Veterinary Microbiology 161 (2013) 286–294

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Antimicrobial resistance and molecular epidemiology of streptococci from bovine mastitis

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ARTICLE INFO

Article history:
Received 17 September 2011
Received in revised form 27 July 2012
Accepted 27 July 2012

Keywords:
Streptococcus agalactiae
Streptococcus dysgalactiae subsp. *dysgalactiae*
Streptococcus uberis
Antimicrobial resistance
Virulence genes
Molecular serotypes

ABSTRACT

Streptococcus agalactiae (Group B *Streptococcus*, GBS), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (Group C *Streptococcus*, GCS) and *Streptococcus uberis* are relevant mastitis pathogens, a highly prevalent and costly disease in dairy industry due to antibiotherapy and loss in milk production. The aims of this study were the evaluation of antimicrobial drug resistance patterns, particularly important for streptococcal mastitis control and the identification of strain molecular features. Antimicrobial resistance was assessed by disk diffusion against amoxicillin–clavulanic acid, cefazolin, cefoperazone, pirlimycin-PRL, rifaximin, streptomycin, chloramphenicol, erythromycin-ERY, gentamicin, tetracycline-TET and vancomycin. Genotypic relationships were identified using pulsed-field gel electrophoresis (PFGE), macrolide and/or tetracycline resistance gene profiling, GBS capsular typing, GBS virulence gene profiling and GBS and *S. uberis* multi locus sequence typing (MLST).

The majority of the isolates were susceptible to all drugs except to aminoglycoside, macrolide, lincosamide and tetracycline. Close to half of the TET resistant isolates have *tetO* and *tetK* and almost all ERY–PRL resistant isolates have *ermB*. A high degree of intra-species polymorphism was found for GCS. The GBS belonged to ST-2, -554, -61, -23 lineages and five new molecular serotypes and human GBS insertion sequences in the *cpsE* gene were found. Also, GBS of serotype V with *scpB* and *lmb* seem to be related with GBS isolates of human origin (same ST-2 and similar PFGE). Overall our results suggested that different therapeutic programs may have been implemented in the different farms and that in most cases clones were herd-specific.

1. Introduction

Streptococcus agalactiae (Group B *Streptococcus*, GBS), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (Group C *Streptococcus*, GCS), and *Streptococcus uberis* are pathogens most frequently associated with bovine mastitis, a highly prevalent and costly disease for the dairy industry due to antibiotherapy, milk production loss and other costs (Erskine et al., 2003). While *S. uberis* and *S. dysgalactiae* subsp. *dysgalactiae* are considered exclusively animal pathogens (Facklam, 2002; Vieira et al., 1998), *S. agalactiae* is also a human pathogen that causes severe invasive neonatal infections, infection in pregnant women and elderly people and causes mortality in immuno-compromised adults (Bisharat et al., 2004; Brochet et al., 2006).

Surveys carried out to investigate temporal changes in the microbial panorama aim to monitor the prevalence of

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0378-1135/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.vetmic.2012.07.043

contagious/environmental agents associated with mastitis (Pitkälä et al., 2004; Bexiga et al., 2005; Ericsson et al., 2009) including monitoring antimicrobial resistance since patterns of resistance may differ among different countries (Hendriksen et al., 2008).

Also, molecular epidemiology tools offer unique opportunities to advance the study of diseases through the investigation of infectious agents at the molecular level in a veterinary context (Muellner et al., 2011). However, studies on the molecular characterization of field streptococci isolates occurring in Portugal, which is of utmost importance in order to implement efficient management practices in herds, are still not documented, with exception of *S. uberis* (Rato et al., 2008) and *S. dysgalactiae* subsp. *dysgalactiae* (Rato et al., 2010).

The aims of this study were the evaluation of antimicrobial drug resistance patterns among *S. agalactiae*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. uberis* and the identification of strain molecular features.

2. Materials and methods

2.1. Bacterial isolates and identification

A total of 60 beta-hemolytic *S. agalactiae* of Lancefield group B (GBS), 18 alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* of Lancefield group C (GCS), and 30 gamma-hemolytic *S. uberis* field isolates from bovine subclinical mastitis were included in the present study. This collection comprised all streptococcal isolates (with the exception of 4 *S. uberis* isolates and 4 *S. agalactiae* isolates that were lost during storage) collected from January 2002 to May 2003, from 444 bovine milk samples of 365 animals with subclinical mastitis, from 11 herds located in the south-western region of Portugal (Bexiga et al., 2005). The original study (Bexiga et al., 2005) involved milk sampling of 459 quarters from 377 animals from a total of 12 dairy herds.

Regarding the incidence of the organisms, among the total of 459 milk samples, 351 were positive for bacterial growth. Of those, *S. dysgalactiae* subsp. *dysgalactiae* was found in 5.1% of the samples, *S. uberis* in 10%, *Staphylococcus aureus* in 10%, Gram negative bacteria (including *Escherichia coli*) in 10%, and *S. agalactiae* in 18.2%.

Further detailed information regarding the streptococcal field isolates that were included in the present work, isolation and identification methods, has been previously described elsewhere (Rato et al., 2008).

Confirmation of identification of *S. agalactiae* isolates was performed by Lancefield grouping with type B antisera (Slidex Strepto Kit, BioMérieux) and by a positive haemolytic result according to the CAMP test (Facklam, 2002). Molecular identification of *S. uberis* and *S. dysgalactiae* subsp. *dysgalactiae* (GCS) field isolates by sequencing of 16S rDNA gene was performed previously (Rato et al., 2008, 2010).

2.2. Pulsed-field gel electrophoresis (PFGE) profiles and cluster analysis

For the identification of clonal lineages, the percentage of similarity among the DNA band patterns, obtained by PFGE, was determined by cluster analysis based in a dendrogram (see Fig. 1). Genotypic related groups or clusters, identified at 80% similarity or above (corresponding to variation up to six bands among patterns) are represented by dashed rectangles in the dendrogram, and determined as described elsewhere (Rato et al., 2008).

2.3. Multilocus sequence typing (MLST)

A group of 18 *S. agalactiae* (GBS) isolates out of 60 were analysed by MLST in the present work. These isolates were chosen taking into account the PFGE band-based dendrogram, and comprised isolates with PFGE patterns sharing approximately 100%, 90%, 80%, 70%, 60% and 50% of similarity. DNA was extracted as described for Group A *Streptococcus* (described at http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm) and the primers used for PCR amplification and sequencing are available at the *S. agalactiae* MLST website (http://pubmlst.org/sagalactiae/). Sequencing was performed by STAB-Vida (Lisbon, Portugal), and sequences were analysed by using the BioEdit sequence alignment editor version 7.0.0 (Hall, 1999). The MLST data was used by eBURST (http://eburst.mlst.net/) for assignment of predicted primary founder(s), clonal complexes or singletons (STs that cannot be assigned to any group).

2.4. Antimicrobial resistance patterns

All *S. uberis*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. agalactiae* isolates were tested by disk diffusion (Oxoid Ltd., Basingstoke, UK) according to the guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2008) for antimicrobial susceptibility tests for bacteria isolated from animals. The following antimicrobials were selected for testing, based on several criteria: (a) licensing for mastitis treatment in cattle [penicillin 10 units (P), cefazolin 30 μg (KZ), cefoperazone 75 μg (CFP), pirlimycin 2 μg (PRL), gentamicin 10 μg (CN), streptomycin 10 μg (S), and amoxicillin–clavulanic acid 30 μg (AMC)]; (b) use in human medicine [rifaximin 40 μg (RAX), erythromycin 15 μg (ERY), vancomycin 30 μg (VA), chloramphenicol 30 μg (CHL), tetracycline 30 μg (TET)]; and (c) determine phenotypes for subsequent search for resistance determinants assumed to be located on genetic mobile elements (TET and ERY).

Resistance was determined by measurement of inhibition of growth around the antimicrobial disk according to the zone diameter interpretative standards of CLSI (2008), and when not available, according to the antimicrobials manufacturers' instructions.

2.5. Macrolide resistance phenotypes

Resistance only to macrolides (M phenotype) or to macrolides, lincosamides and streptogramins-B (MLS_B phenotype), either inducible ($iMLS_B$) or constitutive ($cMLS_B$), were evaluated among all streptococcal isolates from the present study, by a double-disk test with erythromycin 15 μg (ERY) and pirlimycin 2 μg (PRL) disks (Seppälä et al., 1993). Resistance only to lincosamides