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

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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.

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 tumors 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, 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

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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, which 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,