using MALDI Biotyper, and conventional phenotypic methods: a comparison. J Clin Microbiol 2012;50(12):4087-90.

Dekker JP, Lau AF. An Update on the *Streptococcus bovis* group: Classification, Identification, and Disease Associations J Clin Microbiol 2016; pii: JCM.02977-15. [Epub ahead of print].

Durante-Mangoni E, Bradley S, Selton-Suty C, Tripodi MF, Barsic B, Bouza E, et al. Current features of infective endocarditis in elderly patients: results of the International Collaboration on Endocarditis Prospective Cohort Study. Arch Intern Med 2008;168:2095e103.

Edwards U, Rogall T, Blocker H, Emde M, Bottger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 1989;17:7843-53.

Facklam R. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin Microbiol Rev 2002;15:613-630.

Fernandez-Ruiz, M., Villar-Silva, J., Llenas-Garcia, J., Caurcel-Diaz, L., Vila-Santos, J., Sanz-Sanz, F., et al. *Streptococcus bovis* bacteraemia revisited: clinical and microbiological correlates in a contemporary series of 59 patients. J Infect 2010;61:307-13.

García-País MJ, Rabuñal R, Armesto V, López-Reboiro M, García-Garrote F, Coira A, et al. *Streptococcus bovis* septic arthritis and osteomyelitis: A report of 21 cases and a literature review. Semin Arthritis Rheum 2016;45(6):738-46.

Glazunova OO, Raoult D, and Roux V. Partial sequence comparison of the *rpoB, sodA, groEL* and *gyrB* genes within the genus *Streptococcus*. Int J Syst Evol Microbiol 2009;59:2317-22.

Gonzlez-Quintela A, Martínez-Rey C, Castroagudín JF, Rajo-Iglesias MC, Domínguez-Santalla MJ. Prevalence of liver disease in patients with *Streptococcus bovis* bacteraemia. J Infect 2001;42:116-9.

Gupta A, Madani R, Mukhtar H. *Streptococcus bovis* endocarditis; a silent sign for colonic tumour. Colorectal Dis 2010;12:164e71.

Hinse D, Vollmer T, Erhard M, Welker M, Moore ER, Kleesiek K, et al. Differentiation of species of the *Streptococcus bovis/equinus*-complex by MALDI-TOF Mass Spectrometry in comparison to *sodA* sequence analyses. Syst Appl Microbiol 2011;34(1):52-7.

Hoshino T, Fujiwara T, Kilian M. Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. J Clin Microbiol 2005;43:6073-85.

Imperi M, Gherardi G, Berardi A, Baldassarri L, Pataracchia M, Dicuonzo G, et al. Invasive neonatal GBS infections from an area-based surveillance study in Italy. Clin Microbiol Infect 2011;17(12):1834-9.

Isaksson J, Rasmussen M, Nilson B, Stadler LS, Kurland S, Olaison L, et al. Comparison of species identification of endocarditis associated viridans streptococci using *rnpB* genotyping and 2 MALDI-TOF systems. Diagn Microbiol Infect Dis 2015;81(4):240-5.

Jans C, Meile L, Lacroix C, Stevens MJ. Genomics, evolution, and molecular epidemiology of the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC). Infect Genet Evol 2015;33:419-36.

Kärpänoja P, Harju I, Rantakokko-Jalava K, Haanperä M, Sarkkinen H. Evaluation of two matrix-assisted laser desorption ionization-time of flight mass spectrometry systems for identification of viridans group streptococci. Eur J Clin Microbiol Infect Dis 2014;33(5):779-88.

Klein RS, Recco RA, Catalano MT, Edberg SC, Casey JI, Steigbigel NH. Association of *Streptococcus bovis* with carcinoma of the colon. N Engl J Med 1977;297:800-2.

Leclercq, R. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin Infect Dis 2002;34:482-92.

Leclercq R, Huet C, Picherot M, Trieu-Cuot P, Poyart C. Genetic basis of antibiotic resistance in clinical isolates of *Streptococcus gallolyticus* (*Streptococcus bovis*). Antimicrob. Agents Chemother 2005;49:1646-8.

Lee RA, Woo PCY, To APC, Lau SKP, Wong SSY, Yuen KY. Geographical difference of disease association in *Streptococcus bovis* bacteraemia. J Med Microbiol 2003;52:903-8.

López Roa P, Sánchez Carrillo C, Marín M, Romero F, Cercenado E, Bouza E. Value of matrix-assisted laser desorption ionization-time of flight for routine identification of viridans group streptococci causing bloodstream infections. Clin Microbiol Infect 2013;19(5):438-44.

Marmolin ES, Hartmeyer GN, Christensen JJ, Nielsen XC, Dargis R, Skov MN, et al. Bacteremia with the bovis group streptococci: species identification and association with infective endocarditis and with gastrointestinal disease. Diagn Microbiol Infect Dis 2016;85(2):239-42.

Matesanz M, Rubal D, Iñiguez I, Rabuñal R, García-Garrote F, Coira A, et al. *Is Streptococcus bovis* a urinary pathogen? Eur J Clin Microbiol Infect Dis 2015;34(4):719-25.

Matsubara K, Takegawa H, Sakizono K, Nomoto R, Yamamoto G, Osawa R. Transient Bacteremia Due to *Streptococcus gallolyticus Subsp. pasteurianus* in a 3-Year-Old Infant. Jpn J Infect Dis 2015;68(3):251-3.

Noble, C. J. Carriage of group D streptococci in the human bowel. J Clin Pathol 1978;31:1182-6.

Peretz A, Dinisman-Zavulunov E, Koifman A, Brodsky D, Isakovich N, Glyatman T, et al. Susceptibility of 45 *Streptococcus bovis* isolates to five antibiotic agents. Int J Antimicrob Agents 2014;43:298-9.

Poyart C, Quesne G, Trieu-Cuot P. Taxonomic dissection of the *Streptococcus bovis* group by analysis of manganese-dependent superoxide dismutase gene (*sodA*) sequences: reclassification of

‘*Streptococcus infantarius* subsp. *coli* ’ as *Streptococcus lutetiensis* sp. nov. and of *Streptococcus bovis* biotype II.2 as *Streptococcus pasteurianus* sp. nov. Int J Syst Evol Microbiol 2002;52:1247-55.

Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, Seppala H. Nomenclature for macrolide and macrolide-lincosamidestreptogramin B resistance determinants. Antimicrob Agents Chemother 1999;43:2823-30.

Rodriguez-Avial I, Rodriguez-Avial C, Culebras E, Picazo JJ. In vitro activity of telithromycin against viridans group streptococci and *Streptococcus bovis* isolated from blood: antimicrobial susceptibility patterns in different groups of species. Antimicrob Agents Chemother 2005;49:820-3.

Romero B, Morosini MI, Loza E, Rodríguez-Baños M, Navas E, Cantón R, et al. Reidentification of *Streptococcus bovis* isolates causing bacteremia according to the new taxonomy criteria: still an issue? J Clin Microbiol 2011;49:3228-33.

Ruoff KL, Miller SI, Garner CV, Ferraro MJ, Calderwood SB. Bacteremia with *Streptococcus bovis* and *Streptococcus salivarius*: clinical correlates of more accurate identification of isolates. J Clin Microbiol 1989;27:305-8.

Rychert J, Burnham CA, Bythrow M, Garner OB, Ginocchio CC, Jennemann R, et al. Multicenter evaluation of the Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for identification of Gram-positive aerobic bacteria. J Clin Microbiol 2013;51(7):2225-31.

Schlegel L, Grimont F, Ageron E, Grimont PAD, Bouvet A. Reappraisal of the taxonomy of the *Streptococcus bovis*/*Streptococcus equinus* complex and related species: description of *Streptococcus gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *macedonicus* subsp. nov. and S. *gallolyticus* subsp. *pasteurianus* subsp. nov. Int J Syst Evol Microbiol 2003;53:631-45.

Seng P, Rolain J-M, Fournier PE, La Scola B, Drancourt M, Raoult D. MALDI-TOF-mass spectrometry applications in clinical microbiology. Future Microbiol 2010;5:1733-54.

Sheng WH, Chuang YC, Teng LJ, Hsueh PR. Bacteraemia due to *Streptococcus gallolyticus subspecies pasteurianus* is associated with digestive tract malignancies and resistance to macrolides and clindamycin. J Infect 2014;69(2):145-53.

Shibata Y, Tien le HT, Nomoto R, Osawa R. Development of a multilocus sequence typing scheme for *Streptococcus gallolyticus*. Microbiology 2014;160(Pt 1):113-22.

Sommer MO, Dantas G, Church GM. Functional characterization of the antibiotic resistance reservoir in the human microflora. Science 2009;325:1128-31.

Teles C, Smith A, Ramage G, Lang S. Identification of clinically relevant viridans group streptococci by phenotypic and genotypic analysis. Eur J Clin Microbiol Infect Dis 2011;30:243-50.

Teng LJ, Hsueh PR, Ho SW, Luh KT. High prevalence of inducible erythromycin resistance among *Streptococcus bovis* isolates in Taiwan. Antimicrob. Agents Chemother 2001;45:3362-5.

Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33(9):2233-9.

Tripodi MF, Fortunato R, Utili R, Triassi M, Zarrilli R. *Molecular epidemiology of Streptococcus bovis* causing endocarditis and bacteraemia in Italian patients. Clin Microbiol Infect 2005;11(10):814-9.

van Samkar A, Brouwer MC, Pannekoek Y, van der Ende A, van de Beek D. *Streptococcus gallolyticus* meningitis in adults: report of five cases and review of the literature. Clin Microbiol Infect 2015;21(12):1077-83.

Vaska VL, Faoagali JL. *Streptococcus bovis* bacteraemia: identification within organism complex and association with endocarditis and colonic malignancy. Pathology 2009;41:183-6.

Wieser A, Schneider L, Jung J, Schubert S. MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond. Appl Microbiol Biotechnol 2012;93:965-74.

Fig. 1. Phylogenetic analysis of PFGE profiles obtained from 22 *S. gallolyticus subsp. pasteurianus* isolates. The dendrogram was constructed from PFGE profiles by similarity and clustering analysis using the unweighted pair-group method with arithmetic averages and the Dice coefficient. The genetic similarity in percentages is showed above the dendrogram. Strain code, PFGE type and subtype, and resistance genes are marked on the right. S, susceptible to erythromycin and tetracycline.

Table 1. Clinical characteristics of patients positive for *S. gallolyticus subsp pasteurianus* isolation

Strain ID[a]	Sex, Age[b]	Underlying diseases	Source[c]	Date of isolation (mo/yr)
155-11	F, 91	none	urine/UTI	9/11
156-11	M, 71	prostate cancer	urine/UTI	9/11
57-11	F, 55	HCV[d]	urine/UTI	3/11
101-11	M, 65	ischemic cardiopathy, cirrhosis, endocarditis, colonic polyps	blood	5/11
216-10	M, 84	diabetes, ischemic cardiopathy	urine	10/10
230-10	F, 60	ischemic cardiopathy, mieloproliferative disorders	urine	11/10
79-11			urine	4/11
95-11			urine	5/11
5-12	M, 66	diabetes, ischemic cardiopathy	limb ulcer	1/12
185-11	F, 85	colonic adenoma	urine/UTI	11/11
154-11	M, 70	biliary tract cancer	bile	9/11
116-11	F, 85	diabetes, cirrhosis	urine	7/11
110-11	F, 38	ureteral stenosis	urine/UTI	6/11
91-11	M, 80	biliary tract cancer, cholecystitis/cholangitis	bile	5/11
62-11	F, 74	mieloproliferative disorders, breast cancer	urine/UTI	3/11
54-11	F, 68	renal insufficiency	urine/UTI	3/11
53-11	F, 57	diabetes	urine	2/11
19-11	M, 72	diabetes, cirrhosis, endocarditis, lymphoma	blood	1/11
242-10	F, 83	diabetes	urine/UTI	11/10
135-10	F, 82	diabetes, biliary tract cancer, cholecystitis/cholangitis	bile	7/10
124-10	F, 79	none	urine/UTI	5/10
97-09	F, 80	diabetes	urine/UTI	12/10

[a] Strains 230-10, 79-11, and 95-11 were isolated from the same patient.

[b] F, female; M, male.

[c] UTI, urinary tract infection, indicating symptomatic patients with bacteriuria.

[d] HCV, Hepatitis C virus

Table 2. Comparison of different phenotypic, proteomic, and molecular methods used to identify clinical isolates

ID strain	Source	Phoenix100	16S rDNA sequencing[a]	MALDI Biotyper Bruker[b]	Vitek MS[c]
155-11	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus* (99.99%)
156-11	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus* (99.99%)
57-11	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
101-11	blood	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
216-10	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus* (99.99%)
95-11[d]	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
79-11[d]	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
230-10[d]	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
5-12	ulcer	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
185-11	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus* (99.99%)
154-11	bile	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
116-11	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus* (99.99%)
110-11	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
91-11	bile	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
62-11	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
54-11	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
53-11	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
19-11	blood	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
242-10	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
135-10	bile	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
124-10	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus*
97-09	urine	*S. bovis* II	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus*	*S. gallolyticus* subsp *pasteurianus* (99.99%)

[a] Genotypic identification by 16S rDNA partial gene sequencing.

[b] All isolates showed a score > 2, thus providing identification to the species level.

[c] Overall, 6 isolates showed correct ID to the subspecies level, with rate of confidence of 99.99%; the remaining 16 isolates showed correct ID to the species level, as *S. gallolyticus*, with low discrimination to the subspecies level (rate of confidence less than 50%, see text for details).

[d] Strains isolated from the same patient.

Table 3. Clonal relatedness and antibiotic resistance of 22 *Streptococcus gallolyticus subsp. pasteurianus* clinical isolates

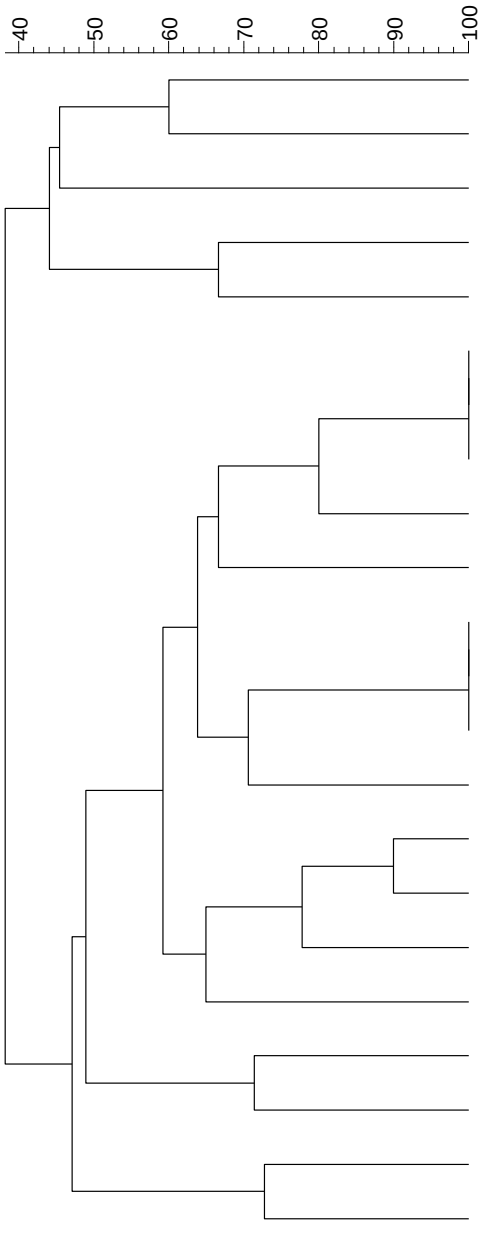
ID strain	Source	PFGE type	ERY/CLI[a] resistance (phenotype, genotype)	TET[a] resistance (phenotype, genotype)	PEN/VAN/ CTX/MER[a]
155-11	urine	1.1	S/S[b]	S	S/S/S/S
156-11	urine	1.1	S/S	S	S/S/S/S
57-11	urine	1.1	R/R[b], *erm*(B)	R, *tet*(O)	S/S/S/S
101-11	blood	2.1	S/S	R, *tet*(M)	S/S/S/S
216-10	urine	2.2	S/S	S	S/S/S/S
230-10[c]	urine	3.1	S/S	R, *tet*(O)	S/S/S/S
79-11[c]	urine	3.1	S/S	R, *tet*(O)	S/S/S/S
95-11[c]	urine	3.1	S/S	R, *tet*(O)	S/S/S/S
5-12	ulcer	4.1	R/R, *erm*(B)	R, *tet*(O)	S/S/S/S
185-11	urine	5.1	S/S	R, *tet*(O)	S/S/S/S
154-11	bile	6.1	S/S	R, *tet*(M)	S/S/S/S
116-11	urine	7.1	S/S	R, *tet*(M)	S/S/S/S
110-11	urine	8.1	R/R, *erm*(B)	R, *tet*(M)	S/S/S/S
91-11	bile	9.1	R/R, *erm*(B)	R, *tet*(O)	S/S/S/S
62-11	urine	10.1	R/R, *erm*(B)	R, *tet*(O)	S/S/S/S
54-11	urine	11.1	S/S	R, *tet*(O)	S/S/S/S
53-11	urine	12.1	R/R, *erm*(B)	R, *tet*(O)	S/S/S/S
19-11	blood	13.1	R/R, *erm*(B)	R, *tet*(O)	S/S/S/S
242-10	urine	14.1	S/S	S	S/S/S/S
135-10	bile	15.1	S/S	S	S/S/S/S
124-10	urine	16.1	S/S	S	S/S/S/S
97-09	urine	17.1	S/S	S	S/S/S/S

[a] ERY, erythromycin; CLI, clindamycin; TET, tetracycline; PEN, penicillin; VAN, vancomycin; CTX, cefotaxime; MER, meropenem.

[b] R, resistance; S, susceptibility.

[c] Clinical strains isolated from the same patient.

Figure



ID strain	PFGE type	R genes
5-12	4.1	erm(B), tet(O)
154-11	6.1	tet(M)
62-11	10.1	erm(B), tet(O)
135-10	15.1	S
97-09	17.1	S
95-11	3.1	tet(O)
79-11	3.1	tet(O)
230-10	3.1	tet(O)
116-11	7.1	tet(M)
91-11	9.1	erm(B), tet(O)
156-11	1.1	S
155-11	1.1	S
57-11	1.1	erm(B), tet(O)
110-11	8.1	erm(B), tet(M)
101-11	2.1	tet(M)
216-10	2.2	S
124-10	16.1	S
54-11	11.1	tet(O)
242-10	14.1	S
19-11	13.1	erm(B), tet(O)
185-11	5.1	tet(O)
53-11	12.1	erm(B), tet(O)

Highlights

- Phenotypic, MALDI, and 16S rRNA gene sequencing were used for *S. bovis* group members identification.

- The combination of these methods identified strains as *S. gallolyticus* subsp. *pasteurianus* (SGSP).

- SGSP isolation was most frequently associated with diabetes, among women, and from urinary tract.

- Approximately 30% of the SGSP were both erythromycin- and clindamycin-resistant, with *erm*(B).

- PFGE analysis revealed a high genetic variability among SGSP isolates.