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Mestre

Epidemiological characterization, antimicrobial resistance and virulence mechanisms of human and animal streptococci

Dissertação para obtenção do Grau de Doutor em Biologia

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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 the dairy industry due to antibiotherapy and loss in milk production. However, molecular characterization of field isolates of *Streptococcus* spp. occurring in Portugal was not known prior to our studies and is important to improve therapeutic and disease control programs. The aims of this study were the identification of strain molecular features, and the evaluation of antimicrobial drug resistance patterns of *S. agalactiae* (*n*=60), *S. dysgalactiae* subsp. *dysgalactiae* (*n*=18) and *S. uberis* (*n*=30) collected from bovine subclinical mastitis between 2002/2003 in Portugal.

Additionally, two *S. dysgalactiae* subsp. *dysgalactiae* strains associated with invasive disease (one collected from cattle and the other from a human), and six *Streptococcus dysgalactiae* subsp. *equisimilis* (group C or group G *Streptococcus* - GCS/GGS) strains from human infection were included in the study, for comparative purposes.

Genotypic relationships were identified using pulsed-field gel electrophoresis (PFGE)/BioNumerics, *S. agalactiae* and *S. uberis* multi-locus sequence typing (MLST), macrolide and tetracycline resistance gene profiling, *S. agalactiae* molecular serotyping, virulence gene profiling, PCR-amplification for screening presence of specific genes and subsequent sequencing for phylogenetic analysis, and reverse transcriptase-PCR (RT-PCR) for gene expression analysis of selected genes. Also, a custom-designed microarray containing 220 virulence genes of the human pathogen *Streptococcus pyogenes* (Group A *Streptococcus* - GAS) was used to test bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* and human GCS/GGS *S. dysgalactiae* subsp. *equisimilis*. Antimicrobial resistance was assessed by disk diffusion against penicillin, gentamicin, streptomycin, amoxicillin-clavulanic acid, cefazolin, cefoperazone, rifaximin, erythromycin, pirlimycin, tetracycline, vancomycin, chloramphenicol and the macrolide-lincosamide resistance phenotypes ($cMLS_B$, $iMLS_B$, M, L).

Among *S. uberis* three PFGE clonal groups (defined by at least 80% similarity) comprised almost half of total isolates, and 50% of GBS isolates were included in four major clonal groups (all farm-associated), which is indicative of a contagious route of transmission between animals. The occurrence of PFGE patterns sharing >82.8% and 100% similarity among *S. dysgalactiae* subsp. *dysgalactiae* isolates collected from different farms suggests an environmental source for this pathogen in our case. By MLST, we observed that all *S. uberis* sequence types (ST) were found to be novel (*n*=14), representing novel genomic backgrounds for this pathogen. Among GBS only three MLST lineages (ST-2, ST-23, and ST-61/ST-554) were detected revealing little heterogeneity among our bovine GBS collection.

Five new *cpsD-cpsE-cpsF* sequences of the *cps* locus (encoding the capsular polysaccharide) were detected in >70% of the bovine GBS, which may represent new serotypes. Human GBS

insertion sequences were found in the *cpsE* gene (of the *cps* locus) in four bovine GBS isolates. Theses inserts probably disrupt the expression of the capsular polysaccharide in theses strains. Bovine GBS isolates (>20%) carry the laminin-binding protein and the C5a peptidase virulence genes (*lmb* and *scpB*), known to be required for human GBS strains to infect the human host. The remaining bovine GBS may have lost these genes during the adaptation to the bovine host. *S. pyogenes* bacteriophage-associated virulence genes (*speC*, *speM*, *speK*, *speL*, *spd1*, *sdn*) encoding superantigens, Dnase, and streptodornase were detected in bovine GCS isolates (72%) but not in the human GCS/GGS isolates. All these genes were shown to be expressed by RT-PCR. Phylogenetic analysis of superantigen gene sequences revealed a high level (>98%) of identity among genes of the bovine GCS, of the horse pathogen *Streptococcus equi* subsp. *equi*, and of the human pathogen GAS. These findings suggest that GAS phages may putatively play a role in the bovine GCS evolution. *S. pyogenes* virulence determinants located in non-bacteriophage-related mobile elements, such as the gene encoding R28 (an adhesin) and the composite transposon Tn*1207.3*/Φ10394.4 were also detected in bovine GCS (100%).
In addition, *S. pyogenes* streptolysin S gene (*sagA*), strongly associated with invasive disease in humans, was detected in the two *S. dysgalactiae* subsp. *dysgalactiae* invasive strains.
The bovine streptococci were susceptible to clinical relevant antimicrobials (namely penicillin, cefoperazone and cefazolin); however resistance levels against erythromycin and pirlimycin were high when compared with data from other countries. Co-resistance to macrolide and lincosamide antimicrobials (corresponding to the $cMLS_B$ phenotype) was detected in 21% of isolates and associated with the presence of *ermB* or *ermA* genes. All pirlimycin-resistant and erythromycin-susceptible isolates (L-phenotype; *n*=11) carry the *linB* gene known to be acquired via a transferable plasmid of *Enterococcus faecium*. Resistance to tetracycline was high (>60% of total isolates) despite it's no longer use for treating bovine infections, and associated with the presence of tetracycline resistance determinants *tetO*, *tetM*, *tetK*, and/or *tetS*.

In conclusion, the presence of relevant GAS virulence genes in *S. dysgalactiae* subsp. *dysgalactiae* may contribute to the increased virulence potential of these strains, and thus, *S. dysgalactiae* subsp. *dysgalactiae* (considered a strictly-animal pathogen) should not be disregarded as a possible human pathogen. Our results also underline the importance of locally monitoring antimicrobial resistance for improvement of antibiotherapy programmes.

Keywords: *Streptococcus agalactiae*; *Streptococcus dysgalactiae* subsp. *dysgalactiae*; *Streptococcus uberis*; Molecular typing; Antimicrobial resistance; Virulence.

Septococcus agalactiae (*Streptococcus* do Grupo B - GBS), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (*Streptococcus* do Grupo C - GCS) e *Streptococcus uberis* são agentes patogénicos relevantes associados a mastite de bovinos, a patologia de maior impacto económico na indústria de lacticínios devido à utilização de antimicrobianos (utilizados em terapia para curar esta doença) e diminuição da produção de leite. No entanto, a caracterização de isolados pertencentes a *Streptococcus* spp. responsáveis por mastite bovina em explorações Portuguesas não era conhecida até à elaboração deste trabalho, o que é fundamental para o melhoramento de programas de terapêutica e controlo desta doença. O presente trabalho visou identificar as características moleculares e perfis de resistência a antimicrobianos de estirpes GBS (*n*=60), GCS (*n*=18) e *S. uberis* (*n*=30) causadoras de mastite subclinica de bovinos, recolhidas entre 2002-2003 em Portugal. Adicionalmente, duas estirpes de *S. dysgalactiae* subsp. *dysgalactiae* associadas a doença invasiva (uma recolhida de um bovino e a outra de um humano), e seis estirpes de *S. dysgalactiae* subsp. *equisimilis* (*Streptococcus* do Grupo C ou G - GCS/GGS) de infecção em humanos, foram incluídas neste estudo para efeitos comparativos.

A genotipagem foi realizada utilizando electroforese de campo pulsado (PFGE)/BioNumerics, sequenciação de múltiplos *loci* (MLST), serotipagem molecular de *S. agalactiae*, amplificação por PCR para rastrear presença de determinados genes, e subsequente sequenciação de genes específicos para efectuar análise filogenética, transcritase reversa (RT-PCR) para análise da expressão genética, e utilização de um microarray contendo 220 genes de virulência do agente patogénico de humanos *Streptococcus pyogenes* (*Streptococcus* do Grupo A - GAS) para testar as estirpes GCS bovinas e as estirpes GCS/GGS humanas. O perfil de resistência a antimicrobianos foi avaliado através do método de difusão em disco aos fármacos: penicilina, gentamicina, estreptomicina, amoxicilina-ácido clavulânico, cefazolina, cefoperazona, rifaximina, eritromicina, pirlimicina, tetraciclina, vancomicina, cloranfenicol, e análise de fenótipos de resistência aos macrólidos e lincosamidas ($cMLS_B$, $iMLS_B$, M, e L).

Por PFGE, observou-se que em *S. uberis* três grupos clonais (definidos >80% em dendrogramas) incluíam 47% dos isolados, e em *S. agalactiae* quatro grandes grupos clonais incluíam 50% dos isolados (todos associados à mesma exploração), o que é indicativo de transmissão directa entre animais. Observaram-se perfis de PFGE idênticos (100% de semelhança) entre isolados de *S. dysgalactiae* subsp. *dysgalactiae* provenientes de explorações de bovinos distintas o que sugere uma fonte ambiental para este agente patogénico. Por MLST, verificou-se que todos os perfis alélicos (ST) encontrados em *S. uberis* são novos (*n*=14), o que representa novas estruturas genómicas em *S. uberis.* Em GBS apenas 3 linhagens (ST-2, ST-23 e ST-61/ST-554) foram identificadas o que indica pouca heterogeneidade na colecção de GBS. Novas sequências *cpsD-cpsE-cpsF* do *locus cps* (que codifica o polissacarídeo capsular) foram identificadas em >70% dos isolados GBS, as quais podem representar novos serótipos.

Observou-se a presença de sequências de inserção (anteriormente descritas em GBS de humanos) no gene *cpsE* do *locus cps* em quatro estirpes de GBS bovinas (7%). Estas inserções provavelmente impedem a expressão do polissacarídeo capsular nestas estirpes.

Isolados de GBS bovinos (>20%) contêm os genes de virulência *lmb* e *scpB* (que codificam a proteina *laminin-binding* e a C5a peptidase, respectivamente), factores considerados necessários para estirpes GBS de humanos infectarem o hospedeiro humano. Os restantes isolados poderão ter perdido a região que inclui os genes *lmb-scpB* durante a adaptação ao hospedeiro bovino.

Genes associados a genomas fágicos de GAS (*speC*, *speM*, *speK*, *speL*, *spd1*, *sdn*) que codificam superantigénios, DNase, e estreptodornase foram detectados nos isolados bovinos GCS (72%) mas não nos isolados GCS/GGS de humanos. Verificou-se por RT-PCR que estes genes são expressos. Através de análise filogenética das sequencias dos genes que codificam superantigénios verificou-se elevado nível (>98%) de identidade entre os genes dos isolados bovinos GCS, do agente patogénico de cavalos *Streptococcus equi* subsp. *equi*, e do agente patogénico de humanos GAS. Estes resultados sugerem que os fagos de GAS contribuem para a evolução de GCS de bovinos. Determinantes de virulência de GAS localizados em elementos genéticos móveis (de origem não-fágica) como o gene que codifica a proteína R28 (adesina) e o transposão composto Tn*1207.3*/Φ10394.4 foram detectados nos isolados bovinos GCS (100%).

Além disso, o gene *sagA* (que codifica para a estreptolisina S) de *S. pyogenes*, associado a doença invasiva, foi detectado nas duas estirpes *S. dysgalactiae* subsp. *dysgalactiae* invasivas.

Os isolados bovinos são susceptíveis aos antimicrobianos clinicamente relevantes (penicilina, cefazolina, cefoperazona), no entanto observou-se perfis de resistência elevados à eritromicina e pirlimicina quando comparados com resultados de outros Países. A resistência à eritromicina e pirlimicina (fenótipo $cMLS_B$) em 21% dos isolados deveu-se à presença dos genes *ermB*/*ermA*. Os isolados resistentes à pirlimicina e susceptíveis à eritromicina (fenótipo L; *n*=11) possuem o gene *linB* que se sabe ser adquirido via um plasmídeo de *Enterococcus faecium*. A resistência à tetraciclina verificou-se elevada (>60%) e associada à presença dos genes *tetO*, *tetM*, *tetK*, *tetS*.

Em conclusão, a presença de genes de virulência de GAS em *S. dysgalactiae* subsp. *dysgalactiae* poderá contribuir para o aumento da virulência nestas estirpes, sugerindo que *S. dysgalactiae* subsp. *dysgalactiae* (considerado agente patogénico exclusivamente de animais) não deveria ser desconsiderado como possível agente patogénico em humanos. Salienta-se ainda que os programas de terapêutica devem ser definidos de acordo com os perfis de resistência a antimicrobianos determinados localmente, e não extrapolando resultados de outros locais.

Palavras-chave: *Streptococcus agalactiae*; *Streptococcus dysgalactiae* subsp. *dysgalactiae*; *Streptococcus uberis*; Tipagem molecular; Resistência a antimicrobianos; Virulência.

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ABC	ATP-binding cassette
ALP	Alpha-like protein
ATCC	American type culture collection
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	Base pair
CAMP	Christie Atkins Munch-Petersen
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
$cMLS_B$	Constitutive resistance to macrolides, lincosamides and streptogramins B
CNS	Coagulase negative staphylococcus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DppA	Dipeptide permease A
EDTA	Ethylenediamine tetraacetic acid
FCT	Fibronectin-binding, collagen-binding, T antigen
GTP	Guanosine 5'-triphosphate
h	Hours
ICEs	Integrative conjugative elements
$iMLS_B$	Inducible resistance to macrolides, lincosamides and streptogramins B
L	Resistance to lincosamides, susceptibility to macrolides
LSA	Resistance to lincosamides, and streptogramins A, susceptibility to macrolides
Mb	Megabase pair
MEGA	Molecular evolutionary genetics analysis
$MgCl_2$	Magnesium chloride
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
MLS_B	Co-resistance to macrolides, lincosamides and streptogramins B
MLST	Multilocus sequence typing
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride

NAD	Nicotine adenine dinucleotide
NA	Not applicable
NCBI	National Center for Biotechnology Information
nd	Not determined
NT	Nontypeable
OD	Optical density
PAM	Plasminogen-binding M-like protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PTS	Phosphotransferase system
Rib	Resistance to proteases, immunity, group B
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SLV	Single locus variant
ST	Sequence type
STSS	Streptococcal toxic shock syndrome
SUAM	*Streptococcus uberis* adhesion molecule
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
THY	Todd-Hewitt broth supplemented with 1% yeast extract
Tris	Tris(hydroxymethyl)aminomethane
UPGMA	Unweighted pair group method with arithmetic mean

Gene abbreviations

arcC	Carbamate kinase
cps	Capsule polysaccharide synthesis
ddl	D-ala-D-ala ligase
dppA	Surface lipoprotein DppA
emm	M-protein
erm	Erythromycin ribosome methylation
fbsB	Fibronectin-binding protein FbsB

gki	Glucose kinase
isp	Immunogenic secreted protein
lbp	Laminin-binding protein Lbp
lin	Lincosamide inactivation nucleotidylation
lmb	Laminin-binding protein Lmb
mef	Macrolide efflux
nga	NAD-glycohydrolase precursor
pheS	Phenylalanyl tRNA synthetase
prtf1	Fibronectin-binding protein F1
prtf2	Fibronectin-binding protein F2
recP	Transketolase
sagA	Streptolysin S
scpA/B	C5A peptidase precursor
sdm	*Streptococcus dysgalactiae* (subsp. *dysgalactiae*) derived mitogen
sdn	Streptodornase
seeL	*Streptococcus equi* subsp. *equi* pyrogenic exotoxin L
seeM	*Streptococcus equi* subsp. *equi* pyrogenic exotoxin M
sfbI	Streptococcal fibronectin-binding protein I
ska	Streptokinase A precursor
sla	Streptococcal phospholipase A_2
slo	Streptolysin O
smeZ	Streptococcal mitogenic exotoxin Z
sodA	Superoxide dismutase
spd1	Streptococcal phage DNase
spe	Streptococcal pyrogenic exotoxin
spegg	*Streptococcus dysgalactiae* subsp. *equisimilis* pyrogenic exotoxin G
ssa	Streptococcal superantigen
szeL	*Streptococcus equi* subsp. *zooepidemicus* pyrogenic exotoxin L
szeM	*Streptococcus equi* subsp. *zooepidemicus* pyrogenic exotoxin M
tdk	Thymidine kinase
tet	Tetracycline resistance protein
tpi	Triosephosphate isomerase
vgaB	ATP-binding protein
yqiL	Acetyl CoA acetyl-transferase

Antimicrobials abbreviations

ERY	Erythromycin
PRL	Pirlimycin
TET	Tetracycline
CN	Gentamicin
S	Streptomycin
CFP	Cefoperazone
AMC	Amoxicillin-clavulanic acid
KZ	Cefazolin
VA	Vancomycin
CHL	Chloramphenicol
P	Penicillin

Species abbreviations

GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
GCS	Group C *Streptococcus*
GGS	Group G *Streptococcus*
SDSD	*Streptococcus dysgalactiae* subsp. *dysgalactiae*
SDSE	*Streptococcus dysgalactiae* subsp. *equisimilis*
SESE	*Streptococcus equi* subsp. *equi*
SESZ	*Streptococcus equi* subsp. *zooepidemicus*
VSU	Veterinary *Streptococcus uberis*

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Márcia G. Rato, Ricardo Bexiga, Carlos Florindo, Lina M. Cavaco, Cristina L. Vilela, and Ilda Santos-Sanches. Antimicrobial resistance and molecular epidemiology of streptococci from bovine mastitis. Vet. Microbiol. (submitted for publication)

Márcia G. Rato, Andreas Nerlich, René Bergmann, Ricardo Bexiga, Sandro F. Nunes, Cristina L. Vilela, Ilda Santos-Sanches and Gursharan S. Chhatwal. 2011. Virulence gene pool detected in bovine group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* using a group A *Streptococcus pyogenes* virulence microarray. J. Clin. Microbiol. **49**: 2470–2479. (doi:10.1128/JCM.00008-11)

Márcia G. Rato, Ricardo Bexiga, Sandro F. Nunes, Cristina L. Vilela, and Ilda Santos-Sanches. 2010. Human Group A Streptococci Virulence Genes in Bovine Group C Streptococci. Emerg. Infect. Dis. **16**:116-119. (DOI: 10.3201/eid1601.090632)

Márcia G. Rato, Ricardo Bexiga, Sandro F. Nunes, Lina M. Cavaco, Cristina L. Vilela, and Ilda Santos-Sanches. 2008. Molecular Epidemiology and Population Structure of Bovine *Streptococcus uberis*. J. Dairy Sci. **91**(12):4542-4551. (doi:10.3168/jds.2007-0907)

This Ph.D. Thesis is organized in six chapters. It includes three published manuscripts and one submitted manuscript.

Chapter 1 provides a general introduction, in particular, it is focused a brief description of the genus, the clinical relevance and host range of the streptococcal species under study, a description of the reported cases of bovine mastitis in Portugal and a state-of-the-art concerning the molecular epidemiology of strains of these bovine species.

Chapter 2 is entitled "Molecular epidemiology and population structure of bovine *Streptococcus uberis*" and includes a published manuscript. This Chapter provides a background knowledge and the experimental work focused the molecular epidemiology and population structure of *S. uberis* from Portugal.

Chapter 3 is entitled "Human group A streptococci virulence genes in bovine group C streptococci" and includes a published manuscript. This chapter gives a background about the virulence traits of the bovine GCS, phage genes of *S.pyogenes* and implication of GAS phages in other human streptococci and in bovine GCS. The experimental work was focused in the search of virulence traits of the human *S. pyogenes* in mastitis GCS isolates.

Chapter 4 is entitled "Virulence gene pool detected in bovine group C *S. dysgalactiae* subsp. *dysgalactiae* isolates by use of a group A *S. pyogenes* virulence microarray" and includes a published manuscript. This Chapter provides a background about the most recent panorama of the pathogenesis of *S. dysgalactiae* subsp. *dysgalactiae* associated with invasive disease. The experimental work comprised the comparison of bovine *Streptococcus dysgalactiae* subsp. *dysgalactiae* and the human GAS, and *S. dysgalactiae* subsp. *equisimilis* using an array of virulence genes from *S. pyogenes*.

Chapter 5 is entitled "Antimicrobial resistance traits and molecular epidemiology of streptococci from bovine mastitis" and consists of one submitted manuscript with modifications. This chapter provides background knowledge in antimicrobial resistance, molecular epidemiology and population structure among streptococci associated with mastitis. The experimental work focused in the comparative antimicrobial resistance genotyping and the molecular epidemiology of the three species, *S. agalactiae*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. uberis*, and, a more detailed characterization of *S. agalactiae* by virulence genotyping and molecular serotyping.

Finally, Chapter 6 provides the conclusions and an outlook for future research in a same topic as presented in Chapter 4.

1

General Introduction

1.1. The genus *Streptococcus* spp.

1.1.1. Brief description of taxonomy and historical perspective

The genus *Streptococcus* comprises numerous clinically significant species which are responsible for wide variety of infections in human and animals. Structurally, streptococci are Gram-positive, non-motile, non-sporeforming, catalase-negative cocci, which due to single and parallel division plan form chains composed of two or more cells (Fig.1.1).

Most streptococci are facultative anaerobes, and some are obligate (strict) anaerobes. Some require blood supplemented agar for in vitro growth, which is often used to observe phenotypic differences among streptococci species (Facklam et al. 2002), (see below).

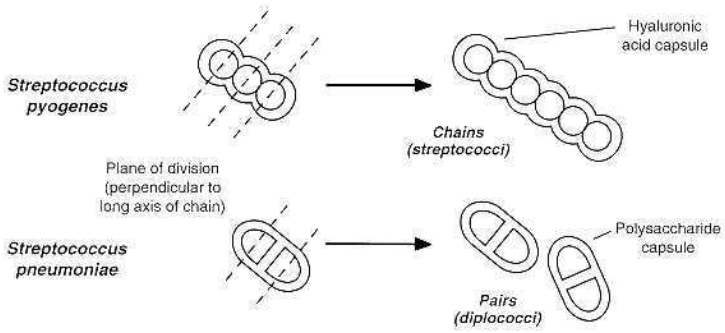


Figure 1.1. Morphology of the streptococci. Streptococci divide in a single plane and tend not to separate, causing chain formation (adapted from Patterson, 1996).

Colony morphology, hemolysis on blood agar plates, biochemical reactions, and serologic specificity consist in phenotypic characteristics of streptococci which are used for their classification and identification (Wyder *et al*., 2011).

Streptococci are divided into three groups based on their type of hemolysis reaction on blood agar plates (Fig. 1.2): beta-hemolysis consisting in complete lysis of red blood cells (clear halo around the colonies); alpha-hemolysis consisting in incomplete lysis of red blood cells (green color halo around the colonies); and no lysis of red blood cells (no hemolysis is observed around the colonies). *Streptococcus agalactiae*, or the Group B *Streptococcus* (GBS), can be presumably identified by the CAMP test, a technique also based on the hemolytic reaction observed in blood agar plates (see Fig. 1.3).

Rapid commercial systems, in particular the API 20 STREP® (BioMérieux, Marcy l'Etoile, France) involving a series of miniaturized biochemical tests, are also used for streptococci identification (Wyder *et al*., 2011).

Serologic grouping for identification of streptococci is based on antigenic differences in cell wall carbohydrate composition, in the cell wall pili-associated protein, and in the polysaccharide capsule (Fischetti, 2000; Mora *et al.*, 2005; Kong *et al*., 2002).

A: *Streptococcus agalactiae* **B:** *Streptococcus dysgalactiae* subsp. *dysgalactiae* **C:** *Streptococcus uberis*

Figure 1.2. Hemolysis reaction of streptococci on blood agar plates: **A**) beta-hemolysis; **B**) alpha-hemolysis; **C**) no hemolysis. Agar plates were supplemented with 5% sheep blood.
(Photos: Márcia G. Rato, FCT/UNL)

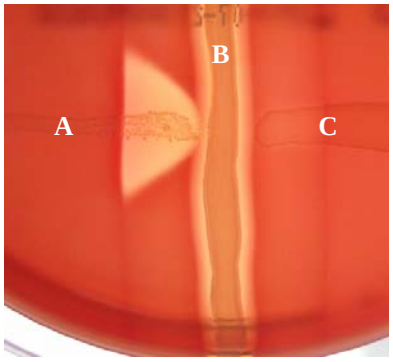
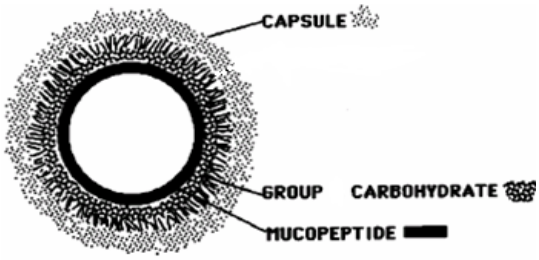


Figure 1.3. The CAMP test for presumably identification of *Streptococcus agalactiae* or group B streptococci (GBS) consists on visible hemolysis on blood agar media (arrow shaped) that occurs when diffusible extracellular CAMP factor of *Streptococcus agalactiae* (streak **A),** reacts with β-hemolysin of *Staphyloccocus aureus* ATCC® 25923 (streak **B**). Negative result for *Streptococcus uberis* (streak **C**).
(Photo: Márcia G. Rato, FCT/UNL)

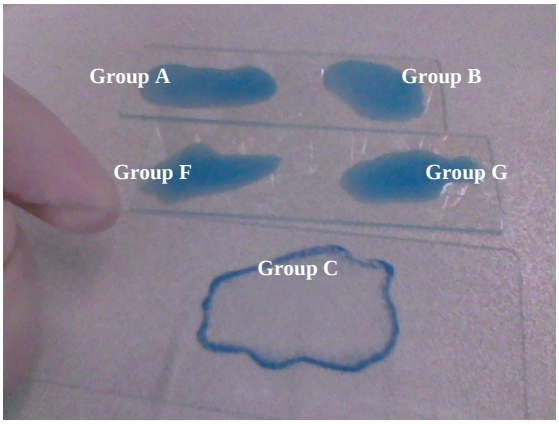
Rebecca Lancefield (1933) systematized the classification of streptococci based on their type of surface antigen, which is a standard technique still used nowadays based in agglutination tests and labelling using capital letters: the Lancefield serological grouping (see Fig. 1.4).

Nomenclature for streptococci in particular in medical use has been largely based on the identification of cell wall components rather than on species names (e.g., *Streptococcus agalactiae* is commonly referred to as group B *Streptococcus* or GBS, and *Streptococcus*

pyogenes is often referred to as group A *Streptococcus* or GAS) (Fischetti, 2008; Fischetti, 2000).



A



B

Figure 1.4. Specific carbohydrates are found in different *Streptococcus* species: **A)** schematic representation of streptococcal cell covered with an outer hyaluronic acid capsule and the group carbohydrate; **B)** photo showing a commercial slide agglutination test (Slidex® Strepto Kit, BioMérieux, Marcy l'Etoile, France) for identification of Lancefield A, B, C, F and G group antigens from colonies, based on co-agglutination of the group-specific antigen and the corresponding antibody. Here is shown *Streptococcus dysgalactiae* subsp. *dysgalactiae* belonging to Lancefield group C.
(Photo: Márcia G. Rato, FCT/UNL).

The first description of streptococcus is though to have been made by Louis Pasteur in 1879 which described them as "chains of beads". The generic name "*Streptococcus*" of the genus was first proposed by Rosenbach in 1884, and the earliest attempt to differentiate streptococci is thought to have been made by Shottmuller in 1903 by using blood agar plates (Evans, 1936; Facklam, 2002).

Nomenclature and taxonomic revisions have been performed in *Streptococcus* genus in the last decades. In the mid-1980s, the genus *Streptococcus* was split into three different genera: *Enterococcus*, *Lactococcus* and *Streptococcus* (Schleifer & Kilpper-Balz, 1984; Schleifer & Kilpper-Balz, 1987). Later, in 1995 the streptococci genus was divided into six different divisions (pyogenic, anginosus, mitis, mutans, salivarius, and bovis) based on their 16S rRNA gene sequence analysis (Kawamura *et al*., 1995), (see Fig. 1.5).

Particularly, in the late 1990s, the genotypic and phenotypic characterization of *S. dysgalactiae* strains from different sources (human and animal) revealed the existence of two subpopulations of strains within the *S. dysgalactiae* species (Vandamme *et al*., 1996; Vieira *et al*., 1998). Thus the term *S. dysgalactiae* subsp. *dysgalactiae* was proposed for the group of strains from animal origin (mostly associated with bovine mastitis) belonging to Lancefield group C, alpha-hemolytic or nonhemolytic, and which did not exhibit streptokinase activity on human

plasminogen (Vieira *et al*., 1998). The name *S. dysgalactiae* subsp. *equisimilis* was proposed for the group of *S. dysgalactiae* strains (from human and animals) which belonged to Lancefield C, G and L, groups, beta-hemolytic and exhibiting streptokinase activity on human plasminogen (Vandamme *et al*., 1996; Vieira *et al*., 1998).

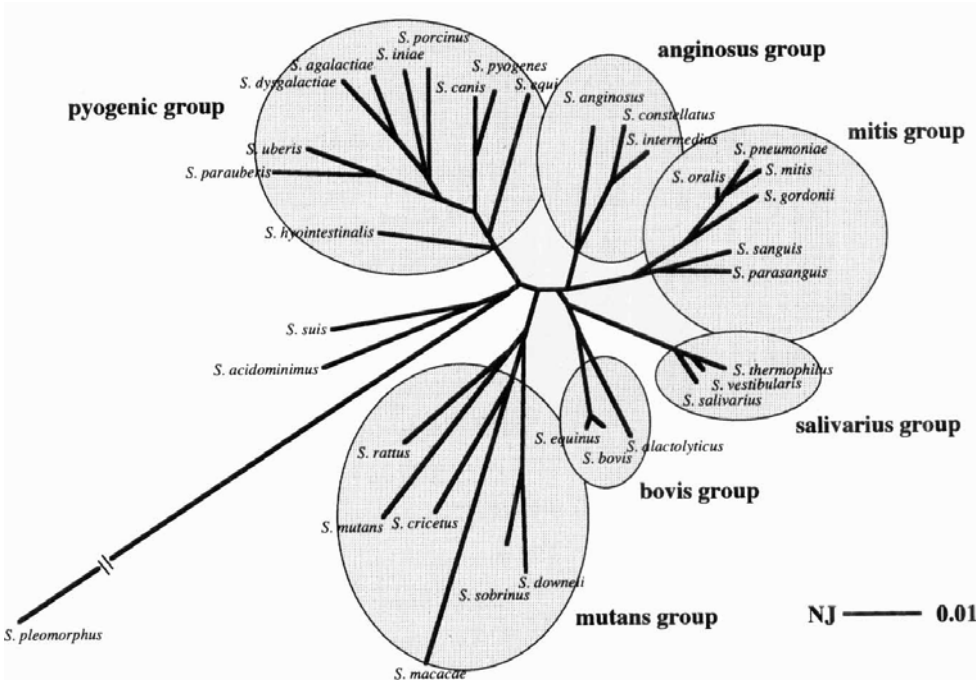


Figure 1.5. Phylogenetic relationships of 34 *Streptococcus* species based on their 16S rRNA gene sequences (Kawamura *et al*., 1995).

Some streptococcal species, namely *Streptococcus pneumoniae* and *Streptococcus uberis* do not possess defined group antigens and thus cannot be classified by the Lancefield serogrouping system, a standardized method for the identification of streptococci, as mentioned above (Facklam, 2002). Non-hemolytic and alpha-hemolytic strains (variants) have been previously reported among human *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* strains, which are beta-hemolytic (Dierksen & Tagg, 2000; Woo *et al*., 2003). These variants may putatively be misidentified in case the identification is primarly based in hemolysis type of colonies on blood agar plates (Dierksen & Tagg, 2000; Woo *et al*., 2003).

For these reasons, molecular techniques are currently used in addition to phenotypic techniques, to complement *Streptococcus* spp. identification, namely the 16S–23S rRNA interspacer region analysis (Chen *et al*., 2004), sequencing of the manganese-dependent superoxide dismutase gene (*sodA*) (Abdelsalam *et al*., 2010) and inner features of 16S rRNA gene analysis (Lal *et al*., 2011). These techniques are based in the analysis of molecular sequences that are generally more revealing of evolutionary relationships than is classical techniques based in phenotypic characteristics (Woese *et al.,* 1990; Wyder *et al*., 2011).

1.1.2. Clinical relevance and host range

Various streptococcal species are involved in colonization of human and other mammals and can cause disease. For several decades, interest was focused in only two species that caused human infection: *S. pyogenes* (GAS) and *S. pneumoniae* (pneumococci). However, the pathogenic potential for humans of other streptococcal species such as those belonging to Lancefield group B, C, or G antigens (GBS, GCS, and GGS), has been documented (Luan *et al*., 2005; Eyre *et al*., 2010). Furtheremore, some streptococcal species are now recognized important animal pathogens such as *S. uberis* (see below). Several phenotypic characteristics of clinical relevant streptococci are listed in Table 1.1.

The major human beta-hemolytic pathogens are included in the pyogenic group. The pyogenic group includes the following pathogens: *Streptococcus pyogenes* (group A *Streptococcus*, GAS), *Streptococcus agalactiae* (group B *Streptococcus*, GBS), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (group C *Streptococcus*, GCS), *Streptococcus dysgalactiae* subsp. *equisimilis* (group G, C, and rarely, group L and group A *Streptococcus*), *Streptococcus equi* subsp. *equi* (GCS), *Streptococcus equi* subsp. *zooepidemicus* (GCS) and *Streptococcus uberis*.

S. pyogenes (GAS) is a strictly-human pathogen and causes a variety of diseases such as pharyngitis, skin and soft tissue infection, impetigo (considered superficial diseases), bacteraemia, necrotizing fasciitis, deep soft tissue infections, cellulitis, pneumonia (considered deep infections), toxic shock-like syndrome, scarletina (toxin mediated diseases), rheumatic fever and post-streptococcal glomerulonephritis (immunologically mediated diseases) (Efstratiou, 2000).

S. agalactiae (GBS) is considered nowadays the leading cause of septicemia and meningitis in human newborn infants and pregnant woman, and associated with bovine mastitis (Liu & Nizet, 2004; Rajagopal, 2009; Barkema *et al*., 2009). Furthermore, GBS has been isolated from dogs, cats, goats and non-mammals such as fish, frogs and crocodiles (Bishop *et al*., 2007).

S. dysgalactiae subsp *equisimilis* (GCS/GGS) is a commensal organism, and causes a spectrum of diseases in humans that resemble GAS infections, which include streptococcal toxic shock syndrome, severe soft tissue infection and poststreptococcal sequelae (Efstratiou, 1997; Takahashi *et al*., 2011). *S. dysgalactiae* subsp *equisimilis* is also a cause of septicaemia, arthritis and valvular endocarditis in pigs, and lymphadenitis and placentitis in horses (Kawata *et al*., 2003; Timoney, 2004).

Streptococcus dysgalactiae subsp. *dysgalactiae* (GCS) is considered to be associated only with animal infections, mostly bovine mastitis (Facklam, 2002). Nevertheless, it has been recently reported associated with severe invasive disease in dogs, bovine, ovine, fish, and in a human in

contact (skin puncture) with raw fish (Vela *et al*., 2006; Chénier *et al*., 2008; Lacasta *et al*., 2008; Koh *et al*., 2009).

Table 1.1. Phenotypic characteristics of β-hemolytic streptococci[b] (adapted from Facklam, 2002)

Species	Lancefield	Bac	PYR	Cam	VP	Hip	Arg	Esc	Str	Sbl	Tre	Rib	Origin
S. pyogenes	A	+	+	-	-	-	+	v	-	-	NA	-	Human
S. agalactiae	B	-	-	+	-	+	+	-	-	-	NA	NA	Human, bovine
S. dysgalactiae subsp. *dysgalactiae*[b]	C	-	-	-	-	-	+	v	-	v	+	+	Animals
S. dysgalactiae subsp. *equisimilis*[c]	A, C, G, L	-	-	-	-	-	+	+	-	-	+	+	Human, animals
S. equi subsp. *equi*	C	-	-	-	-	-	+	v	+	-	-	NA	Animals
S. equi subsp. *zooepidemicus*	C	-	-	-	-	-	+	v	+	+	v	NA	Animals, human,
S. canis[c]	G	-	-	+	-	-	+	+	-	-	v	NA	Dog, human
S. anginosus (group)[d]	A, C, G, F, none	-	-	-	+	-	+	+	-	-	+	NA	Human
S. porcinus	E, P, U, V, none, new	-	+	+	+	v	+	+	-	+	+	NA	Swine, human
S. iniae	None	-	+	+	-	-	-	+	+	-	NA	NA	Dolphin, fish, human
S. phocae	C, F	+	-	-	-	-	-	-	-	-	NA	NA	Seal
S. didelphis	None	-	-	-	-	-	+	-	-	-	+	NA	Opossum

Abbreviations: Group, group carbohydrate antigen; Bac, bacitracin; Pyr, pyrrolidonylarylamidase; Cam, CAMP reaction; VP, Voges-Proskauer reaction; Hip, hydrolysis of hippurate; Arg, deamination of arginine; Esc, hydrolysis of esculin; Str, hydrolysis of starch; Sbl, Tre, and Rib, production of acid in sorbitol, trehalose, and ribose broth, respectively. +, positive reaction (>95%); -, negative reaction (>95%); v, variable reaction (6 to 94% positive); NA, not applicable.

[b] *S. dysgalactiae* subsp. *dysgalactiae* strains are not beta-hemolytic (are alpha-hemolytic) but were included in the Table.

[c] To differentiate between the group G *S. canis* and group G *S. dysgalactiae* subsp. *equisimilis*, testing of α-galactosidase, β-galactosidase, and β-glucoronidase needs to be performed. *S. canis* is positive for α- and β-galactosidase and negative for β-glucoronidase; *S. dysgalactiae* subsp. *equisimilis* gives the opposite reactions.

[d] The *S. anginosus* group includes beta-hemolytic strains of *S. anginosus, S. constellatus*, and *S. intermedius.* There are insufficient data to know the percentage of each of these beta-hemolytic species that contain carbohydrate antigens.

Streptococcus equi subsp. *equi* (GCS) is a horse-restricted pathogen, and considered to have evolved from *Streptococcus equi* subsp. *zooepidemicus* (GCS), a zoonotic pathogen (Holden *et al*., 2009). *Streptococcus equi* subsp. *equi* is the causative agent of equine strangles, and *Streptococcus equi* subsp. *zooepidemicus* is associated with respiratory disease and metritis in horses, respiratory diseases such as pneumonia in dogs, and bacteraemia, endocarditis, meningitis, nephritis, and post-streptococcal glomerulonephritis in humans (Nicholson *et al*., 2000; Timoney, 2004; Beres *et al*., 2008; Priestnall *et al*., 2010).
Streptococcus uberis, as far as we know, has never been reported associated with human infections, and is a recognized pathogen associated with bovine mastitis (Ward *et al*., 2009).

The mitis group/division comprises streptococcal species of which almost all are isolated from the human oral cavity or nasopharynx: *Streptococcus oralis*, *S. mitis*, *S. gordonii*, and *S. pneumoniae*. The latter (*S. pneumoniae*) is associated with otitis media, bronchitis, sinusitis, pneumonia, invasive disease, and meningitis in humans (Kadioglu *et al*., 2008; Mook-Kanamori *et al*., 2011). *S. pneumoniae* is also associated with respiratory insufficiency in horses and meningitis in gerbils and monkeys (Muffat-Jolly *et al*., 1994; Graczyk *et al.,* 1995; Timoney, 2004).
The anginosus and salivarius divisions contain mainly streptococci from the oral cavity from human and animal origin, and the bovis group contains streptococci from human equine and bovine origin (Facklam, 2002). The mutans group, which is the most distant related streptococcal group (Fig. 1.5), includes streptococci from the oral cavities associated with the development of dental caries among humans and other mammals, namely hamsters, rats and monkeys (Facklam, 2002).

1.2. Bovine mastitis

Mastitis is an inflammation of the bovine udder, usually as a consequence of bacterial invasion of the mammary gland (Viguier *et al.*, 2009). These pathogens cause injury to milk secreting tissue and various ducts throughout the mammary gland (Almeida & Oliver, 1995; Tamilselvam *et al.*, 2006). Leukocytes or somatic cells present in the milk are used to define milk quality. Elevated somatic cells in milk, in consequence of mastitis, alter milk composition which undervalues milk, and cause a reduction of milk production (Gröhn *et al*., 2004; Zadoks *et al.*, 2004**)**.
Clinical mastitis is easily detectable and characterized by abnormal milk production and swelling or pain in the bovine udder, and may be accompanied by other systemic signs (Viguier *et al.*, 2009). Subclinical mastitis is characterized by no noticeable changes in the appearance of

the milk or the udder, but bacteria may be present in the secretion and the composition of milk is altered in terms of rise of somatic cells, and milk production decreases (Viguier *et al.*, 2009). Despite the ongoing efforts for the prevention of this disease since the "five point plan" was established (in the 1960s), bovine mastitis remains the most important disease in the dairy industry because of economic loss due to treatment costs (antibiotherapy), and loss in milk production and quality (Neave *et al*., 1966; Bradley, 2002; Seegers *et al*., 2003; Erskine *et al*., 2003; Zadoks *et al.*, 2004; LeBlanc *et al.* 2006; Viguier *et al.*, 2009).

Studying microbial aetiology of mastitis and evaluating antimicrobial resistance patterns has become crucial to better control this disease (Erskine *et al*., 2003; White *et al*., 2006; Muellner *et al.*, 2011). As far as we know, few studies regarding microbial aetiology of bovine mastitis in Portugal have been reported. In the central region of Portugal, a survey has been conducted among 12 farms from February 2002 to May 2003, involving 459 milk samples collected from bovines with subclinical mastitis (Bexiga *et al.*, 2005). No significant differences in the frequency of the pathogens were found when compared to a previous study performed in the same geographical area 20 years earlier (Atalaia, 1983; Bexiga *et al.*, 2005). Both studies reported a higher frequency of "contagious" pathogens (i.e. *S. agalactiae* and *Staphylococcus aureus*) when compared to "environmental" pathogens (i.e. *S. uberis* and *Escherichia coli*).
In contrast, in other countries (where microbial aetiology of bovine mastitis is extensively studied) a major shift in the frequency of udder pathogens was reported in the last years (Bradley, 2002; Pitkälä *et al*., 2004; Pullinger *et al*., 2007; Shum *et al*., 2009; Breen *et al*., 2009). These studies point out that when control measures in herds are effectively implemented, the contagious pathogens (*S. agalactiae* and *S. aureus*) are under control in farms, while environmental pathogens (*S. uberis* and *E. coli*) are increasing in frequency and difficult to eradicate (Bradley, 2002; Pitkälä *et al*., 2004; Pullinger *et al*., 2007; Shum *et al*., 2009; Breen *et al*., 2009). *S. uberis*, in particular, considered an "environmental" pathogen, has become nowadays a major problem in herds where control measures are efficiently implemented (Lopez-Benavides *et al.* 2007).

1.3. *Streptococci* associated with bovine mastitis

S. uberis is traditionally considered an environmental pathogen because it may be found and acquired in the bovine surroundings, such as straw or peat used in bedding materials, in farm soil, nose, vagina, and rumen of the bovines (Zadoks *et al.*, 2005[a]; Lopez-Benavides *et al*., 2007; Ericsson *et al*., 2009). On the other hand, *S. agalactiae* is considered a contagious pathogen because it is spread from infected to healthy udders, between quarters of the same

animal and between animals (e.g., through the milking machine) and is not found in the environment of the bovine (Barkema *et al.*, 2009). *S. dysgalactiae* subsp. *dysgalactiae* is considered either an environmental or contagious pathogen (Ericsson *et al*., 2009).

1.3.1. Population structure and evolution of genomes and its implication in pathogenesis

1.3.1.1. *Streptococcus agalactiae*

Complete genome sequences were obtained for three invasive *S. agalactiae* strains from human origin, and draft sequences have been obtained for another five *S.agalactiae* strains from human origin (COH1, 515, CJB111, H36B, 18RS21) and one *S.agalactiae* strain from bovine mastitis (FSL S3-026), (Tettelin *et a*l., 2002; Glaser *et al*., 2002; Richards *et al*., 2011), (see Table 1.2).

Table 1.2. Characteristics of each streptoccal strain for which complete genome sequences was obtained*

Species and strain ID	Origin	Serot.	Length of the genome (nt)	G+C content (%)	Accession number	Reference
Streptococcus agalactiae 2603V/R	human	V	2160267	35	NC_004116	Tettelin *et a*l., 2002
Streptococcus agalactiae NEM316	human	III	2211485	35	NC_004368	Glaser *et al*., 2002
Streptococcus agalactiae A909	human	Ia	2127839	35	NC_007432	Tettelin *et al*., 2005
Streptococcus uberis 0140J	bovine	NA	1852352	36	NC_012004	Ward *et al*., 2009

*Draft sequences have also been obtained for another five *S.agalactiae* strains from human origin (COH1, 515, CJB111, H36B, 18RS21), one *S.agalactiae* strain from bovine mastitis (FSL S3-026) and one *S. dysgalactiae* subsp. *dysgalactiae* (ATCC 27957) from bovine milk (Tettelin et al, 2005; Richards *et al*., 2011); Serot., serotype; NA, not applicable. Information from the National Center for Biotechnology (NCBI) database (Bethesda, MD, USA).

Virulence factors that have been documented among human *S. agalactiae* isolates include the polysaccharide capsule, the Alp family of proteins, the C5a peptidase, the Lmb protein, fibrinogen-binding FbsA, FbsB, and Spb1, secreted hemolysin, and superoxide dismutase (Franken *et al*., 2001; Liu & Nizet, 2004; Lindahl *et al.*, 2005; Rajagopal, 2009). Few of these virulence determinants have also been described among bovine GBS isolates (Brochet *et al*., 2006). However, bovine *S. agalactiae* isolates are not so extensively studied.

The virulence genes *scpB* and *lmb* (encoding C5a peptidase and Lmb protein, respectively) are present in all human *S. agalactiae* strains, but usually absent in the bovine *S. agalactiae* strains (Franken *et al*., 2001). The *scpB-lmb* intergenic region is known to be a hotspot for the integration of the mobile genetic elements IS1548 and GBSi1, previously described in human GBS belonging to clonal complex 19 (CC19) and CC17, respectively, as assessed by MLST (Safadi *et al*., 2010). Not much is known regarding the distribution (or absence) of these mobile genetic elements in the few bovine GBS that carry the above mentioned genes.

GBS may be serotyped based on their capsular polysaccharides antigens (as previously mentioned). The predominant serotypes in GBS isolates from human origin are Ia, III, and Ib, II and V (Ippolito *et al*., 2010; Tien *et al*., 2011). In contrast, GBS isolates from bovine origin are not serotyped frequently by the conventional serotyping method (based on agglutination with specific serum and labelled with Ia to IX), because they possess antigens which are distinct than those recognized among human isolates. Also, few reports have described so far, bovine GBS isolates studied by molecular serotyping (Kong *et al*., 2002; Zhao *et al*., 2007).
Population structure of GBS isolates from bovine origin is much less described than GBS isolates from human origin. Most bovine GBS, described so far, belong to sequence type 61 (ST-61), ST-2 and ST-23, or are new (Bisharat *et al*., 2004; Brochet *et al*., 2006).
Human *S. agalactiae* strains belonging to clonal complex 17 (CC17) are strongly associated with invasive disease of the central nervous system of neonates, and divergent opinions coexist as to a possible emergence of this highly virulent human lineage (CC17) from a bovine lineage - CC61 (Bisharat *et al*., 2004; Luan *et al*., 2005; Sørensen *et al*., 2010). These two lineages (CC17 and CC61) are close related as assessed by eBURST analysis (see Fig. 1.6).
Human GBS isolates which belong to clonal complex 1 (CC1), CC8 or CC23 are very homogeneous in terms of their prophage content indicating that transduction mechanisms underlining recombination in *S. agalactiae* may be specific to GBS subpopulations of certain lineages (Salloum *et al*., 2011).

A partial genome sequence of a *S. agalactiae* strain from bovine mastitis has been recently published (Richards *et al*., 2011). The bovine GBS genome revealed the presence of an unusual high frequency of insertion sequences, particularly when compared to human GBS genomes known to date, probably in result of the adaptation to the bovine host (Richards *et al*., 2011).

Bisharat *et al*. (2004) observed higher homology among a bovine GBS collection of strains than among a human GBS collection of strains. The author considered the two populations (bovine and human) distinct by using MLST analysis (Bisharat *et al*., 2004). In contrast, Brochet *et al*.

(2006) observed little distinction between two collections of human and bovine *S. agalactiae* strains by using MLST and microarray analysis. Further studies, in particular of bovine GBS isolates, are in need in order to assess if human/bovine GBS represents two subpopulations.

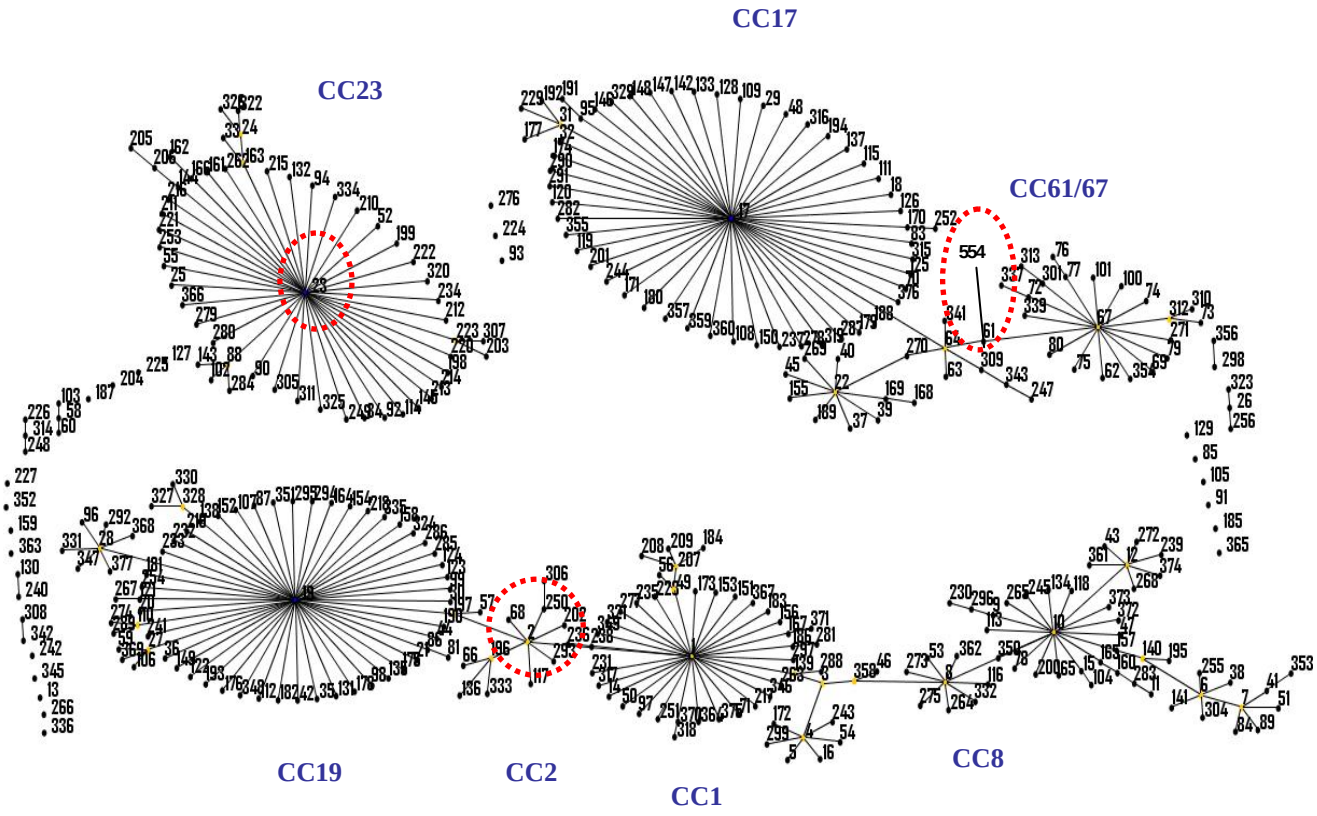


Figure 1.6. MLST/eBURST diagram showing the relationships between *S. agalactiae* strains (http://eburst.mlst.net/). Red circles indicate CC23, CC2, and CC61 (which include ST that are found among bovine GBS).

1.3.1.2. *Streptococcus uberis*

A genome sequence has been obtained for a *S. uberis* strain (Ward *et al*., 2009), (Table 1.2). A lower number of mobile genetic elements in the genome of *S. uberis* were described when compared with other streptococci from the pyogenic group (Ward *et al*., 2009). On the other hand, a great diversity of genes encoding sugar transport and utilization which provide this pathogen to survive in a variety of nutritional habitats has been described, suggesting *S. uberis* as an opportunistic pathogen adapted to changing environmental conditions (Lopez-Benavides *et al*., 2007; Ward *et al*., 2009).

S. uberis is often associated with persistent infections in the bovine udder, and several proteins involved in metabolic activities, which may be implicated in early colonization of *S. uberis* during the infection process, have been described (Tamilselvam *et al*., 2006; Ericsson Unnerstad

et al., 2009; Chen *et al*., 2011). *S. uberis* virulence factors include the hyaluronic acid capsule, plasminogen activator proteins PauA and PauB, the adherence and internalization into epithelial cells mediated by SUAM, and glyceraldehide-3-phosphate dehydrogenase (Rosey *et al*., 1999; Ward *et al*., 2001; Ward & Leigh, 2002; Chen *et al.*, 2011; Reinoso *et al*., 2011). Recently, the *ISS*1-like insertion sequence found in *S. uberis* genome was suggested to have a role in the virulence capacity of *S. uberis*, although further studies are in need to clarify this assumption (Dego *et al*., 2011).

The genetic profiles available in the *S. uberis* MLST database include sequence types (ST) from isolates collected mostly in United Kingdom, Australia, Sweden and Denmark (http://pubmlst.org/suberis/; Pullinger *et al*., 2006). However, information regarding population structure of *S. uberis* in Southern European countries has not been described as far as we know. In particular, population structure among *S. uberis* isolates collected from farms in Portugal was unknown before the present work.

1.3.1.3. *Streptococcus dysgalactiae* subsp. *dysgalactiae*

S. dysgalactiae subsp. *dysgalactiae* (GCS) virulence factors include M-like proteins, which are responsible for the interaction of this pathogen with extracellular matrix proteins of bovine epithelium from the mammary gland (Vasi *et al*., 2000). Indeed, *S. dysgalactiae* subsp. *dysgalactiae* is known to persist inside the bovine mammary epithelial cells (Almeida & Oliver, 1995; Calvinho *et al*., 1998). Recently, this pathogen has been reported associated with invasive disease in a diverse range of hosts, including the human host (koh *et al*.., 2009). However, presence of genes usually associated with invasive disease among *S. dysgalactiae* subsp. *dysgalactiae* isolates was not described before, with exception of *sagA* gene (Chénier *et al.*, 2008; Abdelsalam *et al.*, 2010).

On the other hand, *S. dysgalactiae* subsp. *equisimilis* (GCS, GGS) has been reported associated with human diseases that coincide with the diseases described for the human pathogen *S. pyogenes*, which shares the same host niche in humans. Indeed, *S. dysgalactiae* subsp. *equisimilis* and *S. pyogenes* have the same virulence factors, namely the M-protein, C5a peptidase, and streptokinase (Fischetti, 2000; Geyer & Schmidt, 2000). Furthermore, cross-species acquisition of phage-associated virulence genes, namely *speM*, *ssa*, or *smeZ* (strongly associated with invasive disease) between *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* are known to occur (Igwe *et al*., 2003; Davies *et al*., 2005). However, genomic contributions of the human *S. pyogenes* to the bovine group C *S. dysgalactiae* subsp. *dysgalactiae* (GCS) genome were unknown before the present work.

The complete genome sequencing of several *S. pyogenes* (GAS) strains has revealed the presence of lysogenic bacteriophages in GAS genomes (Beres & Musser, 2007), (see Fig. 1.7). In fact, numerous virulence characteristics in this pathogen are phage associated (Beres & Musser, 2007). *S. pyogenes* phages are members of the *Siphoviridae* family of bacteriophages (Canchaya *et al.*, 2003).

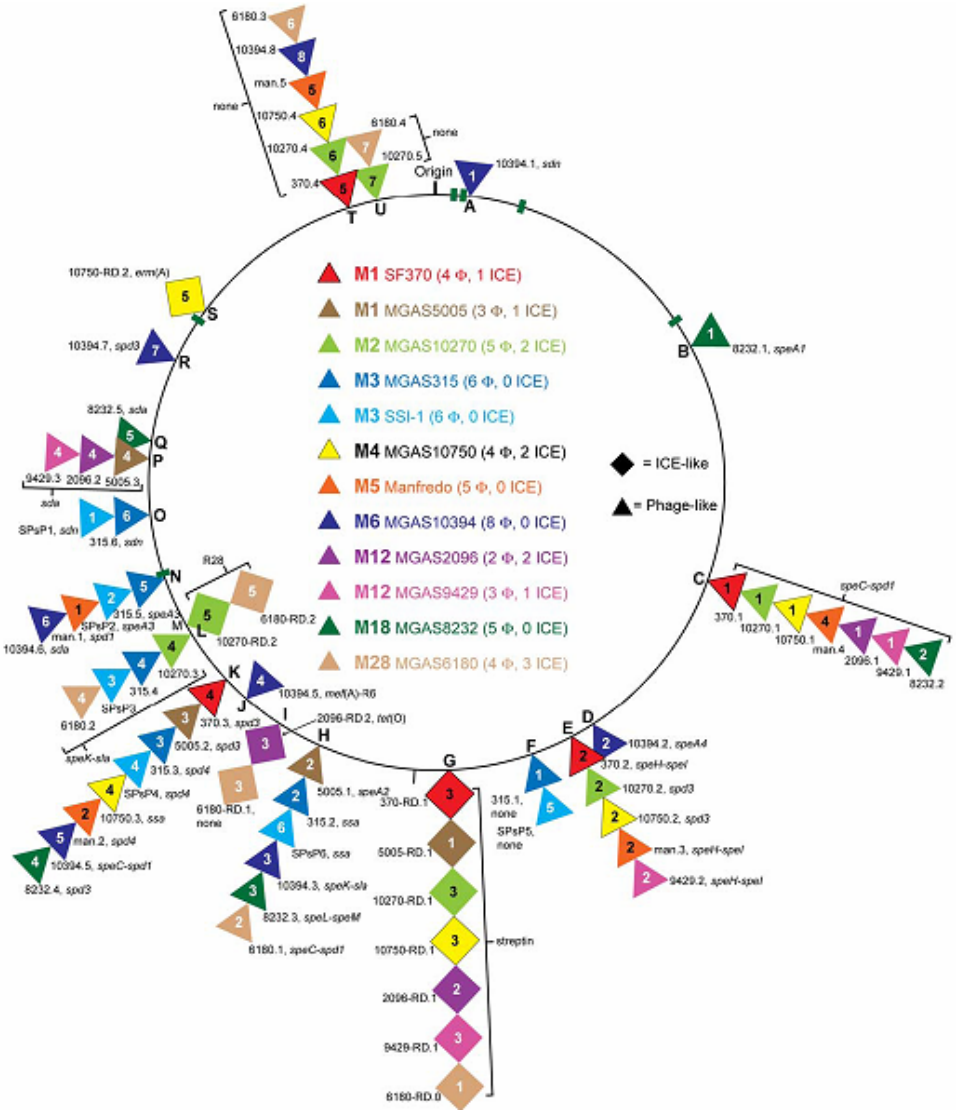


Figure 1.7. Representation of *Streptococcus pyogenes* exogenous elements among 12 *Streptococcus pyogenes* sequenced genomes (Beres & Musser, 2007). Illustrated are the loci of integration of phages and integrative conjugative elements (ICEs) into the core chromosome. Prophages are indicated with triangles and ICEs with squares. Stacked triangles and squares indicate a common integration site. Elements are colour-coded to indicate the source strain. Prophages and ICEs are numbered as they occur clockwise around the core chromosome for each strain. Integration loci are lettered alphabetically as they occur clockwise around the core chromosome. Gene designations are as follows: 1) secreted pyrogenic-toxin-superantigens: *speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM*, and *ssa*; 2) secreted DNAses: *sda*, *sdn*, *spd1*, *spd3*, and *spd4*; 3) secreted phospholipase: *sla*; 4) antimicrobial resistance: *ermA*, *mefA*, and *tet*O; 5) cell surface adhesins: R6 and R28; 6) none, these elements lack a known or obvious virulence gene.

A complete structure of a group G *S. dysgalactiae* subsp. *equisimilis* phage (Φ3396) has been sequenced (Davies *et al.*, 2007). The GGS Φ3396 phage shared overall genomic identity with the *S. pyogenes* 315.1 phage family, but exhibited evidence of recombination from other streptococcal phages (Davies *et al.*, 2007).

Also, comparative genomic analysis between *S. dysgalactiae* subsp. *equisimilis* and *S. dysgalactiae* subsp. *dysgalactiae* revealed that some of the differences in the virulence gene repertoire between these two pathogens could be ascribed to prophage and integrative conjugative elements (Suzuki *et al.*, 2011).

Genomic comparisons between *S. dysgalactiae* subsp. *dysgalactiae*, *S. dysgalactiae* subsp. *equisimilis* and *S. pyogenes*, are likely to provide further information regarding cross-species acquisition of virulence genes that may be involved in the adaptation to new host.

1.3.2. Molecular typing

Molecular epidemiology offers unique opportunities to advance the study of diseases through the investigation of infectious agents at the molecular level. Molecular tools are becoming more widely available to epidemiologists and offer opportunities to increase our understanding of the epidemiology of important pathogens affecting human and animal health (Muellner *et al*., 2011).

Multiple-locus variant-repeat assay (MLVA), multilocus sequence typing (MLST), and Pulsed-Field Gel Electrophoresis (PFGE) are some of the genotyping techniques currently in use for typing and distinguish strains among the streptococcal species under study (Radtke *et al*., 2010; http://www.mlst.net/).

PFGE is generally considered the most discriminatory method to infer relationships between strains (Baseggio *et al*., 1997; Wang *et al*., 1999; Douglas *et al*., 2000). Briefly, PFGE is based on enzymatic DNA restriction of bacterial genome (by using rare-cutting restriction endonucleases) to generate DNA fragments, which are subsequently separated on a gel by applying a specific voltage that is periodically switched among different directions in order to separate the large DNA fragments. The banding pattern obtained from each isolate is then compared with the remaining patterns in order to infer epidemiological relation between the isolates. When two isolates show indistinguishable DNA patterns it is assumed that they are a same strain, and if the patterns differ then its epidemiological relation must be evaluated (Tenover *et al*., 1995; Van Belkum *et al*., 2007).

Different interpretations for analysing of relationships between isolates patterns have been described (Zadoks *et al*., 2002; Van Belkum *et al*., 2007).

MLST allows one to infer evolutionary relationships of strains based on mutations or recombination within housekeeping genes, and detects genetic variation that accumulates slowly in time (Maiden *et al*., 1998). MLST is presently one of the reference methods for genotyping *S. agalactiae* and *S. uberis* (http://pubmlst.org/sagalactiae/, http://pubmlst.org/suberis/).
MLST allows strain comparison between different laboratories and different countries due to public databases which contain the sequence type (ST) information (http://pubmlst.org; Jolley *et al*., 2004).

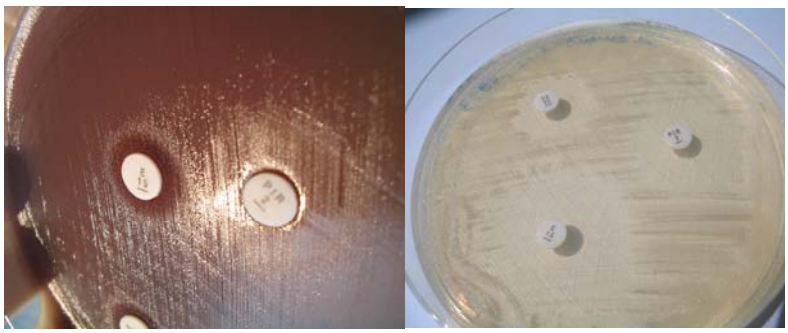
1.3.3. Antimicrobial resistance

Public health issues in particular those involved with the presence of antimicrobial residues in food and dissemination of antimicrobial resistance among bacteria, have determined recommendations for a gradual reduction of antimicrobial use in veterinary medicine (WHO, 2001). The use of antimicrobials in human and veterinary medicine inevitably results in selective pressure for drug resistance in exposed bacteria. Guidelines for a rational use of antimicrobials in veterinary medicine have been described (WHO, 2001).
National recommendations regarding decision on which antimicrobial should be chosen as the first choice in bovine mastitis treatment exist in certain European countries (Ekman *et al*., 1994; Vaarst *et al*., 2002). In Portugal, such as in many other countries, due to restraints in time and financial considerations this choice is usually performed empirically, based on previous experience, national regional recommendations, and also on results obtained by antimicrobial resistance studies performed in other countries. However, resistant patterns to antimicrobials are known to vary between different countries (Kaspar, 2006; Hendriksen *et al*, 2008).

The antimicrobials that are presently used for treating bovine mastitis caused by streptococci in Portugal include penicillin, ampicillin, cloxacillin, pirlimicyn, cefoperazone and cefquinome (http://www.apifarma.pt/simposiumvet/Paginas/Pesquisaavancada.aspx).
Only rare cases of decreased susceptibility to penicillin among bovine mastitis streptococci have been documented (Haenni *et al*., 2010[b]; Kalmus *et al*., 2011). Decrease susceptibility to cephalosporin antimicrobials (such as cefoperazone and cefquinome) is also rare, and cefquinome has been efficiently used for treatment of persistent subclinical mastitis (Tenhagen *et al*., 2006; Kasravi *et al.*, 2011).
On the contrary, resistance to macrolide antimicrobials is a major concern among not only human streptococci but also bovine mastitis streptococci worldwide as previously documented (Rossito *et al.* 2002; Denamiel *et al*., 2005; Duarte *et al.*, 2005; Botrel *et al*., 2010). Erythromycin (which is a macrolide antimicrobial) in particular, is used as first choice treatment

in human therapy in cases where patients are allergic to β-lactam antimicrobials. In some countries such as France and the U.S.A., macrolides are used in the treatment of bovine mastitis (Zwald *et al.* 2004; Schmitt-Van de Leemput & Zadoks, 2007; Botrel *et al.,* 2010); but not in Portugal (http://www.apifarma.pt/simposiumvet/Paginas/Pesquisaavancada.aspx).

Macrolide (such as erythromycin) and lincosamide (such as pirlimycin) antimicrobials have a similar antimicrobial spectra and overlapping targets in their mechanisms of action (see below), which justifies monitoring of resistance patterns against both these antimicrobial classes.
Lincosamide antimicrobials are used in human and animal therapy. Pirlimycin in particular, is available for bovine mastitis treatment in Portugal (http://www.apifarma.pt/simposiumvet/Paginas/Pesquisaavancada.aspx).
Due to the implications of resistance to the above mentioned antimicrobials in human and animal health, surveillance of resistance among veterinary field isolates is of utmost importance, especially in Portugal where such type of information is scarce.
Evaluation of macrolide phenotypes (M, $cMLS_B$, $iMLS_B$, L) can be performed by using a double disk test technique as described previously (Seppälä *et al*., 1993), (see Fig. 1.8). The MLS_B phenotype consists in resistance to macrolide, lincosamide, and streptogramin B antimicrobials, while the M phenotype consists in resistance to macrolide antimicrobials only. The L phenotype consists in resistance to lincosamide antimicrobials (and susceptibility to macrolide).



A **B**

Figure 1.8. Evaluation of macrolide phenotypes: **A**) Double disk test showing the $cMLS_B$ phenotype in a bovine *Streptococcus agalactiae* strain; **B**) control strain *Staphylococcus aureus* ATCC® 25923 used to monitor disk diameter (mm) of erythromycin (macrolide antimicrobial) and pirlimycin (lincosamide antimicrobial) according to the guidelines described before (CLSI, 2008).
Antimicrobial disks used: erythromycin (15µg) and pirlimycin (2µg) (Oxoid®, Basingstoke, England).
(Photos: Márcia G. Rato, FCT/UNL)

The genetic determinants *erm*B and *erm*A encode methylases that confer resistance to erythromycin, and inducible or constitutive resistance to lincosamide, and streptogramin B antimicrobials (macrolide, lincosamide, and streptogramin B phenotype - $iMLS_B$ or $cMLS_B$)

(Weisblum, 1995). The M phenotype is associated with the presence of the genetic determinant *mef*A which encodes a pump conferring resistance to the 14- and 15-membered macrolides.
Resistance to lincosamide and susceptibility to erythromycin (L-phenotype) has been previously observed in streptococci of human and animal origin (Malbruny *et al*., 2004; Haenni *et al*., 2010[a]). The L (or LSA) phenotype is thought to be associated with the presence of *linB* gene, known to be carried by a plasmid of *Enterococcus faecium* from human origin (Bozdogan *et al*., 1999).

Tetracycline is no longer indicated for the treatment of bovine mastitis-associated streptococci in Portugal (http://www.apifarma.pt/simposiumvet/Paginas/Pesquisaavancada.aspx).
Resistance to tetracycline among streptococcal isolates is frequently found in high rates in different countries (Kaspar, 2006; Hendriksen *et al*, 2008).
The genes encoding resistance to tetracycline are usually acquired via transferable plasmids or transposons which also carry erythromycin resistance genes. For example, the association of tetracycline/macrolide determinants *tetM*/*ermB*, *tetO*/*mefA*, *tetO*/*ermA*, or *tetO*/*mefA*, has been previously described to be co-transferred in the same genetic elements among *S. pyogenes* strains (Brenciani *et al*., 2007; Giovanetti *et al*., 2003; D'Ercole *et al*., 2005; Brenciani *et al*., 2010). The *tetS* gene (a tetracycline resistance gene) is known to be carried by integrative conjugative elements among human *S. dysgalactiae* subsp. *equisimilis* strains (Liu *et al*., 2008).
Overall this justifies surveillance for presence of antimicrobial resistance genes among bovine field strains in order to assess the route and dissemination of these genes.

Objectives

The main goal of the study was to provide knowledge about the molecular epidemiology, antimicrobial resistance phenotypes and genotypes and virulence traits of streptococcal species (*S. agalactiae*, *S. dysgalactiae* subsp. *dysgalactiae*, and *S. uberis*) usually associated with bovine mastitis in dairy cows.

The specific aims of this Thesis were:

1. To document the molecular epidemiology and population structure of *S. agalactiae*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. uberis* associated with mastitis to help improvement of bovine health in Portugal. The use of DNA sequencing based methods such as multilocus sequence typing (MLST) for strain characterization (available for *S. agalactiae* and *S. uberis*) will further allow to compare the diversity of strains of these species collected in Portugal and in other countries.

2. To study the antimicrobial resistance and virulence genotypes in order to have a better insight into the incidence, routes and mediators of gene dissemination. The study of antimicrobial susceptibility patterns against antimicrobial drugs used in mastitis control will also contribute to help improvement of bovine health in Portugal.

3. To compare virulence traits, particularly of phage origin, among *S. dysgalactiae* subsp. *dysgalactiae* strains from bovine origin associated with mastitis and severe invasive disease with strains of the human pathogens *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* to further inspect the role of phages and other mobile genetic elements in lateral gene transfer.

2

Molecular epidemiology and population structure of bovine *Streptococcus uberis*

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Abstract

The molecular epidemiology and population structure of 30 bovine subclinical mastitis field isolates of *Streptococcus uberis*, collected from 6 Portuguese herds (among 12 farms screened) during 2002 and 2003, were examined by using pulsed-field gel electrophoresis (PFGE) for clustering of the isolates and multilocus sequence typing (MLST) to assess the relationship between PFGE patterns and to identify genetic lineages. The 30 isolates were clustered into 18 PFGE types, using a similarity cutoff of 80%, and 3 PFGE types accounted for almost half of the isolates (46.6%). These major types were herd specific, suggesting either cow-to-cow transmission or infection with isolates from the same environmental reservoirs. The remaining unrelated PFGE types of isolates were from different herds strongly suggesting environmental sources of *S. uberis* infection. All 30 isolates were analyzed by MLST and clustered into 14 sequence types (ST). These ST were found to be novel, either with 10 new alleles of 6 housekeeping genes or with different combinations of previously assigned alleles. Five of these ST were clustered into 3 clonal complexes (lineages), ST-143, ST-86, and ST-5, known to include bovine isolates from several geographic locations (Australia, New Zealand, United Kingdom, Sweden, and Denmark) and 9 singletons. To our knowledge, this is the first report that documents molecular typing studies of bovine isolates of *S. uberis* from Portugal, which were shown to represent novel genomic backgrounds of this pathogen.

Keywords

Bovine mastitis, *Streptococcus uberis*, pulsed-field gel electrophoresis, multilocus sequence typing

2.1. Introduction

Bovine mastitis remains a major cause of economic loss in the dairy industry worldwide, despite the widespread implementation of mastitis control strategies. Economic losses are caused mainly by decreased milk production, early culling and death of the animals (Seegers *et al*., 2003), and increased antibiotic therapy (Erskine *et al*., 2003).

Contagious mastitis pathogens (such as *S. agalactiae* and *S. aureus*) have been brought under control in dairy herds through use of management practices (Bramley and Dodd, 1984; Bradley, 2002). However, mastitis pathogens traditionally classified as environmental are not well controlled by these methods and are, in several countries, the most frequent cause of subclinical and clinical mastitis in both lactating and nonlactating cows, particularly on well-managed farms (Bradley, 2002). Mastitis due to *S. uberis* infection was generally considered the result of environmental exposure to this pathogen (Lopez-Benavides *et al*., 2007); however, cases of cow-to-cow transmission of *S. uberis* have been reported (Douglas *et al*., 2000; Phuektes et al., 2001; Zadoks et al., 2003).

Methods based on DNA have been successfully used in epidemiological studies aiming to evaluate strain-specific transmission and to improve infection control measures (Khan *et al*., 2003; Wieliczko *et al*., 2002; Zadoks *et al*., 2003). Pulsed-field gel electrophoresis (PFGE) is the most discriminatory method and has been used for typing a broad range of pathogens including bovine (Baseggio *et al*., 1997; Wang *et al*., 1999; Douglas *et al*., 2000) and human pathogens, being most useful in outbreak investigations (Tenover *et al*., 1995; Van Belkum *et al*., 2007). Multilocus sequence typing (MLST) is a valuable tool that allows further investigation of the population structure and evolution of several pathogens (Maiden *et al*., 1998; http://www.mlst.net/), including *S. uberis* (Pullinger *et al*., 2006; Pullinger *et al*., 2007). For *S. uberis*, 2 MLST schemes have been described, one based on sequencing of 6 loci that include virulence genes and housekeeping genes (Zadoks *et al*., 2005[b]) and the other based on the sequencing of 7 housekeeping genes (Coffey *et al*., 2006; http://pubmlst.org/suberis/).

In this study we used PFGE and MLST (using the latter scheme referred to above) to characterize a collection of field isolates of *S. uberis* from subclinical bovine mastitis occurring in Portuguese herds during 2002 and 2003 and to document the molecular epidemiology and population structure of the isolates, which would be useful in providing further insights into the evolutionary and population genetics of this pathogen.

2.2. Materials and Methods

2.2.1. *Streptococcus uberis* collection

A total of 30 *S. uberis* field isolates causing bovine subclinical mastitis were included in this study. This collection comprised all *S. uberis* isolates (with the exception of 4 isolates that were lost during storage) recovered from February 2002 to March 2003, from 261 bovine milk samples of 215 animals with subclinical mastitis from 6 dairy farms (of 12 screened) located in the southwestern region of Portugal (Bexiga *et al*., 2005). The original study (Bexiga *et al*., 2005) involved milk sampling of 459 quarters from 377 animals from 12 dairy herds. Selection criteria for the herds included in the study were geographical area and willingness of the farmers to participate in the study. The 12 herds enrolled in the study had a mean size of 248 lactating cows, a mean bulk tank SCC of 462,000 cells/mL, and a mean milk yield at 305 d of 7,987 kg of milk. Farms were all zero-grazed, which is typical of continental Portuguese herds. The program WinEpiscope 2.0 (Computer-aided Learning In Veterinary Education, CLIVE, University of Edinburgh, UK) was used to calculate the number of cows for milk sampling in each farm. Briefly, a representative number of animals (for an expected prevalence of 50% of subclinical mastitis at cow level, with a margin of error of 10% and a confidence level of 95%) was submitted to a California Mastitis Test, with quarters presenting a score of 3 being chosen for quarter sampling (score 3 on the California Mastitis Test, used to define subclinical mastitis on a scale of 0 to 3, with 0 being negative and 3 being strong positive). Quarters were only sampled once during the study period. Different sampling visits distant in time on the same farm depended on the calculated number of animals to sample.

In total, 351 of 459 samples were positive for bacterial growth. Of those, *S. uberis* and *S. aureus* were found in 10% of the samples, *S. agalactiae* in 18.2%, and CNS in 34.5% (Bexiga *et al*., 2005).

The distribution of the 30 *S. uberis* isolates on the 6 farms (coded F, K, C, D, I, and J) was as follows: 18 isolates from farm F, 6 isolates from farm I, 3 isolates from farm K, and single isolates from farms C, D, and J. All isolates were collected from different animals. Detailed information about the 30 isolates, including source, date of isolation, and, where appropriate, the identification number, breed, and disease status of the cow is available on the *S. uberis* isolates MLST database (http://pubmlst.org/suberis/).

2.2.2. *Streptococcus uberis* identification

All isolates were cultured in sheep blood agar media and were identified as *S. uberis* using the API-20 STREP (bioMérieux, Marcy l'Etoile, France) and BBL Crystal Gram-Positive (Becton Dickinson and Co., Franklin Lakes, NJ) identification systems. Differentiation between *S. uberis* and *S. parauberis*, which are phenotypically indistinguishable (Facklam, 2002), was performed by PCR amplification of the 16S ribosomal DNA (rDNA) gene of all the isolates,

using generic primers for gram-positive bacteria (Takahashi *et al*., 1997) and sequencing with the same primers used for amplification. Automatic sequencing was carried out by Macrogen (Seoul, Korea) or by STAB-Vida (Lisbon, Portugal). Sequence data were edited using SeqMan, DNAstar (DNAstar Inc., Madison, WI). Each of the sequences of the 16S rDNA gene of the 30 isolates was compared with the sequence of the 16S rDNA gene of *S. uberis* reference strain HN1 (GenBank accession no. AB023576) and then compared with the sequences of all the species deposited in the GenBank database using BlastN (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2.3. PFGE typing and cluster analysis

The 30 *S. uberis* isolates were analyzed by PFGE as described previously (Chung *et al*., 2000). Briefly, a 6-mL culture of cells in early stationary phase was harvested and washed in 1 mL of Tris-NaCl buffer (10 mM Tris, pH 8; 1 M NaCl), and resuspended in 200 µL of the same buffer. Concentrations were adjusted to an optical density of 5.0 at 620 nm. This cell suspension was then diluted 1:1 with 150 µL of 1.5% low-gelling temperature agarose (SeaPlaque, FMC Bioproducts, Rockland, ME) in the same Tris-NaCl buffer; disks of 20 µL were allowed to solidify for 5 min at −20°C. The cells were lysed by incubation of the disks at 37°C for 5 h with 50 µg of RNase I, 1 mg of lysozyme, and 5 U of mutanolysine in 1 mL of lysis buffer (6 mM Tris, pH 8, 1 M NaCl, 0.1 M EDTA, pH 8, 0.2% deoxycholate, 0.5% sarkosyl, 0.5% Brij 58). The disks were next incubated in a solution of proteinase K (1 mg/mL) in EDTAsarkosyl buffer (0.5 M EDTA, pH 9, 1% Sarkosyl) at 50°C for 18 h. The agarose disks were washed 8 times in 13 mL of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.5) for 30 min with gentle agitation. The DNA was then considered purified and was stored in approximately 1 mL of Tris-EDTA buffer at 4°C. The DNA was digested with 20 U of *SmaI* (New England BioLabs, Beverly, MA), and PFGE was performed in a CHEF DR-III (contour-clamped homogeneous electric field) apparatus (Bio-Rad, Hercules, CA) for 23 h. The running parameters were as follows: initial pulse = 5 s; final pulse = 35 s; voltage = 200 V; temperature = 11.3°C. Ethidium bromide (0.5 µg/mL) was used to stain the gels. Images were captured using the Gel Doc XR system and the Quantity One 1-D Analysis Software (Bio-Rad). The PFGE lambda marker (New England Biolabs) was used as a molecular weight standard.

All PFGE profiles or patterns were analyzed visually and by computer-assisted cluster analysis using the software BioNumerics v. 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Levels of similarity between fingerprints were calculated by using Dice coefficient, and an unweighted pair group method using arithmetic averages (UPGMA) was used for clustering to produce band-based dendrograms (with a band position tolerance of 1.5% and no optimization). Groups

of patterns with no observed band differences (corresponding to a level of similarity of 100%) were considered indistinguishable and were assigned to the same subtype of a PFGE type. Patterns with variation up to 6 bands were considered related according to previous suggested criteria (Tenover *et al*., 1995) and were clustered in most cases above 80% similarity. These were assigned to different subtypes of a PFGE type. Patterns with variation of 6 or more bands (corresponding to levels of similarity of less than 80%) were not considered related and were assigned to distinct PFGE types. All PFGE types were designated with uppercase letters and each of their subtypes (subclonal variants) was identified in addition by a numeral suffix.

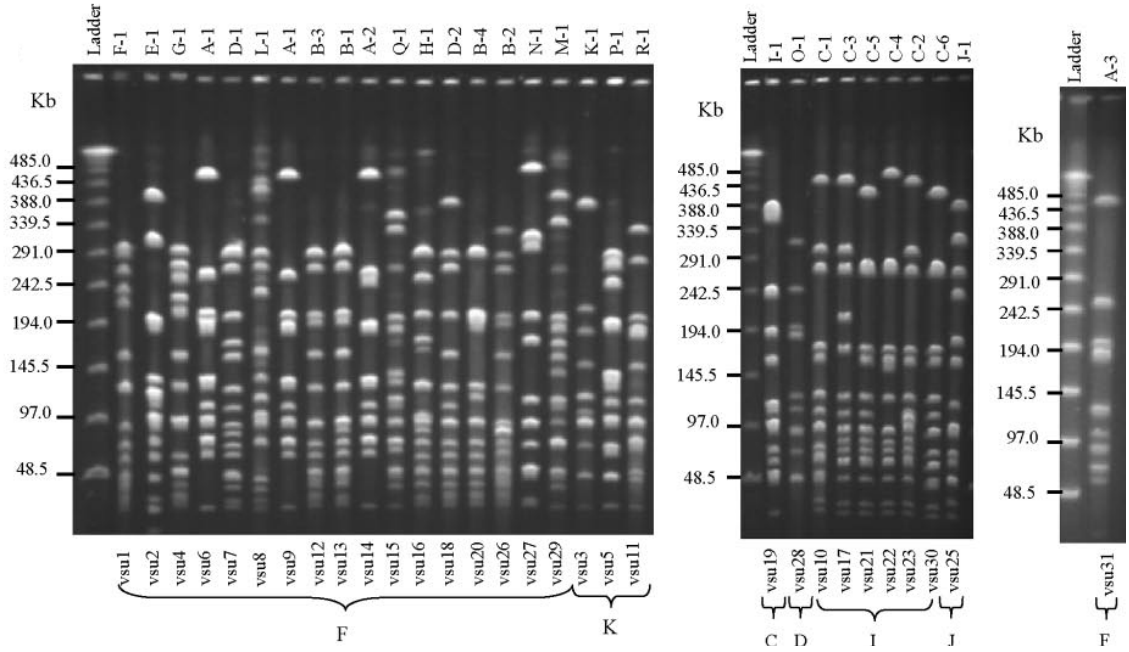

Figure 2.1. Pulsed-field gel electrophoresis (PFGE) profiles of SmaI-digested genomic DNA from *Streptococcus uberis* subclinical mastitis isolates collected at 6 Portuguese dairy herds. Ladder = Lambda PFGE ladder marker (New England BioLabs, Beverly, MA). PFGE type-subtype is designated in top of the images, each isolate code name is designated under the images, as well as the farms from where they were collected, which are designated in capital letters.

2.2.4. MLST and eBURST analysis

All isolates were analyzed by MLST. The DNA was extracted as described for Group A Streptococcus at http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm. The primers used were described previously (www.pubmlst.org/suberis/info/protocol.shtml; Coffey *et al*., 2006). The PCR amplification reactions were adapted from the method available at the *S. uberis* MLST Web site (www.pubmlst.org/suberis/info/protocol.shtml). A 50-µL reaction included 1.25 U of Taq polymerase (MBI Fermentas, Vilnius, Lithuania), 30 pmol of each primer, 1.5

mM MgCl2 (MBI Fermentas), 200 μM of deoxynucleoside triphosphate mix (MBI Fermentas), and 2 μL of DNA. The PCR amplicons were purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI), and sequencing was performed as described above. The sequences were entered at the *S. uberis* MLST database (www.pubmlst.org/suberis/info/protocol.shtml) and novel alleles were assigned new numerals. The allelic profile and sequence type (ST) of each isolate are available at the *S. uberis* MLST database (www.pubmlst.org/suberis/info/protocol.shtml).

The predicted evolutionary descent of the isolates was evaluated by using e-BURST (http://eburst.mlst.net/) of *S. uberis* data set (eBURSTv3 – *Streptococcus uberis*), which included 1 example of each ST assigned at the *S. uberis* MLST database. This tool produces a diagram displaying the way each clonal complex may have emerged and diversified from the predicted founding genotype. This diagram is centered on the predicted founding genotype, and the branches represent the predicted descent to the other genotypes in the group. The eBURST uses lines to show radial links from the founder to each of its single locus variant (initial diversification). Only single locus variant links are shown in an eBURST diagram. All isolates that are directly linked on an e-BURST diagram will therefore differ at only a single locus, and the cluster of linked ST represents a clonal complex.

2.3. Results

2.3.1. 16S rDNA gene sequence analysis for *Streptococcus uberis* identification

Comparative analysis with the 16S rDNA gene sequence of the *S. uberis* reference strain HN1 from the GenBank database showed that out of the 30 isolates, 13 had maximum identity of 100%, 16 isolates differed by 1 bp, corresponding to a maximum identity of 99%, and 1 isolate differed by 3 bp, which also corresponded to a maximum identity of 99%. These variations have been described as microheterogeneity in the 16S rDNA gene sequence within the same species or intraspecies variation of the 16S rDNA gene sequence (Clarridge, 2004). Moreover, sequences of the 16S rDNA gene of streptococcal species other than *S. uberis* (including *S. parauberis*) deposited in the GenBank database were <99% identical to the 16S rDNA gene sequences of the *S. uberis* isolates from the present work. Therefore, we have considered all the isolates as *S. uberis* taking into account the phenotype and this genotypic tool used for identification.

2.3.2. PFGE profiles

Patterns obtained by PFGE ranged from 9 to 15 well-resolved fragments of approximately 10 to 485 kb (Fig. 2.1). Percentage similarity among patterns was determined by cluster analysis in a dendrogram (Fig. 2.2).

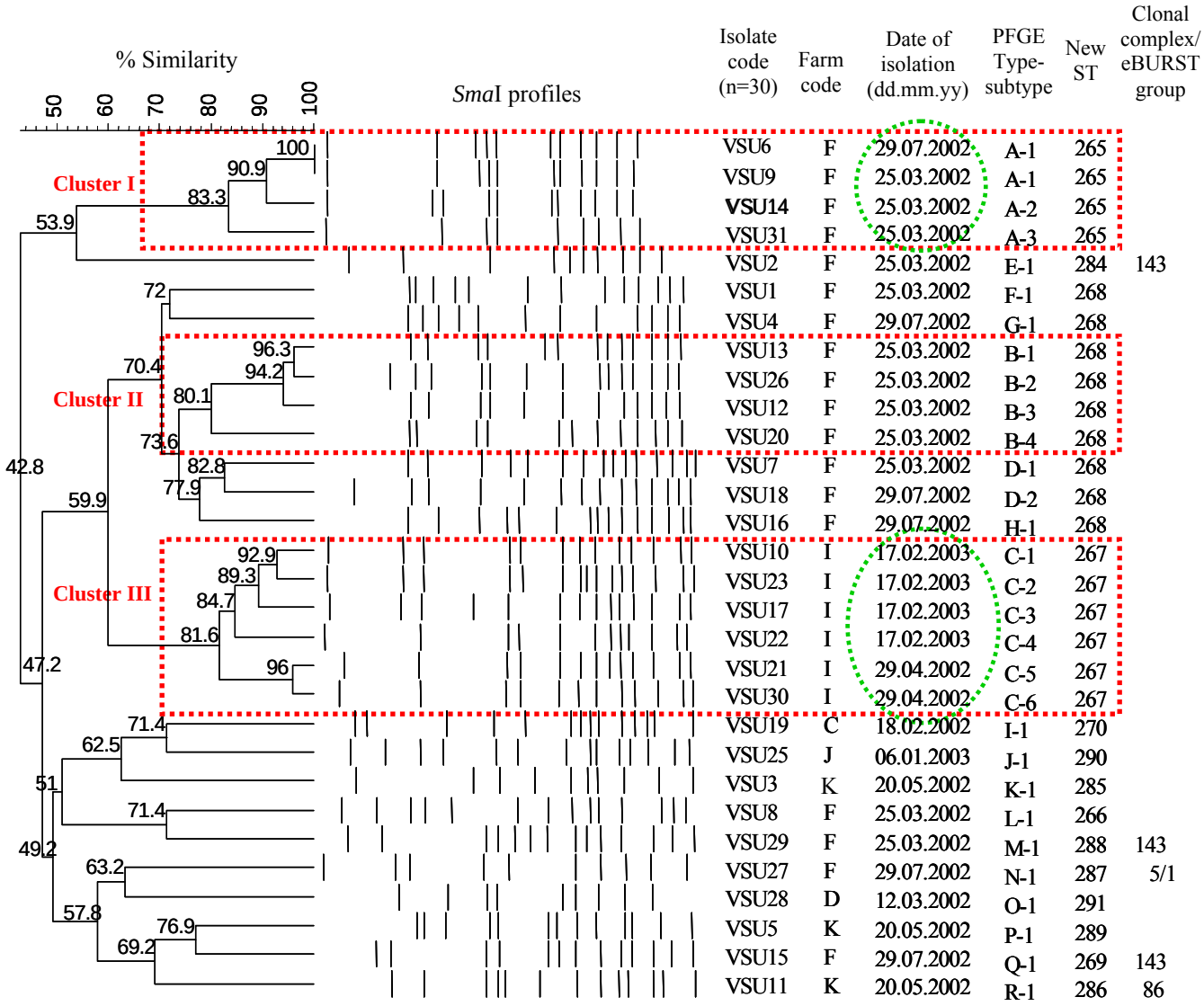

Figure 2.2. Dendrogram of the pulsed-field gel electrophoresis (PFGE) profiles of *Streptococcus uberis* subclinical mastitis isolates, collected at 6 Portuguese dairy herds. Isolate code, farm code, and date of isolation of each strain, and novel multilocus sequence types (ST) and clonal complexes are also designated in the dendrogram. The dendrogram was produced by using Dice coefficients and an unweighted pair group method using arithmetic averages (UPGMA). Default clustering settings of 0.00% optimization (i.e., the relative distance an entire lane is allowed to shift in matching attempts) and 1.5% band position tolerance were used. Plausible related groups (>80% similarity), or clusters, are designated in rectangles. When isolates belonging to a same cluster were collected at different dates, a green circle is indicated. ST, sequence type.

Epidemiologically plausible groups (clusters) were identified using the cut-off of at least 80% similarity as described above. Three major clusters (named I, II, and III) included more than 2 isolates at 80% or greater similarity levels (Fig. 2.2). Cluster I included 4 isolates from farm F. Two isolates (vsu6 and vsu9, collected within a 4 month interval from 2 cows) had indistinguishable patterns (no observed band differences; see Fig. 2.1) corresponding to a level of similarity of 100% and were assigned to a same PFGE subtype (A-1) of PFGE type A. The 2 remaining patterns differed by up to 3 bands between isolate pairs and were assigned to different PFGE subtypes (A-2, A-3). The 3 subtypes have 83.3% similarity. Cluster II included 4 isolates also from farm F, whose patterns differed by 1 to 6 bands between isolate pairs and were assigned to different PFGE subtypes (B-1 to B-4) of PFGE type B with similarity levels ranging from 96.3 to 80.1%. Cluster III included 6 isolates from farm I, whose patterns differed by 1 to 5 bands between isolate pairs and were assigned to different PFGE subtypes (C-1 to C-6) of PFGE type C with similarity levels ranging from 96.0 to 81.6%. A minor cluster comprised 2 other isolates from farm F whose patterns differed by 3 bands and were assigned to different PFGE subtypes (D-1, D-2) of PFGE type D with 82.8% similarity. The remaining isolates were not clustered and were assigned to unique PFGE subtypes.

2.3.3. Allelic profiles and sequence types of Portuguese *Streptococcus uberis* isolates

The sequences of the 7 gene fragments used for the MLST scheme were determined for all 30 isolates confirmed to be *S. uberis*. These isolates were clustered into 14 ST, all found for the first time in this study. These ST were novel because they had different combinations of previously assigned alleles (ST-267, ST-285, ST-286, ST-287, ST-289, and ST-290) or contained 10 new alleles of 6 housekeeping genes (ST-265, ST-266, ST-268, ST-269, ST-270, ST-284, ST-288, and ST-291; Table 2.1). Within the same cluster (I, II, or III), isolates had the same ST: cluster I included 4 isolates of ST- 265, cluster II included 4 isolates of ST-268, and cluster III included 6 isolates of ST-267 (Fig. 2.2). Some of the PFGE subtypes with <80% similarity shared the same ST: PFGE subtypes F-1, G-1, and PFGE subtypes B-1 to B-4 of cluster II with 70.4% of similarity shared the same ST (ST-268); PFGE subtype H-1 and PFGE subtypes D-1 to D-2 with 77.9% of similarity shared the same ST (ST-268; Fig. 2.2).
The 18 isolates of farm F were of seven ST (ST-265, ST-266, ST-268, ST-269, ST-284, ST-287, and ST-288) and 11 PFGE types. The 6 isolates of farm I were of a single ST (ST-267) and a unique PFGE type (with 6 subtypes). The 3 isolates of farm K were of 3 ST (ST-285, ST-286, and ST-289) and of 3 unrelated PFGE types. The single isolates from farms C, J, and D were of ST-270, ST-290, and ST-291, respectively, and of 3 unrelated PFGE types (Table 2.1).

Three clonal complexes or genetic lineages were assigned primarily by the *S. uberis* MLST database curator and all included isolates of single PFGE subtypes. Lineage ST-143 included 3 ST (ST-284, ST-288, and ST-269) of 3 isolates from farm F (vsu2, vsu29, and vsu15) and additionally 122 *S. uberis* strains from dairy herds in Australia, New Zealand, and the UK; lineage ST-5 included the ST-287 of another isolate from farm F (vsu27) and also of 170 *S. uberis* strains from dairy herds in Australia, Denmark, New Zealand, Sweden, and the UK; lineage ST-86 included ST-286 of 1 isolate from farm K (vsu11) and of 41 other *S. uberis* strains from dairy herds in Australia, New Zealand, Sweden, and the UK (data from pubmlst.org/suberis). The remaining 9 ST from this study were singletons.

Table 2.1. Allelic profiles and clonal complexes of *Streptococcus uberis* isolates of bovine subclinical mastitis from Portugal assigned by multilocus sequence typing (MLST).

Farm code	Isolate code	PFGE Type - subtype	Allele							Sequence Types[2] (ST)	Clonal Complex[3] (ST)	e-BURST Group
			arcC	ddl	gki	recP	tdk	tpi	yqil			
F	vsu6	A-1	4	1	26[1]	3	2	2	5	265		
F	vsu9	A-1	4	1	26[1]	3	2	2	5	265		
F	vsu14	A-2	4	1	26[1]	3	2	2	5	265		
F	vsu31	A-3	4	1	26[1]	3	2	2	5	265		
F	vsu8	L-1	2	10	2	1	38[1]	4	27[1]	266		
I	vsu10	C-1	9	1	5	3	17	4	10	267		
I	vsu21	C-5	9	1	5	3	17	4	10	267		
I	vsu23	C-2	9	1	5	3	17	4	10	267		
I	vsu17	C-3	9	1	5	3	17	4	10	267		
I	vsu22	C-4	9	1	5	3	17	4	10	267		
I	vsu30	C-6	9	1	5	3	17	4	10	267		
F	vsu4	G-1	9	1	27[1]	2	39[1]	1	17	268		
F	vsu13	B-1	9	1	27[1]	2	39[1]	1	17	268		
F	vsu26	B-2	9	1	27[1]	2	39[1]	1	17	268		
F	vsu12	B-3	9	1	27[1]	2	39[1]	1	17	268		
F	vsu20	B-4	9	1	27[1]	2	39[1]	1	17	268		
F	vsu1	F-1	9	1	27[1]	2	39[1]	1	17	268		
F	vsu7	D-1	9	1	27[1]	2	39[1]	1	17	268		
F	vsu18	D-2	9	1	27[1]	2	39[1]	1	17	268		
F	vsu16	H-1	9	1	27[1]	2	39[1]	1	17	268		
F	vsu15	Q-1	38[1]	1	2	2	29	4	3	269	143	
C	vsu19	I-1	6	1	5	14[1]	17	1	3	270		
F	vsu2	E-1	9	1	2	2	42[1]	2	3	284	143	
K	vsu3	K-1	6	16	5	2	17	1	3	285		
K	vsu11	R-1	10	1	3	3	13	1	3	286	86	
F	vsu27	N-1	1	1	2	2	16	1	3	287	5	1
F	vsu29	M-1	38[1]	1	2	2	22	4	3	288	143	
K	vsu5	P-1	10	1	3	2	13	1	10	289		
J	vsu25	J-1	1	1	5	2	10	1	3	290		
D	vsu28	O-1	2	27[1]	5	1	43[1]	1	3	291		

[1]PFGE = pulsed-field gel electrophoresis.

[2]Novel allelic profiles or sequence types (ST).

[3]MLST clonal complexes (clusters of linked ST) assigned by the *S. uberis* MLST database curator.

[4]Novel allele.

The e-BURST analysis revealed that only ST-287 from this study of lineage ST-5 was included in a clonal complex described as group 1 by eBURSTv3 – *Streptococcus uberis* software (http://eburst.mlst.net/; Fig. 2.3); ST-287 was found to be a single locus variant of the predicted founder (ST-184) of this major clonal complex (Fig. 2.3), which includes 107 ST of the *S. uberis* MLST database.

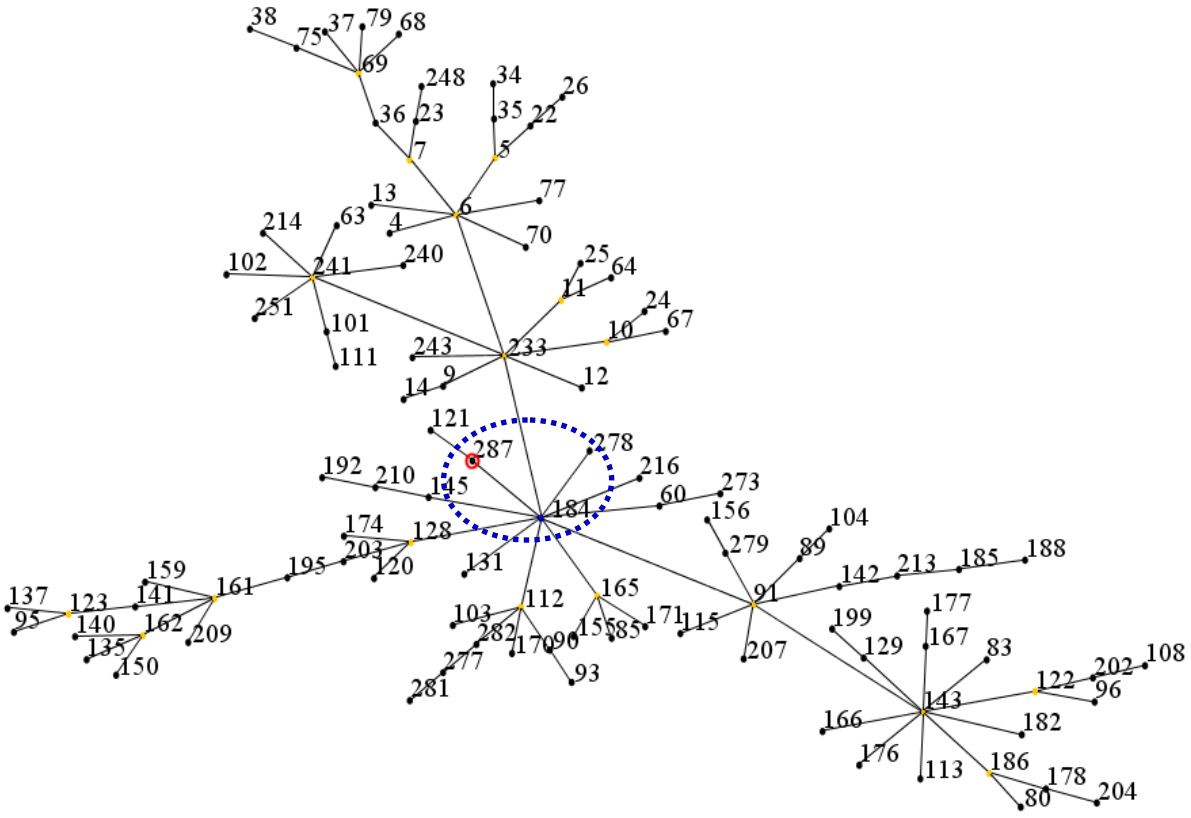

Figure 2.3. e-BURST diagram determined by eBURSTv3 software (http://eburst.mlst.net/) using 1 example of each sequence type (ST) assigned in the *Streptococcus uberis* multilocus sequence typing (MLST) database (http://pubmlst.org/suberis/info/protocol.shtml). Only ST-287 (pointed out in red) from the present work was assigned to the major clonal complex described as group 1, which includes 107 ST of *S. uberis* MLST database; ST-287 is a single locus variant (SLV) of the predicted founder ST-184 (blue circle).

2.4. Discussion

Streptococcus uberis is one of the most prevalent pathogens in dairy herds and is considered one of the principal causative agents of bovine mastitis, a very costly disease in dairy industry (Leigh, 1999; Shim *et al*., 2004). It has been noted that the relative importance of transmission mechanisms and control measures differs between herds and geographical areas (LeBlanc *et al*., 2006). However, studies on the molecular characterization of field *S. uberis* isolates from

Portugal are still not documented and are of utmost importance for a more efficient control of bovine mastitis caused by this pathogen.

This species, together with the contagious pathogen *S. aureus*, accounted for 10% of all milk samples with positive growth, *S. agalactiae* accounted for 18.2%, and CNS accounted for 34.5%, during 2002 and 2003, from a study on bovine subclinical mastitis among 12 commercial dairy farms in a particular region of Portugal (Bexiga *et al*., 2005). Greater prevalence rates of *S. uberis* have been reported in other countries such as New Zealand (Douglas *et al*., 2000) because the dairy industry there is mainly pasture based. However, continental Portuguese herds are typically zero-grazed, which could explain lower rates of *S. uberis* infection. Moreover, among the 12 commercial dairy farms screened, *S. uberis* was found on only 6 farms (50%).

Our aim was to assess if subclinical bovine mastitis episodes were associated with multiple PFGE types or with a limited number of dominant types, which is important information for the implementation of targeted mastitis control programs. Additionally, we aimed to document the genotypic properties of this species in a region not yet surveyed, contributing to increased knowledge about the evolutionary and population genetics of this pathogen by using MLST.

The combined use of the molecular typing techniques mentioned above was a valuable approach to achieve our aims. The prevalence of *S. uberis* varied between herds. The totality of the *S. uberis* isolated in the survey by Bexiga *et al*. (2005) were included in the present study. Close to half (44.4%; n = 8/18) of the *S. uberis* isolates from farm F (n = 18) were clustered into 2 large homogeneous PFGE groups, clusters I and II. Moreover, all the *S. uberis* isolates from farm I (n = 6) were clustered in 1 PFGE homogeneous group (cluster III).

The finding of a limited number of dominant PFGE clusters or PFGE types spread over farms F and I rather than a wide heterogeneity of PFGE types strongly suggested that cows might have been the source of infections or that acquisition from a common environmental source occurred. Despite that, other heterogeneous PFGE types were detected on farm F, which suggests that several environmental sources might have been the source of infections or that the replacement policy on that farm relied more heavily on buying in animals than occurred on other farms.

Related isolates, according to the PFGE data, were seen to share the same ST; however, PFGE and MLST did not correlate in all cases. Isolates of unrelated PFGE types F, G, and B (70.4% similarity) were found to share the same ST (ST-268; Fig. 2.2). In addition, H and D (77.9% similarity) shared ST-268. This result was expected because PFGE is sensitive to microvariation and, therefore, often has more discriminatory power (Van Belkum *et al*., 2007) than MLST, which detects variation that accumulates slowly in housekeeping genes (Maiden *et al*., 1998).

Moreover, different PFGE subtypes of a same type were observed among isolates from different cows within the same herd collected on the same day of sampling. Examples are isolates of

PFGE B-1 to B-4 from farm F or isolates of PFGE C-1 to C-4 from farm I (Fig. 2.2.). In contrast, and of interest, was the finding of 2 isolates with indistinguishable PFGE patterns (100% similarity; PFGE subtype A-1) collected from 2 cows on farm F collected within a 4-month interval, which strongly suggests that infection of both cows was caused by the same strain, either because of transmission during milking or by close contact with the same environmental reservoir. Clarification of the importance of these environmental reservoirs to the incidence of infection by typing isolates from different sites on the cow and environmental sites will be required in the future. Nevertheless, the observed differences in PFGE banding patterns may be due to genetic changes in persisting strains within these herds.

Prevalence of *S. uberis* clones as well as persistent *S. uberis* strains within the same herd have been reported previously (Phuektes *et al*., 2001; Zadoks *et al.*, 2003), although most studies found that the point source of infection is uncommon and the main route of transmission is through environmental sources (Khan *et al*., 2003; Tomita *et al*., 2008). These disparities in the epidemiology of *S. uberis* may reflect differences among production systems (McDougall *et al*., 2004).

More variability was expected between related isolates collected at sampling visits distant in time. In fact, different PFGE subtypes of a same type were observed among isolates from different cows within the same herd collected at 4-month or 10-month intervals between sampling. Examples are isolates of PFGE A-1 to A-3, D-1 and D-2 from farm F, or isolates of PFGE C-1 to C-4 and isolates C-5 or C-6 from farm I (Fig. 2.2).

As noted above, in most cases, the MLST results agreed with the fingerprinting data and allowed us, in addition, to infer a population structure for our collection of isolates. All of the ST found in this study were novel, either with 10 new alleles of 6 housekeeping genes or with different combinations of previously assigned alleles. Presently, the *S. uberis* profile database consists of 291 profiles. Among these, ST-265 to ST-270 and ST-284 to ST-291 were the ones described in this study. Interestingly, the 2 large homogeneous PFGE clusters I and II, which grouped almost half (44.4%) of the *S. uberis* from farm F were judged as singletons (ST-265 and ST-268) by MLST. On the other hand, the remaining isolates that were dispersed by minor and unique PFGE patterns and grouped into 4 sequence types (ST-269, ST-284, ST-288, and ST-287) were clustered into 2 clonal complexes or lineages (ST-5 and ST-143). It was suggested that isolates belonging to clonal complexes ST-5 and ST-143 might possess virulence factors promoting invasion of host tissue, survival in the host environment, evasion of the host immune response, or internalization in the mammary gland (Tomita *et al*., 2008). Moreover, a link was observed between clonal complexes ST-5 and ST-143 and carriage of the hasA virulence gene (Coffey *et al*., 2006; Pullinger *et al*., 2006).

It is intriguing why only the singletons from farm F, rather than the major PFGE cluster I and cluster II (also from farm F), were assigned to these virulent clonal complexes, because these strains might have been hypertransmissible among cows, and therefore would be expected to be more virulent. Our data suggest that strains other than those of clonal complexes ST-5 and ST-143 are also hypervirulent strains.

The occurrence of identical ST between Portugal and other countries was not detected. All ST found in the present work are unique and were found for the first time in this collection, which would be expected because information regarding mastitis isolates from Mediterranean countries is lacking in the MLST database. Nevertheless, ST-287 from the present study was found to be a single locus variant of the predicted founder ST-184 of the major group 1 clonal complex (http://eburst.mlst.net); ST-184 includes one strain from clinical mastitis in Australia and strains from environmental sources in New Zealand.

2.5. Conclusions

In conclusion, it seems that the contagious route of mastitis infection is still a problem in Portugal. High prevalence of the contagious mastitis pathogens *S. agalactiae* and *S. aureus* was found among the subclinical mastitis milk samples collected during the original study period (Bexiga *et al*., 2005). Although *S. uberis* is traditionally classified as an environmental pathogen, it may also be transmitted from cow to cow, probably through the milking process. The PFGE analysis in the present study supports this view, as we observed that a limited number of dominant PFGE types spread in farms F and I (which accounted for 46.6% of all isolates). Nevertheless, acquisition from a common source may also be occurring: all farms were zero-grazed and, therefore, infection with *S. uberis* from environmental reservoirs such as pastures was not expected. These results may reflect a possible defect in the implementation of mastitis control programs on these farms, and better policies for herd management may be needed. Interestingly, presumably more virulent strains (assigned to virulent clonal complexes) were associated with single PFGE types and not with clonal spread. To our knowledge, this is the first report describing genetic relationships among bacterial isolates of bovine *S. uberis* from Portugal, which were shown to represent novel genomic backgrounds and unique genotypes of this pathogen.

Acknowledgments and work contributions

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3

Human group A streptococci virulence genes in bovine group C streptococci

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Abstract

Phage-encoded virulence genes of group A streptococci were detected in 10 (55.6%) of 18 isolates of group C streptococci that had caused bovine mastitis. Bovine isolates carried other genetic determinants, such as composite transposon Tn*1207.3*/Φ10394.4 (100%) and antimicrobial drug resistance genes *ermB*/*ermA* (22.2%), *linB* (16.6%), and *tetM*/*tetO* (66.7%), located on mobile elements.

Keywords

Bacteriophage-virulence genes, Group A streptococci, *Streptococcus pyogenes*, Bovine group C streptococci, *Streptococcus dysgalactiae* subsp. *dysgalactiae*

3.1. Introduction

Strains of *Streptococcus dysgalactiae* subsp. *dysgalactiae* are described as alpha-hemolytic or non-hemolytic (Lancefield group C) and associated only with animal infections (bovine mastitis), a disease with major economic consequences for the dairy industry (Vieira *et al*., 1998). Group A streptococci (GAS) specific phage-associated virulence determinants encoding pyrogenic exotoxins or superantigens (*speM*, *ssa*), which are strongly associated with severe diseases such as scarlet fever, streptococcal toxic shock syndrome, and rheumatic fever, have been described among human group C streptococci (GCS) or group G streptococci (GGS) (*S. dysgalactiae* subsp. *equisimilis*) (Igwe *et al*., 2003) but not among alpha-hemolytic GCS (*S. dysgalactiae* subsp. *dysgalactiae*) of bovine origin. In contrast, M protein or M-like proteins were found in human GGS/GCS (*S. dysgalactiae* subsp. *equisimilis*) and in animal GCS (*S. dysgalactiae* subsp. *dysgalactiae*) but only in beta-hemolytic strains (Zhao *et al*., 2007).
Composite transposons and other genetic determinants also considered to be located in specific mobile elements such as macrolide (either encoding methylases [*erm* genes] or efflux pumps [*mef* genes]) and tetracycline resistance determinants (*tet* genes) have been found among streptococcal species of human origin. We studied a collection of field isolates of bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* to search for genetic determinants, particularly those carried by mobile elements known to be transferred among human GAS and GGS/GCS.

3.2. Materials and Methods

We studied 18 alpha-hemolytic *S. dysgalactiae subsp*. *dysgalactiae* field isolates of Lancefield group C that had caused bovine subclinical mastitis. Isolates were obtained from 304 milk samples of 248 cows from 8 farms in Portugal that were included in the study. Detailed information regarding isolation methods and identification of field isolates by biochemical methods were described in Chapter 3 (identification by biochemical methods was carried as described for *S. uberis*).
To confirm identification of *S. dysgalactiae* subsp. *dysgalactiae*, the 16S rRNA gene was amplified by PCR and sequenced (Weisburg *et al*., 1999). *SmaI*/*cfr9I*-digested DNA banding patterns were obtained by pulsed-field gel electrophoresis for clone identification performed as described for the pathogen *S. uberis* (see Chapter 2).
All virulence genes and antimicrobial resistance genes analyzed by PCR in the present study are described in Table 3.1 and Table 3.2, respectively. The *emm* gene subtyping was performed as described (www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm).
Samples without DNA and strains lacking (negative) or carrying (positive) specific genes were

used as controls in the PCR. Results were consistent in 2 or 3 PCRs that included these controls.

Table 3.1. Virulence genes and primers used for the PCR analysis, among bovine GCS *Streptococcus dysgalactiae* subsp. *dysgalactiae*.

Primer name	Sequence (5'-3')	Product (bp)	Reference
Prophage-associated virulence determinants			
Pyrogenic exotoxins:			
ssa (for.) *ssa* (rev.)	GTGTAGAATTGAGGTAATTG TAATATAGCCTGTCTCGTAC	706	Pires *et al.*, 2009
speA (for.) *speA* (rev.)	CTTAAGAACCAAGAGATGGC ATAGGCTTTGGATACCATCG	200	Pires *et al.*, 2009
speC (for.) *speC* (rev.)	CATCTATGGAGGAATTACGC TGTGCCAATTTCGATTCTGC	246	Pires *et al.*, 2009
speH (for.) *speH* (rev.)	AGATTGGATATCACAGG CTATTCTCTCGTTATTGG	416	Pires *et al.*, 2009
speI (for.) *speI* (rev.)	AAGGAAAAATAAATGAAGGTCCGCCAT TCGCTTAAAGTAATACCTCCATATGAATTCTTT	217	Lingtes *et al.*, 2007
speJ (for.) *speJ* (rev.)	ATCTTTCATGGGTACG TTTCATGTTTATTGCC	535	Pires *et al.*, 2009
speK (for.) *speK* (rev.)	TATCGCTTGCTCTATACACTACTGAGAGT CCAAACTGTAGTATTTTCATCCGTATTAAA	233	Lingtes *et al.*, 2007
speL (for.) *speL* (rev.)	GGACGCAAGTTATTATGGATGCTCA TTAAATAAGTCAGCACCTTCCTCTTTCTC	460	Lingtes *et al.*, 2007
speM (for.) *speM* (rev.)	GCTTTAAGGAGGAGGAGGTTGATATTTATGCTCTA CAAAGTGACTTACTTTACTCATATCAATCGTTTC	411	Lingtes *et al.*, 2007
DNAse1:			
spd1 (for.) *spd1* (rev.)	CCCTTCAGGATTGCTGTCAT ACTGTTGACGCAGCTAGGG	400	Green *et al.*, 2005
Phospholipase A2e:			
slaA (for.) *slaA* (rev.)	CTCTAATAGCATCGGCTACGC AATGGAAAATGGCACTGAAAG	440	Green *et al.*, 2005
Composite transposon:			
Tn*1207.3*/Φ10394.4 R.J.* (for.)	CGAGGAGTTAGTATGGAAAC	473	Figueiredo *et al.*, 2006
Tn*1207.3*/Φ10394.4 R.J.* (rev.)	CCCATAATAGGCAACTGGTCTCCAGC		
Tn*1207.3*/Φ10394.4 L.J.† (for.)	TCTTCGCCGCATAAACCCTATC	453/6,807‡	Figueiredo *et al.*, 2006
Tn*1207.3*/Φ10394.4 L.J.† (rev.)	CCTTTGACCAATGAAGTGACCTTT		
M protein			
emm (for.) *emm* (rev.)	TATTCGCTTAGAAAATTAA GCAAGTTCTTCAGCTTGTTT	variable	**

*R.J.: right junction; †L.J.: left junction

‡The expected ampicon size was 454 bp according to the reported organization of the Tn1207.3 element or 6,807 bp according to the reported sequence of Φ10394.4 (Figueiredo *et al.*, 2006).

**www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm

Sequencing of all virulence gene amplicons was performed with the same primers used for amplification (STAB-Vida, Lisbon, Portugal). All sequences were compared with sequences in GenBank by using the BLAST alignment tool (www.ncbi.nlm.nih.gov/BLAST).

Table 3.2. Antimicrobial resistance genes analyzed by PCR and primers used.

Primer name	Sequence (5'-3')	Product (bp)	Reference
Macrolide Resistance			
mefA (for) *mefA* (rev.)	GACCAAAAGCCACAATTGTGGA CCTCCTGTCTATAATCGCATG	1432	Pires *et al.*, 2005
ermA (for.) *ermA* (rev.)	CCCGAAAAATACGCAAAATTTCAT CCCTGTTTACCCATTTATAAACG	590	Pires *et al.*, 2005
ermB (for.) *ermB* (rev.)	GGAGTGATACATGAACAAAAATA TTCCTTTTAGTAACGTGTAACTTT	531	Pires *et al.*, 2005
Tetracycline Resistance			
tetM (for.) *tetM* (rev.)	TGGAATTGATTTATCAACGG TTCCAACCATACAATCCTTG	1080	Pires *et al.*, 2005
tetO (for.) *tetO* (rev.)	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	515	Ng *et al.*, 2001
tetT (for.) *tetT* (rev.)	AAGGTTTATTATATAAAAGTG AGGTGTATCTATGATATTTAC	169	Aminov *et al*., 2001
tetW (for.) *tetW* (rev.)	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	Aminov *et al*., 2001
tetQ (for.) *tetQ* (rev.)	TTATACTTCCTCCGGCATCG ATCGGTTCGAGAATGTCCAC	904	Ng *et al.*, 2001
tetS (for.) *tetS* (rev.)	GAAAGCTTACTATACAGTAGC AGGAGTATCTACAATATTTAC	169	Aminov *et al*., 2001
tetL (for.) *tetL* (rev.)	TCGTTAGCGTGCTGTCATTC GTATCCCACCAATGTAGCCG	267	Ng *et al.*, 2001
tetK (for.) *tetK* (rev.)	TCGATAGGAACAGCAGTA CAGCAGATCCTACTCCTT	169	Ng *et al.*, 2001
Lincosamide Resistance			
linB (for.) *linB* (rev.)	CCTACCTATTGTTTGTGGAA ATAACGTTACTCTCCTATTC	925	Bozdogan *et al*., 1999
Streptogramin A resistance			
vgaB (for.) *vgaB* (rev.)	AAGTCGACTGACAATATGAGTGGTGG CTGCAGATGCCTCAACAGCATCGAATATCC	1051	Allignet & Solh, 1997

3.3. Results

We detected bacteriophage-associated virulence genes *speM*, *speK*, *speC*, *spd1*, and *speL*. The overall distribution of these GAS virulence genes in the bovine GCS isolates from the present

study, as well as other characteristics of the bovine strains are described in Fig. 3.1. All but 1 of the PCR products showed expected sizes (Table 3.1 and Table 3.2). The Tn*1207.3*/Φ10394.4 composite transposon left junction amplicon showed a size of 380 bp instead of 453–6,807 bp as described for GAS (Figueiredo *et al.*, 2006). No amplification was observed for the right junction of this genetic element.

The *emm* gene encoding the antiphagocytic M surface protein was not amplified in any of the 18 bovine GCS isolates; therefore, no *emm* types were obtained.

Subsets of GCS isolates were erythromycin and pirlimycin resistant (MLS_B phenotype) and contained *ermB* or *ermA* genes (22.2%) or erythromycin susceptible and pirlimycin resistant and contained the *linB* gene (16.6%). All isolates were tetracycline resistant with a subset (66.7%) carrying *tetM* or *tetO* tetracycline resistance determinants. See Chapter 5 for further details regarding results of patterns of antimicrobial drug resistance against macrolides (erythromycin), lincosamides (pirlimycin) and tetracycline, and macrolide phenotypes tested among the bovine GCS.

Sequences of all virulence genes were compared by using the BioEdit sequence alignment editor (Hall, 1999). One different allele was found for each of the following gene sequences: *spd1* (among 6 strains), *speC* (among 6 strains), and *speL* (among 4 strains). Three alleles were found for *speK* (among 8 strains) (*speK*-1, *speK*-2 and *speK*-3), and two alleles were found for *speM* gene sequences (among 2 strains) (*speM*-1, *speM*-2). Bovine alleles had sizes of 386 bp (*spd1*), 222 bp (*speC*), 444 bp (*speL*), 232 bp (*speK*), and 357 bp (*speM*). Examples of alignments between bovine virulence gene alleles with sequences from GenBank (only most similar ones) are shown in Fig. 3.2.

3.4. Discussion

Using PCR, we determined that bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* strains (55.6%) carried >1 GAS-specific bacteriophage virulence-associated genes (*spd1*, *speC*, *speK*, *speL*, and *speM*). This finding suggested that bacteriophages may also play a role in the genetic plasticity and virulence of animal GCS.

The *speL* allele from bovine strains showed higher similarity with the *szeM* allele (99% maximum identity) from *S. equi* subsp. *zooepidemicus* than with the *speL* allele (97% maximum identity) from *S. pyogenes*. The *szeM* gene encodes a superantigen in *S. equi* subsp. *zooepidemicus*, which is primarily a pathogen of nonhuman animal species. This organism causes mastitis in mares and cows and is most frequently found in horses (Alber *et al.*, 2005). We also observed that the *speM* alleles found among bovine strains also showed higher similarity with superantigen-encoding gene *szeL* from *S. equi* subsp. *zooepidemicus* than with

speM gene sequence from *S. pyogenes*. Moreover, another allele (a *speM* allele) showed higher similarity with the *sdm* gene from *S. dysgalactiae* subsp. *dysgalactiae* than with the *speM* gene from *S. pyogenes*.

The remaining alleles (*spd1*, *speC*, *speK*-1, and *speK*-2) from the GCS *S. dysgalactiae* subsp. *dysgalactiae* bovine strains showed high similarity with *S. pyogenes* superantigen genes (98%–99% maximum identity).

This finding supports our hypothesis that GAS prophages may play a role in the genetic plasticity of this pathogen. The *speC* and *spd1* genes are known to be localized on the same GAS prophage (Banks *et al.*, 2002), and both genes were in fact detected in 6 bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates in our study.

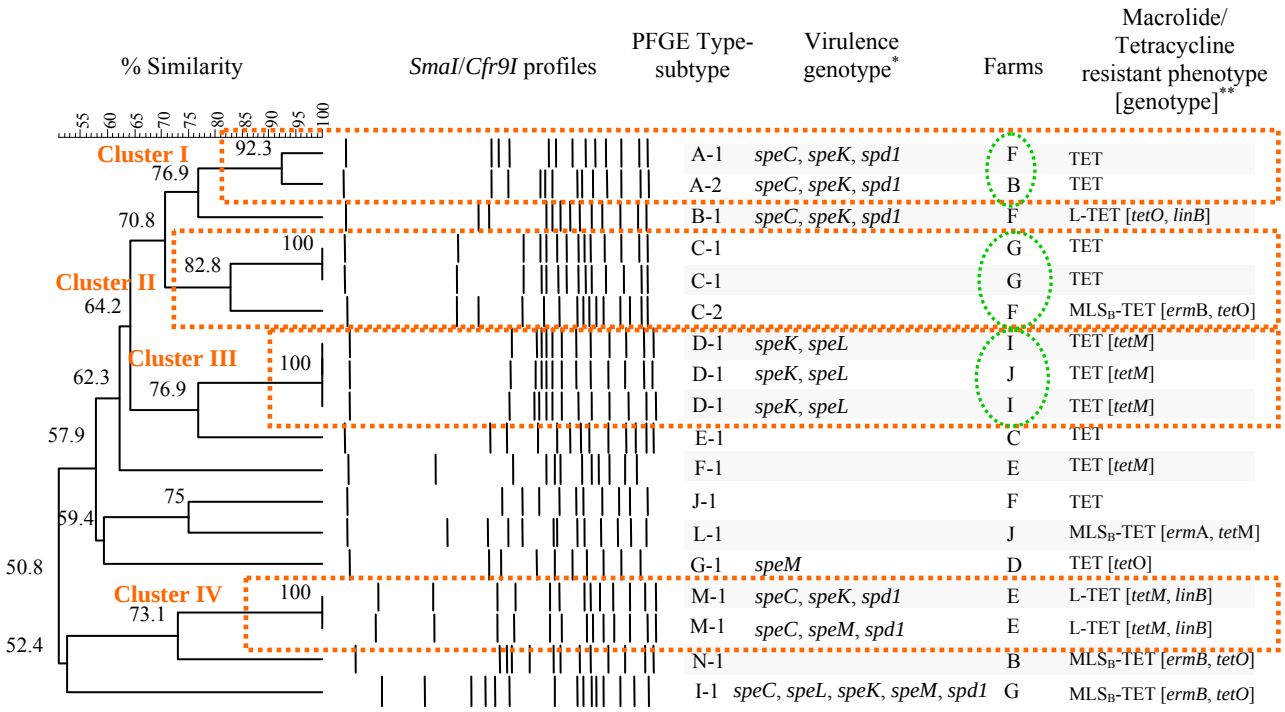

Figure 3.1. Dendrogram and pulsed-field gel electrophoresis (PFGE) profiles of group C streptococci (GCS) *Streptococcus dysgalactiae* subsp. *dysgalactiae* subclinical mastitis isolates from 8 dairy herds, Portugal. PFGE type-subtype, virulence genotype, antimicrobial drug resistance phenotypes, and genotypes of each isolate are indicated. The dendrogram was produced by using Dice coefficients and an unweighted pair group method using arithmetic averages (UPGMA). Default clustering settings of 0.00% optimization and 1.5% band position tolerance were used. Plausible related groups (>80% similarity), or clusters, are designated in rectangles. When isolates belonging to a same cluster were collected at different farms a green circle is indicated. *All isolates were negative for *speA*, *ssa*, *speH*, *speJ*, *speI*, *slaA* genes and for Tn*1207.3*/Φ10394.4 element right junction tested by PCR; **All isolates were negative for *mefA*, *tetT*, *tetW*, *tetL*, *tetQ*, *tetS*, and *tetK* genes; TET, resistance only to tetracycline; MLS_B-TET, resistance to macrolide, lincosamides, streptogramin B and TET; L-TET, susceptibility to macrolides and resistance to lincosamides and TET; Tn*1207.3*/Φ10394.4 element left junction was detected in all isolates.

None of the 18 alpha-hemolytic group C *S. dysglacatiae* subsp. *dysgalactiae* bovine isolates in

this study were typed by *emm*-typing because amplification products in the PCR specific for the M surface protein gene *emm* were not obtained. This result is consistent with those of a report that beta-hemolytic, but not alpha-hemolytic, group C *S. dysglacatiae* subsp. *dysgalactiae* isolates of animal origin contained the *emm* gene (Zhao *et al.*, 2007).

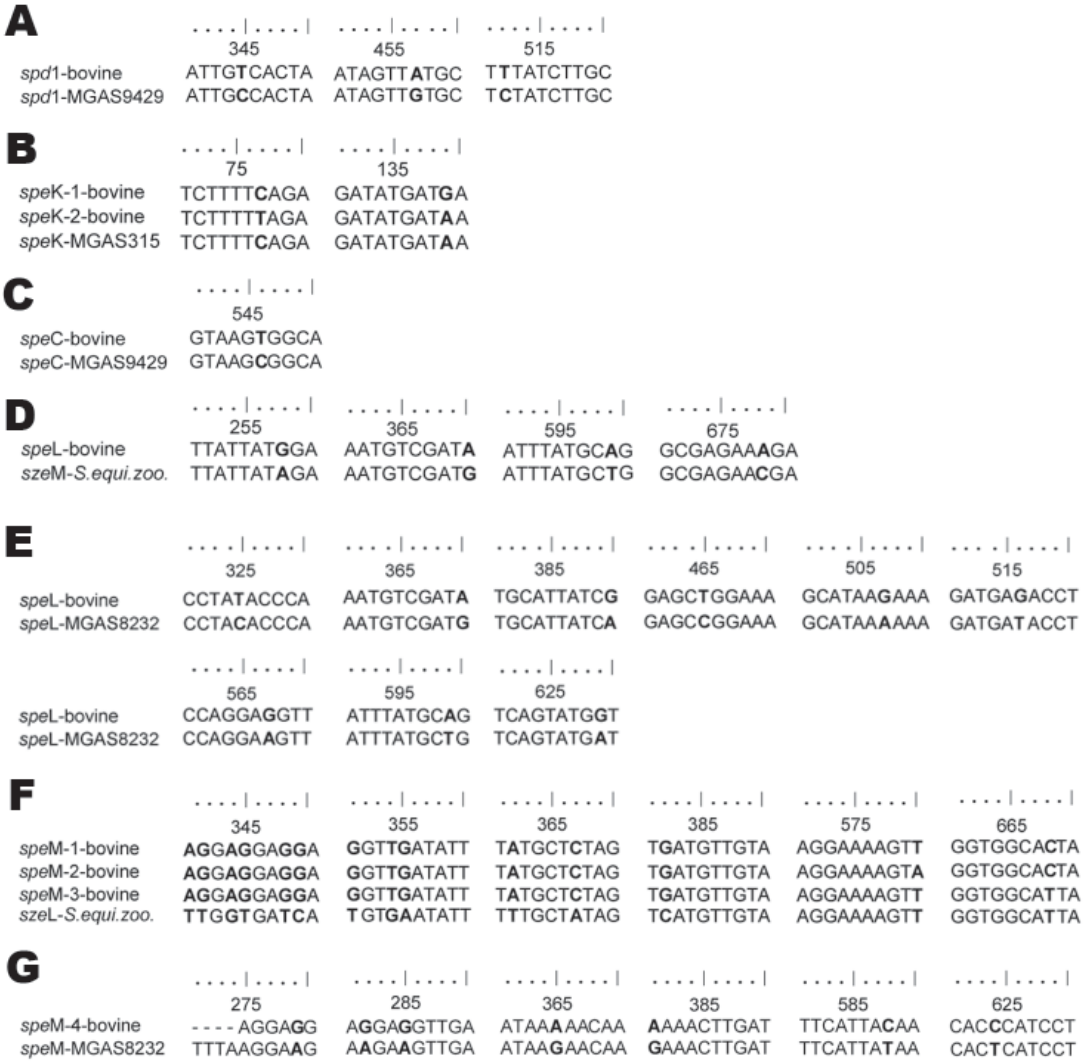

Figure 3.2. Alignments of bovine group C streptococci (*Streptococcus dysgalactiae* subsp. *dysgalactiae*) alleles of virulence genes from 8 dairy herds, Portugal, with sequences from the National Center for Biotechnology (Bethesda, MD, USA) database showing base differences between sequences. The alignments were created by using BioEdit sequence alignment editor (www.mbio.ncsu.edu/BioEdit/bioedit.html). Nucleotide differences are shown in **boldface**. **A)** *spd1* (99% maximum identity); **B)** *speK* (99% maximum identity); **C)** *speC* (99% maximum identity); **D)** *speL–szeM* (99% maximum identity); **E)** *speL* (97% maximum identity; **F)** *speM* alleles 1, 2, and 3 –*szeL* (98%–99% maximum identity); **G)** *spe*M allele 4 (98% maximum identity).
S. equi. zoo.: *S. equi* subsp. *zooepidemicus*; MGAS8232/9429: *S. pyogenes*

The successful amplification (380-bp product) of the left junction of the composite transposon

in bovine isolates suggests that this mobile element may be inserted in a similar location, the *comEC* locus, as mapped in *S. pyogenes* and *S. dysglactiae* subsp. *equisimilis*. Absence or unexpected PCR products specific for any of the junctions of this element have been reported in other studies and attributed to possible lack of homology between the target and primers used (Figueiredo *et al.*, 2006).

Detection of the *linB* gene carried by a large conjugative plasmid (Bozdogan *et al.*, 1999) in 3 of 18 bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates is indicative of horizontal gene transfer.

3.5. Conclusions

Our findings indicate that alpha-hemolytic bovine GCS isolates, which are known to be environmental or contagious pathogens and a cause of bovine mastitis, may be reservoirs of virulence genes encoded by prophages of human-specific GAS. These genes encode exotoxins, superantigens, and streptodornases, which are responsible for GAS virulence and pathogenesis, and may be transferred to other streptococci of human origin by horizontal genetic transfer. Therefore, alpha-hemolytic isolates should not be disregarded as putative infectious disease agents in humans.

Acknowledgments and work contributions:

All experimental work was performed by Márcia G. Rato except collecting bovine strains in farms and the identification of strains by the API-20 STREP and BBL Crystal Gram-Positive which was performed by collaborators at FMV/UTL. This study was supported by grant POCTI/ESP/48407/2002 (Fundação para a Ciência e Tecnologia, Portugal) and Fundo Europeu de Desenvolvimento Regional Project PROC 60839 (Fundação Calouste Gulbenkian, Portugal, Project Faculdade de Medicina Veterinária de Lisboa/46; Bovine mastitis: epidemiology, prophylactic and therapeutic approaches; Centro de Investigação Interdisciplinar em Sanidade Animal/Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Portugal). Márcia G. Rato was supported by PhD grant SFRH/BD/32513/2006 (Fundação para a Ciência e Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior).

4

Virulence gene pool detected in bovine group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* using a group A *Streptococcus pyogenes* virulence microarray

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Abstract

A custom-designed microarray containing 220 virulence genes of *Streptococcus pyogenes* (Group A *Streptococcus* [GAS]) was used to test group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GCS) field strains causing bovine mastitis and group C or group G *Streptococcus dysgalactiae* subsp. *equisimilis* (GCS/GGS) isolates from human infections, with the latter being used for comparative purposes, for the presence of virulence genes. All bovine and all human isolates carried a fraction of the 220 genes (23% and 39%, respectively). The virulence genes encoding streptolysin-S, glyceraldehyde-3-phosphate dehydrogenase, the plasminogen-binding M-Like protein PAM, and the collagen-like protein SclB were detected in the majority of both bovine and human isolates (94 to 100%). Virulence factors, usually carried by human beta-hemolytic streptococcal pathogens, such as streptokinase, laminin-binding protein, and C5a peptidase precursor, were detected in all human isolates but not in bovine isolates. Additionally, GAS bacteriophage-associated virulence genes encoding superantigens, Dnase, and/or streptodornase were detected in bovine isolates (72%) but not in the human isolates. Determinants located in non-bacteriophage-related mobile elements, such as the gene encoding R28, were detected in all bovine and human isolates. Several virulence genes, including genes of bacteriophage origin, were shown to be expressed by reverse transcriptase (RT-PCR). Phylogenetic analysis of superantigen gene sequences revealed a high level (>98%) of identity among genes of bovine GCS, of the horse pathogen *Streptococcus equi* subsp. *equi*, and of the human pathogen GAS. Our findings indicate that alpha-hemolytic bovine GCS, an important mastitis pathogen and considered to be a nonhuman pathogen, carries important virulence factors responsible for virulence and pathogenesis in humans.

Keywords

Streptococcus dysgalactiae subsp. *dysgalactiae*, *Streptococcus dysgalactiae* subsp. *equisimilis* *Streptococcus pyogenes*, Virulence genes, Phage genes

4.1. Introduction

Alpha-hemolytic Lancefield group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GCS) is a pathogen frequently associated with clinical and subclinical bovine mastitis, a disease that causes major economic losses in the dairy industry (Vieira *et al*., 1998; Seegers *et al*., 2003). Virulence determinants have been identified in this pathogen, such as surface proteins which specifically interact with plasma or extracellular matrix proteins of the host, such as alpha-2-macroglobulin, plasminogen, albumin, fibrinogen, fibronectin, vitronectin and collagen (Rantamäki & Müller, 1995; Mamo *et al*, 1987; Leigh *et al*., 1998; Valentin-Weigand *et al.*, 1990) and genes coding for proteins assumed to play a role in mastitis, such as alpha-2-macroglobulin-, immunoglobulin G-, or immunoglobulin A- binding protein Mig (Jonsson & Müller, 1994; Song *et al*., 2004; Jonsson *et al*., 1994), alpha 2-macroglobulin-, or immunoglobulin G- binding protein Mag (Jonsson *et al*., 1994), and fibrinogen- binding M-like protein (Vasi *et al.*, 2000).

Recently, *S. dysgalactiae* subsp. *dysgalactiae* was reported to be associated with toxic shock-like syndrome in cattle (Chénier *et al.*, 2008), suppurative polyarthritis in lambs (Lacasta *et al.*, 2008), bacteremia in dogs (Vela *et al.*, 2006), systemic granulomatous inflammatory disease and severe septicaemia in fish (Hagiwara *et al.*, 2009) and in ascending upper limb cellulitis in humans in contact with raw fish (Koh *et al.*, 2009). The presence of streptococcal pyrogenic exotoxin G gene (*spegg*) and streptolysin S structural gene (*sag*A), which have been associated with invasive disease in the exclusively human pathogen *Streptococcus pyogenes* (group A *Streptococcus*- GAS), has been documented in fish isolates of *S. dysgalactiae* subsp. *dysgalactiae* (Abdelsalam *et al.*, 2010). We have previously reported the presence of GAS phage-carried virulence genes among alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* isolates from bovine mastitis, namely, the streptococcal pyrogenic exotoxin genes *speK*, *speC*, *speL*, *speM*; the phage DNase1 gene *spd1*; and other genes encoding antimicrobial resistance determinants located on mobile genetic elements (MGEs) (see Chapter 4). So far, no more information is available regarding the presence of GAS virulence genes among *S. dysgalactiae* subsp. *dysgalactiae* strains, and nothing is known regarding the presence of GAS prophages in *S. dysgalactiae* subsp. *dysgalactiae*. However, the exchange of lysogenic phages among GAS and other human and animal species, particularly group C *Streptococcus dysgalactiae* subsp. *equisimilis* (a pathogen that colonizes and infects humans with a clinical spectrum of diseases resembling those caused by GAS), *Streptococcus equi* subsp. *equi* (exclusively horse pathogen) and *S. equi* subsp. *zooepidemicus* (a zoonotic pathogen) isolates, was previously reported (Vojtek *et al.*, 2008, Holden *et al.*, 2009).

The aim of the present work was to use a microarray of genes encoding GAS virulence factors (McMillan *et al*., 2007[a]) to have a better insight into the virulence gene pool (encoded or not by mobile genetic elements) shared between GAS and alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* isolates associated with bovine mastitis in comparison with beta-hemolytic *S. dysgalactiae* subsp. *equisimilis* isolates associated with human disease.

4.2. Materials and Methods

4.2.1. Bacterial isolates and identification

A total of 18 alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* field isolates of Lancefield group C (GCS), one of the causative agents of bovine subclinical mastitis in dairy herds in Portugal, were used in the present study. For detailed information regarding these field isolates, including identification and molecular typing data see Chapter 2 and Chapter 4. In addition, six nonduplicated beta-hemolytic *S. dysgalactiae* subsp. *equisimilis* isolates of Lancefield group G (Group G *Streptococcus* [GGS]) (n = 5) and group C (GCS) (n = 1) collected in Portugal, causing pharyngitis (n = 5) and invasive disease (n = 1) in humans, were included in the study for the purpose of comparison. The identification of *S. dysgalactiae* subsp. *equisimilis* isolates was based on colony morphology, hemolysis in blood agar plates, and Lancefield grouping using the Streptex kit (Remel Europe Ltd, Dartford, England) and PCR amplification of the 16S rRNA gene and sequencing (Weisburg *et al.*, 1991).

We have included two GCS alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* invasive strains in the study, which were analyzed for the detection of selected virulence genes by PCR only (see below). One of these strains was associated with toxic shock-like syndrome in cattle (Chénier *et al.*, 2008). The other strain caused ascending upper limb cellulitis in humans in contact with raw fish (Koh *et al.*, 2009). The latter strain was previously identified (Koh *et al.*, 2009) by PCR amplification of the 16S rRNA and *sodA* genes and sequencing (GenBank accession numbers EU693902 and EU719068, respectively). We confirmed the identification of the strain associated with the toxic shock-like syndrome in cattle by PCR amplification of the 16S rRNA (Weisburg *et al.*, 1991) and of the *sod*A (Abdelsalam *et al.*, 2010) genes and sequencing. Sequences were analyzed by using the BioEdit sequence alignment editor (Hall, 1999) and compared with sequences from the National Center for Biotechnology Information (NCBI) database (Bethesda, MD) by using the BLAST alignment tool (www.ncbi.nlm.nih.gov/BLAST).

The new sequences from the invasive *S. dysgalactiae* subsp. *dysgalactiae* strains were deposited in GenBank database (see Annex).

4.2.2. Pulsed-field gel electrophoresis (PFGE) profiles for clonal characterization

A description of the bovine *S. dysgalactiae* subsp. *dysgalactiae* GCS field strains (n=18) including typing by PFGE was described in Chapter 4. The typing of the human *S. dysgalactiae* subsp. *equisimilis* GGS/GCS isolates (*n* = 6) by PFGE was carried out in this study as described for the bovine pathogen *S. uberis* (see Chapter 3). SmaI-digested DNA banding patterns obtained by PFGE were analyzed visually according to previously established criteria (Tenover *et al.*, 1995).

4.2.3. Microarray design and hybridization

The microarray was described previously (McMillan *et al*., 2007[b]) and was used with minor modifications. In brief, the array consists of 70-mer oligonucleotides from the conserved regions of all 'classical' GAS virulence factors and orthologues of virulence factors found in other bacterial species as well as putative virulence genes present in the M1, M3, and M18 genomes. In total, 220 virulence factor/extracellular protein genes, 10 housekeeping genes (positive controls), and 10 negative controls were randomly spotted in six locations on the chip. Genomic DNA was extracted using zirconicum beads in combination with the Qiagen DNeasy kit (Qiagen). Genomic DNA was partially digested with AluI, yielding fragments of between 500 and 1,000 bp, and labeled with biotin. The labeled DNA was purified (PCR Purification Kit; Qiagen) and the labeling efficiency was verified by gel electrophoresis. Array hybridization was performed at 42°C for 16 h, followed by incubation with Streptavidin-Cy5 using a SlideBooster SB800 instrument (Advalytix). Fluorescence signals were obtained with a DNA Microarray Scanner (G2565CA; Agilent Technologies) at a 633-nm excitation wavelength and quantified by using ImaGene software (BioDiscovery).

4.2.4. Microarray data processing

The raw data were corrected for background and transformed to log scale. A two-component normal-mixture model (McLachlan & Peel, 2000) was fitted to the corrected data by a maximum likelihood method adapted from the mclust package (Fraley & Raftery, 2006). A discriminant function was calculated to represent the propensity of a gene for being present or absent. Discriminant values were stored in a signal probability matrix and colored for presentation purposes using the following scheme: black indicates state 0 (not present), green indicates state 1 (present), and yellow indicates indecisive measurement.

Microarray data accession number: Microarray data have been deposited in the public ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-3168.

4.2.5. Confirmation of array data by PCR screening

In order to confirm the results obtained with the GAS array, PCR was carried out on several genes (*speH*, *speC*, *speA*, *speL*, *speK*, *speI*, *speM sdn*, *ssa*, *smeZ*, *sla*, *drs*, *prtf2*, *speG*, *ska*, *dppA*, *lbp*, *scpA*, *emm*, *isp*, *SpyM3_1736*, *slo*, *nga*, *spegg* and *sagA*). The primer sequences, gene description, and amplification length of each reaction are described in Table 4.1. Samples without DNA and strains lacking (negative) or carrying (positive) specific genes were used as controls in the PCR.

4.2.6. Sequence data and phylogenetic analysis of bacteriophage-associated virulence superantigen genes

Sequences of the superantigen-encoding genes *speC*, *speK*, *speL* and *speM* of the bovine mastitis isolates under study, with high levels of identity among them, were chosen to generate an alignment of DNA sequences of the alleles of those genes and homologous sequences deposited in the NCBI database, in particular sequences of the *speC*, *speK, speL* and *speM* genes of *S. pyogenes*; *seeL* and *seeM* genes of *S. equi* subsp. *equi*; *szeL* and *szeM* genes of *S. equi* subsp. *zooeepidemicus*; and the *sdm* genev of *S. dysgalactiae* subsp. *dysgalactiae*.
The alignment (380 bp) was used to construct a phylogenetic tree by using MEGA, version 4, software (Tamura *et al*., 2007). The *p*-distance parameter and neighbor-joining method were used. Bootstrap values were calculated from 1,000 replicates. Deduced amino acid sequences from these bovine alleles were compared with similar sequences from NCBI database by using the BLAST alignment tool (www.ncbi.nlm.nih.gov/BLAST). Sequencing was performed by STAB-Vida (Lisbon, Portugal) using the same primers used for amplification. Nucleotide sequences were analyzed by using the BioEdit sequence alignment editor (Hall, 1999).

4.2.7. *emm*-typing

Determination of *emm* gene (coding the M protein) types was performed as described by the CDC (http://www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm).

Table 4.1. Genes and PCR primers used for screening of virulence determinants among group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* and group C and G *Streptococcus dysgalactiae* subsp. *equisimilis* isolates.

Gene description	Primer target/ Primer sequence	Product (bp)	Source of reference
Antiphagocytic M protein (chromosome)	*emm* (for.) TATTCGCTTAGAAAATTAA *emm* (rev.) GCAAGTTCTTCAGCTTGTTT	variable	—‡
Streptolysin S (chromosome)	*sagA* (for.) TACTTCAAATATTTTAGCTACT *sagA* (rev.) GATGATACCCCGATAAGGATAA	487	Abdelsalam *et al*., 2010
Pyrogenic exotoxin H precursor (phage)	*speH* (for.) AGATTGGATATCACAGG *speH* (rev.) CTATTCTCTCGTTATTGG	416	Schmitz *et al*., 2003
Pyrogenic exotoxin F (chromosome)	*speF* (for.) TACTTGGATCAAGACG *speF* (rev.) GTAATTAATGGTGTAGCC	782	Schmitz *et al*., 2003
Pyrogenic exotoxin B (chromosome)	*speB* (for.) TTCTAGGATACTCTACCAGC *speB* (rev.) ATTTGAGCAGTTGCAGTAGC	300	Jasir *et al*., 2001
Pyrogenic exotoxin C precursor (phage)	*speC* (for.) GCAGGGTAAATTTTTCAACGACACACA *speC* (rev.) TGTGCCAATTTCGATTCTGCCGC	407	This study
Pyrogenic exotoxin A precursor (phage)	*speA* (for.) ATGGAAAACAATAAAAAAGTATTG *speA* (rev.) TTACTTGGTTGTTAGGTAGACTTC	755	Matsumoto *et al*., 2003
Pyrogenic exotoxin L precursor (phage)	*speL* (for.) CTGTTAGGATGGTTTCTGCGGAAGAG *speL* (rev.) AGCACCTTCCTCTTTCTCGCCT	605	This study
Pyrogenic exotoxin K precursor (phage)	*speK* (for.) TACAAATGATGTTAGAAATCCAAGGAACATATATGCT *speK* (rev.) CAAAGTGACTTACTTTACTCATATCAATCGTTTC	656	This study/ Lintges *et al*., 2007
Pyrogenic exotoxin I precursor (phage)	*speI* (for.) AATGAAGGTCCGCCATTTTC *speI* (rev.) TCTCTCTGTCACCATGTCCTG	516	Matsumoto *et al*., 2003
Pyrogenic exotoxin M precursor (phage)	*speM* (for.) CCAATATGAAGATAACAAAGAAAATTGGCACCC *speM* (rev.) CAAAGTGACTTACTTTACTCATATCAATCGTTTC	600	This study/ Lintges *et al*., 2007
Pyrogenic exotoxin J (chromosome)	*speJ* (for.) ATCTTTCATGGGTACG *speJ* (rev.) TTTCATGTTTATTGCC	535	Schmitz *et al*., 2003
Streptodornase (phage)	*sdn* (for.) ACCCCATCGGAAGATAAAGC *sdn* (rev.) AACGTTCAACAGGCGCTTAC	489	Matsumoto *et al*., 2003
Streptococcal Dnase 1 (phage)	*spd1* (for.) CCCTTCAGGATTGCTGTCAT *spd1* (rev.) ACTGTTGACGCAGCTAGGG	400	Green *et al*., 2005
Streptococcal superantigen SSA (phage)	*ssa* (for.) TCCACAGGTCAGCTTTTACAG *ssa* (rev.) TGATCAAATATTGCTCCAGGTG	502	Matsumoto *et al*., 2003

Continued on following page

Table 4.1. *-Continued*

Gene description	Primer target/ Primer sequence	Product (bp)	Source of reference
Phospholipase A2 (phage)	*sla* (for.) CTCTAATAGCATCGGCTACGC *sla* (rev.) AATGGAAAATGGCACTGAAAG	440	Matsumoto *et al*., 2003
Mitogenic exotoxin Z (chromosome)	*smeZ* (for.) CTTCAATATTCATTGCAATAATTTC *smeZ* (rev.) TGTAACTGTTGTTTTGTTAGTTGAT	400	Provided by G. S. Chhatwal
Pyrogenic exotoxin G of *S. pyogenes* (chromosome)	*speG* (for.) TGTATCTTTAGGGATTACTGATCAG *speG* (rev.) CTCGACCTAAAAGCTTATCATCCTT	389	This study
Pyrogenic exotoxin G of *S. dysgalactiae* (chromosome)	*spegg* (for.) GCTTATGATGTTACTCCACTTGA *spegg* (rev.) ATAACGCGATTCCGAATCATAGA	420	This study
Drs protein (chromosome)	*drs* (for.) CAGCAGATGAAGCAAGTAATAGC *drs* (rev.) CTTGTTTGTCAATTTTGCTTTACGACC	760	Hartas & Sriprakash, 1999
Fibronectin binding protein Prtf1 (chromosome)	*prtf1* (for.) TATCAAAATCTTCTAAGTGCTGAG *prtf1* (rev.) AATGGAACACTAACTTCGGACGGG	930	Talay *et al*., 1994
Fibronectin binding protein Prtf2 (chromosome)	*prtf2* (for.) ATAGGATTGTCCGGAGTATCA *prtf2* (rev.) TTATGTTGCTTCTCACCA	2,000	Provided by G. S. Chhatwal
Streptolysin O (chromosome)	*slo* (for.) ACGGCAGCTCTTATCATT *slo* (rev.) GACCTCAACCGTTGCTTTGT	600	Provided by G. S. Chhatwal
C5A peptidase precursor (transposon)	*scpA* (for.) CCAAGACTTCAGCCACAAGG *scpA* (rev.) CAATTCCAGCCAATAGCAGC	591	Provided by G. S. Chhatwal
Streptokinase A precursor (chromosome)	*ska* (for.) CGATCAAAGGGATCATACGG *ska* (rev.) AGGTTCACAGTAACGACGGC	598	Provided by G. S. Chhatwal
NAD-glycohydrolase precursor (chromosome)	*nga* (for.) ATAACGGGAATAAATTGGTCCTC *nga* (rev.) CGCTTTCTTTGTAGACTTGTTTT	408	This study
Laminin-binding protein (transposon)	*lmb* (for.) AACCCCAAACAGCCTACGCAAG *lmb* (rev.) TAAAACGGGATCCGTCCAGGTAT	375	This study
Immunogenic secreted protein (chromosome)	*isp* (for.) CAACTGAAAAAACCCCAGAGCC *isp* (rev.) GGTTGAAGTCAAAGGCACCATAA	429	This study
Surface lipoprotein DppA (chromosome)	*dppA* (for.) CCGTTATGGAGTCCACAATGAA *dppA* (rev.) ACTAGCTTTGAGTTTAATAGTAATC	1,045	This study
Putative ATB-binding cassette transporter protein (chromosome)	SpyM3_1736 (for.) GAGAAGTCAAAGAGGTCTTTGTT SpyM3_1736 (rev.) GGTGTCATACTCTAGTTTACCTTT	392	This study

‡ http://www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm.

4.2.8. Gene expression assays by reverse-transcriptase PCR (RT-PCR)

For RNA extraction, all isolates were grown in Todd-Hewitt broth (Oxoid Limited, Basingstoke, England) supplemented with 1% yeast extract (BD, Franklin Lakes) (THY) at 37ºC until the mid-exponential phase was reached (optical density [OD] at 600 nm of 0.5), and the NucleoSpin RNAII kit (Macherey-Nagel, Dueren, Germany) was used according to the manufacturer's instructions, followed by the addition of 2U/µL of DnaseI (Applied Biosystems/Ambion). RNA quality was confirmed by 1% agarose gel electrophoresis, and images were captured by using the Gel Doc XR system and Quantity One 1-D analysis software (Bio-Rad). To confirm that no remaining DNA was present in RNA samples, PCR assays were performed targeting the housekeeping genes *rpsL* and *rpsB* and the genes under study, *speM*, *speK*, *speL*, *speC*, *spd1*, *sdn*, *slo scpA*, *ska*, *nga*, *lmb*, *isp*, *dppA*, *emm* and SpyM3_1736, using RNA as a template. Reverse transcriptase (RT) reactions for cDNA synthesis were performed by using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen, New Zealand). PCR targeting those genes under study was carried out again using by using cDNA as a template.

4.2.9. Growth curve analysis

In order to ascertain if growth curves (using the same media and conditions of growth) of six of the bovine mastitis GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates from the present study (chosen according to their genotype) were comparable or not, an automated growth curve analysis system (BioScreen C, Piscataway, NJ) was used as described previously (Carlos *et al*., 2009). Briefly, these isolates were grown overnight in THY, and cells were centrifuged in order to collect 10^9 CFU in the pellet. After re-suspended and washed in Phosphate Buffered Saline-PBS (10 mM, pH 7.0), the pellet was resuspended in 100 µl of PBS, and 1% (vol/vol) of this bacterial suspension was used to inoculate each of the wells of the BioScreen plaque containing growth media. Three different growth media were tested: THY, bovine blood serum (Probiologica), and bovine milk serum freshly prepared in the laboratory as previously described (Melchior *et al*., 2009). The growth media were prepared at different pHs (6.0, 6.6, and 7.4), and three different incubation temperatures (37ºC, 38ºC, and 40ºC) were tested for each growth media, which represent different environmental conditions in the bovine udder (e.g., the body temperature of a healthy cow is around 38ºC, which may rise to 40ºC during a mastitis infection).

4.2.10. Virulence gene profiling of invasive *Streptococcus dysgalactiae* subsp. *dysgalactiae*

Eighteen virulence genes (*sagA*, *sla*, *sdn*, *spd1*, *speI*, *speC*, *speA*, *speB*, *speK*, *speF*, *speM*, *speH*, *speL*, *speJ*, *ssa*, *smeZ*, *spegg*, and *prtf*1) were searched for by PCR (see Table 4.1) in the two invasive GCS strains that were included in the study.

4.3. Results

4.3.1. Identification by 16S rDNA and *sod*A genes sequence analyses

The identification of the bovine mastitis *S. dysgalactiae* subsp. *dysgalactiae* isolates was confirmed in a previous work using the 16S rRNA gene sequencing (see Chapter 4). The 16S rRNA gene sequences of all human GCS/GGS isolates from the present study showed 99-100% identity to 16S rRNA gene sequences of *S. dysgalactiae* subsp. *equisimilis* deposited in the NCBI database. Taking into account the phenotypic characteristics of the isolates together with the 16S rRNA gene sequence analysis, we could confirm the six human beta-hemolytic GGS/GCS isolates included in this study as being *S. dysgalactiae* subsp. *equisimilis* isolates.
The 16S rRNA and *sodA* gene sequences of the GCS strain associated with the toxic shock-like syndrome in cattle were deposited in the GenBank database (accession numbers JF789447 and JF789445, respectively), whereas the same above-mentioned sequences of the GCS strain isolated from a case of ascending upper limb cellulitis in humans were already available (accession numbers EU693902 and EU719068, respectively), as described previously (Koh *et al.*, 2009). Both 16S rRNA and *sodA* gene sequences of these two strains showed 99 to 100% identity with *S. dysgalactiae* subsp. *dysgalactiae* 16S rRNA and *sodA* gene sequences available for comparison in the NCBI database.

4.3.2. PFGE profiles

The 18 bovine *S. dysgalactiae* subsp. *dysgalactiae* isolates had 15 PFGE patterns, as shown in Fig. 3.1 (see Chapter 3). The six *S. dysgalactiae* subsp. *equisimilis* isolates had six different PFGE patterns with more than six band differences and were therefore considered unrelated according to established criteria (Tenover *et al.*, 1995).

4.3.3. *emm*-typing

None of the 18 alpha-hemolytic group C *S. dysgalactiae* subsp. *dysgalactiae* bovine isolates were typed by *emm*-typing, since no amplification for this gene could be obtained. The *emm* gene types of the six human *S. dysgalactiae* subsp. *equisimilis* isolates were as follows: *stC839* (GCS isolate; *n* = 1), *stG485*, *stG480*, *stG6792*, and *stG4831* (GGS isolates; *n* = 5).

4.3.4. Microarray data

Of the 220 GAS virulence genes on the array (McMillan *et al.*, 2007[b]), 44 genes (20%) were present in all bovine mastitis GCS (*S. dysgalactiae* subsp. *dysgalactiae*) and in all human GGS/GCS (*S. dysgalatiae* subsp. *equisimilis*) isolates, whereas 66 genes (30%) were not present in any of the isolates tested. The remaining genes (50%) showed variable distributions among isolates of both origins. Relevant genes present in at least one isolate (bovine or human) and genes present in all the bovine isolates (with exception of hypothetical proteins) are shown in Table 4.2. Nine genes (*ska*, *dppA*, *lmb*, *scpA*, *emm*, *isp*, *nga*, *slo*, and SpyM3_1736) were present in all the human isolates and absent in the bovine isolates, whereas only one gene (SpyM3_0345), encoding an uncharacterized protein, was detected in all bovine isolates and absent in all human isolates.

If we restrict the comparison to bacteriophage genes on the array, we observed that 65% of the phage-related genes were present in at least one bovine GCS isolate, whereas 35% were detected in at least one human GCS/GGS isolate. None of the 13 bacteriophage-harbored virulence genes *speC*, *speJ*, *speI*, *speH*, *ssa*, *mf4*, *slaA*, *speA3*, *speK*, *speL*, *speM*, *spd1*, and *sdn* were detected in human GGS/GCS *S. dysgalactiae* subsp. *equisimilis* isolates, whereas at least one of the following six genes *speC*, *speK*, *speL*, *speM*, *spd1*, and *sdn* was detected in 72% (*n* = 13) of the bovine mastitis GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates.

Other GAS genes on the array, located in variable and recombinatory loci of the GAS genomes (McMillan *et al.*, 2007[a]; McMillan *et al.*, 2007[b]), were detected in both human and bovine isolates. These include the Spy2009 gene, encoding a transposase (Sumby *et al.*, 2005), and *sic* (at the *mga* chromosomal location); the spyM18_2055 gene, coding for an amidase (at the *spa* chromosomal location); *malE* (at the maltose chromosomal location); *prtF15* and *sfbI* (at the fibronectin-collagen-T antigen [FCT]-encoding region); *citE* (at the *cit* chromosomal location); and *epf* (at the *sagA* chromosomal location). The gene coding for the adhesin R28 carried by a putative integrative conjugative element was present in all bovine and human isolates.

Table 4.2. Distribution of group A *Streptococcus* virulence factors of the array in bovine mastitis group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates and human non-invasive and invasive group C and G *Streptococcus dysgalactiae* subsp. *equisimilis* isolates[†].

Human Group A *Streptococcus pyogenes* virulence class protein (gene)	Distribution (%) (no. of isolates)	
	Bovine Group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* (*n* = 18)	Human Group C/G *Streptococcus dysgalactiae* subsp. *equisimilis* (*n* = 6)
Adhesins		
Antiphagocytic M protein (*emm*)	0 (n=0)	100 (n=6)
Laminin-binding protein (*lmb*)[a]	0 (n=0)	100 (n=6)
Collagen-like surface protein (*scl*)[a]	89 (n=16)	100 (n=6)
PAM	94 (n=17)	100 (n=6)
Putative adhesion protein (*adcA*)	94 (n=17)	100 (n=6)
Putative extracellular matrix binding protein (*epf*)[a]	94 (n=17)	100 (n=6)
Putative pullulanase (*pulA*)	94 (n=17)	83 (n=5)
Collagen-like protein SclB (*sclB*)	100 (n=18)	100 (n=6)
Glyceraldehyde-3-phosphate dehydrogenase (*plr*)	100 (n=18)	83 (n=5)
R28[a]	100 (n=18)	100 (n=6)
Putative choline binding protein (SpyM3_0025)	100 (n=18)	100 (n=6)
Putative collagen-like protein (Spy1054)	100 (n=18)	100 (n=6)
Putative enolase (*eno*)	100 (n=18)	100 (n=6)
Putative internalin A precursor (*inlA*)	100 (n=18)	100 (n=6)
Fibronectin-binding proteins		
SfbX	6 (n=1)	17 (n=1)
SfbI[a]	89 (n=16)	100 (n=6)
PrtF15[a]	100 (n=18)	100 (n=6)
Putative fibronectin-binding protein-like protein A (SpyM3_0652)	100 (n=18)	67 (n=4)
Proteases		
C5A peptidase precursor (*scpA*)[a]	0 (n=0)	100 (n=6)
Putative C3-degrading proteinase (*cppA*)	22 (n=4)	0 (n=0)
Putative exfoliative toxin (SpyM3_0632)	94 (n=17)	67 (n=4)
Putative serine protease (*degP*)	94 (n=17)	100 (n=6)
Putative protease (SpyM3_0418)	100 (n=18)	100 (n=6)
Toxins		
Streptolysin O (*slo*)	0 (n=0)	100 (n=6)
Putative hemolysin III (*hlyIII*)	83 (n=15)	100 (n=6)
Putative hemolysin (*hlyX*)	94 (n=17)	100 (n=6)
Streptolysin S-associated protein (*sagA*)	100 (n=18)	100 (n=6)
Putative DNA-entry nuclease (*endA*)	100 (n=18)	100 (n=6)
Putative hemolysin (*hlyA*)	100 (n=18)	100 (n=6)
Other virulence factors		
Dipeptide permease complex (*dppA*)	0 (n=0)	100 (n=6)
Immunogenic secreted protein precursor (*isp*)	0 (n=0)	100 (n=6)
Inhibitor of complement-mediated lysis (*sic*)	0 (n=0)	50 (n=3)

Continued on following page

Table 4.2. -*Continued*

Human Group A *Streptococcus pyogenes* virulence class protein (gene)	Distribution (%) (no. of isolates)	
	Bovine Group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* (*n* = 18)	Human Group C/G *Streptococcus dysgalactiae* subsp. *equisimilis* (*n* = 6)
Other virulence factors (*continued*)		
NAD-glycohydrolase (*nga*)	0 (n=0)	100 (n=6)
Pyrogenic exotoxin G precursor (*speG*)	0 (n=0)	83 (n=5)
Streptokinase A precursor (*ska*)	0 (n=0)	100 (n=6)
Transposase (spy2009)[a]	22 (n=4)	83 (n=5)
Putative hyaluronidase (*hyl*)	28 (n=5)	100 (n=6)
Putative 6-phospho-beta-galactosidase (*lacG*)	39 (n=7)	0 (n=0)
Immunogenic secreted protein precursor homologue (*isp.2*)	67 (n=12)	100 (n=6)
Extracellular hyaluronate lyase (*hyl*A)	78 (n=14)	50 (n=3)
Streptococcal protective antigen (*spa*)	78 (n=14)	50 (n=3)
Putative penicillin-binding protein 1A (*pbp1A*)	83 (n=15)	33 (n=2)
Putative glutathione peroxidise (SpyM3_0428)	89 (n=16)	100 (n=6)
Putative GTP-binding protein LepA (*lepA*)	89 (n=16)	100 (n=6)
Arginine deiminase (*arcA*)	100 (n=18)	100 (n=6)
Maltose/maltodextrin-binding protein (*malE*)[a]	100 (n=18)	100 (n=6)
67 kDa myosin-cross-reactive streptococcal antigen (SpyM3_0332)	100 (n=18)	100 (n=6)
Putative acid phosphatase (*lppC*)	100 (n=18)	100 (n=6)
Putative amidase (SpyM18_2055)[a]	100 (n=18)	100 (n=6)
Putative carbamate kinase (*arcC*)	100 (n=18)	100 (n=6)
Putative citrate lyase beta subunit (*citE*)[a]	100 (n=18)	50 (n=3)
Putative cytoplasmic membrane protein (*lemA*)	100 (n=18)	100 (n=6)
Putative dipeptidase (*pepD*)	100 (n=18)	100 (n=6)
Putative dipeptidase (SpyM3_1763)	100 (n=18)	100 (n=6)
Putative ferric uptake regulator (*spf*)	100 (n=18)	100 (n=6)
Putative fructose-1-phosphate kinase (*fruK*)	100 (n=18)	100 (n=6)
Putative lipoprotein (*atmB*)	100 (n=18)	100 (n=6)
Putative manganese-dependent inorganic pyrophosphatase (SpyM3_0278)	100 (n=18)	100 (n=6)
Putative metal binding protein of ABC transporter (*mtsA*)	100 (n=18)	100 (n=6)
Putative peptidyl-prolyl *cis-trans* isomerase (*cypB*)	100 (n=18)	100 (n=6)
Putative protease maturation protein (*prsA*)	100 (n=18)	50 (n=3)
Putative proton-translocating ATPase subunit b (SpyM3_0495)	100 (n=18)	100 (n=6)
Putative PTS system IIB component (SpyM3_1679)	100 (n=18)	100 (n=6)
Putative sugar transporter sugar binding lipoprotein	100 (n=18)	100 (n=6)
Putative two-component sensor histidine kinase (*yesM*)	100 (n=18)	100 (n=6)
Putative uridine kinase (*udk*)	100 (n=18)	100 (n=6)

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Table 4.2. -*Continued*

Human Group A *Streptococcus pyogenes* virulence class protein (gene)	Distribution (%) (no. of isolates)	
	Bovine Group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* (*n* = 18)	Human Group C/G *Streptococcus dysgalactiae* subsp. *equisimilis* (*n* = 6)
Bacteriophage-encoded recognized virulence factors		
Streptodornase, phage associated (*sdn*)	22 (n=4)	0 (n=0)
Putative exotoxin L precursor, phage associated (*speL*)	22 (n=4)	0 (n=0)
Pyrogenic exotoxin C precursor, phage associated (*speC*)	33 (n=6)	0 (n=0)
Putative DNase, phage associated (*spd1*)	33 (n=6)	0 (n=0)
Streptococcal pyrogenic K exotoxin, phage associated (*speK*)	50 (n=9)	0 (n=0)
Putative exotoxin M precursor, phage associated (*speM*)	56 (n=10)	0 (n=0)
Other bacteriophage-encoded factors		
Putative deoxyribonuclease	33 (n=6)	50 (n=3)
Putative lysine, phage associated	89 (n=16)	100 (n=6)
hypothetical phage protein	100 (n=18)	100 (n=6)
Putative endolysin, phage associated	100 (n=18)	100 (n=6)

[a]Genes associated with lateral gene transfer (non-bacteriophage related).
[†]Only genes present in at least one isolate (bovine or human) and genes present in all bovine group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates are listed.
Hypothetical proteins are not listed in the Table.

4.3.5. Confirmation of array data by PCR screening

All PCR results confirmed the GAS microarray data (Table 4.2), except for *speM*, *speK*, and *speG* genes. The *speM* and *speK* genes share high levels of identity among their DNA sequences, which resulted in false-positive results by the array data. In addition, it was not clear how many isolates carried the *speG* gene by both the array and PCR amplification results.By using other primers (designed in this study) targeting the *speM* and *speK* genes of *S. pyogenes* and *spegg* of *S. dysgalactiae* subsp. *equisimilis* (Zhao *et al*., 2007), eight bovine isolates that carried *speK*, one bovine isolate that carried *speM*, one bovine isolate that carried *speK* and *speM*, and five human isolates that carried *spegg* were found.

4.3.6. Sequence data and phylogenetic analysis of bacteriophage-associated virulence superantigen genes

Three *speK* alleles (*speK-1*$_{Bov}$, *speK-2*$_{Bov}$, and *spe*K-3$_{Bov}$) were identified among nine bovine isolates, two *speM* alleles (*speM-1*$_{Bov}$, and *speM-2*$_{Bov}$), were identified in two bovine isolates, one *speC* allele (*speC*$_{Bov}$) was identified in six bovine isolates, and one *speL* allele (*speL*$_{Bov}$) was identified in four bovine isolates (GenBank accession numbers HQ724300 to HQ724305 and HQ696925). The phylogenetic tree based on sequences of those alleles and of homologous sequences available in the NCBI database showed four major groups, each one comprising one of the four *spe* genes (*speK*, *speM*, *speC*, or *speL*) of bovine *S. dysgalactiae* subsp. *dysgalactiae* isolates and of *S. pyogenes*, as shown in Fig. 4.1. The tree also shows that *speM* and *speK* diverged more recently, as these two groups showed higher identities among them than with the *speL* or *speC* groups.

Amino acid sequences deduced from the bovine GCS alleles always showed 98 to 99% identity with the homologous GAS pyrogenic exotoxins gene sequences from the NCBI database, with the exception of bovine SpeL, which showed higher level of identity with SeeM from *S. equi* subsp. *equi* (99%) than with SpeL from *S. pyogenes* (96%). In particular, and interestingly, SpeM from bovine isolates in the present study showed 99% identity with SpeM from GAS and 100% identity with Sdm from *S. dysgalactiae* subsp. *dysgalactiae* (sequences deposited in NCBI database).

The new alleles from the bovine *S. dysgalactiae* subsp. *dysgalactiae* isolates were deposited in GenBank database (see Annex).

4.3.7. Gene expression assays by RT-PCR

Transcriptional analysis revealed that the bacteriophage-associated virulence genes *speM*, *speK*, *speL*, *speC*, *spd1*, and *sdn*, detected in bovine GCS isolates, were transcribed and that the virulence genes *ska*, *dppA*, *lmb*, *scpA*, *emm*, *isp*, SpyM3_1736, *slo*, and *nga*, detected only in human isolates, were also transcribed.

4.3.8. Growth curve analysis

In the present study all the strains under analysis by BioScreen reached the highest OD values when grown in the bovine blood serum media (compared to those when grown in THY and bovine milk serum) and under infection-related conditions (at 40ºC), with the end of the exponential phase achieved relatively fast (prior to 5 h of incubation). In bovine milk serum, the end of the exponential phase was achieved in most cases after 10 h of incubation, whereas in THY, growth curves reached the end of the exponential phase prior to 10 h of growth, followed by long stationary phases (no OD decrease was observed during 30 h or more of growth).

Furthermore, we have observed differences in growth curves among isolates from the present study, suggesting a strain-specific mode of growth.

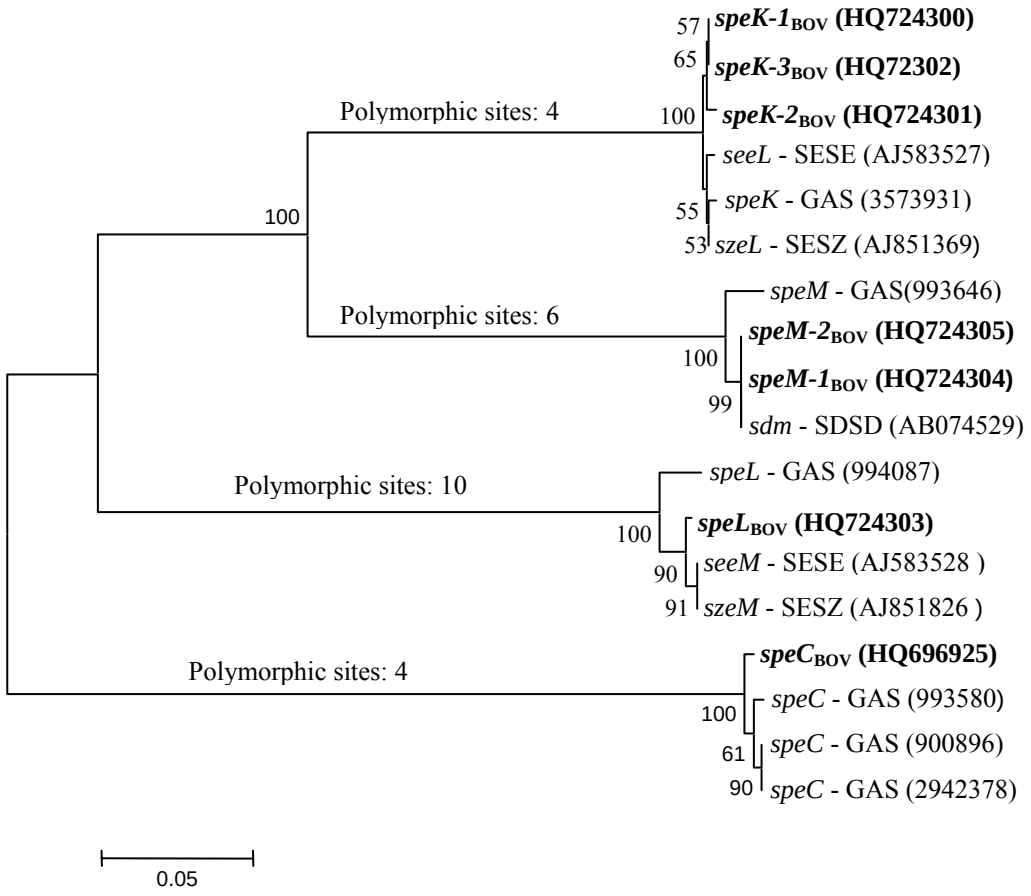

Fig. 4.1. Phylogenetic analysis of superantigen gene sequences of bovine group C *Streptococcus* from the present study and of sequences of *S. pyogenes*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, and *S. dysgalactiae* subsp. *dysgalactiae* available in the National Center for Biotechnology Information (NCBI) (Bethesda, MD) database. Bovine group C *Streptococcus* sequences from the present study are designated in the tree in boldface type. Other sequences are designated as follows: SESE, *S. equi* subsp. *equi* (*seeL* and *seeM*); SESZ, *S. equi* subsp. *zooepidemicus* (*szeL* and *szeM*); SDSD, *S. dysgalactiae* subsp. *dysgalactiae* (*sdm*); GAS, *S. pyogenes* (*speK*, *speM*, *speL*, and *speC*). GenBank accession numbers (for bovine alleles, *seeL*, *seeM*, *szeL*, *szeM*, and *sdm*) and gene ID numbers (for GAS *speK*, *speM*, *speL*, and *speC*) are included in parentheses after the gene names.

4.3.9. Virulence gene profiling of invasive *Streptococcus dysgalactiae* subsp. *dysgalactiae*

Both strains were negative for all the genes tested (n = 18) by PCR, with the exception of the *sagA* gene. According to the sequences available in the NCBI database, the *sagA* of the animal strain (GenBank accession number JF789442) showed 95% identity either with the *sagA* gene of *S. dysgalactiae* subsp. *dysgalactiae* or with that of *S. dysgalactiae* subsp. *equisimilis*. In

parallel, the *sagA* gene of the human strain (accession number JF789443) was 100% identical to the *sagA* gene of *S. dysgalactiae* subsp. *dysgalactiae* and 99% identical to the *sagA* gene of *S. dysgalactiae* subsp. *equisimilis*.
The new sequences from the invasive *S. dysgalactiae* subsp. *dysgalactiae* strains were deposited in GenBank database (see Annex).

4.4. Discussion

4.4.1. Assessment of genes associated with MGEs among *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Streptococcus dysgalactiae equisilimis*

An array containing 220 virulence genes from *S. pyogenes* (group A *Streptococcus* [GAS]) was used to analyze the virulence gene pool among *S. dysgalactiae* subsp. *dysgalactiae* (group C *Streptococcus* [GCS]) strains, associated with bovine mastitis, and among *S. dysgalactiae* subsp. *equisilimis* (group C or group G *Streptococcus* [GCS/GGS]) isolates, associated with human pharyngitis and invasive disease episodes. We previously reported the presence of GAS virulence genes in bovine GCS isolates (see Chapter 4), which motivated us to further analyze the bovine strains in a search for the presence of other GAS virulence genes (either chromosomal or encoded by mobile genetic elements). The genes included in the array used in this study are from M1, M3, and M18 GAS genomes, of which M1 and M3 in particular are usually associated with severe disease in Europe and North America (Luca-Harari *et al.*, 2009; Sierig *et al.*, 2003). The results showed that the genes were unevenly distributed among isolates of different host origins. The bovine GCS and GAS shared 23% of all genes, and both the human GCS/GGS and GAS shared 39% of all genes. A higher content of GAS virulence genes in human GGC/GGS was expected, since both species share the same tissue niche in humans and cause similar spectra of diseases (Davies *et al.*, 2009; Igwe *et al.*, 2003; Takahashi *et al.*, 2010). Nevertheless, and most interestingly, none of the 13 bacteriophage virulence-related genes, all associated with GAS disease, were detected in the human GCS/GGS isolates. However, 6 of those 13 genes were detected in the bovine GCS isolates, and we have observed that these GAS phage-related genes (*speK*, *speL*, *speM*, *speC*, *spd1*, and *sdn*) present in bovine GCS are expressed *in vitro*, suggesting that bacteriophages may also play a role in the genetic plasticity and virulence of bovine mastitis GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates. Specifically, the distribution of genes among the bovine GCS ranged from one phage-related gene (*sdn*) present in three isolates to five genes (*speC-speK-speL-speM-spd1*) present in one isolate. Also, the observed linkage of genes in a same bovine GBS strain, in particular *speC-spd1* (from the M1 phage), *speM-speL* (from the M18 phage) and *speK* (from the M3 phage),

indicates polylysogeny, similarly to what was described previously for GAS (Beres & Musser, 2007). However, the lack of an association of sets of genes carried by complete phage genomes in our bovine isolates strongly suggests a recombinatory mosaic nature of phages, as observed previously for GAS (Beres & Musser, 2007).

Also interesting was the observation that "non-bacteriophage associated" genes previously shown to be located in recombinatorial and mutational hotspots of the GAS genome and thus considered to be associated with lateral gene transfer (McMillan *et al.*, 2007[a]; McMillan *et al.*, 2007[b]), were detected in both the bovine GCS and human GCS/GGS isolates from the present study (see Table 4.2). These genes (such as *prtf15*, *epf*, *citE*, and *sic*) belong to four of the five large chromosomal regions described previously to have variable loci in the GAS genome (McMillan *et al.*, 2007[b]). One of the chromosomal regions includes the FCT locus (Towers *et al.*, 2004), which is considered one of the major location of adhesins in GAS. Another chromosomal region is *sagA*, whereas another region includes the maltose transport and *cit* operons. The fourth region includes the *mga* and *spa* loci.

Furthermore, the gene encoding the cell surface-anchored adhesin R28, carried by a putative integrative conjugative elements in GAS which resemble genetic elements of Group B *Streptococcus agalactiae* (GBS) (Stalhammar-Carlemalm *et al.*, 1999) was detected in all bovine and human isolates. Also, the C5a peptidase precursor *scpA* gene as well as the *lbp* gene, encoding the laminin-binding protein, both known to be carried by a composite transposon of GBS (Franken *et al.*, 2001), were detected in all human isolates and not in bovine isolates. Together, our data highlight the importance of MGEs mediating lateral gene transfer among different streptococcal species, including bovine GCS.

4.4.2. Nonrandom distribution of GAS virulence genes in bovine mastitis GCS isolates

Genes of GAS encoding adhesins, such as glyceraldehyde-3-phosphate dehydrogenase, a putative enolase, PrtF15, R28, a putative internalin A precursor, and putative fibronectin-binding protein-like protein A (Brassard *et al.*, 2004; Cork *et al.*, 2009; Katerov *et al.*, 1998; Stalhammar-Carlemalm *et al.*, 1999; Waldemarsson *et al.*, 2006), detected in all bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates, strongly suggest that these might represent important virulence factors in this particular subspecies. In particular, the *emm* gene, encoding the antiphagocytic M protein, was not present in the bovine isolates, although 94% of these isolates carried the gene encoding PAM, a member of the M protein family.

Also interestingly, streptolysin S, strongly associated with invasive disease caused by GAS and associated with the beta-hemolytic phenotype of GAS and GCS/GGS (*S. dysgalactiae* subsp. *equisimilis*) (Betschel *et al.*, 1998; Humar *et al.*, 2002), was detected in all bovine isolates,

which are alpha-hemolytic (a sequence of this gene from one of the bovine mastitis isolates was deposited in GenBank under accession number JF789444). Furthermore, the presence of the streptolysin S gene (*sagA*) in alpha-hemolytic strains of group G (*S. dysgalactiae* subsp. *equisimilis*) and group C (*S. dysgalactiae* subsp. *dysgalactiae*) streptococci, from human and animal origins, respectively, was reported previously (Abdelsalam *et al.*, 2010; Woo *et al.*, 2003).

Bovine GCS is known to grow well in mammary secretions, either during lactation or from dry animals (Oliver, 1991; Song *et al.*, 2005), which may be necessary to survival and establishment in the specific environmental niche that is the bovine mammary gland. The results obtained in the present study by using the BioScreen suggest environmental adaptability of the bovine GCS strains, which demonstrated the ability to grow fast in bovine blood serum and also, although with lower levels of growth, in the remaining tested media (THY and bovine milk serum).

GAS genes related to housekeeping functions were also detected in the bovine isolates from the present study, such as arginine deiminase, a putative metal-binding protein of the ABC transporter, a putative phosphotransferase system (PTS) IIB component, maltose/maltodextrin-binding protein, and putative ferric uptake regulator (Casiano-Colón & Marquis, 1988; Lei *et al.*, 2003; Lortie *et al.*, 2000; Shelburne *et al.*, 2009; Ricci *et al.*, 2002).

The gene encoding 6-phospho-beta-galactosidase, an enzyme of the glycoside hydrolase family 1 (Cantarel *et al.*, 2008) associated with the capacity of carbohydrate utilization, was detected in only 39% of the bovine isolates (and absent in all human isolates). We have observed differences in growth curves among isolates from the present study, which do not seem to correlate with the presence/absence of the 6-phospho-beta-galactosidase gene. Variable growth pattern of strains in bovine mammary secretions were previously described (Oliver, 1991) and may be related with the presence or absence of genes associated with the capacity for carbohydrate utilization but probably not of this particular gene.

4.4.3. Virulence genes detected only in the human GCS/GGS isolates

In epidemiologically and genetically unrelated *S. dysgalactiae* subsp. *equisimilis* strains from human disease (pharyngitis and invasive), we detected nine genes that were not detected in the bovine isolates. Out of these nine genes, five were previously described for human *S. dysgalactiae* subsp. *equisimilis*: *lbp* (encoding the adhesion Lmb, a laminin-binding protein) (Terao *et al.*, 2002), *ska* (encoding the plasminogen-activating Ska-streptokinase A protein) (McArthur *et al.*, 2008), *slo* (encoding the cytolytic streptolysin O toxin) (Sierig *et al.*, 2003), *emm* (encoding the M protein), as well as *scpA* (encoding a C5a peptidase), with the latter two genes acting on the complement pathway of the host, inhibiting bacterial opsonization and

phagocytosis (Hollingshead *et al.*, 1986; Chen & Cleary, 1990). Both the *lbp* and *scpA* genes have been found in human GBS isolates and are usually absent in bovine GBS isolates (Gleich-Theurer *et al.*, 2009). These latter two genes and also *ska* are known to be carried by a bovine pathogen, *S. uberis*, although Lmb is not required for attachment of *S. uberis* to host epithelial cells, and the Ska locus is devoid of plasminogen activator coding sequences (Ward *et al.*, 2009). The four remaining genes were found for the first time, in our study, in human *dysgalactiae* subsp. *equisimilis* isolates: the *nga* gene (encoding a GAS extracellular enzyme NAD-glycohydrolase), associated with cytotoxicity in host cells (Madden *et al.*, 2001); the *isp* gene (encoding an immunogenic secreted protein), which has an unknown function, although it is known to be expressed by GAS in the human host and generates antibody responses (McIver *et al.*, 1998); the *dppA* gene (encoding a dipeptide permease complex), which is a membrane-associated transporter for dipeptides in GAS and is regulated by the multigene transcriptional regulator Mga (Podbielski *et al.*, 1998); and the SpyM3_ 1736 gene (coding for a putative ABC transporter protein). By gene expression assays (RT-PCR), we observed that all these nine genes from GAS isolates were expressed in human GCS/GGS isolates.

These observations together with the absence of these nine genes in the bovine GCS isolates from this study suggest that they may be more important in human host streptococci than in animal host streptococci.

4.4.4. Sequence data and phylogenetic analysis of superantigen genes of bovine group C *S. dysgalactiae* subsp. *dysgalactiae*

The bovine alleles were distributed by the four clades according to the *S. pyogenes* alleles (Fig. 4.1). In the *speK* clade, the bovine alleles are organized together and separately from the *seeL* and *szeL* genes of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* and the *speK* gene of *S. pyogenes*, which was expected, since the streptococcal pathogens *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, and *S. pyogenes* are known to share a phage pool (Holden *et al.*, 2009). In contrast, within the *speL* clade, the sequences of the *seeM* and *szeM* genes of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*, respectively, were organized closer to the bovine $speL_{Bov}$ allele from the present study and separately to the *speL* sequence of *S. pyogenes*, suggesting a common phage content among the animal species. Also, considering the *speC* clade, we may speculate that the same or a similar phage(s) is shared between bovine *S. dysgalactiae* subsp. *dysgalactiae* and human *S. pyogenes*.

The reason why the *seeL* and *szeL* sequences were not located in the *speL* clade and the *seeM* and *szeM* sequences were not in the *speM* clade may be due to incorrect nomenclature given to

these genes of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*. Therefore, *seeL* and *szeL* should be named *seeK* and *szeK*, whereas *szeM* and *seeM* should be named as *seeL* and *szeL*.
The bovine GCS SpeM amino acid sequence (with a length of 177 amino acid residues) showed 100% identity with *S. dysgalactiae*-derived mitogen (Sdm) sequence available in the NCBI database. Sdm (encoded by the *sdm* gene) was the only superantigen with mitogenic activity described so far for *S. dysgalactiae* subsp. *dysgalactiae* (Miyoshi-Akiyama *et al.*, 2003). In agreement with our findings, the authors of that study (Miyoshi-Akiyama *et al.*, 2003) also noticed high levels of identity between *sdm* (from *S. dysgalactiae* subsp. *dysgalactiae*) and *speM* of *S. pyogenes*. *sdm* and *speM* are probably the same gene, with different nomenclatures.
Our findings underline the role of GAS phages (which are known to be shared with *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*) in the genetic diversity of bovine *S. dysgalactiae* subsp. *dysgalactiae* strains. Additionally, it would be of interest to further search for the presence and expression of virulence factors not represented on the array, which can be either present or not in the genomes of the strains tested. Since several genome sequences of streptococcal species are available, whole-genome and transcriptome comparisons using a pair of *S. dysgalactiae* subsp. *dysgalactiae* strains described in the present study (the putative zoonotic strain and the bovine mastitis strain with a large pool of GAS virulence phage-related genes) would certainly give further insights into the genomic plasticity of this pathogenic subspecies.

4.5. Conclusions

In conclusion, the presence of GAS virulence genes, particularly genes encoded by MGEs, either randomly or nonrandomly distributed among strains of bovine GCS may contribute to the increased virulence potential of these strains, namely, the possibility of dissemination to different tissues of the host and to take advantage of new niches. As we have pointed out previously, *S. dysgalactiae* subsp. *dysgalactiae* should not be disregarded as an infection agent in humans. In fact, this subspecies was associated with invasive disease in humans (Koh *et al.*, 2009) and here was shown to carry the *S. pyogenes* streptolysin S gene, further suggesting that *S. dysgalactiae* subsp. *dysgalactiae* is an emerging zoonotic pathogen.

Acknowledgments and work contributions

All experimental work was performed by Márcia G. Rato except collecting bovine strains in farms and identification of strains by the API-20 STREP and BBL Crystal Gram-Positive which was performed by collaborators at FMV/UTL. Statistical analysis of the microarray data and submission of the array data in the Array-express database was carried out by Andreas Nerlich and technical assistance was given by René Bergmann at HZI. We gratefully acknowledge Marco Coelho (Centro de Recursos Microbiológicos) for his help in the construction and analysis of the phylogenetic tree, Dr. Sonia Chénier (Institut National de Santé Animale, Quebec, Canada) and Dr. Koh Tse Hsien (Singapore General Hospital, Singapore) for providing the two invasive GCS alpha-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* strains included in this work, David McMillan for his help in the design of the microarray, and Robert Geffers for printing the microarray slides. This study was supported by projects POCTI/ESP/48407/2002 (Fundação para a Ciência e Tecnologia, Portugal)- FEDER (Fundo Europeu de Desenvolvimento Regional), PROC 60839 (Fundação Calouste Gulbenkian, Portugal), Project ref.46 (Centro de Investigação Interdisciplinar em Sanidade Animal/Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Portugal), and CREM (Centro de Recursos Microbiológicos, Portugal). Márcia G. Rato was supported by PhD grant SFRH/BD/32513/2006 (Fundação para a Ciência e Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior).

5

Antimicrobial resistance and molecular epidemiology of streptococci from bovine mastitis in Portugal

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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.

Keywords:

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

5.1. 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 *et al*., 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 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.

5.2. Materials and Methods

5.2.1. Bacterial isolates and identification

A total of 60 beta-hemolytic *Streptococcus 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 southwestern 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. Among a 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% (Bexiga *et al*., 2005).

Further detailed information regarding the streptococcal field isolates that were included in the present work, isolation and identification methods has been previously described in the "materials and methods" section of Chapter 2.

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), (see Fig.1.3 and Fig. 1.4; Chapter 1). Molecular identification of *S. uberis* and *S. dysgalactiae* subsp. *dysgalactiae* (GCS) field isolates was performed by sequencing and analysis of 16S rDNA gene as described previously (see Chapter 2 and Chapter 3).

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

For the identification of clonal lineages, the percentage of similarity among DNA band patterns obtained by PFGE was determined by cluster analysis based in a dendrogram. 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 previously (Chapter 2).

5.2.3. 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). The primers used for PCR amplification and sequencing are available at the *S. agalactiae* MLST web site (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 eBURST tool (http://eburst.mlst.net/) was used for assignment of predicted primary founder(s), clonal complexes or singletons (STs that cannot be assigned to any group).

5.2.4. Antimicrobial resistance patterns

All *S. uberis*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. agalactiae* isolates were tested by disk diffusion technique (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 (tetracycline and erythromycin).
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.

5.2.5. 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, 1993), (see Fig. 1.8; Chapter 1). Resistance only to lincosamides (L phenotype) and to lincosamides and streptogramins-A (LSA phenotype) reported previously, was also tested by the double-disk test (Malbruny *et al*., 2004).

5.2.6. PCR detection of antimicrobial resistance genes

The macrolide resistance genes *mefA*, *ermA*, and *ermB*, were searched for by PCR among all isolates presenting either the MLS_B or M phenotypes (Pires *et al*., 2005). Tetracycline resistance genes *tetM*, *tetO*, *tetT*, *tetW*, *tetL*, *tetQ*, *tetK* and *tetS* were tested by PCR among all tetracycline-resistant streptococcal isolates (Pires *et al*., 2005; Ng *et al.*, 2001; Aminov *et al.*, 2001). All isolates were tested for the presence of the lincosamide resistance gene *linB* (Bozdogan *et al*., 1999). All isolates of L phenotype were screened for the presence of the streptogramin A resistance gene *vgaB* by PCR as previously described (Allignet & Solh, 1997).

Antimicrobial resistance genes analyzed by PCR and primers used were described in Table 3.2 (Chapter 3). All negative results were confirmed in at least three independent assays.

5.2.7. Capsular serotyping and genotyping of *S. agalactiae* and phylogenetic analysis of sequence data

The 60 *S. agalactiae* isolates were serotyped by the standard method of slide agglutination using antisera of types Ia, Ib, and II to VIII (Essum, Umeå, Sweden) and by capsule genotyping by PCR amplification and sequencing of *cpsD-cpsE-cpsF* genes as described (Florindo *et al*., 2010). Sequencing was performed by STAB Vida (Lisbon, Portugal) and sequence data was analysed using SeqMan, DNAstar (DNAstar Inc., Madison, WI) and BioEdit sequence alignment editor version 7.0.0 (Hall, 1999).

Each bovine *cpsD-cpsE-cpsF* sequence was aligned with sequences of *S. agalactiae* reference strains of recognized serotypes (Ia, Ib, II, III-1, III-2, III-3, III-4 and IV to VII) in search for nucleotide polymorphisms as described previously (Florindo *et al*., 2010). This alignment (1634 bp) was used to construct a phylogenetic tree by using the MEGA version 5 (Tamura *et al*., 2011). The Maximum Parsimony method was used and Bootstrap values were calculated from 1,000 replicates. The GenBank accession numbers of *S. agalactiae* reference strains used in the analysis are designated in the tree.

Specific primers were designed for sequencing the insertion sequence elements detected in the *cps*E of four bovine GBS isolates from the present study: cpsE2 (rev.) 5'-TTATATCGCTGTCTGTATCTTG-3', cpsE44-1 (for.) 5'-GATCGCGCGATAAATTGTTTTAAA-3', and cpsE44-2 (for.) 5'GGGAATAAATCTCTTTCTAGTGAA'3'.

5.2.8. PCR detection of virulence genes among *S. agalactiae* (GBS)

The 60 bovine *S. agalactiae* isolates were screened by PCR for the presence of the human GBS C5a peptidase precursor gene (*scpB*) and the laminin-binding protein gene (*lmb*) using the primers: *scpB* (for.) 5'-CCAAGACTTCAGCCACAAGG-3', *scpB* (rev.) 5'-CAATTCCAGCCAATAGCAGC-3', *lmb* (for.) 5'-AACCCCAAACAGCCTACGCAAG-3', and *lmb* (rev.) 5'-TAAAACGGGATCCGTCCAGGTAT-3' (see Table 4.1; Chapter 4). The presence of the insertion elements IS*Sag2* (known to flank the *scpB-lmb* region) and IS*1548* or GBSi1 (located in the *scpB-lmb* intergenic region) were searched for by PCR as previously described (Franken *et al*., 2001; Safadi *et al*., 2010). The surface protein Spb gene (*spb*), and the fibrinogen-binding proteins FbsA and FbsB genes (*fbs*A and *fbs*B, respectively) were screened

by PCR as described previously (Brochet *et al*., 2006). All negative results were confirmed in at least three independent assays.

5.3. Results

5.3.1. Pulsed-field gel electrophoresis (PFGE) and cluster analysis among *S. agalactiae, S. dysgalactiae* subsp. *dysgalactiae* and *S. uberis*

The PFGE band-based dendrogram for clonal assessment of the *S. agalactiae* isolates is shown in Fig. 5.1. Other genotypic characteristics of *S. agalactiae* and the farm codes of origin of each individual isolate were also included in Fig. 5.1.

The 60 *S. agalactiae* isolates collected among six farms were resolved into 24 PFGE types. Epidemiologically plausible groups or clusters (identified at 80% of similarity or above) revealed four major clonal groups (see clusters I, VI, IX and X; Fig. 5.1) comprising close to 50% of all bovine GBS isolates (all farm-associated). Other minor clusters, which included two to three isolates, were also identified and comprised 30% of total bovine GBS isolates (all farm associated).

Two bovine GBS PFGE patterns (A-1 and B-1) showed nearly 90% similarity with two human GBS PFGE patterns (see Fig. 5.2), when representative PFGE patterns (38 types) of the bovine GBS isolates were compared in a dendrogram with 135 PFGE patterns of human GBS from a parallel study, involving vaginal and/or rectal swabs of pregnant women at five health institutions in Portugal for assessment of GBS carriage (Santos Sanches I. *et. al*., unpublished data).

5.3.2. Multilocus sequence typing (MLST)

A subset of bovine *S. agalactiae* isolates (n=18) out of a total of 60 (e.g., one isolate representative of each PFGE clonal group) were analysed by MLST. Among this subset of isolates four sequence types (ST) were identified (ST-2, ST-61, ST-23 and ST-554), of which ST-554 was found to be novel due to the identification of a new allele of one of the seven housekeeping genes (*phes*, allele 39). This novel ST-554 is a single locus variant-SLV of ST-61 (Fig. 1.6; Chapter 1). The ST-61 belongs to clonal complex (CC) 61/67, ST-2 belongs to CC2 and ST-23 belongs to CC23 as assessed by eBURST analysis (Fig. 1.6; Chapter 1).

5.3.3. Antimicrobial resistance traits and macrolide/tetracycline resistance phenotypes and genotypes among *S. agalactiae, S. dysgalactiae* subsp. *dysgalactiae* and *S. uberis*

Among the 108 bovine isolates tested the highest antimicrobial resistance patterns were observed for streptomycin and gentamicin (97.2% and 80.6%, respectively) followed by tetracycline (64.8%). None of the streptococcal isolates tested showed resistance to vancomycin, chloramphenicol, penicillin, rifaximin, amoxicillin/clavulanic acid, cefazolin and cefoperazone (see Table 5.1).

All isolates resistant to erythromycin and pirlimycin presented the $cMLS_B$ phenotype (Table 5.2). The phenotype M (resistance to ERY only) was not detected among our streptococcal collection. A group of *S. dysgalactiae* subsp. *dysgalactiae* (n=3) and of *S. uberis* (n=8) isolates showed resistance to pirlimycin and susceptibility to erythromycin (L-phenotype).

All isolates of the $cMLS_B$ phenotype carried either the *ermB* gene (GBS, n=10; GCS, n=3; *S. uberis*, n=8) or the *ermA* gene (GBS, n=1; GCS, n=1), while the macrolide resistance gene *mefA* was not detected. One of $cMLS_B$ (*ermB*$^+$) isolates also carried the *linB* gene. Additionally, all isolates belonging to the L-phenotype (n=11) carried the *linB* gene. None of the isolates that were susceptible to pirlimycin (n=75; among 108 isolates) carry the *linB* gene in our collection. The streptogramin A resistance gene *vgaB* was not detected. All the tetracycline resistant isolates carried at least one of the *tet* genes *tetM*, *tetO*, *tetK* and *tetS*, with exception of six *S. dysgalactiae* subsp. *dysgalactiae* isolates, in which none of the *tet* genes tested were detected. The *tetT*, *tetW*, *tetL* and *tetQ* genes tested were not detected.

5.3.4. Capsular serotyping and genotyping of *S. agalactiae* and phylogenetic tree of sequence data

A great proportion (77%; n=46/60) of the bovine *S. agalactiae* isolates tested by the serologic method were nontypeable (NT), either due to the agglutination with multiple antisera or to the lack of agglutination with any of the antisera, and 14 isolates (23%) were identified as serotype V.

Capsular genotyping of the *cpsD-cpsE-cpsF* region at the *cps* locus confirmed the serotype V of the 14 bovine isolates since sequences matched 100% with the *cpsD-cpsE-cpsF* of *S. agalactiae* reference strain of serotype V (GenBank accession number AF332910). One of the 46 NT isolates was identified as serotype III-3, since the *cpsD-cpsE-cpsF* region matched 100% with the same region of a *S. agalactiae* reference strain of sero-subtype III-3 (GenBank accession number AF332897).

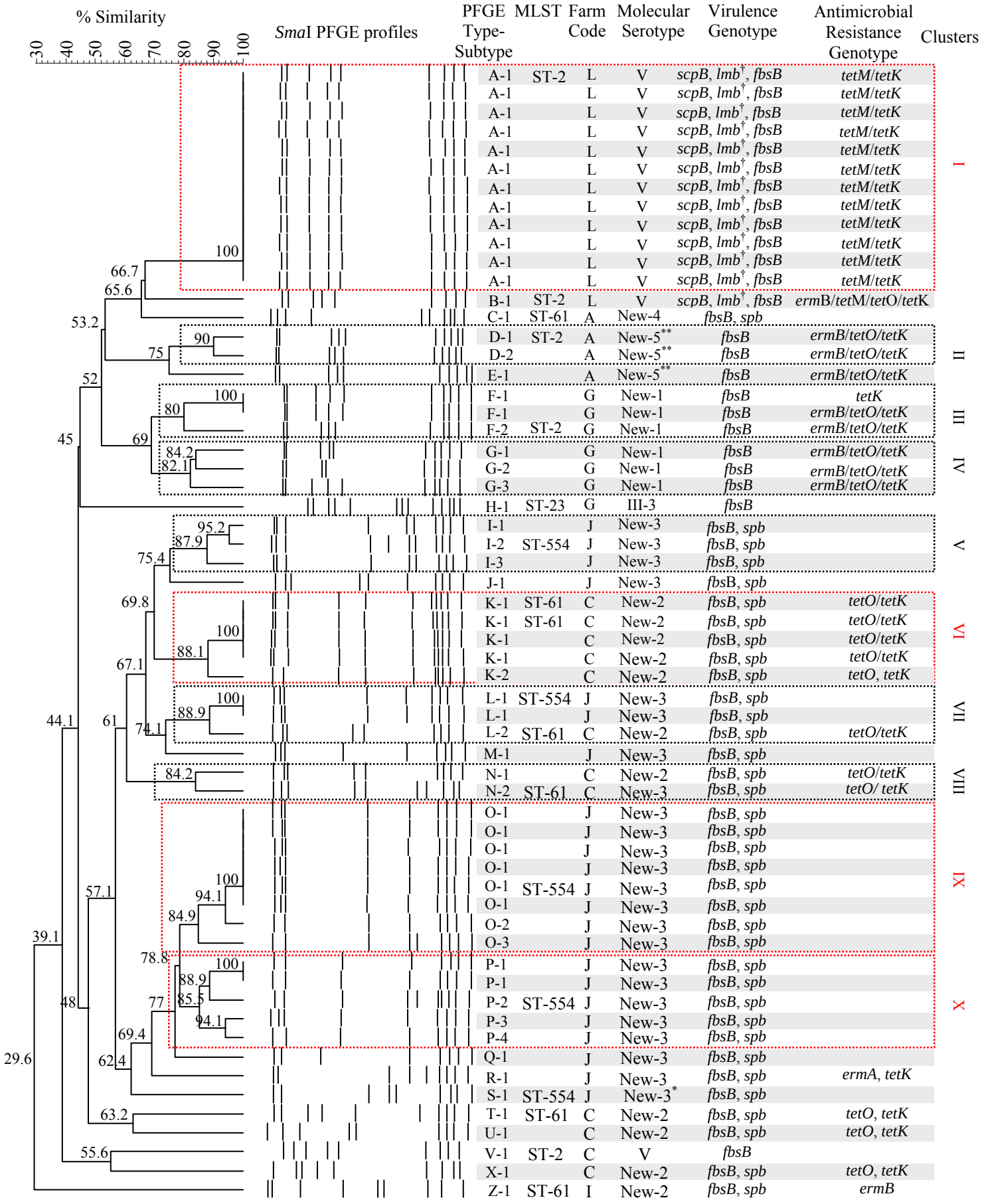


Figure 5.1. Dendrogram of PFGE profiles of group B *Streptococcus* (*Streptococcus agalactiae*) from bovine mastitis, constructed using Dice coefficients and unweighted pair group method using arithmetic averages. Clustering settings of 0.00% optimization and 1.5% band position tolerance were used. *Presence of a transposase in *cpsE* gene; **Presence of an integrase/recombinase in *cpsE* gene (see text); †All *scp*B$^+$ and *lmb*$^+$ isolates carry IS*Sag2*, and do not carry IS*1548* or GBSil elements.

Novel *cpsD-cpsE-cpsF* polymorphisms were detected among the remaining 45 NT isolates, corresponding to five new sequences found: cps_{BOV} New-1 (6 isolates), cps_{BOV} New-2 (11 isolates), cps_{BOV} New-3 (24 isolates), cps_{BOV} New-4 (one isolate), and cps_{BOV} New-5 with an insertion sequence in the *cps*E gene (three isolates). For a better understanding of the polymorphisms, a phylogenetic tree was constructed (see Fig. 5.3).

Additionally, three bovine isolates had a 1051 bp insert in the *cpsE* gene, and one isolate (of cps_{BOV}New-3) had a 1499 bp insert in the *cpsE* gene. By using the BLAST alignment tool (www.ncbi.nlm.nih.gov/BLAST) these inserts were identified as being an integrase/recombinase and a transposase (GenBank accession numbers: EFV96274 and EFV96282, respectively) of a human strain *S. agalactiae* ATCC 13813 from the oral cavity.

The new bovine GBS capsular sequences found in the present study have been deposited in GenBank database under GenBank accession numbers JN384109, JN384110, JN384111, and JN384112 (see Annex). The bovine *cpsD-cpsE-cpsF* sequences containing the above mentioned insertion sequence elements were also deposited in GenBank database under the accession numbers JN384114 and JN384113 (see Annex).

5.3.5. Virulence traits of bovine *S. agalactiae*

The *scpB* and *lmb* genes and the insertion element IS*Sag2* were detected among 13 bovine isolates (of PFGE types A-1 and B-1, all from a same farm). The insertion sequences IS*1548* and GBSi1 tested by PCR were not found in the *scpB-lmb* intergenic region of these 13 isolates.

The *fbsB* gene was present in all bovine *S. agalactiae* isolates while *fbsA* gene was not detected.

The *spb* gene was detected in 60% (n=36) of bovine isolates.

5.4. Discussion

5.4.1. Epidemiological comparison between *S. agalactiae, S. dysgalactiae* subsp. *dysgalactiae* and *S. uberis*

One of our aims was to evaluate the clonal distribution of a collection of streptococcal isolates recovered at different farms. In the present work we found frequent occurrence of indistinguishable PFGE profiles among *S. agalactiae* isolates collected from a same farm, and close to 50% of *S. agalactiae* isolates were included in four major clonal groups as assessed by PFGE (all farm-specific), (see Fig. 5.1). These results suggest widespread strain transmission between animals from the same farm. *S. agalactiae* is considered a contagious mastitis pathogen (Barkema *et al*., 2009) and our results seem to be in agreement with this view.

In contrast, the occurrence of PFGE patterns sharing >82.8% and 100% similarity among *S. dysgalactiae* subsp. *dysgalactiae* isolates collected at different farms (see Fig. 3.1; Chapter 3), suggests an environmental source for this pathogen. *S. dysgalactiae* subsp. *dysgalactiae* is considered a pathogen associated with teat lesions caused by flying insects (Yeruham *et al*., 2002), emphasizing an environmental source for this pathogen in our case.

Heterogeneity in *S. uberis* PFGE patterns was observed between isolates collected from different farms, while three PFGE types accounted for 46.6% of total isolates - all farm-associated (Fig. 2.2; Chapter 2). Our results suggest direct transmission of *S. uberis* among animals within a same farm (Chapter 2). It is plausible that inadequate implementation of management programs for the control of contagious pathogens at farm level, may be promoting the contagious route for *S. uberis* infection within herds, despite it is considered an environmental pathogen (Ericsson *et al.*, 2009).

Table 5.1. Percentages of antimicrobials resistance of *Streptococcus agalactiae*, *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Streptococcus uberis* isolates from the present study against the antimicrobials: erythromycin (ERY), pirlimycin (PRL), tetracycline (TET), gentamicin (CN), streptomycin (S) and cefoperazone (CFP)*.

	Streptococcus agalactiae (n=60)	*Streptococcus dysgalactiae* subsp. *dysgalactiae* (n=18)	*Streptococcus uberis* (n=30)	**Total** (n=108)
ERY	18,3	22,2	26,7	**21,3**
PRL	18,3	38,9	53,3	**31,5**
TE	56,7	100	60	**64,8**
CN	93,3	38,9	80	**80,6**
S	96,7	77,8	100	**97,2**
CFP	3,3	0	3,3	**2,8**

*Resistance was not observed against vancomycin (VA), chloramphenicol (CHL), penicillin (P), amoxicillin-clavulanic acid (AMC), cefazolin (KZ) and rifaximin (RAX).

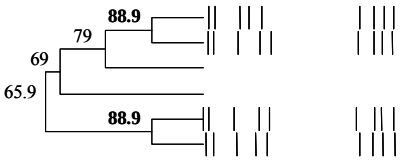
5.4.2. Similar clonal types found among bovine and human *S. agalactiae*

The two PFGE clonal pairs of bovine and human *S. agalactiae* shared the same ST (ST-2) and one of the two PFGE clonal pairs also shared identical serotypes (V) (Fig. 5.2). These results suggest possible bovine-human transmission although this is unlikely in the present study due to geographical distance and lack of epidemiological data regarding these bovine and human isolates in particular. Nevertheless, previous observation of possible transmission of GBS between humans and bovines has been reported (Manning *et al*., 2010).

5.4.3. Multilocus sequence typing (MLST) of *S. agalactiae* and *S. uberis*

Among *S. agalactiae* isolates four STs (ST-61, ST-2, ST-23 and ST-554) were detected, which belong to three clonal complexes (CC2, CC23 and CC61/67), (Fig. 1.6; Chapter 1). These results indicate little heterogeneity among our *S. agalactiae* collection, which was also supported by the PFGE results. Similar results were reported in a previous work (Bisharat *et al*., 2004).

The 30 *S. uberis* isolates tested by MLST revealed 14 different ST (all new) of which almost all are singletons, as determined by e-BURST analysis (Chapter 2). Only ST-287 is a SLV of a predictor founder of a major e-BURST group, which has been previously described in a *S. uberis* isolate from bovine mastitis in Australia, suggesting a common source disseminated to such distant locations in consequence of cattle global trading (Fig. 2.3; Chapter 2). Absence in finding identical ST between *S. uberis* isolates from the present study with those described so far in other countries may be due to the lack of information regarding population structure of *S. uberis* isolates from Southern European countries in the MLST database (http://pubmlst.org/suberis/).



*Sma*I PFGE profiles	Host	Strain name (hum) /PFGE Type-subtype (bov)	Clinical origin	Serotype	Antimicrobial resistance phenotype	MLST
	GBS-Bovine	**B-1**	**Mastitis**	**V**	**cMLS$_B$ + TET**	**ST-2**
	GBS-Human	HAS642	Vaginal/rectal	V	iMLS$_B$	ST-2
	GBS-Human	GO19	Vaginal/rectal	III	TET only	ST-2
	GBS-Bovine	**A-1**	**Mastitis**	**V**	**TET only**	**ST-2**

Figure 5.2. Features of similar clonal types between two bovine *S. agalactiae* (GBS) isolates from the present study and two human *S. agalactiae* (GBS) isolates collected from rectal/ vaginal swabs (colonization) of pregnant woman, among five health institutions in Portugal. The dendrogram included representative PFGE patterns (<100% similarity) of bovine GBS (*n*=38) and 135 representative PFGE patterns of human GBS isolates. Features of the bovine isolates from the present study are designated in **bold** in the figure. Dice coefficients and unweighted pair group method using arithmetic averages (and clustering settings of 0.00% optimization and 1.5% band position tolerance) was used to construct the dendrogram. hum: human; bov: bovine.

5.4.4. Antimicrobial resistance traits and genotypes of *S. agalactiae, S. dysgalactiae* subsp. *dysgalactiae* and *S. uberis*

Updated information regarding published veterinary breakpoints for mastitis pathogens is required to validate the clinical relevance of *in vitro* susceptibility testing results for these

pathogens. In order to have a global view of antimicrobial resistance occurrence among isolates of the three bovine streptococcal species, all isolates from the present study were tested by the *in vitro* disk diffusion method (CLSI, 2008) against 13 antimicrobials, which were chosen taking into account their availability in the Portuguese market for intramamary treatment of mastitis and also in order to monitor the erythromycin and tetracycline resistance phenotypes and genotypes among bovine streptococci.

Interpretative standards (breakpoints) were used according to what is available in the CLSI (2008), and when not available according to the antimicrobials manufacturers' instruction.

Table 5.2. Distribution of erythromycin (ERY), pirlimycin (PRL) and tetracycline (TET) resistance phenotypes and genotypes among bovine *Streptococcus agalactiae* (GBS), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GCS) and *Streptococcus uberis* isolates.

Species (No. total isolates)	Resistance phenotype*	**Total**	No. (%) of resistant isolates carrying resistance genes**						
			ERY resistance genes		PRL resistance gene	TET resistance genes			
			ermA	***ermB***	***linB***	***tetM***	***tetO***	***tetK***	***tetS***
GBS (n=60)	$cMLS_B$ only	**1**	0	1 (100)	0	nd	nd	nd	nd
	$cMLS_B$+TET	**10**	1 (10)	9 (90)	0	1 (10)	9 (90)	10 (100)	0
	TET only	**24**	nd	nd	0	12 (50)	11 (45,8)	24 (100)	0
GCS (n=18)	$cMLS_B$+TET	**4**	1 (25)	3 (75)	0	1 (25)	3 (75)	0	0
	L+TET	**3**	nd	nd	3 (100)	1 (33,3)	2 (66,7)	0	0
	TET only	**11**	nd	nd	0	4 (36,4)	1 (9,1)	0	0
S. uberis (n=30)	$cMLS_B$+TET	**8**	0	8 (100)	1 (13)	0	8 (100)	0	0
	L	**1**	nd	nd	1 (100)	nd	nd	nd	nd
	L+TET	**7**	nd	nd	7 (100)	0	1 (14,3)	0	7 (100)
	TET only	**3**	nd	nd	0	2 (66,7)	0	0	1 (33,3)

*Phenotype M was not found among the bovine isolates.
**The *mefA*, *vgaB*, *tetT*, *tetW*, *tetL*, and *tetQ* antimicrobial-resistant genes were not detected.
$cMLS_B$ only, constitutive MLS_B resistance phenotype only; $cMLS_B$ + TET, constitutive MLS_B resistance phenotype, and resistance to tetracycline; TET only, resistance only to tetracycline; L + TET, resistance to pirlimycin, susceptibility to erythromycin, and resistance to tetracycline; L, resistance to pirlimycin, and susceptibility to erythromycin only;
nd, not determined

Overall, resistance to the aminoglycoside antimicrobials, gentamicin and streptomycin, was high (80.6% and 97.2% respectively) while penicillin, amoxicillin-clavulanic acid, cefazolin, cefoperazone chloramphenicol, vancomycin and rifaximin showed very good *in vitro* efficacy against our streptococcal collection.

High percentages of resistance to tetracycline (64.8 to 100%) were observed among all the isolates, which may be due to the fact that tetracycline had been widely used for treatment of several infections in cattle for many years.

These results were similar to what was described previously in other countries (Hendriksen *et al*., 2008; Petrovski *et al*., 2011), with exception of erythromycin and pirlimycin antimicfronbials. In the presence study we observed that resistance to these antimicrobials was high, which underlines the importance of locally monitoring resistance patterns rather than extrapolating results obtained in other countries.

Co-resistance to erythromycin and pirlimycin observed was mostly due to the presence of *ermB* gene. Also, putative linkage of *ermB* and *tetO* antimicrobial resistance genes in unique strains (considered unrelated as assessed by PFGE) suggested horizontal co-transference of these genes in a same mobile genetic element.

Additionally, resistance to pirlimycin was particularly high among *S. uberis* (53,3%) and *S. dysgalactiae* subsp. *dusgalactiae* (38,9%) due to the presence of *linB* gene, which is carried by a plasmid first described in human strains of *Enterococcus faecium* (Bozdogan *et al*., 1999). *Enterococcus faecium* is also a recognized pathogen associated with bovine mastitis.

These results underline the importance of surveillance of antimicrobial resistance genes among field isolates.

5.4.5. Novel *cps* locus polymorphisms in nontypable bovine *S. agalactiae* and insertion sequence elements in *cpsE*

By using the serologic method we identified 14 bovine *S. agalactiae* isolates as being of serotype V while the remaining 46 isolates (77%) were considered nontypable (NT). Indeed, high percentage of nontypeability among *S. agalactiae* isolates from bovine origin has been reported (Zhao *et al*., 2006).

By molecular serotyping, one of the NT isolates was identified as being of molecular sero-subtype III-3, while the remaining 45 NT isolates showed novel *cpsD-cpsE-cpsF* sequence polymorphisms and did not match completely with any of the recognized molecular serotypes (Ia, Ib, II, III-1, III-2, III-3, III-4 and IV to IX). Further studies are required in the future to assess if the above mentioned polymorphisms observed in the bovine strains are associated with new functional proteins (represent new serotypes).

In the phylogenetic tree (Fig. 5.3), all these novel bovine *cpsD-cpsE-cpsF* sequences clustered together in a clade but separately from the remaining serotypes from human *S. agalactiae* reference strains, suggesting they have diverged within the bovine host and that they do not represent subtypes of known molecular serotypes from human *S. agalactiae*.

The inserts found in the *cpsE* gene (a transposase and an integrase/recombinase of *S. agalactiae* ATCC 13813) of four bovine isolates from the present study illustrate the occurrence of recent genetic events, since only four out of the 60 bovine isolates (7%) carry these inserts.

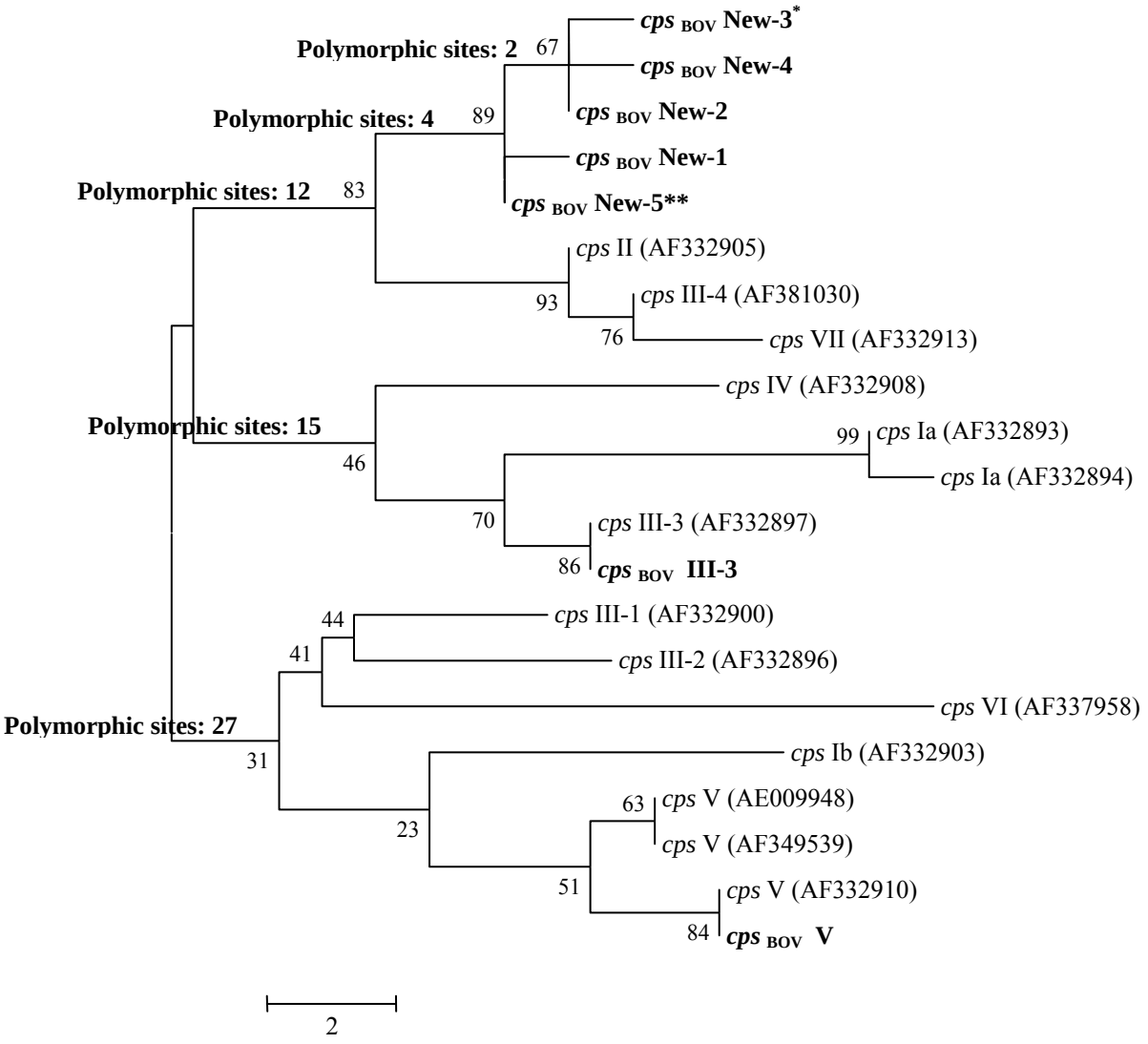


Figure 5.3. Phylogenetic analysis of *cpsD-cpsE-cpsF* sequences of bovine Group B *Streptococcus* (*Streptococcus agalactiae*) isolates and *cpsD-cpsE-cpsF* sequences of *S. agalactiae* reference strains, which were available in National Center for Biotechnology Information (NCBI) (Bethesda, MD). Bovine sequences from the present study are designated in **bold** in the tree. GenBank accession numbers of *Streptococcus agalactiae* reference strains are designated in parentheses in front of serotype designation.
*One bovine isolate of molecular serotype *cps* BOV New-3 has an insert in *cpsE* (mutator family transposase of human *S. agalactiae* ATCC 13813);
**All bovine isolates of molecular serotype *cps* BOV New-5 have an insert in *cpsE* (integrase/recombinase of human strain *S. agalactiae* ATCC 13813), (see text).

Other authors have reported an insert (IS*1381*) in the *cpsE* gene of *cps* locus in a human *S. agalactiae* strain, which resulted in reduced or disrupted expression of the capsule polysaccharide (Ramaswamy *et al*., 2006). Presence of another insert (ISSa4) in the *cpsE* gene

of three human GBS isolates has also been observed (Florindo *et al*., unpublished). Probably the frequency of inserts in *cpsE* among bovine isolates is higher than in human isolates, and may reflect evolutionary adaptation to the bovine host. However, further studies using bovine GBS isolates are needed to elucidate this issue. Few reports have decribed so far the presence of insertion sequences in bovine *S. agalactiae* strains (Shakleina *et al.*, 2004; Richards *et al.*, 2011).

5.4.6. Virulence traits among *S. agalactiae*

The *lmb* and *scp*B virulence genes were found among 13 bovine isolates (22% of total isolates) despite the fact that these genes are described as usually absent in bovine GBS isolates (Franken *et al*., 2001; Safadi *et al*., 2010). Absence of the *scp*B - *lmb* region in the remaining bovine isolates (n=47) from the present work is probably due to deletion during putative adaptation to the bovine host.

An association of *spb* gene with the clonal complex 17 (CC17) isolates has been previously reported (Brochet *et al*., 2006). The CC17 lineage is known to be closely related to CC61 lineage (as assessed by eBURST; Fig. 1.6; Chapter 1), which may help to explain why we have detected the *spb* virulence gene only among GBS isolates of ST-61/ST-554 in the present study.

5.5. Conclusions

In conclusion, our results showed that our bovine streptococci were susceptible to clinical relevant antimicrobials; however resistance levels against erythromycin and pirlimycin in particular, were high when compared with data from other countries.

Also we observed that our bovine *S. agalactiae* are homogenous within herds emphasizing its contagious nature. Few of these bovine isolates have several genotypic characteristics in common with human GBS collected from rectal/vaginal swabs.

Our study highlights novel information regarding features of bovine *S. agalactiae*, namely the presence of insertion sequences in the *cpsE* gene from the *cps* locus which probably prevents expression of the capsule polysaccharide, and new *cpsD-cpsE-cpsF* sequences that may code for new serotypes.

Acknowlegments and work contributions

All experimental work was performed by Márcia G. Rato except collecting bovine strains in farms, identification of strains by the API-20 STREP and BBL Crystal Gram-Positive and antimicrobial resistance tests performed against P, KZ, CFP, CN, S, AMC, and RAX which was performed by collaborators at FMV/UTL. Part of sequencing and technical assistance in molecular serotyping and PCR for search for the presence of GBSi1 and IS1548 mobile genetic elements in GBS was carried out by Carlos Florindo at INSA. We gratefully acknowlege Marco Coelho (Centro de Recursos Microbiológicos) for his help in the construction and analysis of the phylogenetic tree. This study was supported by projects POCTI/ESP/48407/2002 (Fundação para a Ciência e Tecnologia, Portugal). FEDER (Fundo Europeu de Desenvolvimento Regional), PROC 60839 (Fundação Calouste Gulbenkian, Portugal), Project ref.46 (Centro de Investigação Interdisciplinar em Sanidade Animal/Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Portugal), and CREM (Centro de Recursos Microbiológicos, Portugal). Márcia G. Rato was supported by PhD grant SFRH/BD/32513/2006 (Fundação para a Ciência e Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior).

6

General Discussion

6.1. Molecular epidemiology

Continuous surveillance of bovine mastitis pathogens and herd careful management is essential for the well being of dairy cows and prevention of economic loss in the dairy industry. One of the aims of the present study was to study the clonal features of streptococcal field isolates associated with bovine mastitis in Portugal in order to contribute to improvement of bovine health.

The PFGE and MLST approaches were suitable for the comparative analysis. Indeed, among *S. uberis* isolates, three PFGE clonal groups (associated with MLST lineages ST-265, ST-268, and ST-267) comprised >60% of total isolates, and among *S. galactiae* isolates only three MLST lineages (ST-2, ST-23, and ST-61/ST-554) were identified, which is indicative of a contagious route of transmission between animals (Chapter 2 and Chapter 5). These results suggest that control measures for these two pathogens in herds in Portugal deserve more attention.

We identified identical PFGE patterns between *S. dysgalactiae* subsp. *dysgalactiae* isolates from different farms, which suggest an environmental source for this pathogen (see Chapter 3 and Chapter 5).

6.2. Host adaptation

We observed that a group of the bovine *S. agalactiae* isolates (of PFGE types A-1 and B-1) showed features of human *S. agalactiae*, namely carried the *lmb-scpB* virulence genes. These genes are usually absent in *S. agalactiae* isolates from bovine origin but always present in human *S. agalactiae*, and are considered required for human isolates to infect the human host (Safadi *et al.*, 2010). The remaining bovine GBS isolates from the present study lacked the *lmb-scpB* virulence genes, which were probably lost during the adaptation to the bovine host.

We also observed that the above mentioned bovine GBS clones (A-1 and B-1) which carry the *lmb-scpB* virulence genes, showed higher similarity (above 88% between PFGE patterns) with human *S. agalactiae* isolates collected from vaginal/rectal swabs from a parallel study, than with the remaining bovine isolates from the present study (Chapter 5). However, in the present case human-bovine transmission is most unlikely to have occurred due to the geographic distance between these (bovine and human) isolates sharing similar genotypes. Nevertheless, a recent study has documented the occurrence of similar *S. agalactiae* genotypes between isolates collected from humans and their bovines (which were in direct contact), and it was suggested that possible transmission between the two hosts occurs (Manning *et al.*, 2010).

We identified new *cpsD-cpsE-cpsF* sequences (of the *cps* locus encoding the capsular polysaccharide) among the bovine *S. agalactiae*, suggesting novel serotypes not yet described. By phylogenetic analysis, we observed that all the new bovine *cpsD-cpsE-cpsF* sequences clustered together in a clade but separated from the human molecular serotypes, indicating that these new serotypes diverged in the bovine host.

We further detected the presence of insertion sequences (so far described in human *S. agalactiae*) in the *cpsE* gene of the *cps* locus in few bovine *S. agalactiae* strains (Chapter 5). These inserts in *cpsE* probably disrupt the expression of the capsule polysaccharide in these bovine strains (which may result in phenotypic variation); however, further studies are necessary to confirm this hypothesis.

6.3. Phenotypes and genotypes of antimicrobial resistance

Importantly, all streptococcal isolates were susceptible to penicillin, cefoperazone and cefazolin antimicrobials, commonly used in bovine mastitis treatment in Portugal (Chapter 5). However, resistance to pirlimycin was very high (when compared to other countries), which is also an antimicrobial available for use in bovine mastitis treatment in Portugal. This observation points out the importance of locally monitoring antimicrobial resistance patterns for the improvement of antibiotherapy programmes, rather than extrapolating from results obtained in other countries.

The putative linkage between antimicrobial resistance genes, namely *ermB-tetO* observed among unrelated and related bovine streptococcal isolates (as assessed by PFGE), suggests possible co-transference of these antimicrobial resistance determinants via horizontal gene transfer by a same mobile genetic element. Future studies should clarify which mobile genetic elements are putatively involved in such events.

6.4. Phages and other mobile genetic determinants in bovine streptococci evolution

We demonstrated that the bovine *S. dysgalactiae* subsp. *dysgalactiae* strains carried the *S. pyogenes* phage-associated virulence genes *speK*, *speM*, *speL*, *speC*, *spd1*, *sdn*. All these genes were transcribed by RT-PCR (Chapter 3 and Chapter 4). These genes encode pyrogenic-toxin-superantigens, DNAses and streptodornases strongly associated with invasive disease in humans. These results suggest that *S. pyogenes* bacteriophages may play a role in the dynamics of bovine *S. dysgalactiae* subsp. *dysgalactiae* population structure.

Further investigation should be performed in order to clarify the role of phages in the lateral gene transfer between the animal and human streptococci, and in putatively increasing the pathogenesis of the bovine *S. dysgalactiae* subsp. *dysgalactiae*.

Preliminary results in induction of prophages carried out after treatment with mitomycin C in two bovine *S. dysgalactiae* subsp. *dysgalactiae* strains from the present study (which carry *speM*, *speC*, *speK*, *speL*, *spd1* phage-genes) has confirmed that those strains are lysogenic for viable phages (see Fig. 6.1).

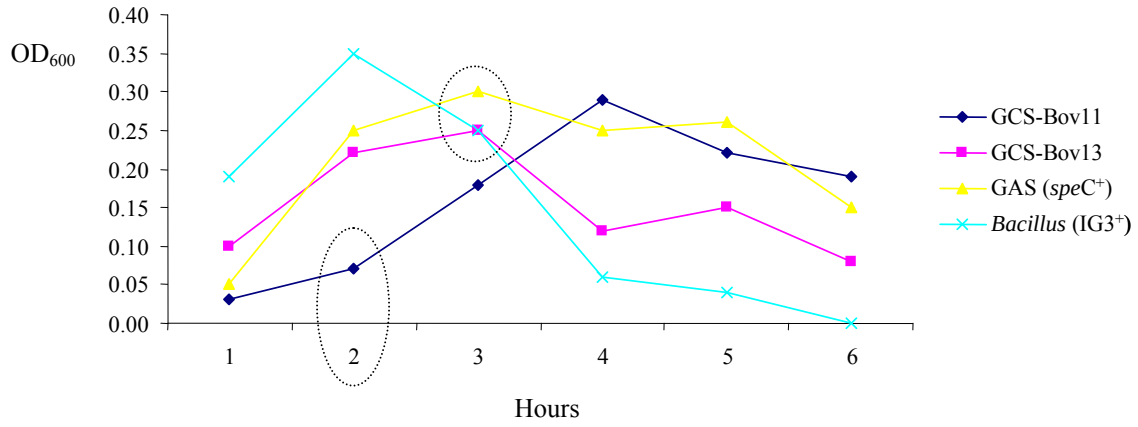


Figure 6.1. Induction of prophages after treating cultures with mitomycin C in bovine *S. dysgalactiae* subsp. *dysgalactiae* strains GCS-Bov11 (which carries the phage-genes *speM*, *speC*, *speK*, *spd1*) and GCS-Bov13 (which carries the phage-genes *speM*, *speC*, *speK*, *speL*, *spd1*), and control strains lysogenic for *speC* phage (*S. pyogenes* or GAS) and lysogenic for IG3 phage (*Bacillus subtillis*).
Mitomycin C was added to cultures incubated at 37ºC in Todd Hewitt broth, after 2 hours of growth (GCS-Bov13, GAS *speC*$^+$ and *Bacillus* IG3$^+$), and after 3 hours of growth (GCS-Bov11).

In addition, by successfully amplifying the left junction of the Tn*1207.3*/Φ10394.4 mobile element in the bovine *S. dysgalactiae* subsp. *dysgalactiae* indicates that a Tn*1207.3*/Φ10394.4-related chimeric element may be inserted in the *comEC* locus as previously described for the human pathogens *S. pyogenes*, *S. dysgalactiae* subsp. *equisimilis* and *S. agalactiae* (Chapter 3).

By using an array of *S. pyogenes* virulence genes, we were able to compare genomic content of the bovine *S. dysgalactiae* subsp. *dysgalactiae* isolates with the human *S. pyogenes*, and human *S. dysgalactiae* subsp. *equisimilis* isolates. Thus, we further demonstrated that other relevant *S. pyogenes* virulence determinants which are transferred by integrative conjugative elements (namely the R28 protein) were also present in the bovine *S. dysgalactiae* subsp. *dysgalactiae* (Chapter 4).

Future perspectives

We have reported for the first time the presence of the human *S. pyogenes* (GAS) phage virulence genes among bovine *S. dysgalactiae* subsp. *dysgalactiae* (GCS). However it is still unclear whether these mobile genetic elements contribute to the increased virulence potential of this bovine streptococcal species. Moreover, conclusive evidence of *S. dysgalactiae* subsp. *dysgalactiae* as an infection agent in humans has not yet been proved.

In the future it would be of utmost interest to further study strains of bovine *S. dysgalactiae* subsp. *dysgalactiae*, as well strains of the other human related subspecies - *S. dysgalactiae* subsp. *equisimilis* and *S. pyogenes* in order to assess the genomic structure of the phages carrying the detected virulence genes. This study would clarify whether the prophages carried by the animal GCS, the *S. dysgalactiae* subsp. *equisimilis*, and *S. pyogenes* strains are the same and thus defining phage(s) host range. The phage(s) isolation and charaterization could also contribute to the clarification of the involvement of those phages in mobile genetic transfer.

It would be of interest to confirm if the bovine isolates carrying GAS virulence genes are expressing these genes at the proteomic level, and to test the bovine strains infection potential in vitro by using human cell lines and in vivo using Zebrafish (as a model for the evaluation of virulence).

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Annex

Nucleotide sequence accession numbers

Sequence data have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers:

JF789447

Streptococcus dysgalactiae subsp. *dysgalactiae* strain 06.1563 16S ribosomal RNA gene, partial sequence

JF789445

Streptococcus dysgalactiae subsp. *dysgalactiae* strain 06.1563 SodA (*sodA*) gene, partial cds

HQ724300

Streptococcus dysgalactiae subsp. *dysgalactiae* pyrogenic exotoxin K (*speK*) gene, *speK-1* allele, partial cds

HQ724301

Streptococcus dysgalactiae subsp. *dysgalactiae* pyrogenic exotoxin K (*speK*) gene, *speK-2* allele, partial cds

HQ724302

Streptococcus dysgalactiae subsp. *dysgalactiae* pyrogenic exotoxin K (*speK*) gene, *speK-3* allele, partial cds

HQ724303

Streptococcus dysgalactiae subsp. *dysgalactiae* pyrogenic exotoxin L (*speL*) gene, *speL-1* allele, partial cds

HQ724304

Streptococcus dysgalactiae subsp. *dysgalactiae* pyrogenic exotoxin M (*speM*) gene, *speM-1* allele, partial cds

HQ724305

Streptococcus dysgalactiae subsp. *dysgalactiae* pyrogenic exotoxin M (*speM*) gene, *speM-2* allele, partial cds

HQ696925

Streptococcus dysgalactiae subsp. *dysgalactiae* strain VSD16 pyrogenic exotoxin C (*speC*) gene, *speC-1* allele, partial cds

JF789444

Streptococcus dysgalactiae subsp. *dysgalactiae* strain VSD1 SagA (*sagA*) gene, partial cds

JF789442

Streptococcus dysgalactiae subsp. *dysgalactiae* strain 06.1563 SagA (*sagA*) gene, partial cds

JF789443

Streptococcus dysgalactiae subsp. *dysgalactiae* strain DB49998/05 SagA (*sagA*) gene, partial cds

JN384109

Streptococcus agalactiae strain VSA12 capsular polysaccharide biosynthesis gene cluster, partial sequence (*cps-1*$_{BOV}$)

JN384110

Streptococcus agalactiae strain VSA1 capsular polysaccharide biosynthesis gene cluster, partial sequence (*cps-2*$_{BOV}$)

JN384111

Streptococcus agalactiae strain VSA3 capsular polysaccharide biosynthesis gene cluster, partial sequence (*cps-3*$_{BOV}$)

JN384112

Streptococcus agalactiae strain VSA17 capsular polysaccharide biosynthesis gene cluster, partial sequence (*cps-4*$_{BOV}$)

JN384113

Streptococcus agalactiae strain VSA44 capsular polysaccharide biosynthesis gene cluster, partial sequence (*cps-3*$_{BOV}$ with insertion sequence)

JN384114

Streptococcus agalactiae strain VSA10 capsular polysaccharide biosynthesis gene cluster, partial sequence (*cps-5*$_{BOV}$ with insertion sequence)