BRUNA GOMES ALVES

Molecular characterization of *Staphylococcus aureus* and *Streptococcus uberis* isolates of intramammary infections of dairy cows

Pirassununga

2020

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Thesis submitted to the Postgraduate Program in Nutrition and Animal Production of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

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Advisor:
Prof. Marcos Veiga dos Santos, Ph.D.

According:

Marcos Veiga dos Santos

Advisor

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CERTIFICADO

Certificamos que a proposta intitulada "CARACTERIZAÇÃO MOLECULAR DE Staphylococcus aureus E Streptococcus uberis ISOLADOS DE INFECÇÕES INTRAMAMÁRIAS DE VACAS LEITEIRAS", protocolada sob o CEUA nº 8178270617 (ID 003944), sob a responsabilidade de **Marcos Veiga dos Santos** *e equipe; Bruna Gomes Alves* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 03/08/2017.

We certify that the proposal "MOLECULAR CHARACTERIZATION OF Staphylococcus aureus and Streptococcus uberis ISOLATES OF INTRAMMARY INFECTIONS OF DAIRY COWS", utilizing 200 Bovines (200 females), protocol number CEUA 8178270617 (ID 003944), under the responsibility of **Marcos Veiga dos Santos** *and team; Bruna Gomes Alves* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 08/03/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2016 a 08/2020 Área: Nutrição E Produção Animal

Origem:	Amostras biológicas estocadas						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	2 a 5 anos	N:	200
Linhagem:	Holandês			Peso:	550 a 650 kg		

Local do experimento: Laboratório Qualileite (Brasil) Departamento de Estudo de Populações, ICBAS Universidade do Porto (Portugal) Università Degli Studi de Milano

São Paulo, 26 de maio de 2020

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenador
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EVALUATION FORM

Author: ALVES, Bruna Gomes

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Date: _____/_____/_____

Committee Members

Prof. Dr._____

Institution:_____ Decision:_____

Prof. Dr._____

Institution:_____ Decision:_____

Prof. Dr._____

Institution:_____ Decision:_____

Prof. Dr._____

Institution:_____ Decision:_____

Prof. Dr._____

Institution:_____ Decision:_____

DEDICATION

Aos maiores incentivadores da minha vida, que mesmo de longe torcem pela minha felicidade e pelo meu sucesso. De onde eu busco forças para nunca desistir e para recomeçar sempre que necessário. Os meus amados pais, Marlene e Hélvio.

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It’s always seems impossible until it’s done…
- Nelson Mandela -

RESUMO

ALVES, B. G. **Caracterização molecular de *Staphylococcus aureus* e *Streptococcus uberis* isolados de infecções intramamárias de vacas leiteiras** [Molecular characterization of *Staphylococcus aureus* and *Streptococcus uberis* isolates of intramammary infections of dairy cows]. 2020. 128 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

A mastite bovina é a doença mais frequente em vacas leiteiras, o que resulta em significativas perdas econômicas. *Staphyloccoccus aureus* e *Streptococcus uberis* estão entre as principais causas de mastite clínica (MC) e subclínica (MSC), pois são de difícil controle por medidas convencionais, já que ambos possuem mecanismos de evasão do sistema imune, aderência e internalização nos tecidos mamários. Sendo assim, esta tese foi organizada em três experimentos: 1) Caracterização genotípica de *S. aureus* isolados de mastite de rebanhos brasileiros e de outros países; 2) Caracterização genotípica e sensibilidade aos antimicrobianos de isolados de *S. aureus* identificados antes e após o tratamento de MSC durante a lactação e 3) Diversidade genotípica e fatores de virulência de *S. uberis* isolados de infecções intramamárias de vacas leiteiras no Brasil. No experimento 1, 70 isolados de *S. aureus* (35 de MC e 35 de MSC) foram isoladas de amostras de leite de quartos mamários de vacas em 16 rebanhos do Brasil. Após a seleção, 15 isolados brasileiros foram agrupados à isolados provenientes da Argentina (n=16), Colômbia (n=15), Alemanha (n=17), Itália (n=17), EUA (n=17), África do Sul (n=11) e Tunísia (n=12) para genotipagem por RS-PCR, baseada na amplificação do espaço intergênico 16S-23S rRNA e investigados em relação a 26 fatores de virulência. No experimento 2, 79 isolados de *S. aureus* foram genotipados por eletroforese em gel de campo pulsado (PFGE) e avaliados quanto à concentração inibitória mínima (CIM) para determinação dos perfis de resistência aos antimicrobianos. No experimento 3, 44 isolados de *S. uberis* foram avaliados por hibridização de DNA através da metodologia *dot blot* para determinação da diversidade genotípica. Os resultados do experimento 1 indicaram alta variabilidade genotípica de *S. aureus* dentre os países avaliados e maior prevalência de genes relacionados à invasão ao organismo do hospedeiro (*clfA*, *cna*, *fmtB* e leucocidinas). Além disso, foi demonstrado que o padrão genotípico de isolados de *S. aureu*s foi específico para cada país, sugerindo que as estratégias de controle devem ser formuladas de acordo com a região em questão e com a virulência das cepas envolvidas na infecção. No experimento 2, foi identificada alta similaridade entre os isolados de um mesmo rebanho indicando especificidade genotípica dentro de uma mesma região, mesmo após os tratamentos durante a lactação. Além disso, todos os isolados avaliados se mostraram suscetíveis à gentamicina, enrofloxacina,

ciprofloxacina e tetraciclina, embora alta resistência frente à amoxicilina e cefalexina tenham sido também observadas. No experimento 3, foram observados nove padrões de *dot blot*, indicando alta heterogeneidade dos isolados de *S. uberis*. Foi possível observar alta prevalência dos genes reguladores de fatores de virulência, como *sua* e *gapC*, responsáveis pela aderência e internalização de *S. uberis* nos tecidos da glândula mamária. Assim, após a conclusão dos três estudos, foi possível concluir que há alta diversidade genotípica em *S. aureus* e *S. uberis* isolados de infecções intramamárias no Brasil. Além disso, foi confirmado que estas duas espécies apresentam inúmeros fatores de virulência que contribuem para permanência na glândula mamária e dificultam o controle da mastite por meio de medidas convencionais. No entanto, estas medidas de controle, tais como utilização de pós-dipping, registro e monitoramento dos casos clínicos e descarte dos animais crônicos devem fazer parte do plano de prevenção da mastite bovina, afim de diminuir a gravidade e a dispersão dos casos. As técnicas moleculares são eficientes na identificação e caracterização genética de isolados bacterianos e podem ser ferramentas auxiliares no diagnóstico e epidemiologia da mastite, direcionando as estratégias de controle e tratamento da doença.

Palavras-chave: concentração inibitória mínima, genotipagem, dot blot, estafilococos, estreptococos.

ABSTRACT

ALVES, B. G. **Molecular characterization of *Staphylococcus aureus* and *Streptococcus uberis* isolates of intramammary infections of dairy cows** [Caracterização molecular de *Staphylococcus aureus* e *Streptococcus uberis* isolados de infecções intramamárias de vacas leiteiras]. 2020. 128 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

The bovine mastitis is the disease more frequent of dairy cows, which results in significant economic losses. *Staphyloccoccus aureus* and *Streptococcus uberis* are among the main causes of clinical (CM) and subclinical (MSC) mastitis, because they are difficult to control by conventional measures, since both have mechanisms of immune system evasion, adherence and internalization in the mammary tissues. Therefore, this thesis was organized in three experiments: 1) Genotypic characterization of *S. aureus* isolated from mastitis from Brazilian herds and from other countries; 2) Genotypic characterization and antimicrobial sensitivity of *S. aureus* isolates identified before and after treatment of MSC during lactation; and 3) Genotypic diversity and virulence factors of *S. uberis* isolated from intramammary infections of dairy cows in Brazil. In experiment 1, 70 *S. aureus* isolates (35 from MC and 35 from MSC) were identified in milk samples from cows' mammary quarters in 16 herds in Brazil. After the selection, 15 Brazilian isolates were grouped with isolates from Argentina (n=16), Colombia (n=15), Germany (n=17), Italy (n=17), USA (n=17), South Africa (n=11) and Tunisia (n=12) for genotyping by RS-PCR, based on the amplification of the 16S-23S rRNA intergenic space and investigated in relation to 26 virulence factors. In experiment 2, 79 *S. aureus* isolates were genotyped by pulsed-field gel electrophoresis (PFGE) and evaluated by minimum inhibitory concentration (MIC) to assess antimicrobial resitance profiles. In experiment 3, 44 isolates of *S. uberis* were evaluated by DNA hybridization using the dot blot methodology, to determine the genotypic diversity. The results of experiment 1 indicated high genotypic variability of *S. aureus* according to the countries evaluated, and high prevalence of genes related to invasion to the host organism (*clfA*, *cna*, *fmtB* and leucocidins). In addition, it was shown that the genotype pattern os *S. aureus* isolates were specific to each country, suggesting that control strategies should be formulated according to the region in question and the virulence of the strains involved in the infection. In experiment 2, a high similarity was identified between the isolates of the same herd, indicating genotypic specificity within the same region, even after treatments during lactation. In addition, all the isolates evaluated were susceptible to gentamicin, enrofloxacin, ciprofloxacin and tetracycline, although high resistance to amoxicillin and cephalexin has also been observed. In experiment 3, nine dot blot patterns were

observed, indicating high heterogeneity of *S. uberis* isolates. It was possible to observe a high prevalence of genes regulating virulence factors, such as *sua* and *gapC*, responsible for the adherence and internalization of *S. uberis* in the mammary gland tissues. Thus, after the conclusion of the three studies, it was possible to conclude that there is a high genotypic diversity in *S. aureus* and *S. uberis* isolated from bovine intramammary infections in Brazil. In addition, it has been confirmed that these two species have numerous virulence factors that contribute to their permanence in the mammary gland and a limit control based on conventional measures. However, these control measures, such as the use of post-dipping, registration and monitoring of clinical cases and the culling chronic cows must be part of the bovine mastitis prevention plan, in order to reduce the severity and dispersion of cases. Molecular techniques are efficient in the identification and genetic characterization of bacterial isolates and can be auxiliary tools in the diagnosis and epidemiology of mastitis, guiding the control and treatment strategies of the disease.

Key words: minimal inhibitory concentration, genotyping, dot blot, staphylococci, streptococci.

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LIST OF ABREVIATIONS

ATM	Antimicrobial
BHI	Brain heart infusion
CAMP	Christie, Atkins, Munch-Petersen test
CLSI	Clinical and Laboratory Standards Institute
CM	Clinical mastitis
CMT	California mastitis test
CNS	Coagulase negative *Staphylococcus*
DDD	Defined daily doses
DIM	Days in milk
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
IMI	Intramammary infection
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry
MH	Muller Hinton
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MQ	Mammary quarter
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	Thiazolyl Blue Tetrazolium Bromide
NMC	National Mastitis Council
PCR	Polimerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RS-PCR	Ribosomal spacer-PCR
SCC	Somatic cell count
SCM	Subclinical mastitis
SUAM	*Streptococcus uberis* adhesion molecule
TBE	Tris/Borate/EDTA
TE	Tris-EDTA Solution
TSA	Trypticase soy agar
TSST	Toxic shock syndrome toxin
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultraviolet

SUMMARY

CHAPTER 1

General introduction and objectives

1 GENERAL INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION AND JUSTIFICATION

Bovine mastitis is a highly prevalent disease in dairy herds and results in losses in quantity and quality of milk produced (DUARTE et al., 2004). In addition, the costs of mastitis include the premature culling of affected cow, discard of milk with antibiotic residues, veterinary services, antimicrobial treatments and diagnostic costs (GRUET et al., 2001). Although mastitis may be caused by different types of microorganisms, bacteria represent the main cause of clinical and subclinical mastitis. In the clinical form, mastitis is diagnosed by color change, presence of clots and lumps in the milk and, depending on the inflammatory process of the udder, systemic clinical signs may occur. The subclinical form, on the other hand, is characterized by increased somatic cell count (SCC) in the affected quarter, which requires the use of specific diagnostic methods, such as the California Mastitis Test (CMT) and cytometry flow (ROYSTER; WAGNER, 2015).

According to the mode of transmission, mastitis causing pathogens could be classified in contagious or environmental. Contagious pathogens have the ability to adapt to the host, especially in the infected quarter; while environmental pathogens are opportunistic in the mammary gland and can survive in the environment. The most frequently isolated contagious mastitis pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis* and *Mycoplasma* spp. (KEEFE, 2012); while environmental ones most frequently isolated comprise enterobacteria (e.g., *Escherichia coli* and *Klebsiella* spp.) and *Streptococcus* species (e.g., *Streptococcus uberis* and *Streptococcus dysgalactiae*) (HOGAN; SMITH, 2012). However, some species may manifest both contagious (cow-to-cow) and environmental mode of transmission, depending on the management conditions or specific strains involved in the infectious process (ZADOKS; SCHUKKEN, 2006).

Staphylococcus aureus is one of the major cause of intramammary infections (IMI) in ruminants that can appear in both clinical and subclinical form (VASUDEVAN et al., 2003), causing major economic losses and increased treatment costs during lactation and / or drying, in addition to the increased risk of premature culling of cows with chronic infections (CREMONESI et al., 2013). Infected mammary quarters, udder skin and milking surface are the main sources of *S. aureus,* although some strains can be isolated in the milking parlor. There is a higher risk of persistence of the subclinical form of mastitis, in which infected cows become sources of infection for other cows, especially during milking (ARTURSSON et al., 2016).

Therefore, control of *S. aureus* is based on management and treatment measures in lactation and at drying off, as well as vaccination and segregation strategies for infected cows. Additionally, molecular characterization of the *S. aureus* strains may also facilitate the identification of specific sourcers of transmission and virulent profiles within and between herds (SOMMERHÄUSER et al., 2003). This pathogen may have surface proteins that aid in tissue colonization like adhesion proteins, biochemical properties that allow bacteria to survive within phagocytes and biofilm, endotoxins and exotoxins production (CREMONESI et al., 2013).

Another highly isolated mastitis causing pathogen in dairy herds is *S. uberis*, which is one of the main environmental pathogens associated with both forms of mastitis manifestation (clinical and subclinical) and may persist in the mammary gland causing a chronic disease (ZADOKS et al., 2003). Conventional control measures for *S. uberis* mastitis, such as housing and milking hygiene, and dry cow therapy are not highly effective because this pathogen is frequently isolated from various sites of the cow and the environment. It can be explained by the high genotypic variability among isolates, the presence of different virulence-associated genes (LANG et al., 2009) and even by identical strains present in different mammary quarters of the same cow and the predominance of some strains in specific herds (PHUEKTES et al., 2001). Because of this diversified transmission profile, new intramammary infections caused by *S. uberis* has increased markedly in modern dairy herds.

The presence of genes associated with virulence factors and antimicrobial resistance has been identified to distinguish cases of persistent and new infections in herds, which makes it possible to classify infections as chronic or recurrent (ZADOKS; SCHUKKEN, 2006). The main molecular identification methods are based on DNA amplification, such as variations of the Polymerase Chain Reaction (PCR) technique, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphism DNA (RAPD), Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST), widely used to evaluate the genetic variability of bacterial species (PERRIG et al., 2015), in addition to DNA hybridization techniques by blotting methods (e.g. dot blot and southern blot).

Although differentiation of strains and description of known clonal complexes of *S. aureus* and *S. uberis* has already been studied, the genotypic variability of these species in different regions of Brazil and the comparison to other countries may contribute to the epidemiological knowledge of these pathogens, which are major causes of mastitis in dairy cows. In addition, epidemiological knowledge of isolates distributed among the herds is a

valuable tool for choosing specific control strategies for the prevention of new infections (SOMMERHÄUSER et al., 2003).

Thus, the hypotheses to be tested in the present study are:

i. There is a high genotypic variability among *S. aureus* isolates in different countries from clinical (CM) and subclinical (SCM) mastitis, as well as differences in virulence factors;

ii. Methicillin resistant *S. aureus* (MRSA) strains have low prevalence among isolates causing bovine mastitis;

iii. Even after treatment, there is great similarity between *S. aureus* isolates within the same herd, or between nearby regions, allowing the occurrence of clonal complexes;

iv. Although some virulence factors may differ across countries, most *S. uberis* virulence factor marker genes are matched regardless of region of origin;

v. There is a considerable resistance of *S. aureus* to most antimicrobials commonly used as antibiotic therapy in herds from Brazil.

1.2 GENERAL OBJECTIVES

The general objective of this thesis was to determine the genotypic variability of *S. aureus* and *S. uberis* isolated from CM and SCM in different regions of Brazil and determine the prevalence of virulence factors and antimicrobial resistance characteristics.

1.3 SPECIFIC OBJECTIVES

This thesis was organized in three experiments, according to the following specific objectives:

Experiment 1 - ***Staphylococcus aureus*** **isolates from bovine mastitis in eight countries: detection of genes encoding different toxins, acquisition of methicillin resistance (*mec*A) and other virulence genes**: To evaluate the variability by genotyping the intergenic space 16S-23S rRNA of *S. aureus* isolated from CM and SCM of cows from some Brazilian herds and from other countries (Argentina, Colombia, Germany, Italy, South Africa, USA and Tunisia). The objective was to compare the molecular profiles of isolates according to three genes related to adhesion and tissue invasion (*clfA*, clumping factor; *cna*, collagen-binding protein and *fmtB*, cell wall-associated protein), 22 genes that can modulate the defense mechanisms (TSST, toxic shock syndrome toxin-1; *scn*, staphylococcal complement inhibitor;

chp, chemotaxis inhibitory protein; *sak*, staphylokinase; *sea* to *sel* enterotoxins; exfoliative toxins *eta* and *etb* and leukocidins *lukE, lukE-lukD*, *lukM*, *lukSF-PV*) and the gene encoding methicillin resistance (*mecA*).

Experiment 2 - **Molecular characterization and antimicrobial resistance pattern of *Staphylococcus aureus* isolated from subclinical mastitis cows treated during lactation**: To evaluate by PFGE the genotypic variability of *S. aureus* isolates from SCM in association with cure rate and antimicrobial susceptibility according to the minimum inhibitory concentration (MIC) within and between herds.

Experiment 3 - **Application of a dot-blot hybridization assay for genotyping *Streptococcus uberis* from Brazilian dairy herds**: To characterize *S. uberis* strains isolated within herd, between herds and between regions of Brazil by dot blot methodology (DNA hybridization) for two taxonomic markers (U1 and U2) and eight markers associated with expression of virulence factors: *nsuR* (regulation gene), *nsuI* (nisin immunity gene), *hasA* and *hasC* (hyaluronic acid operon genes), *gapC* (glyceraldehyde 3-phosphate dehydrogenase gene), *oppF* (oligopeptide permeasse gene), *sua* (*S. uberis* adhesion molecule gene) and *pauA* (plasminogen activator gene).

CHAPTER 2

Literature Review

2 LITERATURE REVIEW

2.1 BOVINE MASTITIS CHARACTERIZATION

Bovine mastitis is an inflammation of the mammary gland caused mainly by bacterial infection (BRADLEY, 2002), in which the inflammatory process of the alveolar cells causes major damage to the glandular tissue impairing milk production. Although many control programs for mastitis prevention have been successfully implemented in recent decades, the disease is still the most common in dairy herds (STEVENS; PIEPERS; DE VLIEGHER, 2016). In a healthy cow, the teat channel closes tightly through the sphincter muscles and is additionally covered by a keratin layer produced by the epithelium itself (VIGUIER et al., 2009). This keratin plug act as a physical barrier against the invasion of potential mastitis causers. However, even with the formation of this plug, some factors interfere with the occurrence of IMI during lactation, such as lactation stage, environmental conditions and management practices established by the herd (STEENEVELD et al., 2008).

The major problems related to mastitis are reduced milk production and quality, unintentional discarding of cows, reduction in the commercial value of these animals, genetic losses on the herd's evolutionary scale and direct spending on medicines and veterinarians. In addition, mastitis causes indirect losses in dairy yield due to physicochemical changes in milk and increased risk of antibiotic residues in milk (COSTA et al., 2013).

Mastitis can be caused by physical trauma or damage to the mammary gland, chemical irritation to some milking compound, or most often by infection of microorganisms, especially bacteria. Infection with mastitis-causing microorganisms occurs by penetration into the teat channel that stimulate the inflammatory response (PHILPOT; NICKERSON, 2000). The defense cells are attracted to the mammary gland and can cause the death of causative agent and some epithelial cells; which are secreted in milk and increase the somatic cell count (SCC) (VIGUIER et al., 2009).

Some factors directly interfere with the aggravation of mastitis and the extent of the losses that may occur, since involves: a) cow resistance, established by age, nutritional status, parity, genetics and anatomical factors; b) the causative agent, virulence factors and how it can evade the immune system; and finally c) the environment (CUNHA et al., 2008). Based on the origin of the infection, mastitis can be classified as environmental or contagious, while by its presentation it can be classified as CM or SCM. Although studies have shown the bacteria ability to be transmitted in both forms (ZADOKS; SCHUKKEN, 2006), the contagious

pathogens are those that infect the mammary gland during milking (cow-to-cow) and are more capable of udder survival; while the environmental, not adaptable to survive in the host, have the ability to colonize the environment. They enter at the teat channel, stimulate the host's immune response, and are eliminated (BRADLEY, 2002).

Regarding the severity of the inflammatory process, mastitis is classified as CM or SCM. In CM, visible changes occur in milk, which can be easily detected by removing the first strips of milk at the milking time. The CM cases can be classified according to severity into three scores: 1 - mild, when only milk has changes in viscosity, color or consistency; 2 - moderate, when visible changes in milk are accompanied by changes in the udder, such as swelling, redness, and pain; and 3 - severe, when there is a combination of abnormal milk, signs of udder inflammation and systemic signs such as fever, dehydration and depression (ROBERSON, 2012). When the disease becomes chronic, the feature of the mammary gland changes, whereas the glandular tissue becomes connective tissue with fibrous aspect (BRADLEY, 2002).

However, IMIs may not generate any visible changes in milk, since the passage of fluids and leukocytes into the mammary gland does not occur at sufficient intensity to alter the appearance of milk, although vascular permeability changes. Thus, the infection is classified as SCM (BRADLEY, 2002). This form is identified by the high SCC of the affected quarter, in which values above 200.000 cells / ml indicate the presence of SCM, either in an isolated mammary quarter or by a pool collection of all functional quarters (ROYSTER; WAGNER, 2015).

2.2 CAUSING AGENTS

Although many mastitis control programs have been developed in recent decades and implemented in many herds, mastitis is still a difficult disease to control because it can be caused by different pathogens, especially bacteria. Strains of various species are capable of infecting the udder and developing infection. Among the contagious pathogens, we highlight *S. aureus*, *S. agalactiae* and *Mycoplasma* spp. Such microorganisms are transmitted during milking and have the host as their reservoir, especially the udder. Among the environmental ones, we highlight *S. uberis*, *S. dysgalactiae* and coliforms, especially *E. coli* and *Klebsiella* spp. (OLIVER; MURINDA, 2012).

Correct identification of mastitis-causing pathogens is extremely important for appropriate antibiotic treatment as well as prevention programs. Pathogen distribution is

different across countries and even between herds, as each site has characteristics that interfere with bacterial reproduction, strain distribution, and virulence factors (GAO et al., 2017). A Swedish study on the etiology of acute cases of CM and individual risk factors analyzed 987 mammary quarters, of which *S. aureus* was the most frequently isolated bacteria (21.3%), followed by *E. coli* (15.9%) and *S. dysgalactiae* (15.6%) (ERICSSON UNNERSTAD et al., 2009).

In New Zealand, coliforms are reported to be less prevalent in CM cases, whereas *S. uberis* is the main pathogen causing mastitis, both clinical and subclinical (MCDOUGALL, 1999). In Brazil, a retrospective 5-year study of 8 dairy herds with CM data reported that of a total of 2,905 cases, 41% of the samples showed no growth, while the remainder had identification of coliforms (19%), environmental *streptococci* (12%), coagulase negative *Staphylococcus* (CNS; 9%) and *S. agalactiae* (4%) (OLIVEIRA et al., 2015). Another Brazilian study analyzed a total of 4,212 cases of CM, of which 44% comprised negative culture samples, and among the positive samples, gram-positive pathogens were the most commonly found (66.3%) (TOMAZI et al., 2018). The same authors reported a high frequency of contagious pathogens and environmental *streptococci*, especially *S. uberis* and *S. agalactiae*, respectively, followed by *S. aureus.*

The types of installation, bedding and seasons are also determinant factors of the mastitis etiologic profile in a region. Organic materials that are often used in beds stimulate a rapid bacterial growth of environmental pathogens due to their easy access and destination, especially when these materials are involved in urine and / or feces (ERICSSON UNNERSTAD et al., 2009). On the other hand, sand is considered as the most recommended material in bed formation, as it does not allow bacterial growth; although studies have reported the presence of environmental coliforms and *streptococci* in clean sand (STOWELL; INGLIS, 2000).

Regarding the seasons of the year, differences can be seen in the manifestations of certain microorganisms; such as high incidence of coliforms in cases of summer CM and *Streptococcus* spp. most commonly isolated in winter (MAKOVEC; RUEGG, 2003). A study with dairy herds in China demonstrated a high frequency of *E. coli*, *Klebsiella* spp., CNS, *S. dysgalactiae*, *S. aureus* and other *streptococci* for CM cases. In this study, the pathogen distribution was associated with the season too; in winter *streptococci* were more present, while in summer coliforms were also more frequently isolated (GAO et al., 2017).

Although mastitis control programs have been better implemented over the years, the prevalence of IMI caused by preferentially contagious or environmental pathogens is still high.

Within this context, we highlight *S. aureus* and *S. uberis*, important etiological agents in the mastitis development that have mechanisms of evasion of the host immune system, as well as genes responsible for their adhesion and internalization in the mammary tissue. This contributes to the high frequency of isolation and difficulties in eliminating both in dairy herds.

2.2.1 *Staphylococcus aureus*

Staphylococcus aureus is a well-known pathogen for causing bovine mastitis in both clinical and subclinical presentation (KEEFE, 2012; CREMONESI et al., 2013). In Brazil, its prevalence has been reported up to 70.9% of the total milk samples analyzed (ZANETTE, SCAPIN, ROSSI, 2010). These bacteria are usually transmitted contagiously (cow-to-cow) during contact with contaminated milk. According to Yang et al. (2016), infection usually results in SCM, which makes the diagnosis difficult to detect, especially in the early stages of the disease.

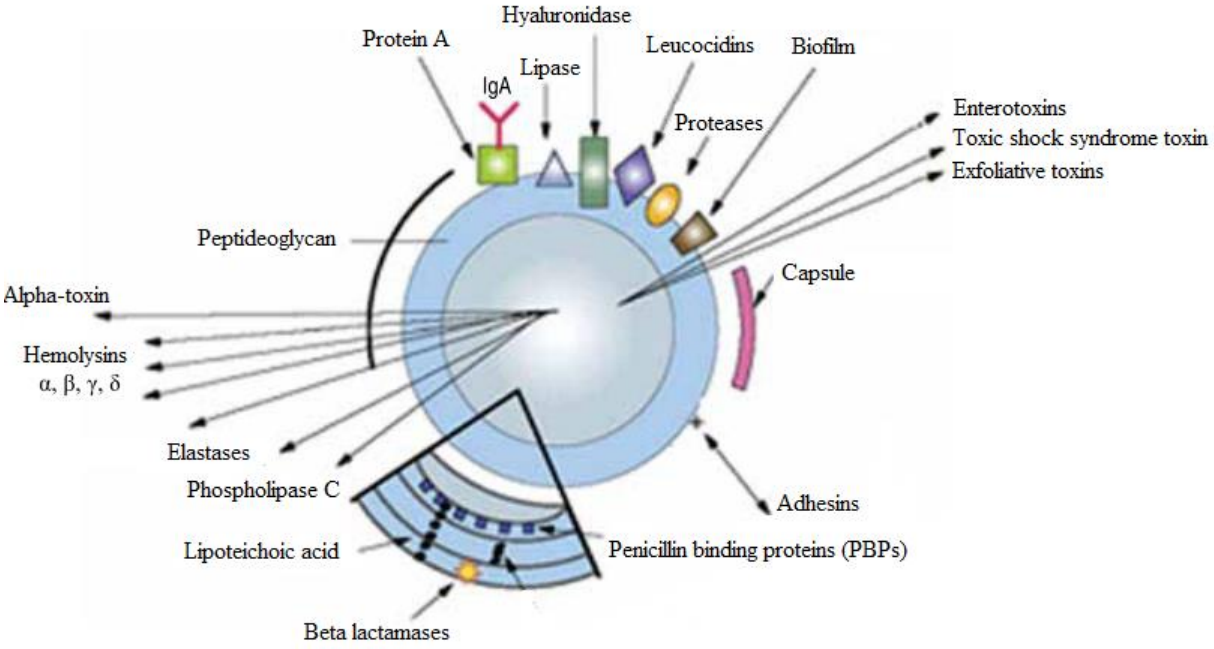
Some studies have discussed the risk of *S. aureus* mastitis becoming more persistent in the subclinical form, as the SCM represent a high percentage in a herd infected with CM and can eliminate *S. aureus* in milk. Therefore, it is recommended to implement a control program that includes vaccination and removal of cows infected with the most pathogenic strains of *S. aureus* (ARTURSSON et al., 2016). The main goal of *S. aureus* treatment is to cure specific cases and reduce the group of infected cows within the herd; what is difficult because of the low cure rate of this pathogen and its increased survival in the udder of infected animals (KEEFE, 2012).

Regarding treatments, mastitis responses caused by *S. aureus* are variable. The minimal inhibitory concentration (MIC) test can be used as therapeutic indications and may increase the treatment efficiency of these infections. Therefore, treatment success for *S. aureus*, especially during lactation may not be expected, as it depends on the immunity of cows, the virulence factors involved, and the antimicrobial therapy recommended (KEEFE, 2012). The adequate knowledge about the epidemiology of *S. aureus* can help in control programs focused on *Staph* infection, prevent clinical cases and identify those strains that are harmful (CREMONESI et al., 2013).

2.2.1.1 Virulence Factors

Tissue colonization capacity accompanied by a high immune evasion (represented by the difficulty of treating *S. aureus*) is in the manifestation of its virulence factors, which are responsible for increasing the pathogenicity of the causative agent. Basically these factors are expressed in three categories: 1) those related to adherence to extracellular matrix or cells, such as aggregation factors (clumping factor) and intracellular adhesion genes like *icaA* and *icaD*; 2) those related to damage to host tissue, like hemolysins (α, β, δ and γ) and 3) those related to fight the immune system (MONISTERO et al., 2018) (Figure 1).

Figure 1 - Virulence factors of *S. aureus*



Source: Adapted from Cervantes-García; García-González; María Salazar-Schettino (2014).

Staphylococcus aureus adheres to the host cell and invades it to multiply, utilizing several virulence factors involved in bacterial pathogenesis, including adhesins, which are fundamental in their spread within and among herds (ZECCONI; SCALI, 2013). The ability to survive in neutrophils (forming small colonies and some variants) and to form biofilm and micro abscesses, contribute to antibiotic therapy not being successful in these cases (KEEFE, 2012).

Virulence factors can be regulated by a single gene or by multiple genes. Among the hemolytic toxin genes is *hlb*, which regulates hemolysins, including beta, which has been shown to be very important for *S. aureus* virulence (DUPIEUX et al., 2015). On the other hand, some genes present in *S. aureus* contribute to the cell adhesion mechanism, which is crucial for the

establishment of infection. These include clumping factors A (*clfA*) and B (*clfB*), fibrinogen binding protein (*fib*), fibronectin binding proteins A (*fnbA*) and B (*fnbB*). Furthermore, *S. aureus* has superantigen coding genes (SAgs), defined as a class of antigens capable of activating up to 20% of host T lymphocytes, generating high cytokine release and causing high severity autoimmune reactions. From this last class, we can identify TSST, enterotoxins, and exfoliative toxins, which, in the context of bovine mastitis, also help in the establishment of infection (GRABER et al., 2009; PICCININI; BORROMEO; ZECCONI, 2010).

In order to investigate which virulence factors contribute to the permanence of *S. aureus* in the herd, a study was carried out with medium to large milk production herds in the Middle East, which aimed to detect genes associated with biofilm and endotoxin production. Six adhesion genes were related to biofilm (*icaD, icaA, fnbA, bap*, *clfA* and *cna*) and five classic enterotoxin genes (*sea*, *seb*, *sec*, *sed* and *see*), with the majority of biofilm isolates (KHORAMROOZ et al., 2016). Another Swedish study aimed to investigate the presence of virulence genes in 70 isolates of acute CM cows and to correlate them with other phenotypic and genotypic traits. The authors described genotypic differences and similarities between the isolates of *S. aureus*, and the most prevalent virulence factors were hemolysin coding genes (α, β, δ and γ), leucocidin components, agglutination factors A and B, fibrinogen binding protein and fibronectin A binding protein (ARTURSSON et al., 2016).

A recent study involving 8 different countries aimed to genotype RS-PCR isolated from bovine mastitis-derived *S. aureus*, distributed in different herds in order to compare molecular profiles and analyze the different lineages present (MONISTERO et al., 2018). In this study, 26 genes involved with cell adhesion and invasion (*clfA, cna, fmtB*), host defense mechanisms (*clfA, cna, fmtB*) and the gene encoding methicillin resistance (*mecA*) were investigated. These authors observed new genotypes as well as new variants of previously identified genotypes. In addition, country-specific genotypic pattern and high frequency of virulence genes (*fmtB, cna, clfA* and leucocidins) were observed.

The success of *S. aureus* infection in causing bovine mastitis is the adaptation of the virulence factors produced, by inhibiting those not necessary and incorporating new genetic sites. Some genes are primarily linked to human infections, while others are strongly associated with bovine mastitis, such as leukocidin-encoding genes, toxins responsible for creating pores in leukocyte membranes and diminishing the host immune response. However, zoonotic transmission of strains may occur primarily from cattle and cause human infections and vice versa. In order to determine the prevalence of *S. aureus* bovine mastitis in Algeria and the

transfer of strains to humans, a study was conducted with samples of milk with mastitis and nasal swabs from workers in contact with cows (AKKOU et al., 2018). In this study the authors analyzed the genetic diversity and its relationships between the two populations, and observed a high prevalence of *S. aureus* in both groups, with distinct epidemiologies and greater diversity among humans. However, this same study confirmed the high prevalence of the CC97 clonal complex in cows, with occasional transmission to humans, as well as the possibility of transfer of the human origin CC22 clonal complex to cows.

To understand the evolutionary process of *S. aureus*, as well as the expression of genes related to virulence factors or antimicrobial resistance, further research is needed in order to elucidate cellular mechanisms of virulence and the possibility of horizontal transmission to humans (CASTAÑÓN -SÁNCHEZ, 2012).

2.2.1.2 Antimicrobial resistance and MRSA

Although several control measures for bovine mastitis are performed, including good milking practices, with pre- and post-milking disinfection and equipment washing, in most cases the severity of the disease and its spread require a control program based on antibiotics. Antimicrobial therapy, however, does not only include the random treatment of the affected mammary quarter; it involves steps such as the detection of the specific pathogen, the treatment itself in the recommended period, the records of the treatments performed, the correct identification of the mammary quarters and animals and the appropriate grace period of the product used (OLIVER; MURINDA, 2012).

There are few studies that quantify the antibiotics used in dairy herds, however, in the United States a low number of antimicrobials are available as intramammary for the treatment of bovine mastitis. Included are β-lactams (penicillins, ceftiofur, cefapyrins, etc.), macrolides (erythromycin) and lincosamides (POL; RUEGG, 2007). In Canada, a survey of 89 dairy herds from 4 regions found that third generation cephalosporins, as well as combinations of penicillin and colistin, are frequently used (SAINI et al., 2012). In Belgium, quantification of antibiotic use in dairy herds showed that fourth-generation cephalosporins are the most widely used, followed by third-generation penicillins and cephalosporins (STEVENS; PIEPERS; DE VLIEGHER et al., 2016). Here in Brazil, Tomazi, (2017) quantified monthly antimicrobial consumption (in daily defined doses - DDD) for the treatment of CM in 19 dairy herds. It was observed in their study an average of 17.7 DDD for each 1000 lactating cows / day, with

aminoglycosides being the most used intramammary antimicrobials and fluorquinolones as the systemic treatment of choice.

Pol and Ruegg (2007) analyzed the relationships between antimicrobial use in dairy farms with the results of antimicrobial susceptibility testing of mastitis-causing pathogens. The MIC was performed for *S. aureus* isolates, CNS and *Streptococcus* spp. from SCM, which showed inhibition at the lowest dilution tested. Penicillin and pirlimycin use was associated with pathogen resistance, with *S. aureus* and CNS being associated with lower susceptibility to penicillin; whereas cefapirine was not associated with reduced susceptibility of either pathogen.

In vitro susceptibility analyzes of 12 antimicrobials were performed with 77 *S. aureus* strains isolated from SCM, which revealed four different resistance patterns, which was predominant for lincomycin (24.7% of strains). The other strains were sensitive to 12 antimicrobials (FERREIRA et al., 2006). Teixeira et al. (2014) determined the MIC of *S. aureus* isolates from 251 bulk tank milk samples for 9 antimicrobials. Of the total samples, 149 isolates of *S. aureus* with 92.54% susceptibility to enrofloxacin and 55 isolates with 83.33% sulfamethoxazole associated with trimethoprim were identified.

Another study with *S. aureus* aimed to evaluate the MIC of this pathogen isolated from clinical (n=58) and subclinical (n=58) cases in order to determine the prevalence of resistance to 12 antimicrobials. Of the total tested, 75% showed no resistance to any antimicrobials, 24.1% showed resistance to one or two antimicrobials, and 0.9% were resistant to multiple active ingredients, with 19% being resistant to tetracycline and 14% resistant. to penicillin (OLIVEIRA et al., 2012).

To determine the extent of MIC association of bulk tank milk-isolated environmental pathogens with housing, management practices, and antimicrobial use, a study was conducted in two dairy cooperatives in California/US. The samples were collected during a one-year period, in which 404 environmental bacteria were isolated, and from these, 337 isolates were submitted to MIC for 10 antimicrobial agents. Four antimicrobial groups with different degrees of resistance to environmental pathogens were found and there were significant associations between antimicrobial resistance and some herd characteristics, such as failure to dry mammary quarters before milking, type of bedding, management, milking practice and antimicrobial treatment for healthy cows (KIRK et al., 2005).

The high resistance to antimicrobials, especially beta-lactams, associated with *S. aureus* strains is mainly due to the production of beta-lactamase enzyme and the production of a penicillin-binding protein (PBP) encoded by the *mecA* gene (FUDA; FISHER; MOBASHERY,

2005). Increasing incidence and increased mortality due to inefficient treatment against methicillin resistant *S. aureus* (MRSA) strains has been increasingly reported in humans (COSGROVE et al., 2005) and in cattle (FEßLER et al., 2010; HARAN et al., 2012).

The identification of *S. aureus* and MRSA strains is important among bovine mastitis cases to guide treatment protocols and optimize control measures, since this pathogen has low response rates to conventional treatments. A study with 150 bulk tank milk samples was conducted to assess the prevalence of *S. aureus* and MRSA, which was 84% and 4%, respectively (HARAN et al., 2012). The same study observed in an antimicrobial susceptibility testing the multiresistance in 5 isolates evaluated, two MRSA. Furthermore, these authors reported staphylococcal enterotoxin production in seven isolates evaluated, as well as the association with hospitals and others communities (HARAN et al., 2012).

Twenty-five MRSA isolates from CM cases were investigated for genetic relationship and virulence properties by PFGE methodology and antimicrobial resistance by broth microdilution (FEßLER et al., 2010). These authors identified 9 distinct pulsotypes while 10 resistance patterns were observed, including some resistance genes such as *blaZ* responsible for penicillin resistance and *erm* gene and its subdivisions (A, B, C and T) responsible for macrolide resistance, lincosamide and streptogramin B. However, the *mecA* expression is induced by beta-lactam antibiotics such as oxacillin and cefoxitin. Mendonça et al. (2012) evaluated 250 *Staphylococcus* spp. isolates by 8 different phenotypic tests based on oxacillin and cefoxitin markers. These authors observed better performance of the cefoxitin disk diffusion test for the prediction of oxacillin resistance, and although no positive *mecA* genes were detected, the *mecI* and *mecRI* genes were also detected in 11.6% (29/250) of the total assessed.

Cefoxitin is now the antibiotic of choice for methicillin susceptibility testing and identification of MRSA strains, as methicillin itself is no longer manufactured (ZURITA; MEJÍA; GUZMAN-BLANCO, 2010). One study aimed to evaluate cefoxitin for MRSA detection in 871 strains of *S. aureus*, collected from humans, by disk diffusion (FERNANDES; FERNANDES; COLLIGNON, 2005) and observed MIC <4mg / L for methicillin sensitive strains. Another study with 11 laboratories was conducted to determine if cefoxitin broth microdilution would be effective in detecting MRSA (SWENSON et al., 2009). Using lower limits of <4 µg / mL and maximum than 8 µg / mL, these authors observed high sensitivity and specificity of the test, showing the ability of microdilution to detect MRSA strains among *S. aureus* isolates.

2.2.2 *Streptococcus uberis*

Streptococcus uberis is considered one of the most prevalent environmental pathogens associated with bovine mastitis, whether clinical or subclinical, in both lactating and non-lactating cows (PERRIG et al., 2015). Still with advances in mastitis control programs in recent years, *S. uberis* is a dairy herd problem as it affects both milk production itself and the welfare of the dairy cows. Although the control of most pathogens has been done by lower exposure of the udder, for *S. uberis* does not seem to be so simple due to their spread in the environment and teat, representing a constant risk of infection. Therefore, milking control procedures such as hygiene and antibiotic therapy are often regular to control IMI caused by *S. uberis* (LEIGH, 1999).

However, Zadoks et al. (2003) molecularly analyzed strains of *S. uberis* and noted that infections by this pathogen can also be contagious. A unique type (clone) of strains was identified in each herd analyzed, which may be the result of failures in strains discrimination or infection of multiple cows from a common source, in this case the milking equipment. In addition, the occurrence of a limited number of strains is generally accepted as evidence of the contagious form. Another Australian study found that out of 62 different strains of *S. uberis* some identical types were identified in different quarters of the same cow and some cows in the same herd, also suggesting direct transmission from an infected quarter to an uninfected quarter, possibly during milking (PHUEKTES et al., 2001).

Streptococcus uberis also deserves attention in IMI during the dry, pre-partum, and early lactation periods, in which the pathogen is not effectively eliminated through mastitis control practices. The number of new infections by *S. uberis* during the dry period and near parturition increases due to the lack of antibiotic effect of dry cow therapy. In addition, many of the new clinical and subclinical infections that originate during early lactation have their onset in the dry period, which should concentrate the control programs and strategies for reducing *S. uberis* from the herd (OLIVER et al., 2004).

Regarding cure rates, mastitis caused by *S. uberis* as well as *S. aureus* have lower rates when compared to IMI caused by other species of staphylococci and streptococci. These differences in response to treatment are related to the presence of specific virulence factors such as toxins, immune evasion mechanisms, or differences in antimicrobial susceptibility (ROYSTER; WAGNER, 2015). *S. uberis* is also relevant in the total bacterial count (TBC) of the herd, whereas its reproduction and dissemination contribute to the increase this variable (DOGAN; BOOR, 2004).

2.2.2.1. Virulence Factors

Due to its high economic impact and difficulty of eradication through conventional mastitis control measures, *S. uberis* has also been the focus of research in recent years regarding the presence of a great number of virulence factors, as well as for *S. aureus*. Such factors also play a critical role in colonizing the mammary gland, as well as in establishing the infection itself. This knowledge about the pathogenesis of *S. uberis* allows strategic control through vaccines or antimicrobial prophylactic therapies (KERRO DEGO et al., 2018).

Over the past 15 years, several *S. uberis*-related virulence factors and its encoding genes have been described as plasminogen activators (*pauA*; ROSEY et al., 1999 and *pauB*; WARD; LEIGH, 2002), streptokininases (*skn*, JOHNSEN et al., 1999), hyaluronic acid capsule (*hasA*, *hasC*, FIELD et al., 2003), lipoprotein receptor (*mtuA*; SMITH et al., 2003), lactoferrin binding protein (*lbp*; MOSHYNSKY et al., 2003), glyceraldehyde-3-phosphate dehydrogenase (*gapC*; FONTAINE et al., 2002), CAMP factor (JIANG et al., 1996), oligopeptide permeases proteins involved in *S. uberis* growth in milk (*opp*, SMITH et al., 2002) and adhesion molecule (SUAM) (*sua*; ALMEIDA et al., 2006). Reinoso et al. (2011) examined by PCR 11 genes associated with virulence in 78 strains of *S. uberis* isolated in Argentina, and observed higher prevalence for the *hasC*, *sua* and *gapC* genes in 89.7%, 83.3% and 79. 4%, respectively. These authors were able to classify 58 different virulence patterns among the isolates, and all detected virulence-associated genes were present in combination. In addition, the same study found different patterns in the same herd and among herds, demonstrating that strains with different virulence patterns were capable of causing mastitis. Perrig et al. (2015) aimed to determine the clonal relationships between 137 isolates of *S. uberis* from mastitis milk (clinical or subclinical), and to evaluate the prevalence of *pauA* and *sua* genes related to infection. The isolates were genotyped by the random DNA polymorphic amplification technique (RAPD) and by PFGE. In the first, 25 different profiles were found, while PFGE found 61 different types of *S. uberis*. The prevalence of *sua* and *pauA* genes were 97.8% and 94.9%, respectively.

Boonyayatra, Tharavichitkul and Oliver (2018) identified 88 isolates of *S. uberis* in 642 milk samples in Thailand, which were analyzed for *pauA, gapC, oppF, mtuA, hasA, hasB, hasC, lbp, sua* and CAMP factor (*cfu*) genes. These authors observed that the most common pattern of virulence-associated genes was *hasA + hasB + hasC + sua + gapC + lbp + pauA + oppF + mtuA* in 34.1% of the isolates analyzed.

These findings emphasize the importance of virulence factors, including SUAM, in the establishment of mastitis and subsequent difficulty in control. Some studies were conducted to

emphasize the importance of SUAM through studies with mutant strains without its gene. Chen et al., (2011) aimed to analyze the role of SUAM in the pathogenesis of *S. uberis* and created a deletion mutant of their gene and evaluated the in vitro ability of this mutant to adhere to and internalize bovine mammary epithelial cells. These authors observed that adhesion and internalization of the mutant strain was significantly reduced, which confirms the role of SUAM in the pathogenesis of *S. uberis*. Almeida et al., (2015) created a mutant clone to exclude their gene, unable to express SUAM. These authors observed that when the mammary gland was infused with the mutant clone there was less infection compared to the wild strain. In addition, the clone-infused mammary glands showed milder clinical symptoms compared to those infused with the wild strain, suggesting that the mutant was less virulent.

Some research has identified specific genes or amount of genes expressed in pathogen-host interaction using mRNA sequencing (RNA-seq), allowing to identify which is used during the pathogenesis of the microorganism (WARD et al., 2001; OGUNNIYI et al., 2012). The knowledge of the pathogen genetic composition as well as the expression of the specific genes involved during the disease allows identifying possible targets for vaccine development or treatment indications.

A US study aimed to evaluate by RNA-seq if *S. uberis*, when in contact with mammary tissue, expresses host adaptation and colonization factors that could suppress its defense mechanisms as well as determine the immunogenic effect of a vaccine (KERRO DEGO et al., 2018). These authors identified *S. uberis* genes (*exsbP1, iihK, iirR, exsbP2*) that appear to be regulators of the early stages of bacterial-host interaction, in addition to the *slp* gene, a regulator of surface lipoprotein that aids in membrane stability during the stationary phase. In addition, the same authors observed that the recombinant proteins reacted with the serum of a *S. uberis* infected cow, confirming their immunogenic effect. Another recent study aimed to determine the presence, conservation and distribution of 6 potential virulence genes and their relationship with molecular profiles in 34 isolates of *S. uberis*, as well as the distribution of virulence patterns (FESSIA et al., 2019). This study reported that all genes are conserved and present in most isolates, although high clonal heterogeneity was found.

2.3 MOLECULAR IDENTIFICATION TECHNIQUES

With the advent of molecular biology techniques, it has become possible to identify and classify mastitis pathogens with greater precision, as well as to determine the genes involved in infection, especially those related to virulence factors. Thus, with precise and improved

techniques, the occurrence of diagnostic errors decreased, favoring specific treatment for the pathogen that causes the infection. These molecular tools have helped to better understand the epidemiology and pathology of an IMI, as they can identify the biological relationships between phylogenetic groups and major transmission pathways (GURJAR et al., 2012).

Most genotyping methods are based on restriction of specific regions of DNA by restriction enzymes or by electrophoretic amplification and separation of DNA fragments, which have different molecular sizes. This separation is represented by specific band patterns in a gel, since DNA (negatively charged) moves across an electric field, allowing fragment separation. However, due to the difficulty of standardizing gel runs, the comparison of isolates occurs in the same gel (ZADOKS; SCHUKKEN, 2006). Some factors such as the technique feasibility, the cost and the time required for the result should be carefully evaluated in the chosen method, which will also depend on the laboratory resources, the researcher's need and the technification level (GURJAR et al., 2012). Here, three techniques are best described, RS-PCR, PFGE and DNA hybridization by blotting techniques.

2.3.1 RS - PCR

The rRNA gene locus is a genetic unit found in prokaryotic and eukaryotic organisms. Due to its conservation and low evolution rates, the rRNA region is used for phylogenetic reconstruction (WOESE; KANDLER; WHEELIS, 1990) and can be used for the identification of bacterial species in a short time (CREMONESI et al., 2006). In prokaryote organisms, the rRNA locus contains three genes, the 16S, 23S and 5S which are separated by spacer regions, with variations in sequence and length, which will differ for each species and genus (JENSEN; WEBSTER; STRAUS, 1993).

The fastest way to visualize the polymorphism of the intergenic regions is by PCR amplification, using conserved sequence primers and the amplification product will allow the recognition of genera and species present in the sample. In addition, a restriction enzyme may be used and fragments observed by electrophoretic separation. The 16S rRNA and 23S rRNA gene sequences have already been used to identify several bacterial species, but the intergenic spacer region between the two units presents greater variability between species within the same genus, allowing for better differentiation within one species and another (GARCÍA-MARTÍNEZ et al., 1996; HASSAN et al., 2003).

To identify the genus and species of a variety of bacteria, a study was developed with the amplification of the intergenic region between the 16S and 23S genes by PCR (RS-PCR;

JENSEN; WEBSTER; STRAUS, 1993). A total of 300 bacterial strains belonging to 8 genera and 28 species were amplified, and the patterns could be used to distinguish all species from the group tested. They produced characteristics of amplification profiles with diversity level that could be identified, and the gender characteristics allowed the diagnosis of the bacteria in a simple and straightforward way. To determine if a 16S-23S rRNA set previously described for *S. agalactiae* could define a similar amplicon for *Streptococcus difficile*, a study was performed using RS-PCR, and an amplicon production was observed, supporting the genetic relationship between them (BERRIDGE; BERCOVIER; FRELIER, 2001).

A study aimed to genotyping *S. aureus* isolates from buffaloes (bulk tank milk, mammary quarters and udder tissue) and fourth small ruminant quarter milk (CREMONESI et al., 2013). The 16S-23S rRNA intergenic region was amplified, able to identify different genotypes of *S. aureus*, besides genes related to virulence factors (n = 19). These authors observed an association of specific endotoxins between buffalo *S. aureus* and those linked to small ruminants, allowing an understanding of *S. aureus* epidemiology on these species. Milk samples from SCM cows in a Brazilian herd were submitted to PCR tests for the same amplification of the intergenic region in 77 *S. aureus* isolates and the results revealed the occurrence of 9 different genotypic patterns (FERREIRA et al., 2006). Furthermore, the results of this study indicated high genetic heterogeneity of *S. aureus* within the same herd evaluated. This diversity of genotypic patterns must be studied in order for mastitis control programs to be applied correctly.

In order to investigate if existing *S. aureus* subtypes differ in their contagion and pathogenicity properties, Fournier et al. (2008) analyzed strains of *S. aureus* isolated from IMI for their virulence genes and genotypes, obtained by PCR amplification of the 16S-23S intergenic region. There were found 17 different genotypes associated with some epidemiological and clinical data from 26 herds, which showed high association with virulence genes. In addition, the method had high performance, ease of execution and economy.

2.3.2 Pulsed Field Gel Electrophoresis (PFGE)

Agarose gel electrophoresis is still the best known technique for separating various sizes of DNA molecules. However, the concentration of agarose used is relative to the size of the DNA fragments to be separated, since it forms a network through which the molecules migrate. The higher the concentration of agarose, the smaller the DNA molecules that run the gel. Therefore, in order to separate very large DNA fragments, the concentration of agarose in the

gel has to be small, which makes the gel fragile and breakable. For this, the pulsed field gel electrophoresis (PFGE) technique was developed with the intuition of separating fragments of various sizes for a longer execution period (MAGALHÃES et al., 2005).

In PFGE, the bacterial genome remains more stable than protein markers, has good discriminatory power between strains analyzed and ease of interpretation, and allows the detection of highly polymorphic DNA (BANNERMAN et al., 1995). Thus, due to their high reproducibility, the comparison of strains between laboratories is facilitated, making possible the best genomic investigation of certain pathogens (PHUEKTES et al., 2001).

The method is useful for the epidemiological study of bacterial pathogens because of its rapid detection of genetic changes, allowing the visualization of epidemiological changes in a short time (ABUREEMA et al., 2014). However, the DNA fragments generated by this method have large molecular size, requiring specialized equipment of electric fields. This may be a limiting factor in the availability and execution of PFGE by diagnostic laboratories (ZADOKS; SCHUKKEN, 2006).

However, reliable PFGE results depend on some factors such as the correct composition and concentration of agarose for the desired DNA fragment sizes, the buffer solution in which the gel is immersed, the voltage of the electric current (inversely proportional to the size of the fragments), the pulse time (which makes the reordering of the DNA molecules) and the running time. In addition, the uniformity of amplified DNA, as well as its integrity and running temperature can also affect the resolution of the technique (MAGALHÃES et al., 2005).

Rabello et al. (2005) evaluated the antimicrobial susceptibility and genotypic patterns of 107 *S. aureus* isolates from SCM cows in Brazilian herds. Genotypic diversity was analyzed by PFGE, which identified 16 types and 24 subtypes of the pathogen. Two types (A and C) and 5 subtypes (A1, A2, A4, A12 and C2) corresponded to 39.3% of the isolates, while 60.7% represented types and subtypes of isolates from only one of the herds. At the end of the study, all isolates were grouped with 52% similarity pattern (RABELLO et al., 2005). Another study with 25 isolates of MRSA *S. aureus* from CM cows was carried out in 17 dairy herds in Germany, where the genetic relationships between isolates, antimicrobial resistance and virulence factors were investigated (FEßLER et al., 2010). By the PFGE technique it was possible to identify several resistance genes and 16 different types among MRSA isolates with high variability standard. This indicates the spread of various types of MRSA in the herds, as well as the existence of a specific type for each herd, which makes it difficult to apply mastitis control measures.

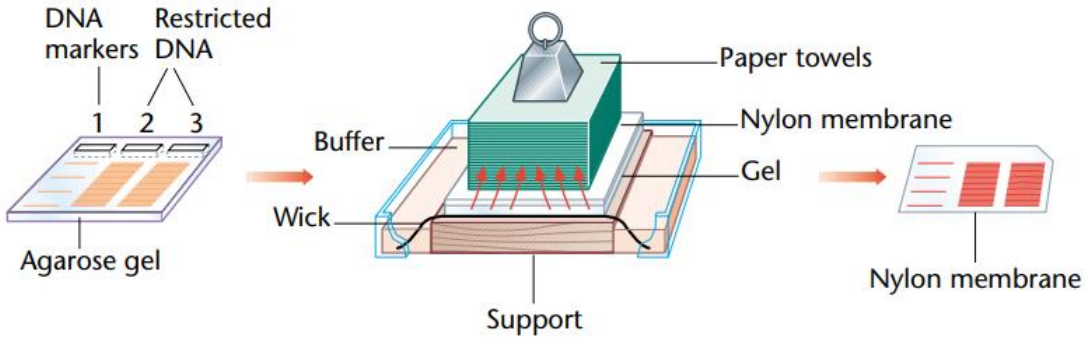
Also with *S. aureus* isolates from 50 farms, one study analyzed 150 bulk tank milk samples collected during three seasons (spring, summer and fall). It was possible to observe a high prevalence of MRSA (84%) in a total of 93 isolates, in which the *spa* gene (protein A coding found in *S. aureus* cell wall) was also identified. Therefore, it is highly recommended to correctly monitor the status of *S. aureus* in the herd, as well as its transmission dynamics and cross-transmissions (HARAN et al., 2012).

2.3.3 DNA hybridization

DNA hybridization is a macroarray technique in which DNA filled points are hybridized with specific probes corresponding to the gene of interest. Probes are known DNA fragments, sequences corresponding to the genes of interest, while target samples contain labeled complementary DNA. Target samples are usually immobilized on a solid matrix and labeled with fluorophores or radioisotopes. Strong hybridization between the target molecule and the probe results in an increase in fluorescence, which is measured by an analyzer. Macroarrangements using nylon or nitrocellulose membranes are more cost-effective compared to microarray techniques, but due to the different hybridizations can generate background noise that can cause confusion to interpret the results.

Techniques based on the use of fluorophores or radioisotopes markers offer robust and reproducible protocols, and the quantification of them is possible after exposure of a signal to an image capture device. Non-radioactive methods are also widely used and work for both direct and indirect markers. Probes are widely used for various applications between different techniques and the choice of their type depends on the sensitivity and resolution required. Among the DNA hybridization techniques, we highlight the blotting techniques, including Southern Blot, Northern Blot, Western Blot and Dot Blot. In the first one there is the transfer of DNA fragments from an electrophoresis gel to a nitrocellulose or nylon membrane, while the gel band pattern is reproduced on the membrane (Figure 2). This transfer of fragments can be by capillarity, which requires a longer execution time or by vacuum equipment, which has recently optimized the technique for a few minutes. On the other hand, the Northern Blot identifies specific mRNA sequences in a pool of RNA molecules in a technique similar to Southern Blot.

Figure 2 - Southern blotting technique



Source: Brown (1999).

The Western Blot technique is used for proteins separation and identification from different sources and molecular weights, which, by gel electrophoresis, produce a band (the thickness band is proportional to the amount of protein in the sample) that is also transferred to a protein membrane. Then, the membrane is incubated with targeting antibodies specific for the target protein, and those that don't bind to proteins are washed away (MAHMOOD; YANG, 2012). The last technique is dot blot, based on the fixation of the target DNA on a nitrocellulose or nylon membrane with the aid of a vacuum system (Figure 3).

Figure 3 - Hybridization using Bio-Dot platform macroarrays

Source: Alves, B.G. (2020).

Labeled spots are formed on the membrane which, in contact with known DNA probes, result in positive or negative marks, given by the probability of correlation with the positive and negative controls (CARIDADE et al., 2015). The dot blot hybridization technique is a practical and economical alternative among microarray, and allows a high number of results (EL-SAYED et al., 2017).

A study was performed to detect viruses that infect cucurbit cultures by dot blot hybridization with three PCR-synthesized probes of different digoxigenin-labeled lengths (MENG et al., 2007). The results of this study showed good sensitivity, specificity and reproducibility in viral detection, with fast and accurate identification. Another dot blot assay was developed for detection of *Mycobacterium tuberculosis* gene mutations with 12 oligonucleotide probes based on wild type and pathogen mutant genotype sequences (GUO et al., 2015). The authors observed high sensitivity and specificity of the method, as well as high accuracy, positive predictive value (PPV) and negative predictive value (NPV) compared to DNA sequencing.

In mastitis, detection and typing of pathogens may be limited by time-consuming molecular techniques requiring culture. One study aimed to establish a specific marker for *Streptococcus* genus and specific markers for *S. agalactiae* and *S. uberis* by dot blot (ALMEIDA et al., 2013). A total of 12 markers were validated for 50 reference strains analyzed, confirming the specificity of the selected markers. In addition, the same authors found specific markers for virulence factors belonging to *S. agalactiae* and *S. uberis*, suggesting that the dot blot methodology presents rapid and economic discrimination of mastitis-causing pathogens. In a recent study, Albuquerque et al. (2017) aimed to evaluate the population structure of *S. uberis* from bovine mastitis by dot blot methodology compared to multilocus sequence analysis (MLSA) genotyping. Positive hybridization signals were converted to probability values, which allowed confirming the identity of the isolates using taxonomic markers and determining the presence of genes related to virulence and antibiotic resistance. The same authors identified the most prevalent *S. uberis* clonal strains and environmental or contagious transmission to *S. uberis*.

CHAPTER 3

Staphylococcus aureus isolates from bovine mastitis in eight countries: detection of genes encoding different toxins, acquisition of methicillin resistance (MECA) and other virulence genes

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3. *Staphylococcus aureus* ISOLATES FROM BOVINE MASTITIS IN EIGHT COUNTRIES: DETECTION OF GENES ENCODING DIFFERENT TOXINS, ACQUISITION OF METHICILLIN RESISTANCE (*mecA*) AND OTHER VIRULENCE GENES

3.1 ABSTRACT

Staphylococcus aureus is recognized worldwide as one of the major agents of dairy cow intramammary infections. This microorganism can express a wide spectrum of pathogenic factors used to attach, colonize, invade and infect the host. The present study evaluated 120 isolates from eight different countries that were genotyped by RS-PCR and investigated for 26 different virulence factors to increase the knowledge on the circulating genetic lineages among the cow population with mastitis. New genotypes were observed for South African strains while for all the other countries new variants of existing genotypes were detected. For each country, a specific genotypic pattern was found. Among the virulence factors, *fmtB*, *cna*, *clfA* and leucocidins genes were the most frequent. The *sea* and *sei* genes were present in seven out of eight countries; *seh* showed high frequency in South American countries (Brazil, Colombia, Argentina), while *se*l was harboured especially in one Mediterranean country (Tunisia). The *etb*, *seb* and *see* genes were not detected in any of the isolates, while only two isolates were MRSA (Germany and Italy) confirming the low diffusion of methicillin resistance microorganism among bovine mastitis isolates. This work demonstrated the wide variety of *S. aureus* genotypes found in dairy cattle worldwide. This condition suggests that considering the region of interest might help to formulate strategies for reducing the infection spreading.

3.2 INTRODUCTION

Staphylococcus aureus continues to be one of the most prevalent pathogens causing intramammary infections (IMI) in dairy cows. It's a worldwide pathogen recognized as a cause of subclinical infections, resulting in increased somatic cell count (SCC), but may also cause clinical mastitis. Staphylococcal mastitis is a major problem in dairy industry, affecting animal health and causing economic losses of up to 300 per cow per year, due to the reduced milk quality and production (DEB et al., 2013, GOMES et al.,2016). The main reservoir of *S. aureus* seems to be the infected quarter, and transmission usually occurs from cow to cow during milking.

Successful infection depends on virulence factors produced by *S. aureus*. A wide spectrum of secreted and cell surface-associated virulence factors can be expressed to promote adhesion to the host extracellular matrix components, damage host cells, and fight the immune system (FOSTER, 2005). At least 25 different toxins (such as enterotoxins SEA to SEQ, toxic shock syndrome toxin-1 TSST-1, exfoliative toxins Eta, Etb), 15 microbial surface components recognizing adhesive matrix molecules, which are important for adhesion to tissues (such as clumping factor A *clfA*, intercellular adhesion genes *icaA* and *icaD*), 20 immune evasion molecules (such as protein A, coagulase, haemolysins and leucocidins, factors associated with suppressing innate immunity) and several other *S. aureus* virulence factors are known. Some virulence factors are expressed by genes that are located on mobile genetic elements called pathogenicity islands (i.e., TSST and some enterotoxins) or lysogenic bacteriophages (i.e., Panton-Valentine Leucocidin, PVL) and others such as the staphylococcal complement inhibitor, *scn*, the chemotaxis inhibitory protein, *chp*, and staphylokinase, *sak*, are integrated in the bacterial chromosome (VAN WAMEL et al., 2006). Furthermore, *S. aureus* can also acquire the staphylococcal cassette chromosome *SCCmec*, giving rise to methicillin-resistant *S. aureus* (MRSA; PANTOSTI, 2012). In fact, the expression of the *mecA* or *mecC* gene in *S. aureus* confers resistance to most of β-lactams, drugs which are frequently used for treatment of mastitis (SAWANT; SORDILLO; JAYARAO, 2005).

The determination of the origin of the *S. aureus* isolates involved in the aetiology of bovine mastitis is highly relevant from the epidemiological point of view. In such a context, the precise characterization of this pathogen provides monitoring of the bacterial strains dissemination among animal populations.

Over the past two decades, a wide range of phenotyping and genotyping methods have been used or developed for *S. aureus* including, but not limited to, ribotyping, RAPD-typing, PFGE, MLST, spa-typing, RS-PCR, coagulase gene RFLP, Multiple loci VNTR analysis (MLVA), micro-arrays and whole genome comparisons (FITZGERALD et al., 1997; SOMMERHÄUSER et al., 2003; HERRON-OLSON et al., 2007; SUNG, LLOYD, LINDSAY et al., 2008; IKAWATY et al., 2009). Many molecular epidemiological studies have been based on the use of selected targets in the genome, giving rise to banding patterns based on restriction- or primer binding sites, or to allelic profiles for housekeeping or virulence genes (ZADOCKS et al., 2011). Such studies continue to be useful diagnostic tools when the aim is to understand pathogen sources and transmission mechanisms. Moreover, among the genotyping methods, the RS-PCR, based on amplifying the 16S-23S rRNA intergenic spacer region by PCR

Target gene	Primer sequence (5'-3')	Amplification size	Reference
sec	ACCAGACCCTATGCCAGATG TCCCATTATCAAAGTGGTTTCC	371 bp	Cremonesi et al., 2006
sed	TCAATTCAAAAGAAATGGCTCA TTTTTCCGCGCTGTATTTTT	339 bp	Cremonesi et al., 2006
see	TACCAATTAACTTGTGGATAGAC CTCTTTGCACCTTACCGC	170 bp	Jarraud et al., 2001
seg	CCACCTGTTGAAGGAAGAGG TGCAGAACCATCAAACTCGT	432 bp	Cremonesi et al., 2006
seh	TCACATCATATGCGAAAGCAG TCGGACAATATTTTTCTGATCTTT	463 bp	Cremonesi et al., 2006
sei	CTCAAGGTGATATTGGTGTAGG CAGGCAGTCCATCTCCTGTA	529 bp	Cremonesi et al., 2006
sej	GGTTTTCAATGTTCTGGTGGT AACCAACGGTTCTTTTGAGG	306 bp	Cremonesi et al., 2006
sel	CACCAGAATCACACCGCTTA CTGTTTGATGCTTGCCATTG	240 bp	Cremonesi et al., 2006
*mec*A	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	310 bp	Syring et al., 2012

Source: Monistero et al., (2018).

Grouping of the RS-PCR profiles and the virulence factors was obtained with the BioNumeric 5.0 software package (Applied Maths, Kortrjik, Belgium) using the UPGMA (unweighted pair group method using arithmetic averages) cluster analysis.

3.4 RESULTS

In this study, a total of 120 isolates collected from eight different countries were genotyped by RS-PCR and analyzed for 26 virulence factors related to *S. aureus* pathogenicity, such as genes related to host adhesion and invasion (*clfA*, *cna*, *fmtB*), genes that have the potential to interfere with host defense mechanisms (*tsst*, *scn*, *chp*, *sak*, enterotoxins from *sea* to *sel* and leukotoxins), and the gene encoding the acquisition of methicillin resistance (*mecA*).

3.4.1 RS-PCR Genotyping

Table 2 - Primer used in this study for *S. aureus* strains characterization

Target gene	Primer sequence (5'-3')	Amplification size	Reference
coa	ATAGAGATGCTGGTACAGG GCTTCCGATTGTTCGATGC	polymorphique size	Hendriksen et al., 2008
*clf*A	GGCTTCAGTGCTTGTAGG TTTTCAGGGTCAATATAAGC	1000 bp	Hendriksen et al., 2008
nuc	AGTTCAGCAAATGCATCACA TAGCCAAGCCTTGACGAACT	400 bp	Cremonesi et al., 2006
*luk*E	AATGTTAGCTGCAACTTTGTCA CTTTCTGCGTAAATACCAGTTCTA	831 bp	El-Sayed et al., 2006
*luk*M	TGGATGTTACCTATGCAACCTAC GTTCGTTTCCATATAATGAATCACTAC	780 bp	Graber, 2016
*luk*E-*luk*D	TGAAAAAGGTTCAAAGTTGATACGAG TGTATTCGATAGCAAAAGCAGTGCA	269 bp	Graber, 2016
lukS/F-PV	ATCATTAGGTAAAATGTCTGGACATGATCA GCATCAAGTGTATTGGATAGCAAAAGC	433 bp	Syring et al., 2012
scn	ATACTTGCGGGAACTTTAGCAA TTTTAGTGCTTCGTCAATTTCG	320 bp	Herron-Olson et al., 2007
chp	TTTTTAACGGCAGGAATCAGTA TGCATATTCATTAGTTTTTCCAGG	404 bp	Herron-Olson et al., 2007
fmtb	AATGAAGATGCGAATCATGTTG CATCCATTTTTGTTTGCGTAGA	725 bp	Herron-Olson et al., 2007
sak	TGAGGTAAGTGCATCAAGTTCA CCTTTGTAATTAAGTTGAATCCAGG	403 bp	Herron-Olson et al., 2007
cna	AAAGCGTTGCCTAGTGGAGA AGTGCCTTCCCAAACCTTTT	192 bp	Akineden et al., 2001
spa	CAAGCACCAAAAGAGGAA CACCAGGTTTAACGACAT	polymorphique size	Hendriksen et al., 2008
tsst	ATGGCAGCATCAGCTTGATA TTTCCAATAACCACCCGTTT	300 bp	Hendriksen et al., 2008
eta	CTAGTGCATTTGTTATTCAA TGCATTGACACCATAGTACT	120 bp	Hendriksen et al., 2008
etb	ACGGCTATATACATTCAATT TCCATCGATAATATACCTAA	200 bp	Hendriksen et al., 2008
sea	TAAGGAGGTGGTGCCTATGG CATCGAAACCAGCCAAAGTT	180 bp	Cremonesi et al., 2006
seb	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	478 bp	Cremonesi et al., 2005

of the PCR products was then used for the miniaturized electrophoresis (Agilent) performed as described by the manufacturer of the system. New genotypes were named and extended according to Fournier et al., 2008 leading to the genotypes GTA to GTZ, followed by the genotypes GTAA to GTAZ, GTBA to GTBZ, and GTCA. An electrophoretic pattern differing in one band from the one of a known genotype was considered as a genotypic variant. It was indicated with roman numerals superscripted after the name of the genotype (e.g., GTR^{I}, GTR^{II}). To identify the genotypes and their variants of the present strains, a freely available, in-house computer program, calculating the corresponding Mahalanobis distance of informative peak sizes and by comparing it to those of the prototype strains using the "Mahalanobis Distances of *Staph. aureus* Genotypes" software (SYRING et al., 2012). Finally, genotypes and their variants were combined into genotypic clusters (CL; COSANDEY et al., 2016).

3.3.4 Molecular Isolates Characterization

The DNA was amplified to investigate the presence of 26 factors that can contribute in diferente ways to *S. aureus* pathogenicity and therefore influence the management of the disease. In this study genes encoding enterotoxins (from *sea* to *sel*), leucocidins (*lukE, lukSF-PV, lukE-lukD, lukM*), the acquisition of methicillin resistance (*mec*A) and genes related to host invasion (*clfA, fmtB, cna, eta, etb*) or to factors that have the potential to interfere with host defense mechanisms (*tsst, scn, chp, sak*) were analyzed using primers and protocols described in literature and listed in Table 2. The amplified PCR fragments were visualized on 2% agarose gel electrophoresis (GellyPhor, Euroclone, Milan, Italy), stained with ethidium bromide (0.05 mg/mL; Sigma Aldrich, Milan, Italy), and visualized by UV transilluminator (BioView Ltd., Nes Ziona, Israel). A 100 bp DNA ladder (Finnzymes, Espoo, Finland) was included in each gel.

Table 1 - World survey on *S. aureus* cow strains: participating countries, total strains analyzed per country, number of isolated from clinical mastitis or high somatic cell count (SCC) samples, and type of sample collection (C = composite milk sample; Q = quarter milk sample)

Country	Total strains analyzed per country			
	Clinical mastitis	High SCC (H)	Number of farms	Type of Sample
Argentina	16		10	C
Brazil	15		12	Q
Colombia		15	11	Q
Germany	17		17	Q
Italy	17		15	Q
South Africa	11		9	Q
Tunisia		12	10	C
New York State (USA)	17		13	Q
Total	93	27	97	

Source: Monistero et al., (2018)

After samples thawing, 10 µL were streaked on blood agar plate. The plates were then incubated aerobically at 37ºC and examined after 24h. The colonies were provisionally identified based on morphology and hemolysis patterns and confirmed by coagulase test.

3.3.2 DNA Extraction

DNA was extracted from isolates using the protocol previously described by Cremonesi et al., 2006. The amount and quality of DNA were measured using a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), and DNA was stored at -20 C until use.

3.3.3 Genotyping

All the 120 *nuc* positive isolates (=*S. aureus*) were then genotyped by RS-PCR and a miniaturized electrophoresis system (Agilent Technologies, Santa Clara, CA, USA) as previously described (Fournier et al., 2008; GRABER, 2016) where a detailed working protocol is given. The method is based on amplification of the 16S–23S rRNA intergenic spacer region. Each reaction contained (total volume 25 L) 1HotStarTaq Master Mix (Qiagen, Hilden Germany), 800 nM of each primer (G1 and L1 primer) (Fournier et al., 2008) and 7 L of DNA (originally extracted DNA diluted 1:100 in water). The PCR profile was: 95 ºC for 15 min, followed by 27 cycles comprising 94 ºC for 1 min, followed by a 2 min ramp and annealing at 55 ºC for 7 min. After a further 2 min ramp, extension was done at 72 ºC for 2 min. PCR was terminated by incubating at 72 ºC for 10 min followed by cooling down to 4 ºC. One µL of each

(ribosomal spacer-PCR), showed to be accurate, rapid and inexpensive with a discriminatory power like the other more-recognized genotyping methods (CREMONESI et al., 2015).

The aim of this study was to genotype by RS-PCR and compare the molecular-epidemiologic profiles of a large world collection of *S. aureus* isolates to deepen the knowledge on the circulating genetic lineages among the cow population with mastitis. The isolates were investigated for three genes related to host adhesion and invasion (*clfA*, clumping factor; *cna*, collagen-binding protein; *fmtB*, cell wall-associated protein), 22 genes that have the potential to interfere with host defence mechanisms (*tsst*, toxic shock syndrome toxin-1; *scn*, staphylococcal complement inhibitor; *chp*, chemotaxis inhibitory protein; *sak*, staphylokinase; enterotoxins from *sea* to *sel*; exfoliative toxins *eta*, *etb* and leucocidins *lukE*, *lukE-lukD*, *lukM*, *lukSF-PV*), and the gene encoding the acquisition of methicillin resistance (*mec*A).

3.3 MATERIALS AND METHODS

3.3.1 Sample Collection and Bacteriological Analysis

A total of 120 *S. aureus* isolates from eight countries Argentina, Brazil, Colombia, Germany, Italy, New York State, South Africa, Tunisia, were selected for this study (Table 1). Isolates of *S. aureus* were taken from the authors' bacterial culture collections (BC) and they included isolates previously collected (Argentina: from April 2015 to June 2017; Brazil: from July 2014 to May 2015; Colombia: from November 2016 to March 2017; Germany: from May 2012 to August 2016; Italy: from September 2012 to December 2016; New York State: from January 2017 to April 2017; South Africa: from August 2016 to February 2017; Tunisia: from September 2015 to December 2016) from clinical mastitis and from high somatic cell count (H) samples. The milk collection was made from quarters (Q) or composite milk samples (C). The isolates were stored at -20 ºC until they were transported to the Italian laboratory (University of Milan) where storage was continued at - 20 ºC until further use. During transport to the laboratory, they were kept frozen using styrofoam boxes and dry ice (for long distances) or wet ice (for short distances).

For the RS-PCR genotyping analysis, the genotypes, were named and extended according to a previous study (COSANDEY et al., 2016) leading to the genotypes GTA to GTZ, followed by the genotypes GTAA to GTAZ, GTBA to GTBZ, and so on. A genotypic variant, differing in only 1 band of a known genotype, was indicated with roman numerals superscripted after the name of the genotype (e.g., GTR^{I}, GTR^{II}). Variation in more than one band, between profiles, was regarded as a new genotype. Finally, genotypes and their variants (e.g., genotype GTB and its variants GTB^{I}, GTB^{II}, GTB^{III}), encompassing at least 5% of all the strains, were combined into genotypic clusters (CL).

New genotypes comprising GTAR, GTBZ, and GTCA were observed for South African and Tunisian strains (Table 3). For all the other countries, at maximum new variants of existing genotypes were detected. They included GTI^{V}, GTI^{VI} (Argentina), $GTAQ^{I}$, $GTBN^{I}$, $GTBN^{II}$, $GTBY^{I}$ (Brazil), $GTAO^{I}$, $GTAO^{II}$ (Colombia), GTR^{XIII} (Italy), GTC^{V} and GTI^{V} (New York State). For each country, a specific genotypic pattern was found. Major genotypes with their variants were combined into genotypic clusters (CL) (COSANDEY et al., 2016) and showed in Figure 4. For Argentina (Table 3, Figure 4) it mainly consisted of CLI (56% of GTI variants) and CLR (25% of GTR variants), whereas for Brazil CLBN (20% of GTBN plus variants) and CLBY (40% of GTBY plus a variants) weremost prominent. The Colombian strains were mainly positive for GTAO and its variants (CLAO, 60%). In the case of Germany and Italy, the most prevalent genotypes were GTC^{I}, GTR plus variants, and GTB, combined into CLC (30%), CLR (64.7%) and CLB (29.4%), respectively. Finally, the main genotypes observed for the South African and Tunisian strains were GTR and its variants (CLR, 45%), whereas the American strains were mainly positive for GTC and variants of it (CLC, 70.6%). In conclusion, cluster C was observed mostly in Germany and New York State, while CLR was widely disseminated in seven countries; especially it was frequently detected in Argentina, Germany, Italy, South Africa and Tunisia but less in Colombia and New York State.

All the existing genotypes including their variants such as GTC and GTC^{I} had been previously isolated from bovine intramammary infection or bovine milk. Exceptions were GTBH (sandwich with Mozzarella) and GTAO (human nasal carriage).

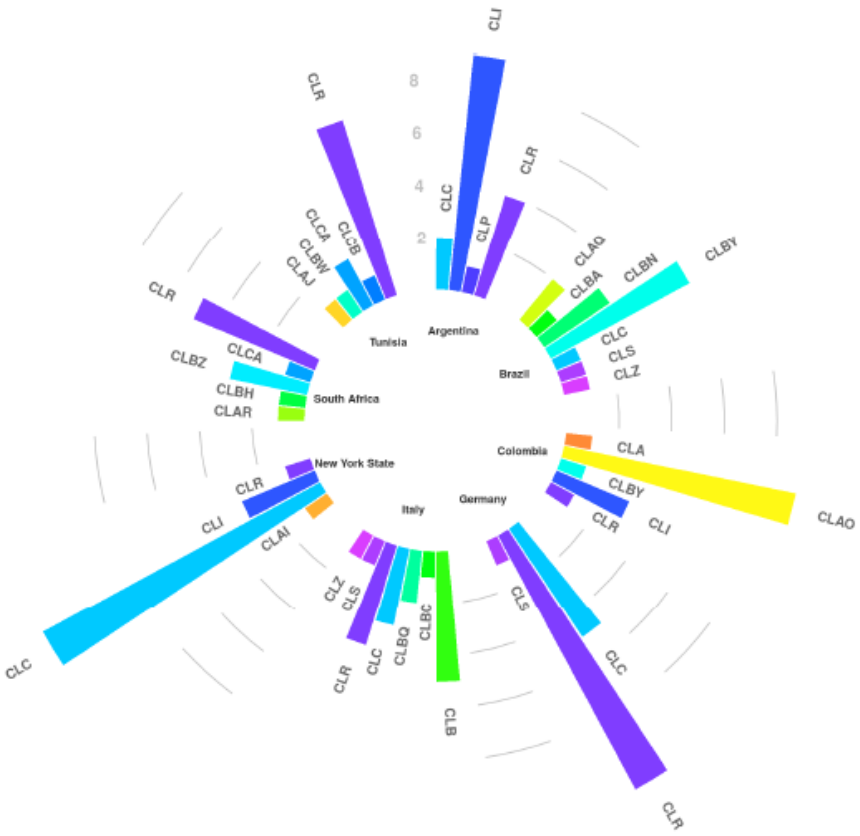
Table 3 - Distribution of genotypes in the eight countries

Country	Genotypic Cluster	Genotype (Isolate No.)	New Genotypes or Variants	Total Strains
Argentina	CLC	GTC (6, 15)	GTI^{V}, GTI^{VI}	16
	CLI	GTI^{I} (1, 4, 5, 7)		
		GTI^{II} (10, 11, 14)		
		GTI^{V} (9)		
		GTI^{VI} (12)		
		GTP (8)		
	CLP	GTR^{I} (2, 3, 16)		
	CLR	GTR^{VI} (13)		
Brazil	CLAQ	GTAQ (31)	$GTBN^{I}$, $GTBN^{II}$, $GTBY^{I}$, $GTAQ^{I}$	15
		$GTAQ^{I}$ (30)		
	CLBA	GTBA (17)		
	CLBN	GTBN (29)		
		$GTBN^{I}$ (20)		
		$GTBN^{II}$ (23)		
	CLBY	GTBY (18, 19, 21, 28)		
		$GTBY^{I}$ (24, 25)		
	CLC	GTC^{III} (26)		
	CLS	GTS^{I} (22)		
	CLZ	GTZ (27)		
Colombia	CLA	GTA^{I} (33)	$GTAO^{I}$, $GTAO^{II}$	15
	CLAO	GTAO (39, 40, 41)		
		$GTAO^{I}$ (38, 43, 44, 46)		
		$GTAO^{II}$ (32, 42)		
	CLBY	GTBY (45)		
	CLI	GTI^{I} (35, 36, 37)		
	CLR	GTR (34)		
Germany	CLC	GTC^{I} (54, 55, 56, 57, 59)		17
	CLR	GTR (47, 48, 49, 51)		
		GTR^{I} (58, 60, 61, 63)		
		GTR^{II} (50, 62)		
		GTR^{VI} (52)		
	CLS	GTS (53)		
Italy	CLB	GTB (64, 65, 66, 78, 80)	GTR^{XIII}	17
	CLBG	GTBG (70)		
	CLBQ	$GTBQ^{I}$ (73, 79)		
	CLC	GTC^{I} (69, 75)		
		GTC^{II} (76)		
	CLR	GTR^{I} (67, 68)		
		GTR^{XIII} (72)		
		GTR^{VI} (71)		
	CLS	GTS (77)		
	CLZ	GTZ (74)		

Country	Genotypic Cluster	Genotype (Isolate No.)	New Genotypes or Variants	Total Strains
NY State	CLAI	GTA^{I} (93)	GTC^{V}, GTI^{V}	17
	CLC	GTC (82, 83, 85, 86, 88, 94, 96)		
		GTC^{I} (81, 87, 91)		
		GTC^{III} (90)		
		GTC^{V} (95)		
	CLI	GTI^{I} (89)		
		GTI^{V} (92, 97)		
	CLR	GTR^{I} (84)		
South Africa	CLAR	GTAR (101)	GTAR, GTBZ, GTCA	11
	CLBH	GTBH (98)		
	CLBZ	GTBZ (99, 100, 105)		
	CLCA	GTCA (103)		
	CLR	GTR (102, 104, 107, 108)		
		GTR^{VI} (106)		
Tunisia	CLAJ	GTAJ (111)	GTCA	12
	CLBW	$GTBW^{II}$ (110)		
	CLCA	GTCA (113, 114)		
	CLCB	GTCB (119)		
	CLR	GTR^{I} (109)		
		GTR^{VI} (112, 115, 116, 117, 118, 120)		

Source: Monistero et al., (2018).

Figure 4 - Representation of the major genotypes with their variants combined into genotypic clusters (CL)



Source: Monistero et al., (2018).

3.4.2 Virulence Genes

All the 120 isolates analyzed in this study were positive for coagulase (*coa*) and thermonuclease (*nuc*) genes, but negative for a gene involved in host cell invasion, the exfoliative toxin (*etb*), and for SEB and SEE enterotoxins. The distribution of the virulence genes for each country is described in detail below.

3.4.2.1 Argentina

All the 16 Argentinian isolates were positive for a leucocidin (*lukE-lukD*) and for an enterotoxin (*sei*), but negative for the gene encoding exfoliative toxin (*eta*), for *mecA, sel* and *sej* (Table 4). All strains were also negative for two mobile genetic element genes (*chp*, *scn*), while 5 carried sak. Out of 16 isolates, 15 (93.7%) had the genes encoding for *lu*kE and *clfA*, 14 (87.5%) for a cell wall-associated protein (*fmtB*), 13 (81.2%) harboured the genes encoding for collagen-binding protein (*cna*), lukM and Panton-Valentine leucocidin *lukSF-PV*, whereas 5 (37.5%) were positive for *sak* and/or for *tsst*, respectively.

All the 16 isolates were enterotoxigenic, harbouring at least one of the genes coding for A, C, D, G and H enterotoxins. Three isolates from 3 different farms were positive for 5 different enterotoxins (combination of *sea, sec, seg, seh* and *sei* or *sea, sed, seg, seh* and *sei* or *sea, sed, seg, seh* and *sei*) while 8 isolates from 8 different farms were positive for 4 enterotoxins (combination of *sed, seg, seh* and *sei* or *sea, seg, seh*). Four isolates, collected in 4 different farms, were positive for 3 enterotoxins genes (combination of *sea, seg* and *sei* or *seg, seh* and *sei*) and 1 isolates for 2 different enterotoxins genes (*seh, sei*).

3.4.2.2 Brazil

Isolates collected from Brazil were all positive for *fmtB, can, clfA* and for the genes encoding leucocidins (*lukE, lukE-lukD, lukM, lukSF-PV*) (Table 5). All the Brazilian isolates were negative for genes carried on mobile genetic elements and usually present in strains involved in human infections such as *chp, scn* and *sak*. Moreover, they were negative for *tsst, eta, mec*A, and *sec, sed, sel, sej* enterotoxins. Out of 15 isolates, 5 (33.3%) were positive for *seh*, 8 (53.3%) for both *sea* and *seh*, while a single isolate (6.6%) harboured other 2 enterotoxin genes (*seg, sei*).

3.4.2.3 Colombia

As shown in Table 6, all the Colombian isolates were positive for *lukE-lukD* and *cna*, but negative for *chp*, *tsst*, *eta*, *mecA* and *sec*, *sel*, *sej*. Out of 15 isolates, 14 (93.4%) were positive for *clfA* and *fmtB* genes, 13 (86.7%) for *lukSF-PV*, 10 (66.7%) for *sak* and *lukM*, and 7 (46.7%) for *scn*. Fourteen (93.3%) isolates were enterotoxigenic harbouring at least one of the genes *sea*, *sed*, *seg*, *sei* or *seh*. The most frequently detected genes were *seh* (93.3%) and *sea* (86.6%), followed by *sei* (26.6%) and *seg* (20%). One isolate harboured all the 5 enterotoxin genes (*sea*, *sed*, *seg*, *seh* and *sei*); 2 other isolates coming from 2 different farms harboured 4 enterotoxin genes (*sea*, *seg*, *seh* and *sei*) and 1 isolate 3 enterotoxin genes (*sea*, *seh* and *sei*). Finally, 9 isolates, from 6 different farms, had the combination of genes encoding for SEA and SEH.

3.4.2.4 Germany

All the German isolates were positive for *lukE* and *cna*, but negative for the mobile genetic element genes (*chp*, *scn*, *sak*), for *eta*, *lukSF-PV* and for enterotoxin genes *sed*, *seh*, *sel*, *sej* (Table 7). Out of 17 isolates, one (6%) harboured the *mecA* gene, 4 (23.5%) the *tsst*, 13 (76.5%) the *fmtB*, 15 (88.2%) the *lukM* and 16 (94.1%) both *clfA* and *lukE-lukD* genes.

Fifteen isolates out of 17 (88.2%), collected from 15 different farms, were enterotoxigenic, harbouring at least one of the genes coding for A, C, G and I enterotoxins. The most frequently detected genes were *sea* (88.2%) and *seg* (58.8%), followed by *sei* and *sec* (29.4%). Two isolates harboured all the 4 enterotoxin genes (*sea*, *sec*, *seg*, and *sei*); 3 and 8 other isolates harboured 3 (*sea*, *sec*, and *seg*) or 2 genes (combination of s*ea* and *seg*, or *sea* and *sei*), respectively.

Table 4 - Molecular characteristics of strains isolated in Argentina

Isolates	*RS-PCR*	*clfA*	*fmtB*	*cna*	*lukE*	*lukM*	*lukE-lukD*	*lukS/F-PV*	*scn*	*chp*	*sak*	*eta*	*tsst*	Enterotoxins positive	*spa*	*coa*	*mecA*
1		+	+	+	+	-	+	+	-	-	-	-	+	*sea, seg, sei*	286	894	-
2		+	+	+	+	+	+	+	-	-	+	-	-	*sed, seg, seh, sei*	286	894	-
3		+	-	+	+	+	+	+	-	-	-	-	-	*sed, seg, seh, sei*	286	894	-
4		-	-	+	+	+	+	+	-	-	-	-	+	*sea, seg, sei*	286	894	-
5		+	+	+	+	+	+	+	-	-	-	-	-	*sed, seg, seh, sei*	320	894	-
6		+	+	+	+	+	+	-	-	-	+	-	+	*sea, sec, seg, seh, sei*	108	617	-
7		+	+	+	+	+	+	-	-	-	+	-	+	*sea, seg, seh, sei*	292	894	-
8		+	+	+	-	+	+	+	-	-	+	-	-	*sea, sed, seg, seh, sei*	132	894	-
9		+	+	+	+	+	+	+	-	-	-	-	-	*sed, seg, seh, sei*	286	443	-
10		+	+	-	+	+	+	+	-	-	+	-	-	*sea, seg, seh, sei*	286	894	-
11		+	+	-	+	-	+	+	-	-	-	-	-	*sea, seg, seh, sei*	270	894	-
12		+	+	+	+	+	+	+	-	-	-	-	+	*seg, seh, sei*	270	443	-
13		+	+	-	+	+	+	+	-	-	-	-	-	*seg, seh, sei*	337	894	-
14		+	+	+	+	-	+	+	-	-	-	-	-	*sea, sed, seg, seh, sei*	270	879	-
15		+	+	+	+	+	+	+	-	-	-	-	+	*sea, seg, seh, sei*	106	605	-
16		+	+	+	+	+	+	-	-	-	-	-	-	*seh, sei*	270	879	-

Source: Monistero et al., (2018).

Table 5 - Molecular characteristics of strains isolated in Brazil

Isolates	*ClfA*	*lukE*	*lukM*	*lukE-lukD*	*lukS/F-PV*	*scn*	*chp*	*fmtb*	*sak*	*cna*	*tsst*	*eta*	Enterotoxins positive	*spa*	*coa*	*mecA*
17	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seh*	225	630	-
18	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seh*	225	630	-
19	+	+	+	+	+	-	-	+	-	+	-	-	*seh*	225	630	-
20	+	+	+	+	+	-	-	+	-	+	-	-	*seh*	108	630	-
21	+	+	+	+	+	-	-	+	-	+	-	-	*seh*	108	630	-
22	+	+	+	+	+	-	-	+	-	+	-	-	*seh*	108	630	-
23	+	+	+	+	+	-	-	+	-	+	-	-	*seh*	108	540	-
24	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seh*	225	630	-
25	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seh*	225	630	-
26	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seh*	225	630	-
27	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seg, seh, sei*	225	630	-
28	+	+	+	+	+	-	-	+	-	+	-	-	-	225	630	-
29	+	+	+	+	+	-	-	+	-	+	-	-	-	108	630	-
30	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seh*	225	630	-
31	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seh*	225	630	-

Source: Monistero et al., (2018).

Table 6 - Molecular characteristics of strains isolated in Colombia

Isolates	*ClfA*	*lukE*	*lukM*	*lukE-lukD*	*lukS/F-PV*	*scn*	*chp*	*fmtb*	*sak*	*cna*	*tsst*	*eta*	Enterotoxins positive	*spa*	*coa*	*mecA*
32	-	+	-	+	+	-	-	+	-	+	-	-	-	312	925	-
33	+	+	+	+	+	+	-	+	+	+	-	-	*sea, seh*	129	621	-
34	+	+	+	+	+	+	-	+	+	+	-	-	*seh*	311	913	-
35	+	+	+	+	+	+	-	+	+	+	-	-	*sea, seh*	311	913	-
36	+	+	-	+	-	+	-	+	+	+	-	-	*sea, seh*	334	913	-
37	+	+	+	+	-	+	-	+	+	+	-	-	*sea, seh*	267	913	-
38	+	+	+	+	+	+	-	+	+	+	-	-	*sea, seh*	288	913	-
39	+	+	+	+	+	-	-	+	+	+	-	-	*sea, seh*	311	913	-
40	+	+	+	+	+	-	-	+	+	+	-	-	*sea, seh*	334	913	-
41	+	+	-	+	+	+	-	-	+	+	-	-	*sea, seh*	311	913	-
42	+	+	+	+	+	-	-	+	-	+	-	-	*sea, sed, seg, seh, sei*	311	913	-
43	+	+	+	+	+	-	-	+	+	+	-	-	*sea, seg, seh, sei*	288	913	-
44	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seg, seh, sei*	311	913	-
45	+	-	-	+	+	-	-	+	-	+	-	-	*sea, seh*	127	617	-
46	+	+	+	+	+	-	-	+	-	+	-	-	*sea, seh, sei*	286	894	-

Source: Monistero et al., (2018).

Table 7 - Molecular characteristics of strains isolated in Germany

Isolates	*ClfA*	*lukE*	*lukM*	*lukE-lukD*	*lukS/F-PV*	*scn*	*chp*	*fmtb*	*sak*	*cna*	*tsst*	*eta*	Enterotoxins positive	*spa*	*coa*	*mecA*
47	+	+	+	+	-	-	-	+	-	+	-	-	*sea*	290	540	-
48	+	+	+	+	-	-	-	+	-	+	-	-	*sea*	290	540	-
49	+	+	+	+	-	-	-	+	-	+	-	-	*sea, seg*	290	540	-
50	+	+	+	+	-	-	-	+	-	+	-	-	*sea, seg*	132	930	-
51	+	+	+	+	-	-	-	+	-	+	-	-	*sea, seg*	290	630	-
52	+	+	-	+	-	-	-	+	-	+	-	-	-	247	930	-
53	-	+	-	-	-	-	-	+	-	+	-	-	-	225	830	+
54	+	+	+	+	-	-	-	+	-	+	-	-	*sea, sec, seg, sei*	108	605	-
55	+	+	+	+	-	-	-	-	-	+	+	-	*sea, sec, seg*	108	630	-
56	+	+	+	+	-	-	-	-	-	+	+	-	*sea, sec, seg*	108	630	-
57	+	+	+	+	-	-	-	-	-	+	+	-	*sea, sec, seg, sei*	108	630	-
58	+	+	+	+	-	-	-	+	-	+	-	-	*sea, sei*	130	930	-
59	+	+	+	+	-	-	-	-	-	+	+	-	*sea, sec, seg*	108	630	-
60	+	+	+	+	-	-	-	+	-	+	-	-	*sea, seg*	130	930	-
61	+	+	+	+	-	-	-	+	-	+	-	-	*sea, seg*	130	930	-
62	+	+	+	+	-	-	-	+	-	+	-	-	*sea, sei*	130	930	-
63	+	+	+	+	-	-	-	+	-	+	-	-	*sea, sei*	130	930	-

Source: Monistero et al., (2018).

3.4.2.5 Italy

All the Italian isolates were positive for *lukE, lukE-lukD, cna* and *fmtB*, but negative for *chp, eta, lukSF-PV* and *seh, sel* enterotoxin genes (Table 8). Out of 17 isolates, 14 (82.3%) were positive for *clfA* and 9 (53%) had the gene encoding *lukM*. One isolate (6%) was positive for both *scn* and *sak* genes, and other two different isolates were positive for *tsst* (6%) and *mecA* (6%), respectively.

Fourteen isolates out of 17 (82.3%) were enterotoxigenic, harbouring at least 1 of the genes coding for A, C, D, G, I and J enterotoxins. The most frequently detected genes were *sed* (82.3%) and *seg* (70.5%), followed by *sej* (64.7%), *sea* (58.8%) and *sei* (47%). Six isolates harboured 5 enterotoxin genes (combination of *sea, sed, seg, sei* and *sej*, or *sea, sed, seg, sec* and *sej*); 4 other isolates harboured 4 enterotoxin genes (combination of *sea, sei, sed* and *seg*, or *sei, sed, seg* and *sej* or *sea, sed, sej* and *seg*). Moreover, 2 isolates harboured 3 different enterotoxins (*sea, sed* and *seg*) and 2 isolates, from the same farm, a combination of *sed* and *sej*.

3.4.2.6 New York State

As reported in Table 9, all the New York State isolates were positive for *lukE-lukD*, but negative for *chp, scn, sak, tsst, eta, mecA* and *sec, sel, seh, sej*. Out of 17 isolates, 15 (88.2%) were positive for *cna* and *lukE*, while 13 (76.4%) and 9 (53%) were positive for *lukM* and *clfA* genes, respectively. In addition, 6 isolates (35.2%) and 2 (12%) had the *fmtB* and *lukSF-PV* genes, respectively. Only one isolate was not enterotoxigenic; the remaining 16 isolates (95%) harboured at least one of the genes encoding SEA, SED, SEG, SEI enterotoxins. Five isolates, collected from 5 different farms, had all the enterotoxin genes (*sea, sed, seg, sei*); 6 isolates, from 6 different farms, harboured 3 genes (combination of *sea, sed* and *seg* or *sea, seg* and *sei* or *sed, seg* and *se*i). Five isolates, from 4 different farms, had 2 enterotoxin genes (combination of *sed* and *seg* or *seg* and *sei* or *sed* and *sei*).

Table 8 - Molecular characteristics of strains isolated in Italy

Isolates	*ClfA*	*lukE*	*lukM*	*lukE-lukD*	*lukS/F-PV*	*scn*	*chp*	*fmtb*	*sak*	*cna*	*tsst*	*eta*	Enterotoxins positive	*spa*	*coa*	*mecA*
64	+	+	-	+	-	-	-	+	-	+	-	-	-	270	620	-
65	+	+	-	+	-	-	-	+	-	+	-	-	*sed, sej*	270	620	-
66	-	+	-	+	-	-	-	+	-	+	-	-	*sed, sej*	200	620	-
67	+	+	-	+	-	-	-	+	-	+	-	-	-	319	893	-
68	+	+	-	+	-	-	-	+	-	+	-	-	-	319	893	-
69	+	+	+	+	-	-	-	+	-	+	-	-	*sed, seg, sei, sej*	109	619	-
70	+	+	+	+	-	-	-	+	-	+	-	-	*sed, seg, sei, sej*	132	691	-
71	+	+	+	+	-	-	-	+	-	+	-	-	*sea, sed, seg, sei, sej*	109	881	-
72	+	+	+	+	-	-	-	+	-	+	-	-	*sea, sed, seg, sei*	132	893	-
73	+	+	-	+	-	-	-	+	-	+	-	-	*sea, sed, seg, sei, sej*	151	893	-
74	+	+	-	+	-	-	-	+	-	+	-	-	*sea, sed, seg, sei, sej*	200	619	-
75	+	+	+	+	-	-	-	+	-	+	-	-	*sea, sed, seg*	109	619	-
76	+	+	+	+	-	-	-	+	-	+	+	-	*sea, sec, sed, seg, sej*	106	637	-
77	-	+	+	+	-	-	-	+	-	+	-	-	*sea, sed, seg*	266	846	+
78	+	+	+	+	-	+	-	+	+	+	-	-	*sea, sed, seg, sei, sej*	291	637	-
79	+	+	+	+	-	-	-	+	-	+	-	-	*sea, sed, seg, sej*	291	846	-
80	-	+	-	+	-	-	-	+	-	+	-	-	*sea, sed, seg, sei, sej*	201	637	-

Source: Monistero et al., (2018).

Table 9 - Molecular characteristics of strains isolated in New York State

Isolates	*ClfA*	*lukE*	*lukM*	*lukE-lukD*	*lukS/F-PV*	*scn*	*chp*	*fmtb*	*sak*	*cna*	*tsst*	*eta*	Enterotoxins positive	*spa*	*coa*	*mecA*
104	+	+	-	+	-	-	-	-	-	+	-	-	*sea, sed, seg, sei*	105	637	-
105	-	+	+	+	-	-	-	-	-	+	-	-	*sea, sed, seg, sei*	105	637	-
106	+	+	+	+	-	-	-	+	-	+	-	-	*sed, seg*	105	637	-
107	+	+	-	+	-	-	-	+	-	+	-	-	*sed, seg*	198	846	-
108	+	+	+	+	-	-	-	-	-	+	-	-	*sed, seg, sei*	108	637	-
109	+	+	+	+	-	-	-	-	-	+	-	-	*seg, sei*	108	637	-
110	+	+	+	+	-	-	-	-	-	+	-	-	*sea, sed, seg*	108	630	-
111	+	+	+	+	-	-	-	-	-	+	-	-	*sea, sed, seg, sei*	108	630	-
112	+	+	+	+	-	-	-	+	-	+	-	-	*sed, seg, sei*	269	925	-
113	-	-	+	+	-	-	-	-	-	-	-	-	*sea, sed, seg*	108	630	-
114	-	+	+	+	-	-	-	-	-	+	-	-	*sea, sed, seg, sei*	108	630	-
115	-	+	+	+	-	-	-	+	-	+	-	-	*sed, sei*	291	925	-
116	+	-	-	+	-	-	-	-	-	+	-	-	*sea, sed, seg, sei*	108	630	-
117	-	+	+	+	-	-	-	-	-	+	-	-	*sea, seg, sei*	108	630	-
118	-	+	+	+	+	-	-	-	-	-	-	-	*sea, seg, sei*	108	630	-
119	-	+	+	+	-	-	-	+	-	+	-	-	*seg, sei*	107	630	-
120	-	+	-	+	+	-	-	+	-	+	-	-	-	291	925	-

Source: Monistero et al., (2018).

3.4.2.7 South Africa

As reported in Table 10, all the South African isolates were positive for *sak, cna, lukE-lukD, lukE* genes. All the isolates were negative for *chp, mecA, tsst* and for *sec, sed, seg, sej* and *sel*. In addition, 10 (90.9%) out of 11 isolates were positive for *fmtB*, 7 (63.7%) for *clfA*, 3 (27.3%) for *lukSF-PV*, 2 (18.2%) for *lukM* and 1 (9%) for *eta* genes, respectively. Ten isolates, recovered in 9 different farms, were enterotoxigenic and positive for both *sea* and *seh* genes; out of them, 3 isolates from 2 different farms, harboured also the *sei* gene.

3.4.2.8 Tunisia

The Tunisian isolates were all positive for *fmtB, cna* and *clfA* genes, but negative for *eta, mecA, lukSF-PV* and *sea, sed, seg, sei, sej* (Table 11). Out of 12 isolates, 11 (91.6%) harboured leucocidin genes (*lukM, lukE, lukE-lukD*). Six isolates (50%) were positive for at least one gene of the immune evasion cluster with the combination of *chp, scn* and *sak* for 2 isolates, *scn* and *sak* or *chp* and *scn*, respectively, while the remaining 2 isolates harboured only the *chp* gene. Moreover, 4 isolates from 4 different farms, were enterotoxigenic harbouring *sec* and *sel* (2 isolates) or *seh* genes (2 isolates).

Table 10 - Molecular characteristics of strains isolated in South Africa

Isolates	*ClfA*	*lukE*	*lukM*	*lukE-lukD*	*lukS/F-PV*	*scn*	*chp*	*fmtb*	*sak*	*cna*	*tsst*	*eta*	Enterotoxins positive	*spa*	*coa*	*mecA*
81	+	+	-	+	+	-	-	+	+	+	-	-	*sea, seh*	225	630	-
82	-	+	-	+	-	-	-	+	+	+	-	-	*sea, seh, sei*	248	880	-
83	-	+	-	+	-	-	-	+	+	+	-	-	*sea, seh, sei*	248	880	-
84	-	+	-	+	-	-	-	+	+	+	-	-	*sea, seh, sei*	246	630	-
85	+	+	+	+	-	-	-	+	+	+	-	-	*sea, seh*	177	930	-
86	+	+	-	+	-	+	-	+	+	+	-	+	*sea, seh*	317	630	-
87	+	+	-	+	-	-	-	+	+	+	-	-	*sea, seh*	248	930	-
88	-	+	-	+	-	-	-	-	+	+	-	-	*sea, seh*	248	880	-
89	+	+	+	+	-	-	-	+	+	+	-	-	*sea, seh*	200	930	-
90	+	+	-	+	+	-	-	+	+	+	-	-	*sea, seh*	200	930	-
91	+	+	-	+	+	-	-	+	+	+	-	-	-	266	846	-

Source: Monistero et al., (2018).

Table 11 - Molecular characteristics of strains isolated in Tunisia

Isolates	*ClfA*	*lukE*	*lukM*	*lukE-lukD*	*lukS/F-PV*	*scn*	*chp*	*fmtb*	*sak*	*cna*	*tsst*	*eta*	Enterotoxins positive	*spa*	*coa*	*mecA*
92	+	-	-	+	-	-	-	+	-	+	-	-	-	309	780	-
93	+	+	+	+	-	-	-	+	-	+	+	-	*sec, sel*	222	780	-
94	+	+	+	+	-	-	-	+	-	+	+	-	*sec, sel*	222	780	-
95	+	+	+	+	-	+	+	+	+	+	-	-	-	190	780	-
96	+	+	+	+	-	-	+	+	-	+	-	-	*seh*	170	600	-
97	+	+	+	+	-	+	-	+	+	+	-	-	*seh*	170	600	-
98	+	+	+	+	-	-	-	+	-	+	-	-	-	190	780	-
99	+	+	+	+	-	-	+	+	-	+	-	-	-	309	780	-
100	+	+	+	+	-	-	-	+	-	+	-	-	-	170	780	-
101	+	+	+	+	-	-	-	+	-	+	-	-	-	309	780	-
102	+	+	+	+	-	+	+	+	-	+	-	-	-	222	510	-
103	+	+	+	-	-	+	+	+	+	+	-	-	-	280	780	-

Source: Monistero et al., (2018).

3.5 DISCUSSION

Pathogenic factors of *S. aureus* enable this bacterium to attach, colonize, invade and infect the host tissue. In this study, *S. aureus* isolates, collected from eight different countries, were investigated using RS-PCR genotyping and PCR analysis for the carriage of different virulence factors to examine the epidemiology of this microorganism.

The samples were obtained from collections of the collaborators, allowing a first overview about the presence of the various staphylococcal subtypes among countries. Three new genotypes were observed for South Africa whereas new variants were found in Argentina, Brazil, Colombia, Italy and New York State. As previously described (COSANDEY et al., 2016), GTB was observed only in Europe (Italy) while CLR and CLC clusters were observed throughout America, Europe and Africa; particularly CLR, which forms a large cluster containing 13 variants, was detected in each country involved, except for Brazil. It is quite well demonstrated (COSANDEY et al., 2016) that *S. aureus* CLC and CLR clusters are "dairy cattle specific" whose spreading process must have been started a long time ago, with the spreading of breeding cattle from Europe to the other countries. On the contrary, GTB derives from a more recent bovine adaptation due to a new human-to-cow host jump (CREMONESI et al., 2015). Certainly, further studies will be necessary to explain the different geographic distribution especially for the minor genotypes.

As previously described (CREMONESI et al., 2013), *S. aureus* isolates harbouring genes coding for clumping factor (*clfA*), a cell wall-associated protein (*fmtB*), and collagen-binding protein (*cna*) have a greater capability to adhere to extracellular matrix proteins, essential for colonization and the establishment of infections. Our results indicated that, except for the American isolates with a lower presence of *fmtB* and *clfA* genes, in the other seven countries these genes were widely present in the circulating isolates particularly in Brazilian and Tunisian ones. The presence of these genes, necessary for host invasion, could improve the persistence of the microorganism in the host, ensuring the probability of survival in the population.

And more, according to previous studies (IKAWATY et al, 2009; CREMONESI et al., 2013; CREMONESI et al., 2015), except for Brazil, Germany and USA, the remaining countries showed isolates encoding at least 2 virulence factors out of staphylococcal complement inhibitor (*scn*), chemotaxis inhibitory protein of *S. aureus* (*chp*) and staphylokinase (*sak*). These virulence factors show activity prevalently against the human innate immune system but their presence among isolates recovered in herds with high

prevalence of *S. aureus* mastitis suggests their involvement also in bovine mammary gland immune response (MAGRO et al., 2017), and should be further studied, especially in Colombia and Tunisia where this gene cluster is quite common (BEN SAID et al., 2016). In a previous study (VAN WAMEL et al., 2006), human strains were grouped in 7 immune evasion cluster (IEC) types, depending on the presence of 2 out of the 3 genes, in association or not with *sea* or *sep*. Unlike Colombian, Italian, South African strains and Tunisian isolates, the Argentinian ones carried only one gene, *sak*, showing a clear distance from human strains. Among the isolates from the other countries, uniquely the Tunisian strains testing positive for IEC, did not harbor *sea*.

Superantigens, especially enterotoxins, have been suggested to play a role in the development of mastitis, for instance by creating an attractive environment for colonization (PICCININI; BORROMEO; ZECCONI, 2010) since they are more often identified in *S. aureus* isolated from cows with mastitis than in isolates from healthy cows or from the environment (PIECHOTA et al., 2014). As a result, enterotoxins support the pathogenesis of *S. aureus* compromising mammary gland immune response and susceptibility to antibiotics resulting in the onset of many diseases (EL-SAYED et al., 2006). In this study, *sea* and *sei* were the main enterotoxin genes present in all countries except for Tunisia (prevalence between 50% and 90%). While *seh* gene had a frequency higher than 90% in Argentinian, Brazilian, Colombian and South African isolates, *sej* and *sel* genes were carried only by Italian and Tunisian isolates, respectively. Among the 120 isolates analyzed, only 17 (14%) were not enterotoxigenic (1 from Argentina, 1 from Colombia, 2 isolates from Germany, 3 from Italy, 1 from New York State, 1 from South Africa, and 8 from Tunisia). The remaining 103 isolates (86%) harboured a combination of at least 2 up to 5 enterotoxins with the linkages between *sea*, *sed*, *seg* and *seh* confirming their predominance in cows, as previously described (HAVERI et al., 2007; FOURNIER et al., 2008; ARTUSSON et al., 2016; SHARMA et al., 2017). The absence of the enterotoxin genes *seb* and *see* in our isolates was in accordance with previous results (FOURNIER et al., 2008; BYSTRO´ N et al., 2009; OTE et al., 2011; CREMONESI et al., 2013).

Here, among all the isolates we did not find the presence of *etb* exfoliative gene and only one isolate from South Africa was positive for *eta* gene. These results agree with previous studies conducted in different countries (DARWISH; ASFOUR, 2013; SILVEIRA-FILHO et al., 2014; AKINDOLIRE, BABALOLA, ATEBA, 2015), showing that *S. aureus* isolates from animals with mastitis were rarely positive for exfoliative toxins. On the contrary, in Europe,

Kot and coworkers reported a 14.5% of *S. aureus* harbouring the *eta* gene from bovine mastitis (KOT et al., 2016). In our study, the presence of *tsst* gene was more relevant, being carried by 37% of Argentinian, 23% of German, 16% of Tunisian and 6% of Italian isolates. All these isolates were also positive at least for a combination of *sec* and *sel*, or *sec, seg,* and *sei* or *sec, seg* and *sej* or *sec, seg* and *sel* genes which are located on the same bovine staphylococcal pathogenicity island (SaPIbov), confirming a positive correlation between *sec, sei* or *sej* and *tsst*, as previously reported (ZSCHÖCK et al., 2005).

Panton-Valentine leucocidin, encoded by 2 co-transcribed genes located on a prophage, causes leukocyte destruction and tissue necrosis (BHATTA et al., 2016). The presence of PVL-encoding genes in *S. aureus* is reported to be associated with increased disease severity (SHARIATI et al., 2016). In the present study, the presence of PVL gene was lower than 20% in South Africa and New York State, higher than 80% in Argentina, Colombia and Brazil, while in Germany, Italy and Tunisia none of the *S. aureus* isolates carried the gene. For European countries, previously published results were in accordance with this study (FUEYO et al., 2005; PARISI et al., 2016). Additionally, genes encoding the bicomponent leucotoxin *lukE-lukD* were observed in all isolates, and, except for South Africa with only 2 isolates, most of the other isolates harboured *lukM*, a gene encoding one operon like the one of PVL. The high rates of *lukE-lukD* and *lukM* found in this study agree with other reports (FUEYO et al., 2005; SCHLOTTER et al. 2015; PARISI et al., 2016;). Additionally, only 2 isolates, one from Germany and one from Italy were positive for *mecA*, confirming the low diffusion of MRSA among bovine mastitis isolates (LUINI et al., 2015; HENDRIKSEN et al., 2008) interestingly, they are both GTS, in accordance with previous results (CREMONESI et al., 2015).

3.6 CONCLUSION

Knowledge about the epidemiology of *S. aureus* genotypes in dairy species and herds might help to formulate strategies for reducing the infection spreading and for focused treatments. In our work we found that CLR and CLC clusters and some virulence factors related to host invasion, such as *fmtB, cna, clfA* or immune defense impairment such as leukocidin genes, were the most frequent ones. These genes combination could be related to the *S. aureus* ability to colonize the host. Further, *fmtB* gene has been shown to be related to the resistance of *S. aureus* to β-lactam antibiotics (SUNG; LLOYD; LINDSAY, 2008). Therefore, due to the prevalence of these genes worldwide, it might be useful screening them in *S. aureus* isolates to

help predicting clinical outcomes and specially to identify harmful strains. Meanwhile, our work demonstrated also that each country had a specific genotypic pattern and in some countries the isolates harboured some virulence factors, such as PVL-encoding genes, with high prevalence, recommending a close surveillance of *S. aureus* isolates in the animals of these countries to avoid the wide spreading of these genes. Finally, it is notable that most of the isolates worldwide were negative for *mecA*, confirming the evidence of the low diffusion of MRSA among bovine mastitis isolates, as previously described (LUINI et al., 2015; HENDRIKSEN et al., 2008).

In conclusion, this study confirms the wide variety of *S. aureus* genotypes found in dairy cattle worldwide and that genetic differences are related to geographical origin of the isolates, suggesting that considering the region of interest and the strain virulence might help to formulate strategies directed to reduce the infection spreading and to set up control measures according to pathogen and host features. Therefore, based the characterization of the circulating strain, the farmer would be able to decide to segregate positive cows applying hygienic milking procedures and a suitable milking order, or even to cull the infected animals.

3.7 REFERENCES

AKINDOLIRE, M.A.; BABALOLA, O.O.; ATEBA, C.N. Detection of Antibiotic Resistant Staphylococcus aureus from Milk: A Public Health Implication. **Int. J. Environ. Res. Public Health**, v. 12, p.10254–10275, 2015.

AKINEDEN, O.; ANNEMULLER, C.; HASSAN, A.A.; LAMMLER, C.; WOLTER,W.; ZSCHOCK, M. Toxin genes and other characteristics of Staphylococcus aureus isolates from milk of cows with mastitis. **Clin. Diagn. Lab. Immunol**, v.8, p.959–964, 2001.

ARTURSSON, K.; SÖDERLUND, R.; LIU, L.; MONECKE, S.; SCHELIN, J. Genotyping of Staphylococcus aureus in bovine mastitis and correlation to phenotypic characteristics. **Vet. Microbiol**, v.193, p.156–161, 2016.

BEN SAID, M.; ABBASSI, M.S.; BIANCHINI, V.; SGHAIER, S.; CREMONESI, P.; ROMANÒ, A.; GUALDI, V.; HASSEN, A.; LUINI, M.V. Genetic characterization and antimicrobial resistance of Staphylococcus aureus isolated from bovine milk in Tunisia. **Lett. Appl. Microbiol.**, v.63, p.473–481, 2016.

BHATTA, D.R.; CAVACO, L.M.; NATH, C.; KUMAR, K.; GAUR, A.; GOKHALE, S.; BHATTA, D.R. Association of Panton Valentine leukocidin (PVL) genes with methicillin-

resistant Staphylococcus aureus (MRSA) in Western Nepal: A matter of concern for community infections (a hospital based prospective study). **BMC Infect. Dis**., 16, 199, 2016.

BYSTRO´ N, J.; BANIA, J.; LIS, E.; MOLENDA, J.; BEDNARSKI, M. Characterisation of Staphylococcus aureus strains isolated from cows' milk. **Bull. Vet. Inst. Pulawy**, v.53, p.59–63, 2009.

COSANDEY, A.; BOSS, R.; LUINI, M.; ARTURSSON, K.; BARDIAU, M.; BREITENWIESER, F.; HEHENBERGER, E.; LAM, T.; MANSFELD, M.; MICHEL, A.; MÖSSLACHER, G.; NASKOVA, J.; NELSON, S.; PODPEČAN, O.; RAEMY, A.; RYAN, E., SALAT, O.; ZANGERL, P.; STEINER, A.; GRABER, H.U. Staphylococcus aureus genotype B and other genotypes isolated from cow milk in European countries. **J. Dairy Sci.,** v.99, p.529–540, 2016.

CREMONESI, P.; CASTIGLIONI, B.; MALFERRARI, G.; BIUNNO, I.; VIMERCATI, C.; MORONI, P.; MORANDI, S.; LUZZANA, M. Technical Note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. **J. Dairy Sci**., v.89, p.163–169, 2006.

CREMONESI, P.; LUZZANA, M.; BRASCA, M.; MORANDI, S.; LODI, R.; VIMERCATI, C.; AGNELLINI, D.; CARAMENTI, G.; MORONI, P.; CASTIGLIONI, B. Development of a multiplex PCR assay for the identification of Staphylococcus aureus enterotoxigenic strains isolated from milk and dairy products. **Mol. Cell. Probes**, v.19, p.299–305, 2005.

CREMONESI, P.; POZZI, F.; RASCHETTI, M.; BIGNOLI, G.; CAPRA, E.; GRABER, H.U.; VEZZOLI, F.; PICCININI, R.; BERTASI, B.; BIFFANI, S.; CASTIGLIONI B.; LUINI, M. Genomic characteristics of Staphylococcus aureus strains associated with high within-herd prevalence of intramammary infections in dairy cows. **J. Dairy Sci**., v.98, p.6828–6838, 2015.

CREMONESI, P.; ZOTTOLA, T.; LOCATELLI, C.; POLLERA, C.; CASTIGLIONI, B.; SCACCABAROZZI, L.; MORONI, P. Identification of virulence factors in 16S-23S rRNA intergenic spacer genotyped Staphylococcus aureus isolated from water buffaloes and small ruminants. **J. Dairy Sci**, v.96, p.7666–7674, 2013.

DARWISH, S.F.; ASFOUR, H.A. Investigation of biofilm forming ability in Staphylococci causing bovine mastitis using phenotypic and genotypic assays. **Sci. World J.** Article ID 378492, 9 pages, 2013.

DEB, R.; KUMAR, A.; CHAKRABORTY, S.; VERMA, A.K.; TIWARI, R.; DHAMA, K.; SINGH, U.; KUMAR, S. Trends in diagnosis and control of bovine mastitis: A review. **Pak. J. Biol. Sci**., v.16, p.1653–1661, 2013.

EL-SAYED, A.; ALBER, J.; LAMMLER, C.; JAGER, S.; WOTER, W.; VÁZQUEZ, H. Comparative study on genotypic properties of Staphylococcus aureus isolated from clinical and subclinical mastitis in Mexico. **Vet. Mex.**, 37, 165–179., 2006.

FITZGERALD, J.R.; MEANEY, W.J.; HARTIGAN, P.J.; SMYTH, C.J.; KAPUR, V. Fine-structure molecular epidemiological analysis of Staphylococcus aureus recovered from cows. **Epidemiol. Infect.**, v.119, p.261–269, 1997.

FOSTER, T.J. Immune evasion by staphylococci. **Nat. Rev. Microbiol**, v.3, p,948–958, 2005.

FOURNIER, C.; KUHNERT, P.; FREY, J.; MISEREZ, R.; KIRCHHOFER, M.; KAUFMANN, T.; STEINER, A.; GRABER, H.U. Bovine Staphylococcus aureus: Association of virulence genes, genotypes and clinical outcome. **Res. Vet. Sci**., v.85, p.439–448, 2008

FUEYO, J.M.; MENDOZA, M.C.; RODICIO, M.R.; MUÑIZ, J.; ALVAREZ, M.A.; MARTÍN, M.C. Cytotoxin and pyrogenic toxin superantigen gene profiles of Staphylococcus aureus associated with subclinical mastitis in dairy cows and relationships with macrorestriction genomic profiles. **J. Clin. Microbiol**., v.43, p.1278–1284, 2005.

GOMES, F.; HENRIQUES, M. Control of Bovine Mastitis: Old and Recent Therapeutic Approaches**. Curr. Microbiol**., v.72, p.377–382, 2016.

GRABER, H.U. Genotyping of Staphylococcus aureus by Ribosomal Spacer PCR (RS-PCR). **J. Vis. Exp.** 2016.

HAVERI, M.; ROSLÖF, A.; RANTALA, L.; PYÖRÄLÄ, S. Virulence genes of bovine Staphylococcus aureus from persistent and nonpersistent intramammary infections with different clinical characteristics. **J. Appl. Microbiol**., v.103, p.993–1000, 2007.

HENDRIKSEN, R.S.; MEVIUS, D.J.; SCHROETER, A.; TEALE, C.; MEUNIER, D.; BUTAYE, P.; FRANCO, A.; UTINANE, A.; AMADO, A.; MORENO, M.; GREKO, C.; STÄRK, K.; BERGHOLD, C.; MYLLYNIEMI, A.L.; WASYL, D.; SUNDE, M.; AARESTRUP, F.M. Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. **Acta Vet. Scand.**, 50, 28, 2008.

HERRON-OLSON, L.; FITZGERALD, J.R.; MUSSER, J.M.; KAPUR, V. Molecular correlates of host specialization in Staphylococcus aureus. **PLoS ONE**, 2, e1120, 2007.

IKAWATY, R.; BROUWER, E.C.; JANSEN, M.D.; VAN DUIJKEREN, E.; MEVIUS, D.; VERHOEF, J.; FLUIT, A.C. Characterization of Dutch Staphylococcus aureus from bovine

mastitis using a Multiple Locus Variable Number Tandem Repeat Analysis. **Vet. Microbiol.**, v.136, p.277–284, 2009.

JARRAUD, S.; MOUGEL, C.; THIOULOUSE, J.; LINA, G.; MEUGNIER, H.; FOREY, F.; NESME, X.; ETIENNE, J.; VANDENESCH, F. Relationships between Staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. **Infect. Immun**., v.70, p.631–641, 2001.

KOT, B.; SZWEDA, P.; FRANKOWSKA-MACIEJEWSKA, A.; PIECHOTA, M.; WOLSKA, K. Virulence gene profiles in Staphylococcus aureus isolated from cows with subclinical mastitis in eastern Poland. **J. Dairy Res**., 83, 228–235, 2016.

LUINI, M.; CREMONESI, P.; MAGRO, G.; BIANCHINI, V.; MINOZZI, G.; CASTIGLIONI, B.; PICCININI, R. Methicillin-resistant Staphylococcus aureus (MRSA) is associated with low within-herd prevalence of intra-mammary infections in dairy cows: Genotyping of isolates. **Vet. Microbiol**., v.178, p.270–274, 2015.

LUINI, M.; CREMONESI, P.; MAGRO, G.; BIANCHINI, V.; MINOZZI, G.; CASTIGLIONI, B.; PICCININI, R.; MAGRO, G.; BIFFANI, S.; MINOZZI, G.; EHRICHT, R.; MONECKE, S.; LUINI, V.; PICCININI, R. Virulence genes of S. aureus from dairy cow mastitis and contagiousness risk. **Toxins**, 9, 195, 2017.

MCCLURE, J.A.; CONLY, J.M.; LAU, V.; ELSAYED, S.; LOUIE, T.; HUTCHINS, W.; ZHANG, K. Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and sim ultaneous discrimination of methicillin-susceptible from -resistant staphylococci. **J. Clin. Microbiol**., 44, 1141–1144, 2006.

MONDAY, S.R.; BOHACH, G.A. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. **J. Clin. Microbiol**., 37, 3411–3414, 1999.

OTE, I.; TAMINIAU, B.; DUPREZ, J.N.; DIZIER, I.; MAINIL, J.G. Genotypic characterization by polymerase chain reaction of Staphylococcus aureus isolates associated with bovine mastitis. **Vet. Microbiol**., v.153, p.285–292, 2011.

PANTOSTI, A. Methicillin-Resistant Staphylococcus aureus associated with animals and its relevance to human health. **Front. Microbiol**., 3, 127, 2012.

PARISI, A.; CARUSO, M.; NORMANNO, G.; LATORRE, L.; SOTTILI, R.; MICCOLUPO, A.; FRACCALVIERI, R.; SANTAGADA, G. Prevalence, antimicrobial susceptibility and

molecular typing of methicillin-resistant Staphylococcus aureus (MRSA) in bulk tank milk from southern Italy. **Food Microbiol**., v.58, p.36–42, 2016.

PICCININI, R.; BORROMEO, V.; ZECCONI, A. Relationship between Staphylococcus aureus gene pattern and dairy herd mastitis. **Vet. Microbiol**., v.145, p.100–105, 2010.

PIECHOTA, M.; KOT, B.; ZDUNEK, E.; MITRUS, J.; WICHA, J.; WOLSKA, M.K.; SACHANOWICZ, K. Distribution of classical enterotoxin genes in staphylococci from milk of cows with- and without mastitis and the cowshed environment. **Pol. J. Vet. Sci**., v.17, p.407–411, 2014

SAWANT, A.A.; SORDILLO, L.M.; JAYARAO, B.M. A survey on antibiotic usage in dairy herds in Pennsylvania. **J. Dairy Sci**., v.88, p.2991–2999, 2005.

SCHLOTTER, K.; EHRICHT, R.; HOTZEL, H.; MONECKE, S.; PFEFFER, M.; DONAT, K. Leukocidin genes lukF-P83 and lukM are associated with Staphylococcus aureus clonal complexes 151, 479 and 133 isolated from bovine udder infections in Thuringia, Germany. **Vet. Res.**, 43, 42, 2012.

SHARIATI, L.; VALIDI, M.; HASHEMINIA, A.M.; GHASEMIKHAH, R.; KIANPOUR, F.; KARIMI, A.; NAFISI, M.R.; TABATABAIEFAR, M.A. Staphylococcus aureus isolates carrying Panton-Valentine leucocidin genes: Their frequency, antimicrobial patterns, and association with infectious disease in Shahrekord city, Southwest Iran. **Jundishapur J. Microbiol.**, 9, e28291, 2016.

SHARMA, V.; SHARMA, S.; DAHIYA, D.K.; KHAN, A.; MATHUR, M.; SHARMA, A. Coagulase gene polymorphism, enterotoxigenecity, biofilm production, and antibiotic resistance in Staphylococcus aureus isolated from bovine raw milk in North West India. **Ann. Clin. Microbiol. Antimicrob**, 16, 65, 2017.

SILVEIRA-FILHO, V.M.; LUZ, I.S.; CAMPOS, A.P.; SILVA, W.M.; BARROS, M.P.; MEDEIROS, E.S.; FREITAS, M.F.; MOTA, R.A.; SENA, M.J.; LEAL-BALBINO, T.C. Antibiotic resistance and molecular analysis of Staphylococcus aureus isolated from cow's milk and dairy products in northeast Brazil. **J. Food Prot**., 77, 583–591, 2014.

SOMMERHÄUSER, J.; KLOPPERT, B.; WOLTER, W.; ZSCHÖCK, M.; SOBIRAJ, A.; FAILING, K. The epidemiology of Staphylococcus aureus infections from subclinical mastitis in dairy cows during a control programme. **Vet. Microbiol**, 96, 91–102, 2003.

SUNG, J.M.; LLOYD, D.H.; LINDSAY, J.A. Staphylococcus aureus host specificity: Comparative genomics of human versus animal isolates by multi-strain microarray. **Microbiology**, v.154, p.1949–1959, 2008.

SYRING, C.; BOSS, R.; REIST, M.; BODMER, M.; HUMMERJOHANN, J.; GEHRIG, P.; GRABER, H.U. Bovine mastitis: The diagnostic properties of a PCR-based assay to monitor the Staphylococcus aureus genotype B status of a herd, using bulk tank milk. **J. Dairy Sci**., 95, 3674–3682, 2012.

VAN WAMEL, W.J.; ROOIJAKKERS, S.H.; RUYKEN, M.; VAN KESSEL, K.P.; VAN STRIJP, J.A. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of Staphylococcus aureus are located on beta-hemolysin-converting bacteriophages. **J. Bacteriol**, v.188, p.1310–1315, 2006.

ZADOKS, R.N.; MIDDLETON, J.R.; MCDOUGALL, S.; KATHOLM, J.; SCHUKKEN, Y.H. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. **J. Mammary Gland Biol. Neoplasia**, v.16, p.357–372, 2011.

ZECCONI, A.; CESARIS, L.; LIANDRIS, E.; DAPRA, V.; PICCININI, R. Role of several Staphylococcus aureus virulence factors on the inflammatory response in bovine mammary gland. **Microb. Pathog.**, v.40, p.177–183, 2006.

ZSCHÖCK, M.; KLOPPERT, B.; WOLTER, W.; HAMANN, H.P.; LAMMLER, C.H. Pattern of enterotoxin genes seg, seh, sei and sej positive Staphylococcus aureus isolated from bovine mastitis. **Vet. Microbiol**., v.108, p.243–249, 2005.

CHAPTER 4

Molecular characterization and antimicrobial resistance pattern of *Staphylococcus aureus* isolated from subclinical mastitis cows treated during lactation

4. MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL RESISTANCE PATTERN OF *Staphylococcus aureus* ISOLATED FROM SUBCLINICAL MASTITIS COWS TREATED DURING LACTATION

4.1 ABSTRACT

The aim of the study was to evaluate distribution of clonal groups and the antimicrobial resistance profiles of *Staphylococcus aureus* isolated from cows with subclinical mastitis (SCM) treated with antibiotics and vaccination during lactation. A total of 4 dairy herds with a high prevalence of SCM caused by *S. aureus* were selected to evaluate the efficiency of a three treatment protocols during lactation (no treatment; combination of intramammary and intramuscular antimicrobial therapy associated or not with vaccination). Based on species level identification using mass spectrometry (MALDI-TOF), 79 *S. aureus* isolates (63 pre-treatments and 16 post-treatment) were submitted for Pulsed-Field Gel Electrophoresis (PFGE) analysis, and minimum inhibitory concentration (MIC) antimicrobial susceptibility to 13 antimicrobials. According to the band patterns generated by PFGE, two dendograms were made to compare *S. aureus* pulsotypes. Considering the pre-treatment isolates, 53 were grouped into 7 distinct pulsotypes and 10 isolates could not be grouped. Considering only the mammary quarter with pre- and post-treatment evaluation, five different pulsotypes were observed and in three cows, *S. aureus* was isolated from the same quarter before and after treatment. In 4 cases, pre- and post-treatment isolates presented the same pulsotypes. All *S. aureus* isolates were susceptible to gentamicin, enrofloxacin, ciprofloxacin and tetracycline. On the other hand, resistance was observed in 100% of the isolates for amoxicillin, 96.2% for erythromycin and 77.2% for both ampicillin and penicillin. Enrofloxacin and ciprofloxacin had the lowest MIC_{90} values (≥ 0.125 μg / mL for both), while the highest MIC_{90} values were observed for amoxicillin, ampicillin, cephalexin and penicillin (≥ 128 μg / mL). One methicillin-resistant *S. aureus* (MRSA) was identified based on the resistance to cefoxitin. Our results demonstrated a considerable genetic diversity of *S. aureus* causing SCM, and their persistence in the quarter even after the antimicrobial treatment. Also, a high antimicrobial resistance to β-lactam antibiotics was identified in the MIC test.

4.2 INTRODUCTION

Staphylococcus aureus is a commensal skin pathogen, capable of infecting humans, cattle and a variety of other species. This microorganism is frequently isolated from raw milk (BOUFAIDA ASNOUNE et al., 2012; SAIDI; KHELEF; KAIDI, 2013) and its within-herd prevalence on Brazilian dairy farms was reported to be 7.4% (DITTMANN et al., 2017) and of 70.3% among herds (MESQUITA et al., 2019). Intramammary infections (IMI) caused by *S. aureus* usually present a cyclic pattern of somatic cell count (SCC) and bacterial shedding in milk. Considering that SCC are not regularly elevated, infected cows are usually not identified, allowing the transmission of the pathogen to other cows (ZECCONI et al., 2005). Therefore, *S. aureus* causing persistent-IMI in cows represent a high risk of transmission of new infections for healthy cows.

The mechanisms by which *S. aureus* induces chronic infections involve the modulation and evasion of the immune system such as the adherence and internalization in mammary epithelial cells, as well as the high level of antimicrobial resistance (ZECCONI; SCALI, 2013). Antimicrobial resistance represents an enormous challenge for the treatment of IMI caused by *S. aureus*, and more specifically the methicillin-resistant *S. aureus* (MRSA) strains, which are often resistant to other classes of antimicrobials and β-lactams (LEE, 2003; VAN DUIJKEREN et al., 2004).

The success of antimicrobial therapy against *S. aureus* can vary considerably because it depends on the cow factors, the bacterial strain and the treatment protocol (BARKEMA et al., 2006). Due to the presence of multiple virulence factors and antimicrobial resistance to conventional antimicrobial treatments, low cure rates for *S. aureus* IMI is usually observed. Approaches to increase the cure of *S. aureus* IMI have been studied whether using the combination of antimicrobial treatment with vaccines (CUNHA et al., 2020; HAMBALI et al., 2018; MISRA et al., 2018; PEREIRA et al., 2011) or by extended therapy (OLIVER et al., 2004; SKOULIKAS et al., 2018). However, it is not known whether there are genotypic differences in *S. aureus* isolates that cause mastitis during lactation before and after a treatment period.

Thus, the present study aimed to assess the distribution of clonal profiles and the antimicrobial resistance of *S. aureus* isolated from cows with subclinical mastitis (SCM) submitted to treatment protocols using antimicrobials and vaccination during lactation.

4.3. MATERIAL AND METHODS

All the experimental procedures were performed according the National Council of Control of Animal Experimentation (CONCEA) and approved by the Animal Use Ethics Committee of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA/FMVZ), n 8178270617.

4.3.1 Characterization of previous study and origin of isolates

A total of 79 strains of cryopreserved *S. aureus* isolated from mammary quarters (MQ) of subclinically infected dairy cows were selected from a companion study (PINHEIRO, 2016), to evaluate the genotypic diversity and antimicrobial resistance. Briefly, in the study by Pinheiro, (2016), *S. aureus* isolates were obtained from 47 cows with SCM distributed in 4 herds, which had been identified as having high prevalence of *S. aureus* (about 40% of lactating cows). Three treatment protocols during lactation were evaluated: 1) Control, no treatment (n = 30); 2) Combined antimicrobial therapy [ATMT – intramammary (IMM) plus systemic treatment]: IMM twice a day for 5 days using 75 mg of ampicillin with 200 mg of cloxacillin (Bovigam L, Bayer) along with single dose systemic enrofloxacin (7.5 mg / kg of body weight; Kinetomax, Bayer, Brazil) on day 1 (n = 36); and 3) ATMT combined with vaccination (TopVac, HIPRA, Brazil) (n = 13): 3 doses (14 days prior to, at antibiotic treatment day and 14 days' post-treatment).

Microbiological cultures were performed on milk samples collected at the day of treatments and three consecutive collections at 7-day intervals (7, 14 and 21d). The MQ that had no isolation of *S. aureus* in all three post-treatment sampling were considered cured; and the MQ that showed at least one positive isolation of *S. aureus* during post-treatment samplings were considered not cured. All *S. aureus* isolates were cryopreserved in 1.5 mL of brain heart infusion (BHI) broth and after 24 hours at 37 °C in aerobic condition, a 20% glycerin solution was added for later cryopreservation (-80 °C).

4.3.2 *S. aureus* identification by MALDI-TOF MS

After thawing, an aliquot of all cryopreserved isolates was firstly inoculated on blood agar plates (35 °C/24 h) and then a single colony was streaked onto the surface of Tryptic Soy

Agar (TSA) medium, followed by the incubation at 35 °C/24 h. After, one colony was applied to the MALDI-TOF steel plate spot with a disposable loop (BARCELOS et al., 2019). A volume of 1.0 µL of formic acid (70%) was applied to the spot and dried at room temperature. After drying, 1.0 µL of matrix solution, consisting of α-cyano-4-hydroxycinnamic acid (HCCA) diluted in 50% acetonitrile and 2.5% trifluoroacetic acid (Sigma-Aldrich Canada Inc., Oakville, ON, Canada) was applied, and again left to dry at room temperature. The plate reading was performed according to the specifications for ribosomal bacteria protein identification (Bruker Daltonik, Bremen, Germany) and the spectral data processing was done using the MALDI Biotyper 4.1.70 (Bruker Daltonik, Bremen, Germany) computer software for microorganism identification (MBT version 7311 MPS library). A standard protein solution (Bacterial Test Standard, BTS; Bruker) was used for MALDI-TOF MS calibration. A positive control (*Escherichia coli*) and a negative control (only formic acid and matrix) were analyzed on each plate. Scores ≥ 1.7 were considered reliable for genus identification, and scores ≥ 2.0 were considered reliable for genus and species identification. For the present study, isolates with identification score > 2 were selected (n = 79).

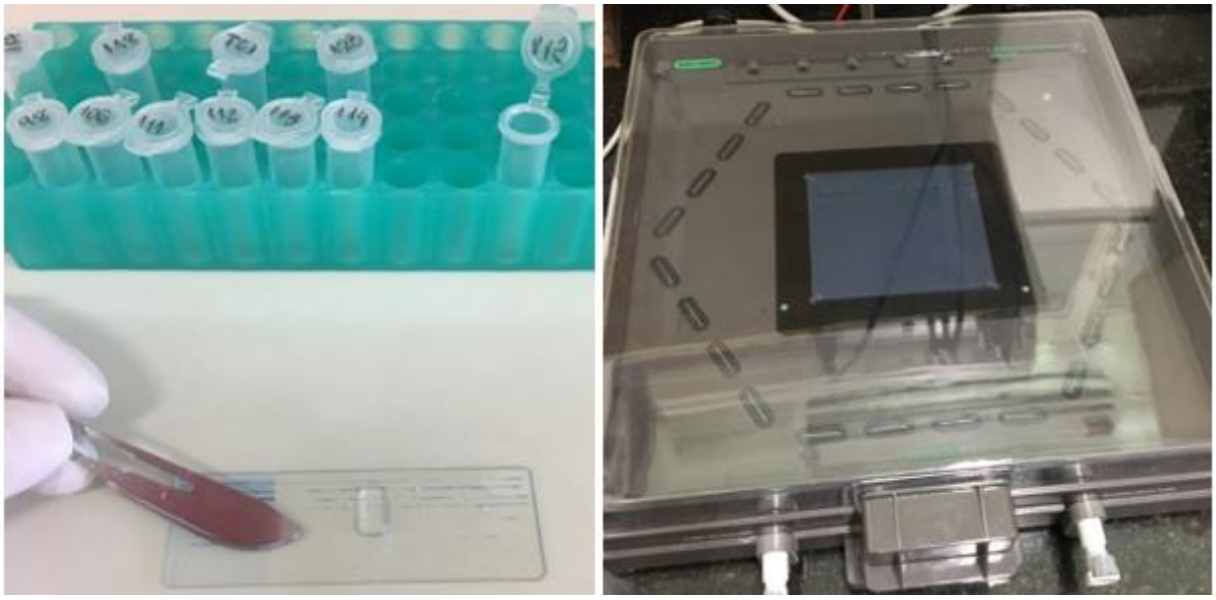
4.3.3 Pulsed-field gel electrophoresis (PFGE) genotyping

PFGE analyzes were performed according to a protocol defined by the Center for Disease Control and Prevention (CDC, 2016). After a 24-hour incubation in the TSA medium, one loop-full of colonies were inoculated in Brain Heart Infusion (BHI) broth and incubated at 37 °C for approximately 18 hours. After this period, the plugs were prepared by adding 2.5 µL of lysostaphin and 150 µL of 1.8% low melting agarose (BioRad, France) previously diluted in 0.5x TBE buffer. This mixture was quickly dispensed in a mold and the plugs were dried at room temperature. After drying, the plugs were soaked in EC solution (Tris HCl 1M; NaCl 2M; EDTA tetrasodium 1M; sodium deoxycholate 2%; sarcosil 5%) and incubated overnight at 37 °C. Subsequently, the plugs were washed and digested with 30 U of SmaI restriction enzyme (Fast Digest Thermo Fisher), according to the manufacturer's recommendations.

The gel was made in a 15-well mold (12 samples + 3 standards) and PFGE was performed in a CHEF-DR III system (Bio-Rad Laboratories, Inc., CA, USA) according to the following parameters: 6 V / cm, temperature 14 °C, initial pulse 5 s, final pulse 40 s, angle included 120 °C and operating time 22 h (Figure 5). After running, the gel was stained in ethidium bromide solution (final concentration 1 mg / ml) for 30-40 min in a covered container. The images were captured by an UV transilluminator and the DNA fragments of ATCC BAA-

664 (*Salmonella* ser. Braenderup H9812) were used as molecular weight markers for XbaI digestion standards.

Figure 5 - Plugs with *Staphylococcus aureus* DNA and CHEF-DR III System for PFGE



Source: Alves, B.G. (2020).

4.3.4 Antimicrobial resistance analyses

The minimum inhibitory concentrations (MIC) analyzes were performed for *S. aureus* isolates using microdilution broth method (CLSI, 2013). Seventy-nine isolates and the *S. aureus* ATCC 29213 strain were used to determine the MIC of 13 ATMs (amoxicillin, ampicillin, ceftiofur, gentamicin, penicillin, enrofloxacin, oxytetracycline, tetracycline, ciprofloxacin, cephalexin, lincomycin, erythromycin and cefoxitin). Twelve serial dilutions were performed (concentrations ranging from 0.03 to 64 μg / mL) using stock solutions at a concentration of 1mg / mL prepared using specific solvents for each antimicrobial, according CLSI (2013) (Figure 6).

Briefly, selected colonies were inoculated on blood agar and after 24 hours at 37 ºC, they were transferred to BHI broth and reincubated under the same conditions. Upon growth, the colonies were suspended in 2 mL of sterile 0.9% saline and, turbidity was adjusted to 0.5 McFarland scale, containing approximately 1.5×10^8 cfu / mL, using a nephelometer (Uniscience, São Paulo, Brazil).

For microdilution assay, sterile microplates with 96 wells were used, filled with Mueller Hinton broth (MH) adjusted to pH 7.2 ± 0.2 (CLSI, 2013). For each isolate, 2 microplates (A

and B) were used, and the control rows were distributed along with the 13 ATM (Figure 7). A total of two-fold serial dilutions were made to obtain concentrations of the twelve antimicrobials ranging from 64 to 0.03 µg / mL. After dilutions, 12.5 µL of the inoculum of *S. aureus* isolates were added to each well (one plate per inoculum) and incubated aerobically for 24 hours at 37 °C.

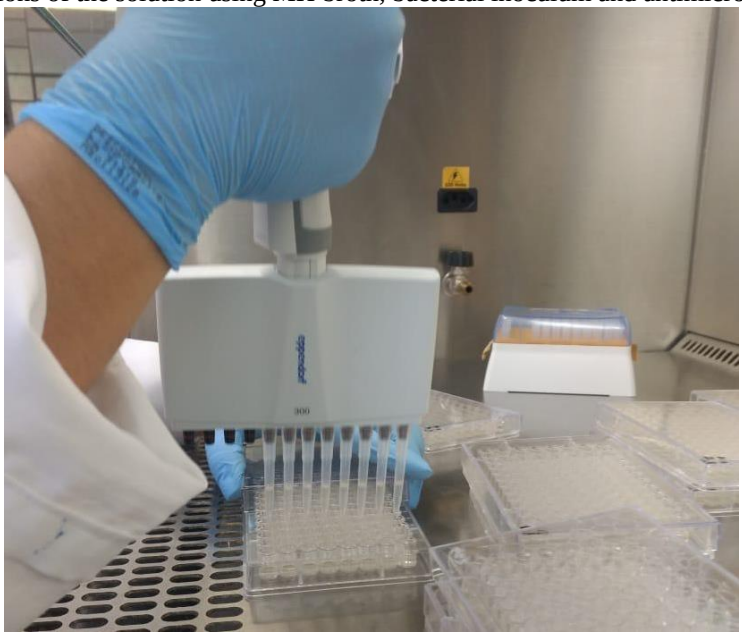
Figure 6 - Arrangement of antimicrobials in the microplate used to determine the minimum inhibitory concentration

Dilutions (µg/mL) – Plate A													
A	Amoxicillin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
B	Ampicillin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
C	Cephalexin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
D	Ceftiofur	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
E	Ciprofloxacin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
F	Cefoxitin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
G	Enrofloxacin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
H	Control	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31

Dilutions (µg/mL) – Plate B													
A	Erytromicin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
B	Gentamicin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
C	Lincomycin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
D	Oxytetracycline	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
E	Penicillin	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
F	Tetracycline	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
G	Control	64	32	16	8	4	2	1	0,5	0,25	0,125	0,62	0,31
H													

Source: Alves, B.G. (2020).

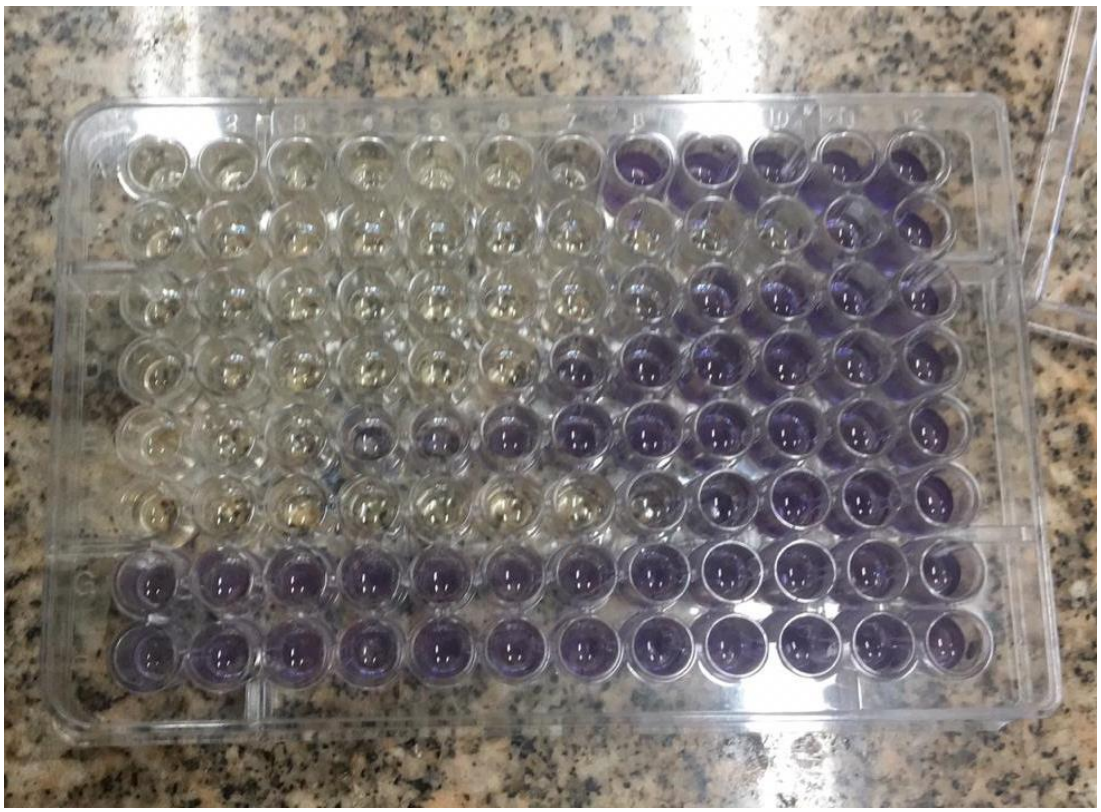
Figure 7 - Serial dilutions of the solution using MH broth, bacterial inoculum and antimicrobials



Source: Alves, B.G. (2020).

Following the 24-hour incubation, 20 μL of the MTT reagent (Thiazolyl Blue Tetrazolium Bromide, Sigma) was pipetted into each well, and after three hours they were visually read. The purple staining generated by MTT indicated the presence of viable cells (Figure 8). The MIC was defined as the lowest concentration of each antimicrobial that inhibited the visible growth of *S. aureus*. The dilution ranges for each ATM and the interpretation criteria for determining the susceptible, intermediate and resistant are shown in Table 12. Isolates with intermediate susceptibility were considered resistant (TOMAZI et al., 2018). Strains of *S. aureus* resistant to cefoxitin were classified as MRSA (FERNANDES; FERNANDES; COLLIGNON, 2005).

Figure 8 - Evaluation of the minimum inhibitory concentration of *S. aureus* by the broth microdilution method



Source: Alves, B.G. (2020).

Table 12 - Dilution ranges and susceptibility limits used for 13 antimicrobials to determine susceptibilities of *S. aureus* isolated from subclinical mastitis

Antimicrobial Classes		MIC Breakpoints (μg/mL)		
		Susceptible	Intermediate	Resistant
Penicillin and derivatives	Amoxicillin [1]	≤ 0.25	-	≥ 0.5
	Ampicillin [1]	≤ 0.25	-	≥ 0.5
	Penicillin[1]	≤ 0.12	-	≥ 0.25
Cephalosporins	Ceftiofur[1]	≤ 2	4	≥ 8
	Cephalexin[2]	≤ 4	-	≥ 8
	Cefoxitin[1]	≤ 4	-	≥ 8
Tetracyclines	Oxytetracycline[1]	≤ 4	8	≥ 16
	Tetracycline[1]	≤ 4	8	≥ 16
Aminoglycosides	Gentamicin [1]	≤ 4	8	≥ 16
Macrolides	Erythromycin[1]	≤ 0.5	1-4	≥ 8
Lincosamides	Lincomycin[1]	≤ 0.5	1-2	≥ 4
Quinolones	Enrofloxacin[3]	≤ 0.25	-	≥ 4
	Ciprofloxacin[2]	≤ 1	-	> 1

[1]Susceptibility limits defined according to CLSI, 2013. The standards were based on humans, with the exception of ceftiofur, based on bovine mastitis data.[2]Susceptibility limits defined according to EUCAST v.10 (2020).[3]Susceptibility limit defined according to Rubin; Ball; Chirino-Trejo (2011).
Source: Alves, B.G. (2020).

4.3.5 Statistical Analysis

4.3.5.1 PFGE-genotyping

The DICE coefficient and the Average Association Method (UPGMA) were used to compare the fingerprints obtained by PFGE, using a 2% tolerance according the BioNumerics software version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Two different comparisons were made, according to the evaluated treatments and persistence of the same profile in the same MQ, both following the 80% similarity criteria for the clusters, considered the gold standard (MCDOUGAL et al., 2003; VAN BELKUM et al., 2007). In addition, a minimum of 2 isolates was used to classify a cluster.

4.3.5.2 Antimicrobial resistance

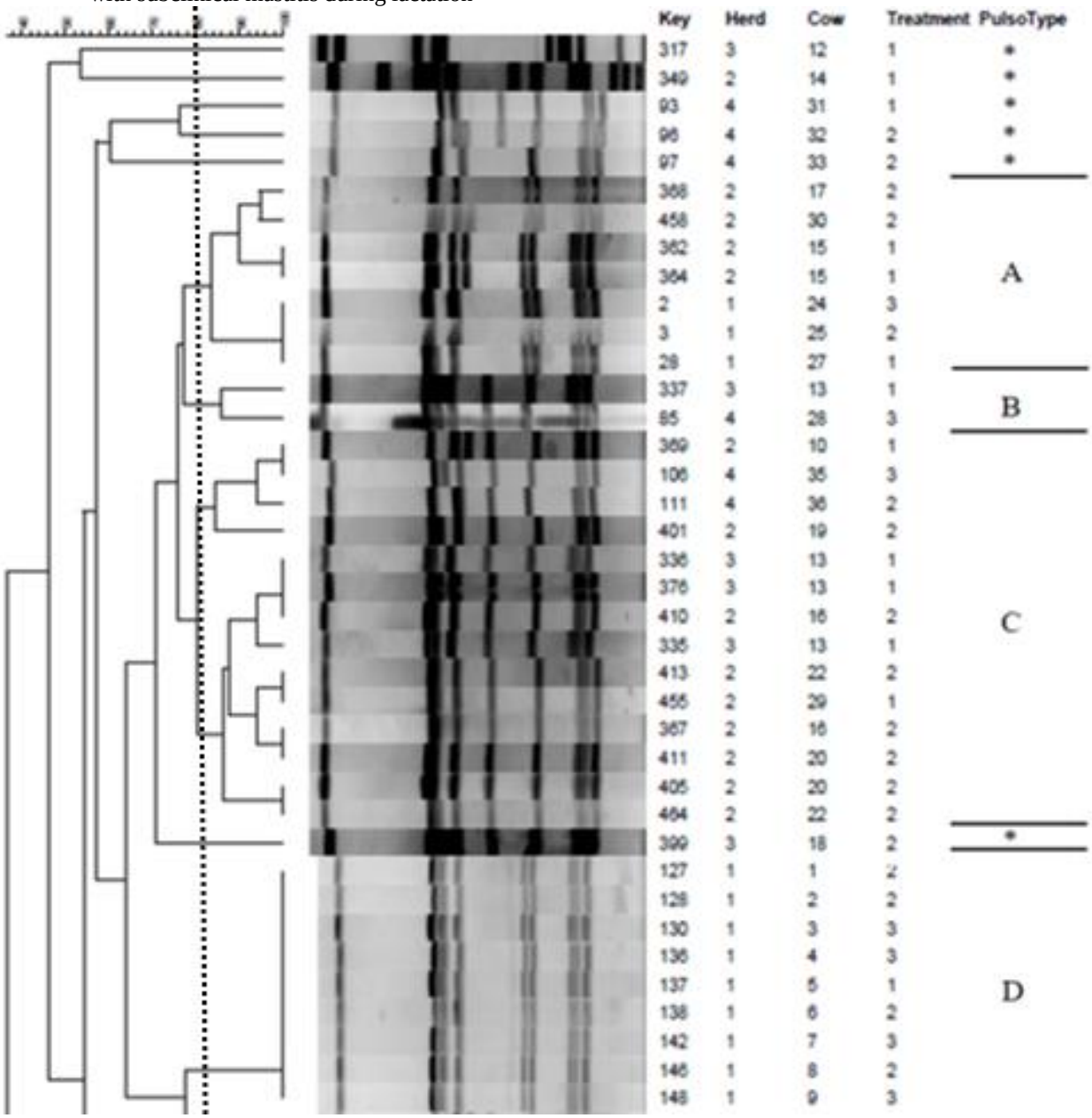
Survival analysis was performed using PROC LIFETEST (SAS ver. 9.4, SAS Institute Inc., Cary, NC) to determine MIC and concentrations tested as a time variable (CORTINHAS et al., 2013). The isolates that grew in the highest concentration tested (100 μg / mL) were considered resistant. The MIC value was given by the lowest concentration of each antimicrobial that visibly inhibited the growth of *S. aureus* isolates. MIC_{50} and MIC_{90} were determined based on inhibition of 50 and 90% of the tested isolates, respectively. The antimicrobial concentration was defined as variable over time, while the non-difference between the survival curves was tested using the Log-rank and Wilcoxon tests, on the Kaplan-Meier curves. Values of $P < 0.05$ were considered significant.

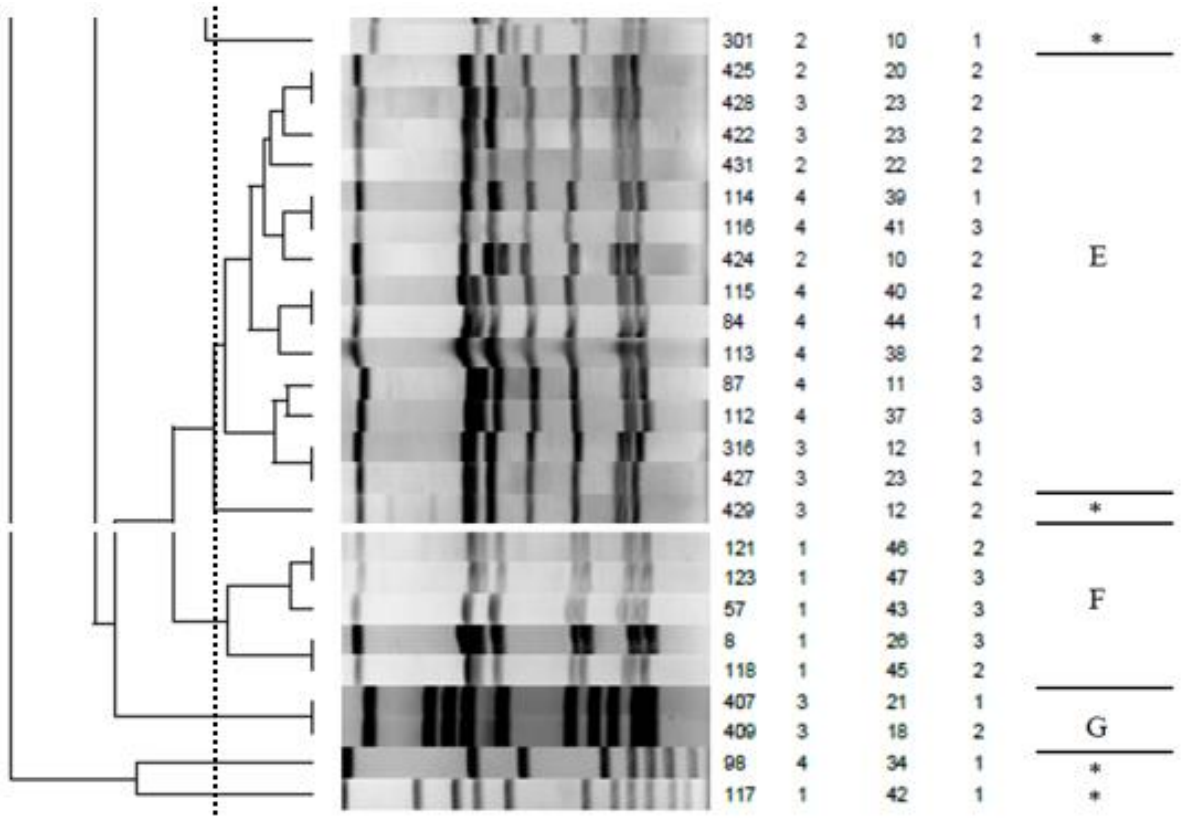
4.4 RESULTS

4.4.1 PFGE analysis

Figure 9 shows the dendrogram of *S. aureus* isolates that were collected prior to treatments (n=63). A total of 53 isolates were grouped into 7 distinct pulsotypes (A-G), in which pulsotypes C and E being the most prevalent (n = 14), followed by D, (n = 9). The isolates designated as pulsotypes C and E originated from herds 2, 3 and 4, whereas that of pulsotype D originated only from herd 1. Of the total evaluated, 10 isolates had unique PGFE pattern.

Figure 9 - Pulsed Field Gel Electrophoresis (PFGE) dendrogram of 63 isolates of *S. aureus* obtained from cows with subclinical mastitis during lactation

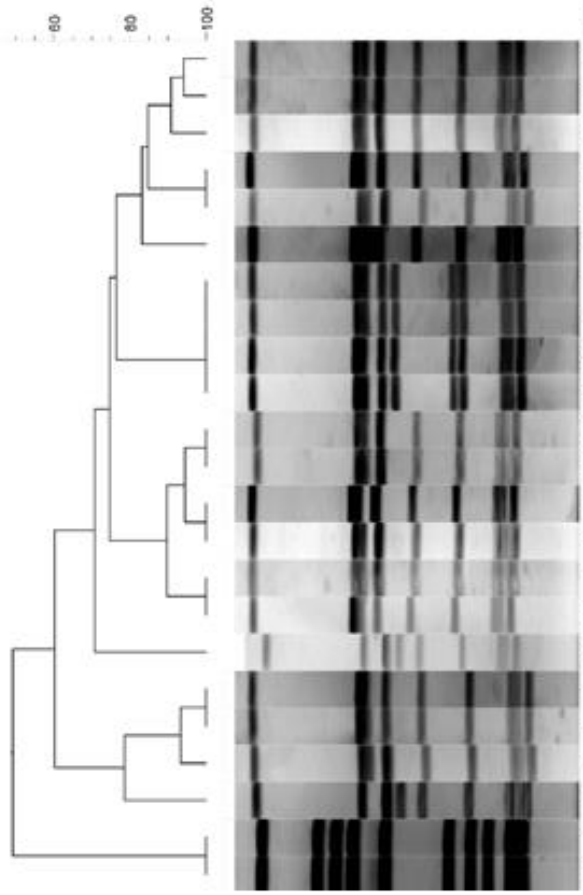




Legend: The dotted vertical line indicates the cutoff point (80% similarity). Key = isolates. The treatments correspond to: 1) Control, no treatment; 2) antibiotic therapy (combined intramammary (IMM) and systemic): IMM treatment given twice a day for 5 days using 75 mg of ampicillin with 200 mg of cloxacillin along with single dose IM enrofloxacin (7.5 mg / kg of body weight) on day 1; and 3) antibiotic therapy combined with vaccination: 3 doses (14 days prior to, at treatment and 14 days' post-treatment). * = isolates that had unique PGFE pattern. Source: Alves, B.G. (2020).

To assess the frequency of pulsotypes of persistent *S. aureus* IMI, a second comparison was made, considering only those isolates from the same uncured MQ, collected before and after treatment protocols during lactation. A total of seven cows which had no bacteriological cure were selected and 23 isolates were classified into 5 different pulsotypes (A-E) (Figure 10). In 10 MQ it was possible to identify pulsotypes before and after treatment protocols. Of these, 6 MQ (60%) presented a change in the genotypic profile of the infecting strain after the treatments applied (represented by the letter B after the treatment number). For 4 MQ of 3 cows (26, 45 and 46) there was no change in the classification of the pulsotype before and after the treatment applied. Two isolates had unique PGFE pattern.

Figure 10 - Pulsed Field Gel Electrophoresis (PFGE) dendrogram of *S. aureus* isolates obtained from milk samples from uncured cows with subclinical mastitis during lactation submitted to antimicrobial treatment and vaccination



Key	Herd	Cow	Treatment	Quarter	Cure	PulsoType
335	3	45	1	RF	No	A
336	3	45	1	LR	No	A
419	3	45	1-B	LR	No	A
406	2	19	1-B	RF	No	A
417	3	43	2-B	RR	No	A
337	3	45	1	RR	No	A
357	2	26	1-B	LF	No	B
358	2	26	1-B	LR	No	B
362	2	26	1	LF	No	B
364	2	26	1	LR	No	B
329	3	46	1-B	RF	No	C
330	3	46	2-B	RR	No	C
316	3	46	1	RF	No	C
418	3	45	1-B	RF	No	C
420	3	45	1-B	RR	No	C
433	2	25	2-B	LF	No	C
301	2	23	1	LF	No	*
413	2	25	2	LF	No	D
455	2	19	1	RF	No	D
438	3	46	1-B	LF	No	D
404	2	23	1-B	LF	No	*
407	3	46	1	LF	No	E
409	3	43	2	RR	No	E

Legend: The dotted vertical line indicates the cutoff point (80% similarity). The isolates are identified by 'key'. Letter B indicates isolate collected after treatment during lactation while its absence indicates isolate collected prior to treatments. 1) Control, no treatment; 2) antibiotic therapy (combined intramammary (IMM) and systemic): IMM treatment given twice a day for 5 days using 75 mg of ampicillin with 200 mg of cloxacillin along with single dose IM enrofloxacin (7.5 mg / kg of body weight) on day 1; and 3) antibiotic therapy combined with vaccination: 3 doses (14 days prior to, at treatment and 14 days' post-treatment). * = isolates that had unique PGFE pattern. Source: Alves, B.G. (2020).

4.4.2 MIC analysis

The antimicrobial susceptibility testing of *S. aureus* isolates showed 100% of susceptibility to gentamicin, enrofloxacin, ciprofloxacin and tetracycline, followed by oxytetracycline (98.7%) and ceftiofur (94.9%; Table 13). On the other hand, lower antimicrobial susceptibilities (both with 22.7%) were observed for ampicillin and penicillin. For amoxicillin and erythromycin, resistance was observed in 100% and 96.2% of evaluated isolates, respectively. Only one isolate showed cefoxitin resistance.

Table 13 - Antimicrobial susceptibilities of 79 strains of *Staphylococcus aureus* isolated from subclinical mastitis in 4 dairy herds in Brazil

Antimicrobial	Res.[2] (%)	Frequency (%) of isolates at each indicated MIC (µg/ mL)													MIC_{50} [3]	MIC_{90} [4]
		0.01	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128		
Amoxicillin	92.4	-	-	-	-	-	2.5	1.3	0.0	0.0	1.3	1.3	2.5	91.1	≥128	≥128
Ampicillin	46.8	17.7	3.8	0.0	1.3	3.8	0.0	0.0	0.0	3.8	5.0	7.6	11.4	45.6	≥64	≥128
Cefalexin	46.8	-	-	-	7.6	14.0	19.0	7.6	1.3	2.5	0.0	3.8	6.4	37.8	≥2	≥128
Cefoxitin	0	-	-	-	-	-	11.4	74.7	12.6	1.3	-	-	-	-	≥2	≥4
Ceftiofur	0	-	-	-	-	1.3	51.9	41.8	5.0	-	-	-	-	-	≥1	≥2
Ciprofloxacin	0	-	20.3	73.4	5.0	1.3	-	-		-	-	-	-	-	≥0.125	≥0.125
Enrofloxacin	0	14.0	64.5	20.2	1.3	-	-	-	-	-	-	-	-	-	≥0.06	≥0.125
Erythromycin	0	-	-	-	-	3.8	78.4	17.8	-	-	-	-	-	-	≥1	≥2
Gentamicin	0	-	-	8.9	63.3	25.3	2.5	-	-	-	-	-	-	-	≥0.25	≥0.5
Lincomycin	0	-	-	-	5.0	46.8	36.7	6.4	2.5	1.3	1.3	-	-	-	≥0.5	≥2
Oxytetracycline	0	-	-	-	-	-	1.3	56.9	40.5	1.3	-	-	-	-	≥2	≥4
Penicillin	60.7	20.2	1.3	1.3	0.0	0.0	0.0	1.3	0.0	0.0	1.3	2.5	11.4	60.7	≥128	≥128
Tetracycline	0	1.3	1.3	0.0	7.6	54.4	34.1	1.3	-	-	-	-	-	-	≥0.5	≥1

**P value* Log-Rank = <.0001, *P value* Wilcoxon = 0.0015

MIC: Minimum Inhibitory Concentration

[1]The results were interpreted according to CLSI (2013); EUCAST v.10 (2020) and Rubin; Ball; Chirino-Trejo (2011). The resistant category included those isolates classified as intermediate or resistant. Light shading represents the sensitivity zone and dark shading represents the resistant zone.

[2]Isolates resistant to the highest concentration of antimicrobial tested

[3]MIC_{50} = MIC (µg/mL) that inhibited 50% of the isolates.

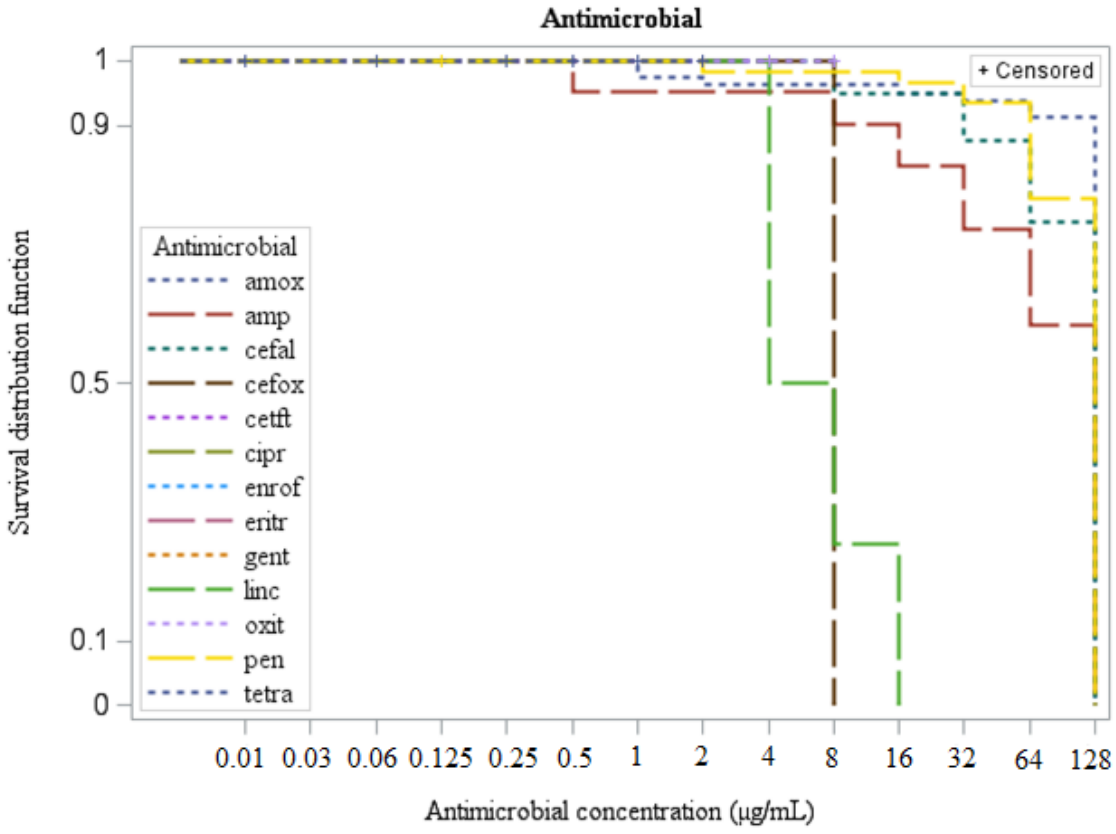
[4]MIC_{90} = MIC (µg/mL) that inhibited 90% of the isolates.

Source: Alves, B.G. (2020).

The lowest MIC_{90} values for *S. aureus* were observed for enrofloxacin and ciprofloxacin, both ≥ 0.0125 µg / mL; while amoxicillin, ampicillin, cephalexin and penicillin had the highest MIC_{90} values (≥ 128 µg / mL). According to the MIC_{50}, enrofloxacin had the lowest value (≥ 0.06 µg / mL), while amoxicillin and penicillin had the highest values (≥ 128 µg / mL).

The Kaplan-Meier survival curves showed homogeneous curves between ceftiofur, ciprofloxacin, enrofloxacin, erythromycin, gentamicin, oxytetracycline and tetracycline. For the others antimicrobials, heterogeneous curves were observed between the ATMs used against *S. aureus* (*P* <.0001 for Log-Rank test and *P* = 0.0015 for Wilcoxon test; Figure 11).

Figure 11 - Kaplan-Meier survival curves for 79 *S. aureus* isolated from subclinical mastitis and stratified based on 13 antimicrobials tested in susceptibility testing



Source: Alves, B.G. (2020).

4.5 DISCUSSION

In this study, *S. aureus* isolates collected from cows with SCM were submitted to PFGE to characterize the genotypic diversity before and after treatments during lactation. A great

diversity of strains was observed within each herd and between herds, which corroborates to the hypothesis of the high genetic variability of *S. aureus* that causes IMI in high prevalence herds, although high frequent pulsotypes (C, D and E) were identified within herds and among herds. In addition, high levels of ATM resistance were observed, especially for β-lactam antibiotics.

This specific predominance of some pulsotypes within a specific herd showing that the transmission between cows occurred possibly from a common source. Similar to our study, Rabello et al., (2005), Vitale et al., (2018) and Srednik et al., (2018) also reported a wide genotypic diversity of *S. aureus* when PFGE was used. Rabello et al., (2005) found 16 distinct pulsotypes (15%) and 24 subtypes considering 107 *S. aureus* isolates, among nine dairy herds. A greater genetic variability of *S. aureus* (29%) was reported in a study using 80 *S. aureus* isolates from clinical or subclinical mastitis, in which 23 distinct pulsotypes were observed, using 80% of similarity (SREDNIK et al., 2018), similarly to the present study indicating a high capacity of transmission of specific pulsotypes within dairy herds. Even using 100% of similarity, Dorneles et al., (2019) also observed high genotypic variability in 79 *S. aureus* isolates (34 different pulsotypes).

In the present study, 84.1% of the isolates were grouped into 7 distinct pulsotypes, considering only *S. aureus* isolated before treatments, with pulsotypes C and E being more prevalent. A large proportion of pulsotypes C (64.2%) and E (100%) were observed in only a single herd. This high prevalence of specific pulsotypes C and E suggests a common source of infected cows, which could spread from cow to cow during milking. This prevalence of the same *S. aureus* pulsotypes within a specific herd could be partially explained by the pathogen ability of adapting to a specific condition of the herd (GOERKE; WOLZ, 2004; TUCHSCHERR et al., 2010). Five pulsotypes were observed from 23 *S. aureus* isolated from uncured IMI in the same MQ (isolated before and after treatment). Pulsotypes A and C were the most prevalent (26%), mostly represented by isolates belonging to the same herd. Of the 10 MQ evaluated from 6 cows, 40% (n = 4 MQ) had the same pulsotypes before and after treatment protocols, which suggests the persistence of the same strain and the ineffectiveness of the treatment protocols. All of these isolates (n = 8) showed the same pattern in MIC, being resistant to ampicillin and sensitive to enrofloxacin, the antimicrobial bases used in the in vivo experiment. However, this sensitivity to enrofloxacin was not sufficient to modify the pattern of the infecting strain.

The high percentage of strains resistant to β-lactams in the present study was similar to that observed in other previous studies such as Daka et al., (2012), who observed that 67.9% of 78 *S. aureus* strains of MQ was resistant to penicillin G. In Brazil, resistance to β-lactams has also been observed between 18 to 95% in *S. aureus* isolated from bovine milk (CARVALHO et al., 2018; FERREIRA et al., 2016; GIRARDINI et al., 2016; MEDEIROS et al., 2009; SILVA et al., 2012). Also, a high percentage of strains in the present study were resistant to erythromycin, which results are similar to those reported in a previous study in which 17% of the strains were classified as MRSA (n = 16/93), and all were resistant to erythromycin and carried at least one resistance gene *ermA*, *ermB* or *ermC* (GATERMANN; KOSCHINSKI; FRIEDRICH, 2007; TÜRKYILMAZ et al., 2010). A total of 22.6% resistance to erythromycin was observed in a study that evaluated the phenotypic susceptibility against 93 *S. aureus* isolates (MONISTERO et al., 2020).

In the present study, the resistance to cefoxitin was used as a predictor of MRSA strains, as it was used in susceptibility tests to identify strains resistant to methicillin (FERNANDES; FERNANDES; COLLIGNON, 2005), since methicillin is no longer manufactured (CLSI, 2013; HO et al., 2016; SWENSON et al., 2009; ZURITA; MEJÍA; GUZMAN-BLANCO et al., 2010). We observed only 1 (1.2%) isolate resistant to cefoxitin, classified as MRSA, which indicated a low MRSA diffusion among bovine mastitis isolates as previously reported (MONISTERO et al., 2018). Differently of our study, Papadopoulos et al., (2019) observed 11 MRSA strains in samples of bulk tank milk, dairy products and swabs from workers and equipment surfaces (3.6%). In addition, in the later study it was described 99% resistance of the isolates for at least 1 of the tested ATMs, 22% being classified as multidrug-resistant. Although high rates of MRSA are not usually found in bovine mastitis samples, some factors can be discussed as variations in responses to MRSA, mainly due to the presence of the *mecA* gene sequence, known to generate PBP, the protein that reduces the body's affinity to connect to β-lactam antibiotics. This lack of analyses for the presence of genes was a limitation of our study, although the test with cefoxitin as a marker for the detection of MRSA is very accurate, both with disc diffusion and agar dilution methods (FERNANDES; FERNANDES; COLLIGNON, 2005).

Finally, the survival analysis of antimicrobials susceptibility testing suggested that all *S. aureus* isolates were susceptible to gentamicin, enrofloxacin and tetracycline hydrochloride, as observed in previous studies (DORNELES et al., 2019; TEIXEIRA et al., 2014; YANG et al., 2016). In Brazil, Teixeira et al. (2014) evaluated the MIC of 278 *S. aureus* isolates from

bulk tank milk and observed 94% and 92.54% of sensitivity to gentamicin and enrofloxacin, respectively. Similarly to the present study, high susceptibility of *S. aureus* isolated from bovine mastitis (n = 44) was observed for gentamicin (90.91%) and tetracycline (81.82%) in an ATM resistance study in China (YANG et al., 2016). More recently, Dorneles et al. (2019) observed a high sensitivity of gentamicin (97.2%) and enrofloxacin (98.6%) against *S. aureus* (n = 71) mastitis-causing.

Although it was possible to observe a high cure rate for antibiotic treatments and vaccination by Pinheiro (2016), it is suggested that this high rate is attributed due to the systemic application of enrofloxacin, since 100% of the isolates evaluated were resistant to the ampicillin used in the intramammary treatment. On the other hand, almost all strains were shown to be sensitive to enrofloxacin by the MIC test. With very similar results, a Brazilian study also aimed to evaluate the efficacy of enrofloxacin in the treatment of subclinical bovine mastitis caused by *S. aureus* (LANGONI et al., 2000) and observed that of the 184 MQ infected by *S. aureus*, enrofloxacina had cure rates of 72% and 75%, via intramammary and systemic routes, respectively. Another more recent study, however, with sheep clinical mastitis, found a higher cure rate (82%) than that found in the present study, also with enrofloxacin injectable against clinical mastitis by *S. aureus* (ATTILI et al., 2016). In addition, the extended duration of therapy during lactation also afected cure rate, as demonstrated by Roy; Keefe (2012). Of the MQ evaluated before and after the treatment protocol, it was possible to observe that there were no differences in the sensitivity of cured and uncured isolates, all of them showed resistance to ampicillin, applied intramammary. However, all of isolates showed sensitivity to enrofloxacin, which may have been the reason for the responses of the cured. A limitation was the lack of a group with only intramammary ampicillin, and thus the success of the treatment would be isolated from the intramuscular application of enrofloxacin.

The use of vaccination in combination with antimicrobial therapy increased the cure of treatment against *S. aureus* during lactation, by 17.9 percentage points when compared to treatment with antibiotics alone. Another study, which evaluated the efficacy of the same vaccine used in the present study, found differences in the rate of cure depending on the farm and the number of lactation of the animals, which on average was around 45% (SCHUKKEN et al., 2014). However, this authors only used the vaccine and no antimicrobials treatment was done in combination. And the same authors point out that the use of the vaccine must be carefully implemented in excellent conditions for milking and handling infected animals.

In the present study, 100% of the isolates showed sensitivity to ciprofloxacin, which was similar to the results reported by Zanette, Scapin, Rossi (2010) and Sun et al., (2018), 97.4% and 86.5%, respectively. Although ciprofloxacin use is restricted in some countries and not approved for use in food producing animals (JACOBY, 2005), it's approved in some developing countries as an antimicrobial option for treatment of mastitis due to its effectiveness (JOSHI; GOKALE, 2006), alone or combined with anti-inflammatory drugs. A possible explanation for these high rates of sensitivity to ciprofloxacin is due to its ability, as well as the other antimicrobials of the quinolone group, to accumulate in the phagocytic cell, possibly increasing the death of the mastitis-causing pathogen (QIN; SUN, 2009).

Even so, more epidemiological studies are certainly needed to be able to search for expression of resistance genes among *S. aureus* isolates distributed in dairy herds, especially the presence of methicillin-resistant, which constitute a risk factor for public health.

4.6 CONCLUSION

It was observed that even after antimicrobial treatment during lactation, there was a great diversity of *S. aureus* distributed within and among herds, although a specific pattern per herd tends to be formed. Still, there was persistence of pulsotypes observed in the same MQ. Susceptibility tests indicate variation in resistance profiles, although with great susceptibility to quinolones and aminoglycosides.

4.7 REFERENCES

ATTILI, A.R.; PREZIUSO, S; NGU NGWA, V.; CANTALAMESSA, A.; MORICONI, M.; CUTERI, V. Clinical evaluation of the use of enrofloxacin against Staphylococcus aureus clinical mastitis in sheep. **Small Ruminant Res**, v.136, p.72-77, 2016.

BARCELOS, M.M.; MARTINS, L.; GRENFELL, R.C.; JULIANO, L.; ANDERSON, K.L.; DOS SANTOS, M. V.; GONÇALVES, J.L. Comparison of standard and on-plate extraction protocols for identification of mastitis-causing bacteria by MALDI-TOF MS. **Brazilian J. Microbiol**. 2019.

BARKEMA, H.W.; SCHUKKEN, Y.H.; ZADOKS, R.N. Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine Staphylococcus aureus mastitis. **J. Dairy Sci.** v.89, p.1877–95, 2006.

BOUFAIDA ASNOUNE, Z.; BUTEL, M.J.; OUZROUT, R. Prévalence des principales bactéries responsables de mammites subcliniques des vaches laitières au nord-est de l'Algérie. **Rev. d'élevage médecine vétérinaire des pays Trop**. 65, 5., 2012.

CARVALHO, A.S.S.; SERRA, J.L.; RODRIGUES, L.C.; RODRIGUES JUNIOR, L.S.; MOUCHREK, A.N.; FERREIRA, E.M. Susceptibility of staphylococcus aureus isolated from raw milk to commercial antibiotics. **Cienc. anim. bras** v.19, p.1–8, 2018.

CLSI. **Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals: Approved Standard**. 4th ed. CLSI document Vet 01-A4. Clinical and Laboratory Standards Institute, Wayne, PA, 2013.

CORTINHAS, C.S.; OLIVEIRA, L.; HULLAND, C.A.; SANTOS, M.V.; RUEGG, P.L. Minimum inhibitory concentrations of cephalosporin compounds and their active metabolites for selected mastitis pathogens. **AJVRAm J Vet Res** v.7474, p.683–690, 2013.

CUNHA, A.F.; ANDRADE, H.M.; SOUZA, F.N.; FIALHO JÚNIOR, L.C.; ROSA, D.L.S.O.; RAMOS SANCHEZ, E.M.; GIDLUND, M.; GOTO, H.; BRITO, M.A.V.P.; GUIMARÃES, A.S.; LAGE, A.P.; REIS, L.C.; DELLA LIBERA, A.M.M.P.; HEINEMANN, M.B.; CERQUEIRA, M.M.O.P. Comparison of antibody repertories against Staphylococcus aureus in healthy and infected dairy cows with a distinct mastitis history and vaccinated with a polyvalent mastitis vaccine. **J. Dairy Sci.** v.103, p.4588–4605, 2020.

DAKA, D.; GSILASSIE, S.; YIHDEGO, D. Antibiotic-resistance Staphylococcus aureus isolated from cow's milk in the Hawassa area, South Ethiopia. **Ann. Clin. Microbiol. Antimicrob**. 11, 26. 2012.

DITTMANN, K.K.; CHAUL, L.T.; LEE, S.H.I.; CORASSIN, C.H.; DE OLIVEIRA, C.A.F.; DE MARTINIS, E.C.P.; ALVES, V.F.; GRAM, L.; OXARAN, V., Staphylococcus aureus in some Brazilian dairy industries: Changes of contamination and diversity. **Front. Microbiol**. 8. 2017.

DORNELES, E.M.S.; FONSECA, M.D.A.M.; ABREU, J.A.P.; LAGE, A.P.; BRITO, M.A.V.P.; PEREIRA, C.R.; BRANDÃO, H.M.; GUIMARÃES, A.S.; HEINEMANN, M.B. Genetic diversity and antimicrobial resistance in Staphylococcus aureus and coagulase-negative Staphylococcus isolates from bovine mastitis in Minas Gerais, Brazil. **Microbiology open** 8, 1–7, 2019.

EUCAST. **The European Committee on Antimicrobial Susceptibility Testing**. Breakpoint Tables for Interpretation ofMICs and Zone Diameters. Version 10.0, 2020. Available at: http://www.eucast.org

FERNANDES, C.J.; FERNANDES, L.A.; COLLIGNON, P. Cefoxitin resistance as a surrogate marker for the detection of methicillin-resistant Staphylococcus aureus. **J. Antimicrob. Chemother**. v.55, p.506–510, 2005.

FERREIRA, M.A.; BERNARDO, L.G.; NEVES, L.S.; CAMPOS, M.R.H.; LAMARO-CARDOSO, J.; ANDRÉ, M.C.P. Virulence profile and genetic variability of Staphylococcus aureus isolated from artisanal cheese. **J. Dairy Sci**. v.99, p.8589–8597, 2016.

GATERMANN, S.G.; KOSCHINSKI, T.; FRIEDRICH, S. Distribution and expression of macrolide resistance genes in coagulase-negative staphylococci. **Clin. Microbiol. Infect.** v.13, p.777–781, 2007.

GIRARDINI, L.K.; PAIM, D.S.; AUSANI, T.C.; LOPES, G. V.; PELLEGRINI, D.C.P.; BRITO, M.A.V.P.; CARDOSO, M. Antimicrobial resistance profiles of Staphylococcus aureus clusters on small dairy farms in southern **Brazil. Pesq. Vet. Bras** v.36, p.951–956, 2016.

GOERKE, C.; WOLZ, C. Regulatory and genomic plasticity of Staphylococcus aureus during persistent colonization and infection. **Int. J. Med. Microbiol**. v.294, p.195–202, 2004.

HAMBALI, I.U.; BHUTTO, K.R.; JESSE, F.F.A.; LAWAN, A.; ODHAH, M.N.; WAHID, A.H.; AZMI, M.L.M.; ZAKARIA, Z.; ARSALAN, M.; MUHAMMAD, N.A.; JEFRI, M.N. Clinical responses in cows vaccinated with a developed prototype killed Staphylococcus aureus mastitis vaccine. **Microb. Pathog.** v.124, p.101–105, 2018.

HO, C.M.; LIN, C.Y.; HO, M.W.; LIN, H.C.; CHEN, C.J.; LIN, L.C.; LU, J.J. Methicillin-resistant Staphylococcus aureus isolates with SCCmec type V and spa types t437 or t1081 associated to discordant susceptibility results between oxacillin and cefoxitin, **Central Taiwan. Diagn. Microbiol. Infect. Dis**. v.86, p.405–411, 2016.

JACOBY, G.A. Mechanisms of Resistance to Quinolones. **Clin. Infect. Dis.** v.41, p.S120–S126, 2005.

JOSHI, S., GOKALE, S. Status of Mastitis as an Emerging Disease in Improved and Periurban Dairy Farms in India. **Ann. N. Y. Acad. Sci**. v.1081, p.74–83, 2006.

LANGONI, H.; CABRAL, K. DE G.; DOMINGUES, P.F.; PULGA, M.E.; MARINHO, M.; PARDO, R.B. Utilization of enrofloxacina (Baytril®) on staphylococcal bovine mastitis. **Ciência Rural** v.30, p.167–170, 2000.

LEE, J.H. Methicillin (Oxacillin)-Resistant Staphylococcus aureus Strains Isolated from Major

Food Animals and their Potential Transmission to Humans. **Appl. Environ. Microbiol**. v.69, p.6489–6494, 2003.

MCDOUGAL, L.K; STEWARD, C.D.; KILLGORE, G.E.; CHAITRAM, J.M.; MCALLISTER, S.K.; TENOVER, F.C. Pulsed-field gel electrophoresis typing of oxacillin-resistant Staphylococcus aureus isolates from the United States: establishing a national database. **J. Clin. Microbiol.** v.41, p.5113–20. 2003.

MEDEIROS, E.S.; MOTA, R.A.; SANTOS, M. V.; FREITAS, M.F.L.; PINHEIRO JÚNIOR, J.W.; TELES, J.A.A. In vitro antimicrobial sensitivity to Staphylococcus spp. Isolates from dairy cows with subclinical mastitis. **Pesqui. Vet. Bras.** v.29, p.569–574. 2009.

MESQUITA, A.A.; ROCHA, C.M.B.M.; BRUHN, F.R.P.; CUSTÓDIO, D.A.C.; BRAZ, M.S.; PINTO, S.M.; SILVA, D.B.; COSTA, G.M. Staphylococcus aureus and streptococcus agalactiae: Prevalence, resistance to antimicrobials, and their relationship with the milk quality of dairy cattle herds in minas gerais state, **Brazil. Pesqui. Vet. Bras**. v.39, p.308–316. 2019.

MISRA, N.; WINES, T.F.; KNOPP, C.L.; HERMANN, R.; BOND, L.; MITCHELL, B.; MCGUIRE, M.A.; TINKER, J.K. Immunogenicity of a Staphylococcus aureus-cholera toxin A2/B vaccine for bovine mastitis. **Vaccine** v.36, p.3513–3521, 2018.

MONISTERO, V.; BARBERIO, A.; BISCARINI, F.; CREMONESI, P.; CASTIGLIONI, B.; GRABER, H.U.; BOTTINI, E.; CEBALLOS-MARQUEZ, A.; KROEMKER, V.; PETZER, I.M.; POLLERA, C.; SANTISTEBAN, C.; VEIGA DOS SANTOS, M.; BRONZO, V.; PICCININI, R.; RE, G.; COCCHI, M.; MORONI, P. Different distribution of antimicrobial resistance genes and virulence profiles of Staphylococcus aureus strains isolated from clinical mastitis in six countries. **J. Dairy Sci.** 103. 2020.

MONISTERO, V.; GRABER, H.; POLLERA, C.; CREMONESI, P.; CASTIGLIONI, B.; BOTTINI, E.; CEBALLOS-MARQUEZ, A.; LASSO-ROJAS, L.; KROEMKER, V.; WENTE, N.; PETZER, I.M.; SANTISTEBAN, C.; RUNYAN, J.; VEIGA DOS SANTOS, M.; ALVES, B.G; PICCININI, R.; BRONZO, V.; ABBASSI, M.; SAID, M.; MORONI, P. Staphylococcus aureus Isolates from Bovine Mastitis in Eight Countries: Genotypes, Detection of Genes Encoding Different Toxins and Other Virulence Genes. **Toxins (Basel)**. 10, 247, 2018.

OLIVER, S.P.; ALMEIDA, R.A.; GILLESPIE, B.E.; HEADRICK, S.J.; DOWLEN, H.H.; JOHNSON, D.L.; LAMAR, K.C.; CHESTER, S.T.; MOSELEY, W.M. Extended Ceftiofur Therapy for Treatment of Experimentally-Induced Streptococcus uberis Mastitis in Lactating Dairy Cattle. **J. Dairy Sci.** 87, 3322–3329, 2004.

PAPADOPOULOS, P.; PAPADOPOULOS, T.; ANGELIDIS, A.S.; KOTZAMANIDIS, C.; ZDRAGAS, A.; PAPA, A.; FILIOUSSIS, G.; SERGELIDIS, D. Prevalence, antimicrobial

susceptibility and characterization of Staphylococcus aureus and methicillin-resistant Staphylococcus aureus isolated from dairy industries in north-central and north-eastern Greece. **Int. J. Food Microbiol**. v.291, p.35–41, 2019.

PEREIRA, U.P.; OLIVEIRA, D.G.S.; MESQUITA, L.R.; COSTA, G.M.; PEREIRA, L.J. Efficacy of Staphylococcus aureus vaccines for bovine mastitis: A systematic review. **Vet. Microbiol.** v.148, p.117-24 2011.

PINHEIRO, E.C.S. **Treatment and vaccination efficacy against bovine Staphylococcus aureus subclinical mastitis**. 2016. 98p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

QIN, J.; SUN, Y. Curative Effect Observation of Ciprofloxacin Injection in the Treatment of Acute Mastitis on Dairy Cows. **Mod. Apllied Sci.** 3, 2009.

RABELLO, R.F.; SOUZA, C.R.V.M.; DUARTE, R.S.; LOPES, R.M.M.; TEIXEIRA, L.M.; CASTRO, A C.D. Characterization of Staphylococcus aureus isolates recovered from bovine mastitis in Rio de Janeiro, Brazil. **J. Dairy Sci**. v.88, p.3211–9, 2005.

ROY, J.P.; KEEFE, G. Systematic Review: What is the Best Antibiotic Treatment for Staphylococcus aureus Intramammary Infection of Lactating Cows in North America? **Vet. Clin. North Am**. **Food Anim. Pract**, v.28, p.39-50, 2012.

RUBIN, J.E.; BALL, K.R.; CHIRINO-TREJO, M. Antimicrobial susceptibility of Staphylococcus aureus and Staphylococcus pseudintermedius isolated from various animals. **Can. Vet. J.** v.52, p.162–164, 2011.

SAIDI, R.; KHELEF, D.; KAIDI, R. Subclinical mastitis in cattle in Algeria: Frequency of occurrence and bacteriological isolates. **J. S. Afr. Vet. Assoc.** v.84. 2013.

SCHUKKEN, Y.H.; BRONZO, V.; LOCATELLI, C.; POLLERA, C.; ROTA, N.; CASULA, A.; TESTA, F.; SCACCABAROZZI, L.; MARCH, R.; ZALDUENDO, D.; GUIX, R.; MORONI, P. Efficacy of vaccination on Staphylococcus aureus and coagulase-negative staphylococci intramammary infection dynamics in 2 dairy herds. **J. Dairy Sci.** v.97, p.5250–5264, 2014.

SILVA, E.R., PEREIRA, A.M.G., MORAES, W. DA S., SANTORO, K.R., SILVA, T.R.M., Perfil de sensibilidade antimicrobiana in vitro de Staphylococcus aureus isolado de mastite subclínica bovina. **Rev. Bras. Saude e Prod. Anim.** v.13, p.701–711,2012.

SKOULIKAS, S.; DUFOUR, S.; HAINE, D.; PERREAULT, J.Y.; ROY, J.P. Early-lactation extended pirlimycin therapy against naturally acquired Staphylococcus aureus intramammary infections in heifers: A randomized controlled trial. J. Dairy Sci. 101, 1419–1427. 2018.

SREDNIK, M.E.; USONGO, V.; LÉPINE, S.; JANVIER, X.; ARCHAMBAULT, M.; GENTILINI, E.R. Characterisation of Staphylococcus aureus strains isolated from mastitis bovine milk in Argentina. **J. Dairy Res.** v.85, p.57–63. 2018.

SUN, X.; LIN, Z.; HU, X.; YAO, W. MING; BAI, B.; WANG, H. YAN; LI, D. YUN; CHEN, Z.; CHENG, H.; PAN, W. GUANG; DENG, M. GUI; XU, G. JIAN; TU, H. PENG; CHEN, J. WEN; DENG, Q. WEN; YU, Z. JIAN; ZHENG, J. XIN. Biofilm formation in erythromycin-resistant Staphylococcus aureus and the relationship with antimicrobial susceptibility and molecular characteristics. **Microb. Pathog.** v.124, p.47–53. 2018.

SWENSON, J.M., BRASSO, W.B., FERRARO, M.J., HARDY, D.J., KNAPP, C.C., LONSWAY, D., MCALLISTER, S., RELLER, L.B., SADER, H.S., SHORTRIDGE, D., SKOV, R., WEINSTEIN, M.P., ZIMMER, B.L., PATEL, J.B. Correlation of cefoxitin MICs with the presence of mecA in Staphylococcus spp. **J. Clin. Microbiol**. 47, 1902–5. 2009.

TEIXEIRA, J.P.; SILVA, N. DA; FONSECA, L.M. DA; COSTA, G.M. Detection of femA and mecA genes in Staphylococcus aureus isolated from bovine raw milk using duplex PCR and determination of the minimum inhibitory concentration of the isolates. **Rev. Inst. Adolfo Lutz** v.73, p.272–279, 2014.

TOMAZI, T.; DE SOUZA FILHO, A.F.; HEINEMANN, M.B.; SANTOS, M.V. Molecular characterization and antimicrobial susceptibility pattern of Streptococcus agalactiae isolated from clinical mastitis in dairy cattle. **PLoS One** 13, e0199561, 2018.

TUCHSCHERR, L.; LÖFFLER, B.; BUZZOLA, F.R.; SORDELLI, D.O. Staphylococcus aureus adaptation to the host and persistence: role of loss of capsular polysaccharide expression. **Future Microbiol**. v.5, p.1823–1832, 2010.

TÜRKYILMAZ, S.; TEKBIYIK, S.; ORYASIN, E.; BOZDOGAN, B. Molecular epidemiology and antimicrobial resistance mechanisms of methicillin-resistant staphylococcus aureus isolated from bovine milk**. Zoonoses Public Health** v.57, p.197–203, 2010.

VAN BELKUM, A.; TASSIOS, P.T.; DIJKSHOORN, L.; HAEGGMAN, S.; COOKSON, B.; FRY, N.K.; FUSSING, V.; GREEN, J.; FEIL, E.; GERNER-SMIDT, P.; BRISSE, S.; STRUELENS, M. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. **Clin. Microbiol. Infe**ct. v.13, p.1–46, 2007.

VAN DUIJKEREN, E.; WOLFHAGEN, M.J.H.M.; BOX, A.T.A.; HECK, M.E.O.C.; WANNET, W.J.B.; FLUIT, A.C. Human-to-dog transmission of methicillin-resistant Staphylococcus aureus. **Emerg. Infect. Dis**. v.10, p.2235–2237, 2004.

VITALE, M.; GAGLIO, S.; GALLUZZO, P.; CASCONE, G.; PIRAINO, C.; DI MARCO LO PRESTI, V.; ALDUINA, R. Antibiotic Resistance Profiling, Analysis of Virulence Aspects and Molecular Genotyping of Staphylococcus aureus Isolated in Sicily, Italy. **Foodborne Pathog. Dis**. v.15, p.177–185, 2018.

YANG, F.; WANG, Q.; WANG, X.; WANG, L.; LI, X.; LUO, J.; ZHANG, S.; LI, H. Genetic characterization of antimicrobial resistance in Staphylococcus aureus isolated from bovine mastitis cases in Northwest China. **J. Integr. Agric**. v.15, p.2842–2847, 2016.

ZANETTE, E.; SCAPIN, D.; ROSSI, E.M. Suscetibilidade antimicrobiana de Staphylococcus aureus isolados de amostras de leite de bovinos com suspeita de mastite. **Unoesc Ciência - ACBS** v.1, p.65–70, 2010.

ZECCONI, A.; BINDA, E.; BORROMEO, V.; PICCININI, R. Relationship between some Staphylococcus aureus pathogenic factors and growth rates and somatic cell counts. **J. Dairy Res.** v.72, p.203–208, 2005.

ZECCONI, A.; SCALI, F. Staphylococcus aureus virulence factors in evasion from innate immune defenses in human and animal diseases. **Immunol. Lett.** v.150, p.12–22. 2013.

ZURITA, J.; MEJÍA, C.; GUZMAN-BLANCO, M. Diagnosis and susceptibility testing of methicillin-resistant Staphylococcus aureus in Latin America. **Brazilian J. Infect. Dis.** v.14, p.97–107, 2010.

CHAPTER 5

Application of a dot-blot hybridization assay for genotyping *Streptococcus uberis* from Brazilian dairy herds

5. APPLICATION OF A DOT-BLOT HYBRIDIZATION ASSAY FOR GENOTYPING *Streptococcus uberis* FROM BRAZILIAN DAIRY HERDS

5.1 ABSTRACT

Streptococcus uberis is one of the main causes of environmental mastitis, and it continues as a matter of concern despite current knowledge regarding contagious transmission patterns in several dairy herds. Dot blot hybridization analysis allows rapid identification of *S. uberis* population structures within and between herds, identifying the main route of transmission as well as possible clonal lineages, which directly affects the control of bovine mastitis caused by this pathogen. The aim of this study was to evaluate the diversity of *S. uberis* isolates obtained from clinical (n = 22) and subclinical (n = 22) cases of mastitis in dairy herds (n=13) in Brazil during a 12-month interval. Forty-four *S. uberis* isolates were submitted to dot blot hybridization followed by automatic data analysis. Nine different hybridization patterns were identified through the genetic markers associated with virulence factors and taxonomy, indicating diversity within the population of *S. uberis* suggestive of environmental transmission. However, the evidence of identical dot-blot pattern in different mammary quarters of the same animal suggested local contagious transmission. Of the virulence genes evaluated, a high prevalence of the genes *sua*, *pauA* and *gapC* were obtained, highlighting the importance of these virulence factors for the adhesion, invasion and multiplication of *S. uberis* in subclinical and clinical intramammary infections.

5.2. INTRODUCTION

Streptococcus uberis is considered one of the most prevalent environmental pathogens causing clinical and subclinical bovine mastitis, both in lactating and dry cows (PERRIG et al., 2015). This pathogen causes a high rate of new infections during dry, pre-partum and lactation periods, as *S. uberis* is widely distributed in the environment and difficult to control with conventional mastitis control measures. The risk of intramammary infections (IMI) increases with teat end contamination, incomplete formation of keratin plug in the teat and immunosuppression before and after calving (BRADLEY; GREEN, 2004). In addition, new clinical and subclinical infections caused by *S. uberis* that occur during early lactation begin during the dry period, when an increased risk is observed (TIMONEN et al., 2018).

S. uberis can be transmitted from both environmental sources and other potential reservoirs, such as cows with chronic IMI. Previous studies have molecularly characterized strains of *S. uberis* and identified a mode of contagious transmission, since similar strains were isolated from different cows in a single herd, indicating a common reservoir in chronic infected cows (ZADOKS et al., 2003). In addition, mastitis caused by *S. uberis* is less likely to be cured when compared to IMI caused by other species of streptococci, due to the presence of specific virulence factors, such as toxins and immune evasion mechanisms (ROYSTER; WAGNER, 2015). The presence of theses virulence factors can provide some competitive advantages in comparison to other bacteria, due to increased invasion and ability to survive in the udder. Some genes associated with virulence characteristics are nisin U operon (Wirawan et al., 2006), responsible for the immunity system and regulation; plasminogen activator protein (pauA) (ROSEY et al., 1999), that facilitate the growth of *S. uberis* by its nutrient acquisition; oligopeptide permeases (SMITH et al., 2002) and *S. uberis* adhesion molecule (SUAM) (ALMEIDA et al., 2006), that contribute to adhesion and internalization; hyaluronic acid, that plays in phagocytosis (FIELD et al., 2003) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), involved in glycolysis in different functions (REINOSO et al., 2011).

The identification of population structure patterns of *S. uberis* can be used for epidemiological investigation among and within herds. Several DNA-based typing techniques such as restriction fragment length polymorphism (RFLP), DNA random amplification reaction, pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (PERRIG et al., 2015) were already described for *S. uberis* characterization. However, methods based on specific probes derived by PCR (hybridization) present higher accuracy and lower cost for virus and bacterial discrimination (MENG et al., 2007; ZHANG et al., 2014; GUO et al., 2015). Different than PCR, which searches for specific or random genes, DNA hybridization depends on the labeling of the DNA probes on the target samples. The dot blot is more practical and economical among microarray techniques, besides providing specific results and being used for the simultaneous detection of different targets or pathogens (EL-SAYED et al., 2017). In bovine mastitis studies, dot blot hybridization methods have been used for identification of *S. uberis* isolates, to infer its population structure, and determine the presence of virulence- and antibiotic resistance-related genes (ALBUQUERQUE et al., 2017).

The objective of the present study was to evaluate the genotypic diversity of *S. uberis* isolated from bovine mastitis in two different regions from Brazil using dot blot hybridization.

In addition, we aimed to identify associations between epidemiological distribution of the isolates and virulence factors associated with *S. uberis*.

5.3 MATERIAL AND METHODS

Experimental procedures were performed according to the ethical principles of animal experimentation, as well as the norms edited by the National Council of Control of Animal Experimentation (CONCEA). It was approved by the Animal Use Ethics Committee of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA/FMVZ), n 8178270617.

5.3.1 *Streptococcus uberis* collection and DNA preparation

Isolates presumably classified as *S. uberis* (n = 44) were obtained from the Bacterial Isolates Collection of the Qualileite Laboratory - FMVZ / USP, which were cryopreserved in BHI solution and 20% glycerin at -80 ° C. Twenty-two isolates of *S. uberis* causing subclinical mastitis (SCM) from 4 herds were randomly selected. In addition, 22 isolates of *S. uberis* were selected from clinical mastitis (CM) in 9 herds, collected during rainy and dry seasons. Clinical cases were defined when a cow had visible changes in milk, accompanied or not by signs of inflammation in the udder and systemic signs (ROBERSON, 2012) whereas subclinical cases were defined by the absence of visible signs and individual somatic cell count (SCC) > 200,000 cells / ml (SCHUKKEN et al., 2003; NAQVI et al., 2018). Regardless of the mastitis presentation (clinical or subclinical), all isolates of *S. uberis* were obtained by individual samples of mammary quarters.

The isolates were inoculated in blood agar at 37 °C for 24 h and a single colony was selected, then cultured in trypticase soy agar (TSA) and characterized by Christie, Atkins e Munch-Petersen (CAMP), esculin and bile-esculin tests. Morphological evaluation and Gram identification of *S. uberis* was carried out as recommended by National Mastitis Council (OLIVER et al., 2004).

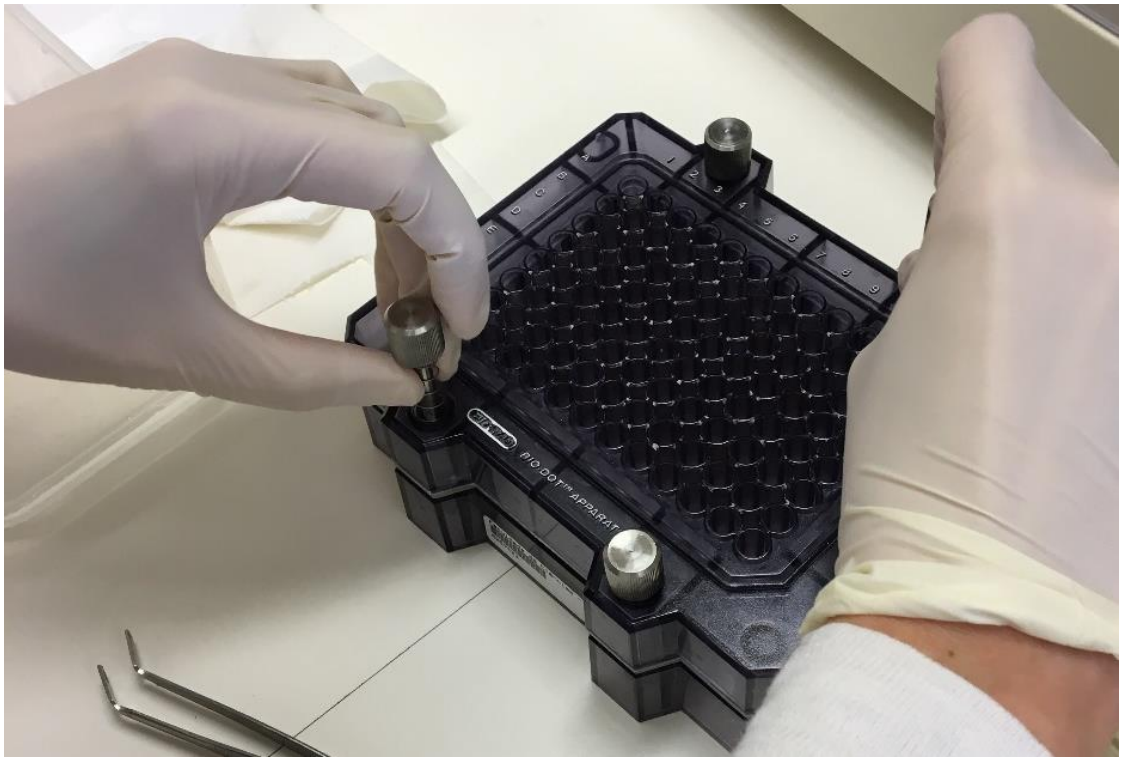
After positive identification and biochemical characterization, *S. uberis* isolates were submitted to DNA extraction, essentially according to the methodology described by Fan et al., (1995), and resuspending the DNA in Tris-EDTA buffer (TE; 10mM Tris HCl, 5mM EDTA,

pH = 8.0). After extraction, the DNA samples were refrigerated at -20 °C. DNA quantification was carried out with the Qubit 2.0 Fluorometer HS Assay (Invitrogen, Carlsbad, CA, USA).

5.3.2 Dot Blot Hibridization and PCR

The dot blot hybridization was performed on a 96-well platform, 8 of which were used for positive and negative controls. For each selected isolate, sample 100 ng of DNA was diluted in 200 µL TE solution and spotted onto a nylon membrane using a BioDot apparatus (Bio-Rad, Hercules, USA) (Figure 12). The DNA probes used and displayed in Table 14 were prepared as described by Albuquerque et al., (2017) and included two *S. uberis* taxonomic markers and eight functional markers corresponding to two nisin operon genes and six virulence factors.

Figure 12 - Production of nitrocellulose membrane in Bio-Dot (Bio-Rad)



Source: Alves, B.G. (2020)

Table 14 - DNA probes used in dot blot hybridization for *S. uberis* identification

Type	DNA marker	Description	References
Taxonomic	U1	Taxa-specific (*S. uberis*)	Almeida et al., 2013
	U2	Taxa-specific (*S. uberis*)	Almeida et al., 2013
Nisin Operon	NU1	Regulation gene (*nsuR*)	Almeida et al., 2013
	NU3	Nisin immunity gene (*nsuI*)	Almeida et al., 2013
Virulence Related	V1	Hyaluronic acid operon gene (*hasC*)	Ward et al., 2001
	V2	Glyceraldehyde 3-phosphate dehydrogenase gene (*gapC*)	Reinoso et al., 2011
	V3	Oligopeptide permease gene (*oppF*)	Smith et al., 2002
	V4	*S. uberis* adhesion molecule gene (*sua*)	Albuquerque et al., 2017
	V6	Plasminogen activator gene (*pauA*)	Albuquerque et al., 2017
	V7	Hyaluronic acid operon gene (*hasA*)	Field et al., 2003

Source: Alves, B.G. (2020)

Hybridization was carried out overnight at 68 °C. The membranes were washed according to the manufacturer's instructions. The hybridization signals were detected using a chemiluminescent reagent (CDP-Star; Roche Diagnostics, Basel, Switzerland) and the dot blot images were captured using the Molecular Imager ChemiDoc system (Bio-Rad, Hercules, CA). The exposure time was measured according to the pixel saturation, in which a probabilistic number was assigned to each dot according the control dots (positive and negative) (CARIDADE et al., 2015) and this number represented the hybridization signal for the combination of the *S. uberis* DNA sample and the known DNA probe. Probability values higher than 0.5 were considered as positive results. Considering dot blot results, dot-blot patterns were created according the number of positive values (> 0.5). The positive response to at least one taxonomic probe (U1 or U2) classified the isolate like *S. uberis*.

5.3.3 Epidemiological Criteria

Four epidemiological patterns defined as chronic, clinical, contagious and mixed were identified, based on the different dot-blot patterns and according the clinical evaluation of the animals. Persistent infections, i.e. chronic cases, were defined as those that showed the same dot-blot pattern in the same mammary quarter on different collection days. The inference about the contagious transmission occurred when the same dot-blot pattern was found in the same cow, on the same day of collection, in different mammary quarters. The clinical pattern was

defined for dot-blot patterns present only in clinical cases, while the mixed pattern was defined for dot-blot patterns that were observed in both clinical and subclinical cases.

5.4 RESULTS

5.4.1 Characterization of milk samples

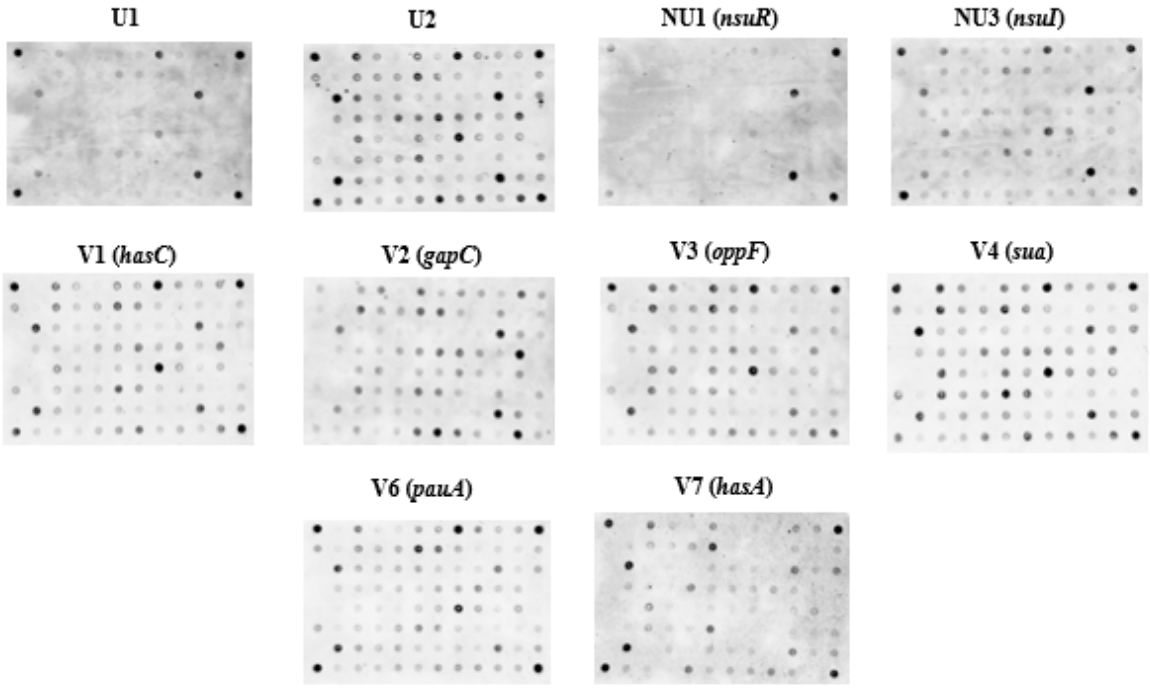
Of the total isolates initially selected for the collection of mammary quarters milk samples, 22 were isolated from subclinical infections while 22 isolates came from clinical infections, comprising 12 of mild cases (54.5%), 9 of moderate cases (40.9%) and 1 of severe cases (4.5%).

5.4.2 Dot Blot Analysis

Ten DNA markers were analyzed in the present study, two taxonomic markers (U1 and U2, specific for *S. uberis*) to confirm the identity of the isolates, two markers of nisin operon (NU1-nsuR and NU3-nsuI), and six markers of expression genes of virulence factors (V1-hasC, V2-gapC, V3-oppF, V4-sua, V6-pauA and V7-hasA). The presence of these 10 genes in the 44 selected samples was evaluated by dot blot hybridization (Figure 13).

Figure 13 - Scheme of distribution of *S. uberis* isolates for hybridization in a dot blot platform and images resulting from DNA genomic hybridization membranes

C+	1su	8su	15su	16su	19su	24su	26su	27su	29su	30su	C+
31su	32su	33su	34su	35su	38su	41su	43su	44su	45su	48su	49su
6tsu	10tsu	15tsu	19tsu	20tsu	21tsu	40tsu	45tsu	46tsu	51tsu	54tsu	55tsu
C-	62tsu	63tsu	65tsu	69tsu	71tsu	74tsu	84tsu	85tsu	88tsu	93tsu	C-
C-	1su	8su	15su	16su	19su	24su	26su	27su	29su	30su	C-
31su	32su	33su	34su	35su	38su	41su	43su	44su	45su	48su	49su
6tsu	10tsu	15tsu	19tsu	20tsu	21tsu	40tsu	45tsu	46tsu	51tsu	54tsu	55tsu
C+	62tsu	63tsu	65tsu	69tsu	71tsu	74tsu	84tsu	85tsu	88tsu	93tsu	C+



Legend: The distribution of the isolates in the nylon membrane was performed in duplicate, whereas the membrane had 88 sample wells, in addition to 4 positive controls (C +), represented by known DNA responsive to the probes; and 4 negative controls (C-), compounds of TE buffer. Source: Alves, B.G. (2020).

Based on the results of the imaging software (CARIDADE et al., 2015), dot blot images were converted into probability values (P) of each dot to be positive for the hybridization between theta DNA probe with the DNA of the sample (Table 15). Considering that $P > 0.5$ values were positives, it was possible to confirm the identity of 79.5% of the isolates analyzed (35/44) for *S. uberis*, according the results obtained with U1 or U2 taxonomic probes.

Table 15 - Average probability values of dot blot hybridization results for *S. uberis*

Strain	Taxonomic		Nisin Operon		Virulence Factors						Dot blot pattern
	U1	U2	NU1 *(nsuR)*	NU3 *(nsuI)*	V1 *(hasC)*	V2 *(gapC)*	V3 *(oppF)*	V4 *(sua)*	V6 *(pauA)*	V7 *(hasA)*	
51tsu	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**0.99**	**0.97**	A
8su	0.29	**0.99**	0.00	**0.97**	**0.97**	**1.00**	**1.00**	**1.00**	**0.52**	**0.90**	B
26su	**0.95**	**1.00**	**0.62**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	0.05	B
30su	0.29	**0.84**	0.00	**0.60**	**0.85**	**1.00**	**0.92**	**0.96**	**0.84**	**0.88**	B
38su	**0.62**	**1.00**	0.00	**0.99**	**1.00**	**1.00**	**0.98**	**1.00**	**0.97**	**1.00**	B
10tsu	**0.91**	**1.00**	0.10	**0.98**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	B
55tsu	0.33	**0.94**	0.50	**0.56**	**0.62**	**0.94**	**0.93**	**0.97**	**0.87**	**0.92**	B
69tsu	0.00	**0.86**	0.00	**0.52**	**0.75**	**0.96**	**0.87**	**0.99**	**0.62**	**0.96**	B
19su	0.22	**0.98**	0.00	**0.63**	**0.73**	**1.00**	**1.00**	**1.00**	**0.60**	0.46	C
27su	0.12	**0.87**	0.09	**0.83**	**0.88**	**1.00**	**0.94**	**0.97**	**0.90**	0.03	C
33su	0.06	**0.95**	0.00	**0.82**	**0.71**	**1.00**	**1.00**	**1.00**	**0.60**	0.28	C
35su	0.06	**0.89**	0.00	**0.66**	**0.64**	**1.00**	**0.95**	**0.96**	**0.81**	0.32	C
41su	0.22	**0.83**	0.00	**0.95**	**0.95**	**1.00**	**0.91**	**0.99**	**0.91**	0.00	C
49su	0.48	**0.88**	**0.64**	0.26	0.45	**1.00**	**0.82**	**0.89**	**0.52**	**0.80**	C
74tsu	0.06	**1.00**	0.00	**0.66**	**0.99**	**1.00**	**1.00**	**0.99**	**0.86**	0.39	C
85tsu	0.06	**0.97**	0.00	0.45	**0.83**	**1.00**	**0.88**	**0.97**	**0.92**	**0.78**	C
93tsu	0.06	**1.00**	0.26	**0.56**	**0.98**	**1.00**	**1.00**	**1.00**	**0.59**	0.04	C
15su	0.06	**0.72**	0.00	**0.62**	**0.63**	**1.00**	**1.00**	**0.94**	0.06	0.06	D
31su	0.00	**0.86**	0.00	0.40	**0.89**	**0.92**	**0.89**	**0.98**	**0.88**	0.00	D
43su	0.08	**0.70**	0.00	**0.67**	0.39	**1.00**	**0.59**	**0.62**	**0.54**	0.00	D
19tsu	0.05	**0.79**	0.00	**0.55**	0.21	**0.98**	**0.85**	**0.91**	**0.53**	0.01	D
84tsu	0.06	**0.99**	0.00	0.49	**0.55**	**1.00**	**0.97**	**0.95**	**0.84**	0.41	D
15tsu	0.00	**0.98**	0.16	0.36	**0.68**	**0.63**	0.05	**0.81**	**0.83**	0.00	E
20tsu	0.00	**0.87**	0.00	0.19	0.45	**0.51**	**0.82**	**0.60**	**0.76**	0.00	E
54tsu	0.01	**0.70**	0.10	**0.53**	0.34	**1.00**	**0.90**	**0.89**	0.21	0.38	E
71tsu	0.00	**0.98**	0.01	0.42	**0.90**	**1.00**	**0.95**	**0.85**	0.31	0.17	E
88tsu	0.04	**0.87**	0.43	**0.69**	0.27	0.50	**0.53**	**0.78**	**0.64**	0.32	E
29su	0.41	**0.78**	0.13	0.19	0.40	**0.87**	**0.90**	**0.94**	0.34	0.48	F
34su	0.03	**0.88**	0.00	0.34	0.50	**1.00**	**0.90**	**0.94**	0.43	0.00	F
45su	0.10	**0.66**	0.19	0.40	0.24	**1.00**	**0.77**	**0.57**	0.13	0.18	F
21tsu	0.00	**0.56**	0.00	0.23	0.47	0.48	**0.78**	**0.87**	**0.59**	0.26	F
63tsu	0.06	**0.98**	0.02	0.31	0.41	**0.98**	**0.94**	**0.85**	0.39	0.01	F
62tsu	0.06	**0.94**	0.08	**0.59**	0.34	**0.75**	0.44	0.48	0.29	0.43	G
40tsu	0.00	**0.71**	0.00	0.32	0.13	0.44	**0.57**	0.35	0.32	0.10	H
65tsu	0.08	**0.71**	0.00	0.30	0.14	0.48	0.27	**0.83**	0.37	0.00	H
1su	0.03	0.08	0.05	0.07	0.00	0.00	0.07	0.03	0.02	0.00	I
16su	0.05	0.17	0.00	0.24	0.03	0.14	0.15	0.11	0.06	0.00	I
24su	0.02	0.38	0.00	**0.64**	**0.65**	**0.98**	**0.95**	**0.95**	**0.76**	0.00	I
32su	0.01	0.30	0.01	0.23	0.04	0.24	0.31	0.15	0.08	0.00	I
44su	0.28	0.03	0.08	0.13	0.07	0.00	0.11	0.05	0.06	0.00	I
48su	0.08	0.03	0.00	0.15	0.02	0.33	0.17	0.10	0.06	0.26	I
6tsu	0.00	0.00	0.00	0.03	0.00	0.09	0.00	0.01	0.01	0.00	I

45tsu	0.00	0.40	0.00	0.09	0.08	0.03	0.20	0.47	0.30	0.04	I
46tsu	0.01	0.46	0.01	**0.79**	0.07	0.49	0.29	0.38	0.12	0.00	I

*Probability values higher than 0.5 was considered as positive result and are highlighted in bold.

* Negative answer for taxonomic markers = dot blot pattern I

* Positive response for taxonomic markers (at least 1) = A (8 positives for virulence factors + nisin operon); B (7 positives); C (6 positives); D (5 positives); E (4 positives); F (3 positives); G (2 positives) and H (1 positive).

Source: Alves, B.G. (2020).

The nisin operon marker evaluated by probes NU1 and NU3, responsible for the regulation of the *nsuR* and *nsuI* genes, respectively, was identified in 8.5 and 60% of the isolates, respectively. Only two isolates (26su and 51tsu) had positive results for both *nsuR* and nsuI markers: one each of CM and SCM.

Of the six probes associated with the virulence factors, five were positive to at least 50% of the isolates analyzed. The *sua* gene, targeted by marked V4, was identified in 33 of the 35 *S. uberis* isolates (94.2%) whereas the V7 probe, *hasA* gene marker was identified in only 25.7% (9/35) of the isolates. However, the V2 probe, associated with the *gapC* gene was identified in 88.5% (31/35) of *S. uberis* isolates, indicating the activity of the glyceraldehyde-3-phosphate dehydrogenase enzyme associated with the pathogen's adhesion mechanism.

5.4.3 Dot Blot Patterns

Considering dot blot results obtained for all markers, a total of 9 different dot-blot patterns were observed for the 44 isolates of *S. uberis* (A-I). Of the total (n= 44), 8 had no response to the taxonomic probes (U1 or U2) and were assigned as dot-blot pattern I. Only one isolate (51tsu) was positive for all virulence markers, [V1 (hasC), V2 (gapC), V3 (oppF), V4 (sua), V6 (pauA), V7 (hasA)], two nisin operon markers [NU1 (nsuR), NU3 (nsuI)] and for at least one taxonomy marker (pattern A). The dot-blot pattern C was the most common among isolates (20.45%), being positive to 7 of the 10 DNA probes analyzed, including taxonomic (U2), nisin factor (NU3) and virulence factors (V1, V2, V3, V4, V6). For the epidemiological patterns analysis, only the isolates identified as *S. uberis* by the taxonomic markers were taken into consideration. Therefore, those with a dot-blot pattern I were not assigned to any epidemiological class. After the exclusion of isolates classified as dot-blot pattern I, 4 different patterns for subclinical isolates and 8 different patterns for clinical isolates (Table 16) were observed.

Table 16 - Complete list of *Streptococcus uberis* isolates selected for dot-blot pattern evaluation

Strain	Herd	Animal ID	DIM[1]	Parity	Severity Score[3]	Collection Day	MQ[4]	Mastitis Form[5]	Dot blot Pattern
31su	1	1	143	4	.	30/06/2015	LF	SC	D
45su	1	2	239	3	.	14/07/2015	RR	SC	F
30su	2	3	483	1	.	17/06/2015	LR	SC	B
29su	2	3	483	1	.	17/06/2015	RF	SC	F
8su	3	4	419	3	.	28/05/2015	RF	SC	B
19su	3	4	426	3	.	11/06/2015	RF	SC	C
38su	3	4	447	3	.	02/07/2015	RF	SC	B
15su	3	5	109	1	.	11/06/2015	RR	SC	D
33su	3	5	130	1	.	02/07/2015	RR	SC	C
35su	3	5	130	1	.	02/07/2015	LR	SC	C
34su	3	5	130	1	.	02/07/2015	LF	SC	F
49su	3	7	277	3	.	16/07/2015	LR	SC	C
26su	4	8	100	2	.	13/06/2015	RF	SC	B
27su	4	8	100	2	.	13/06/2015	LR	SC	C
43su	4	9	390	1	.	05/07/2015	LF	SC	D
41su	4	10	113	3	.	05/07/2015	LF	SC	C
69tsu	5	12	122	5	2	18/02/2015	LR	C	B
88tsu	5	13	147	5	1	12/04/2015	RR	C	E
10tsu	6	14	301	2	2	02/05/2014	RF	C	B
19tsu	6	15	270	3	1	08/07/2014	RR	C	D
21tsu	6	16	87	4	1	31/07/2014	LF	C	F
15tsu	7	17	70	2	1	15/05/2014	RR	C	E
20tsu	8	18	16	3	2	14/07/2014	LF	C	E
51tsu	8	19	180	3	3	04/11/2014	LF	C	A
71tsu	8	20	364	3	1	01/02/2015	RR	C	E
74tsu	8	21	42	4	1	05/02/2015	RF	C	C
40tsu	9	22	148	8	2	30/11/2014	LR	C	H
84tsu	10	25	5	4	2	28/03/2015	LR	C	D
54tsu	11	26	3	1	1	24/12/2014	LF	C	E
55tsu	11	27	125	2	1	06/12/2014	RF	C	B
65tsu	11	28	247	1	1	02/03/2015	RR	C	H
62tsu	12	29	97	3	2	06/02/2015	LF	C	G
63tsu	12	29	112	3	1	21/02/2015	LF	C	F
93tsu	12	30	284	1	2	24/07/2015	LR	C	C
85tsu	13	31	257	5	2	29/03/2015	RF	C	C

[1]DIM = Days in milk

[2] SCC, somatic cell count (cells/mL)

[3] Severity score: 1 = mild; 2 = moderate; 3 = severe

[4] Mammary quarter from which the isolate was obtained: front right/rear right/front left/rear left (FR/RR/FL/RL)

[5] Mastitis Form, C = clinical and SC = subclinical

Source: Alves, B.G. (2020).

5.4.4 Epidemiological patterns

Three distinct epidemiological patterns were observed for the 35 isolates evaluated. The clinical epidemiological pattern included dot pattern A, E, G and H, which were only detected in clinical isolates. Cow 5 showed the same dot-blot pattern (C) in different mammary quarters on the same collection occasion, showing a contagios pattern of infection. Finally, the mixed epidemiological pattern was characterized by dot patterns B, C, D and F, which presented clinical and subclinical isolates. Another relevant observation is the diversity of patterns circulating within the same herd, reinforcing the hypothesis of dominant environmental origin among the *S. uberis* strains studied.

5.5 DISCUSSION

The results of the present study revealed high heterogeneity within herds and between neighboring geographic regions, with the identification of nine different dot-blot patterns among isolates evaluated. This high diversity of *S. uberis* strains may indicate the relatively low efficiency of milking practices and other conventional hygienic measures for the control of mastitis caused by this pathogen in dairy herds. Although in this study most of the clinical cases were classified as mild, about 18% of isolates had seven of the eight virulence factors studied, which are associated with adhesion to mammary gland tissue and immune system evasion.

Eight of the 44 isolates evaluated by the dot blot methodology had no hybridization with any of the probes, indicating that they probably did not correspond to *S. uberis*. The absence of hybridization with at least one of the taxonomic probes may indicate that the conventional microbiological culture was not able to accurately identify all *S. uberis* isolates. This fact emphasizes the accuracy of the dot blot methodology in characterizing a strain taxonomically, since the positive dot hybridization indicates the presence of the corresponding target gene in the *S. uberis* strain. In addition, the low response for the U1 probe may suggest a different pattern from the isolates compared to those evaluated by Albuquerque et al. (2017) by the same technique. However, the U2 probe had a positive response among Brazilian isolates, allowing the taxonomic classification as *S. uberis*.

In this study, the V4 probe encoding the *sua* virulence gene (*S. uberis* adhesion molecule) was identified in 94.2% of isolates, indicating that this gene, associated with the adhesion and internalization of *S. uberis* in the mammary gland was present in almost all isolates evaluated. This result was similar to that found by Perrig et al. (2015) when they

evaluated the clonal relationship between 137 *S. uberis* isolates of clinical and subclinical mastitis from Argentina dairy herds. Based on analyses of PFGE and RAPD, that study reported a prevalence of 97.8% for *sua* gene and 94.9% for *pauA* gene among the tested *S. uberis* isolates. Using PFGE, Fessia et al. (2019) determined the presence of six potential adhesion genes in 34 *S. uberis* isolates from bovine mastitis in Argentina. These authors reported a lower prevalence of the *sua* gene (79.41%) among the isolates evaluated, although they also revealed a high level of heterogeneity among them (26 pulse-types). Furthermore, the prevalence of the *pauA* gene among *S. uberis* isolates was 71.4%, lower than that reported by Perrig et al. (2015) (94.9%) and Fessia et al. (2018) (94.11%). However, only two isolates that had a positive response to taxonomical probes (U1 and U2) were negative for both genes (*sua* and *pauA*), in addition to the fact that this difference can be explained by the high heterogeneity of the isolates used in the present study, which come from 13 different herds. Despite the clonal diversity among strains of *S. uberis*, the *sua* and *pauA* genes specifically indicate the presence of *S. uberis* in the herd.

Based on the evaluation of the dot-blot patterns created from the response to DNA markers, the dot blot profile C were identified in different mammary quarters of the same cow (cow ID 5, Table 16). These results indicate the contagious transmission pattern of *S. uberis* between mammary quarters of the same cow during milking, which may be the one significant route of transmission of this pathogen, depending on the conditions of the herd (DAVIES et al., 2015). Similar results were described in a study with 138 *S. uberis* isolates submitted to PFGE, in which 62 different strains were found and identical strains were detected in different mammary quarters of the same cow and among cows within the same herd, suggesting the contagious transmission mode (PHUEKTES et al., 2001). Abureema et al. (2014) also found evidence of direct transmission of *S. uberis* among cows using the PFGE technique. Additionally, the study of Abureema et al. (2014) showed that contagious frequency from *S. uberis* among cows, mammary quarters and environmental sources depends on hygiene conditions in milking and environment. Therefore, the results of this study corroborate the *S. uberis* contagious transmission pattern, although the wide variability of dot-blot patterns also indicates the environmental transmission profile.

Of the 44 isolates of *S. uberis* evaluated by dot blot in this study, 35 were positive for at least one of the evaluated taxonomic markers (U1 or U2), and from 9 that were negative for the taxonomic probes, 7 were also negative for the other evaluated markers. The high prevalence of the *hasC*, *gapC*, *sua*, *oppF* and *pauA* genes found in the present study was similar to a recent

study that used dot blot hybridization for evaluation of *S. uberis* strains (ALBUQUERQUE et al., 2017). Fifty-four *S. uberis* isolates from two dairy herds with persistent IMI in distinct regions of Portugal were evaluated and 100% prevalence was observed for *hasC*, *gapC*, *oppF*, *sua* and *pauA* genes. However, Albuquerque et al. (2017) observed that only the *hasA* gene was absent in some isolates evaluated, which differed from the present study that found a prevalence of this gene in only 22.22% of the isolates.

Although *S. uberis* isolates used in the present study were collected in different regions, the presence of some markers such as *sua*, *gapC* and *pauA* specifically were found. Nevertheless, the genetic heterogeneity of the isolates observed in the present study may suggest that the profiles observed for the dot blot are very different from those presented by Albuquerque et al. (2017), who focused only on two dairy herds. Although the antimicrobial resistance markers have not been evaluated in the present study, the dot blot technique allows the evaluation of resistance genes for antimicrobial compounds, as described by Albuquerque et al. (2017). The epidemiological evaluation allowed a diagnosis that half of the dot blot profiles were classified as mixed epidemiologically, since they were capable of causing clinical and subclinical infections. Also, although *S. uberis* is primarily an environmental pathogen, it was possible to identify a contagious pattern, which increases its potential infection. Further research is needed to assess the potential of these markers to predict the epidemiological behavior at the farm.

Thus, the results of the present study suggest a potential applicability of dot blot hybridization in epidemiological studies to identify specific profiles isolated from a herd or between herds, as well as in the characterization of the *S. uberis* population in specific regions.

5.6 CONCLUSION

A high prevalence of the genes *sua*, *pauA* and *gapC* was obtained, suggesting the importance of these virulence factors for the adhesion, invasion and multiplication of *S. uberis* in intramammary infections. Also, the presence of the same dot pattern in different mammary quarters of the same cow suggests the contagious transmission mode in some herds. Our results suggest that Brazilian *S. uberis* strains present high heterogeneity that reinforces that the contamination comes from several places. So, the application of hygiene measures is very important, such as the use of pre and post dipping and the correct cleaning of milking equipment.

5.7 REFERENCES

ABUREEMA, S.; SMOOKER, P.; MALMO, J.; DEIGHTON, M. Molecular epidemiology of recurrent clinical mastitis due to Streptococcus uberis: evidence of both an environmental source and recurring infection with the same strain. **J. Dairy Sci**. v.97, p.285–90, 2014.

ALBUQUERQUE, P.; RIBEIRO, N.; ALMEIDA, A.; PANSCHIN, I.; PORFIRIO, A.; VALES, M.; DINIZ, F.; MADEIRA, H. Application of a Dot Blot Hybridization Platform to Assess Streptococcus uberis Population Structure in Dairy Herds. **Front. Microbiol**. v.8, p.1–11. 2017.

ALMEIDA, R.A.; LUTHER, D.A.; PARK, H.M.; OLIVER, S.P. Identification, isolation, and partial characterization of a novel Streptococcus uberis adhesion molecule (SUAM). **Vet. Microbiol**. v.115, p.183–191, 2006.

ALMEIDA, A.; ALBUQUERQUE, P.; ARAUJO, R.; RIBEIRO, N.; TAVARES, F. Detection and discrimination of common bovine mastitis-causing streptococci. **Vet. Microbiol**., v.164(3-4), p.370–377, 2013.

BRADLEY, A.J.; GREEN, M.J. The importance of the nonlactating period in the epidemiology of intramammary infection and strategies for prevention. **Vet. Clin. North Am. Food Anim**. **Pract**. v.20, p.547–568. 2004.

CARIDADE, C.M.R.; MARCAL, A.R.S.; ALBUQUERQUE, P.; MENDES, M.V.; TAVARES, F. Automatic Analysis of Dot Blot Images. **Intell. Autom. Soft Comput.** v.21, p.607–622. 2015.

DAVIES, P.L.; ARCHER, S.C.; EMES, R.D.; BRADLEY, A.J.; LEIGH, J.A.; GREEN, M.J. Molecular Epidemiology of Streptococcus uberis clinical Mastitis in Dairy Herds: Strain Heterogeneity and Transmission. **J. Clin. Microbiol**. v.54, JCM.01583-15. 2015.

EL-SAYED, A.; AWAD, W.; ABDOU, N.E.; CASTAÑEDA VÁZQUEZ, H. Molecular biological tools applied for identification of mastitis causing pathogens. **Int. J. Vet. Sci. Med**. v.5, p.89–97. 2017.

FAN, H.H.; KLEVEN, S.H.; JACKWOOD, M.W. Application of Polymerase Chain Reaction with Arbitrary Primers to Strain Identification of Mycoplasma gallisepticum. **Avian Dis**. v.39, p.729. 1995.

FESSIA, A.S.; DIESER, S.A.; ODIERNO, L.M. Identificación de Streptococcus uberis aislados de muestras de leche bovina. **Rev. Científica FAV-UNRC Ab Intus** v.1, p.82–87, 2018.

FESSIA, A.S.; DIESER, S.A.; RASPANTI, C.G.; ODIERNO, L.M. Genotyping and study of adherence-related genes of Streptococcus uberis isolates from bovine mastitis. **Microb. Pathog.** v.130, p.295–301. 2019.

FIELD, T.R.; WARD, P.N.; PEDERSEN, L.H.; LEIGH, J.A. The hyaluronic acid capsule of Streptococcus uberis is not required for the development of infection and clinical mastitis. **Infect. Immun.** v.71, p.132–9. 2003.

GUO, Q.; YU, Y.; ZHU, Y.L.; ZHAO, X.Q.; LIU, Z.G.; ZHANG, Y.Y.; LI, G.L.; WEI, J.H.; WU, Y.M.; WAN, K.L. Rapid Detection of Rifampin-resistant Clinical Isolates of Mycobacterium tuberculosis by Reverse Dot Blot Hybridization. **Biomed. Environ. Sci**. v.28, p.25–35. 2015.

MENG, J.; GU, Q.; LIN, S.; PENG, B.; LIU, L.; TIAN, Y.; LI, L. Dot-Blot Hybridization for Detection of Five Cucurbit Viruses by Digoxigenin-Labelled cDNA Probes. **Agric. Sci. China** v.6, p.1450–1455. 2007.

NAQVI, S.A.; DE BUCK, J.; DUFOUR, S.; BARKEMA, H.W. Udder health in Canadian dairy heifers during early lactation. **J. Dairy Sci**. v.101, p.3233–3247. 2018.

OLIVER, S.P.O.; GONZÁLEZ, R.N.; HOGAN, J.S.; JAYARAO, B.M.; OWENS, W.E. Microbiological Procedures for the Diagnosis of bovine udder infection and determination of milk quality. Pages 1–40, 44–46 in National Mastitis Council Inc, **Proceedings..** Verona, USA, 2004.

PERRIG, M.S.; AMBROGGIO, M.B.; BUZZOLA, F.R.; MARCIPAR, I.S.; CALVINHO, L.F.; VEAUTE, C.M.; BARBAGELATA, M.S. Genotyping and study of the pauA and sua genes of Streptococcus uberis isolates from bovine mastitis. **Rev. Argent. Microbiol**. v.47, p.282–294. 2015.

PHUEKTES, P.; MANSELL, P.D.; DYSON, R.S.; HOOPER, N.D.; DICK, J.S.; BROWNING, G.F. Molecular Epidemiology of Streptococcus uberis Isolates from Dairy Cows with Mastitis. **J. Clin. Microbiol.** v.39, p.1460–1466. 2001.

REINOSO, E.B.; LASAGNO, M.C.; DIESER, S.A.; ODIERNO, L.M. Distribution of virulence-associated genes in Streptococcus uberis isolated from bovine mastitis. **FEMS Microbiol. Lett.** 318:183–188. 2011.

ROBERSON, J.R. Treatment of Clinical Mastitis. **Vet. Clin. North Am. Food Anim. Pract.** v.28, p.271–288. 2012.

ROSEY, E.L.; LINCOLN, R.A.; WARD, P.N.; YANCEY, R.J.; LEIGH, J.A. PauA: a novel plasminogen activator from Streptococcus uberis. **FEMS Microbiol. Lett.** 178:27–33. 1999.

ROYSTER, E.; WAGNER, S. Treatment of Mastitis in Cattle. **Vet. Clin. North Am. - Food Anim. Pract**. v.31, p.17–46. 2015.

SCHUKKEN, Y.; WILSON, D.; WELCOME, F.; GARRISON-TIKOFSKY, L.; GONZALEZ, R.; SCHUKKEN, Y.H.; WILSON, D.J.; GONZALEZ, R.N. Monitoring udder health and milk quality using somatic cell counts. **Vet. Res.** v.34, p.579–596. 2003.

SMITH, A.J.; KITT, A.J.; WARD, P.N.; LEIGH, J.A. Isolation and characterization of a mutant strain of Streptococcus uberis, which fails to utilize a plasmin derived beta-casein peptide for the acquisition of methionine. **J. Appl. Microbiol.** v.93, p.631–9. 2002.

TIMONEN, A.A.E.; KATHOLM, J.; PETERSEN, A.; ORRO, T.; MÕTUS, K.; KALMUS, P. Elimination of selected mastitis pathogens during the dry period**. J. Dairy Sci.** v.101, p.1–7. 2018.

WARD, P. N.; FIELD, T. R.; DITCHAM, W. G.; MAGUIN, E.; LEIGH, J. A. Identification and disruption of two discrete loci encoding hyaluronic acid capsule biosynthesis genes hasA, hasB, and hasC in Streptococcus uberis. **Infect Immun**, v.69(1), p.392–399, 2001.

WIRAWAN, R.E.; KLESSE, N.A.; JACK, R.W.; TAGG, J.R. Molecular and Genetic Characterization of a Novel Nisin Variant Produced by Streptococcus uberis. **Appl. Environ. Microbiol.** v.72, p.1148–1156. 2006.

ZADOKS, R.N.; GILLESPIE, B.E.; BARKEMA, H.W.; SAMPIMON, O.C.; OLIVER, S.P.; SCHUKKEN, Y.H. Clinical, epidemiological and molecular characteristics of Streptococcus uberis infections in dairy herds. **Epidemiol. Infect. v.**130(2), p.335-49, 2003.

ZHANG, C.Y.; CHEN, G.F.; LIU, Y.; WANG, Y.Y.; XU, Z.; ZHANG, B.Y.; WANG, G.C. Simultaneous detection of harmful algae by multiple polymerase chain reaction coupled with reverse dot blot hybridization. **Harmful Algae** v.35, p.9–19. 2014.

CHAPTER 6

Final Considerations

6. FINAL CONSIDERATIONS

Taking into the consideration the results reported in this thesis, we confirmed our hypothesis of the high genotypic variability for *S. aureus* and *S. uberis* isolated from bovine mastitis. In chapter 3, the genotypic variability of *S. aureus* isolated in 8 different countries was evaluated by genotyping the 16S-23S rRNA intergenic space. Using this methodology, it was possible to find new genotypes or variants of existing genotypes, and a specific genotype pattern in each country. In addition, the study investigated the presence of 26 virulence factors, among genes related to cell adhesion and invasion, host defense system, enterotoxins and exfoliative toxins. It was possible to notice a great dispersion of genes encoding enterotoxins in all countries; however, a low diffusion of MRSA strains has been isolated from bovine mastitis.

In the chapter 4, the objective was to evaluate the antimicrobial resistance profiles and clonal diversity of *S. aureus* strains isolated from cows submitted to a treatment protocol against subclinical mastitis during lactation. The PFGE, considered the gold standard of molecular typing, allowed a high discrimination of genotypic profiles, with two evaluations performed to better understand the results. First, all the pre-treatment isolates were considered, and 7 distinct pulsotypes were identified, and a second evaluation to compare the profiles of isolates from the same mammary quarter, before and after clinical treatment. This study allowed us to observe a high diversity of strains, within the same herd, even after the application of antibiotics combined with vaccination.

The broth microdilution test was used to assess resistance to antimicrobials, and our study identified that some compounds were highly effective in vitro for inhibiting the *S. aureus* growth. For example, gentamicin, enrofloxacin, ciprofloxacin and tetracycline had the lowest MIC. On the other hand, a high proportion of the isolates in our study were resistant to erythromycin, ampicillin and penicillin, while amoxicillin was resistant to all *S. aureus* evaluated, confirming our hypothesis about the considerable resistance to some antimicrobials in Brazil. In the same analysis, we identified a low occurrence of MRSA strains in our study, since the MIC test identified only 1 methicillin-resistant isolate.

Finally, in chapter 5 we described the genotypic diversity in *S. uberis* using the hybridization of DNA. The presence of some genetic markers was evaluated and thus, it was possible to establish which were the virulence factors commonly found in *S. uberis* isolated in bovine mastitis. The Dot-Blot technique allowed a rapid confirmation of the isolates through taxonomic markers and it was possible to observe a high prevalence of the *sua* gene, responsible

for *S. uberis* adhesion and *pauA* gene, related with mechanisms for obtaining nutrients by the bacteria, necessary for its growth. Although its environmental transmission, it is known that *S. uberis* also has a contagious transmission profile, which was also confirmed by our study, while identical patterns were observed in different mammary quarters of the same animal.

In general, our study allowed to obtain molecular and epidemiological data about *S. aureus* and *S. uberis*, using different molecular techniques. *S. aureus* remains a pathogen with a difficult control, due to its resistance to antimicrobials and production of virulence factors. Despite of the low bacteriological cure rates of *S. aureus* IMI to different antimicrobial treatments, our results suggest that almost half of non-cured IMI are new infections caused by a different genotypic profile. Measurement of resistance to antimicrobials by MIC test is essential for selecting the most effective therapy, and can be combined with molecular analysis in the search for resistance genes. In the case of *S. uberis*, we described that this pathogen has both an environmental and contagious transmission pattern, and that molecular methodos helps in the identification of transmission patterns and pathogenicity of the infecting strains.

Although the number of isolates per study was possibly a limitation of the present study, it was possible to infer some important characteristics of these pathogens isolated from Brazil and other countries. In addition, it was possible to determine which are the main virulence factors associated with both species and identified specific patterns for certain regions. Future studies should be done, to evaluate a greater number of isolates, as well as assisting in the control strategies of *S. aureus* and *S. uberis* mastitis, minimizing the acquisition of resistance to antimicrobials and increasing the success rate of treatments against bovine mastitis.

REFERENCES

ABUREEMA, S.; SMOOKER, P.; MALMO, J.; DEIGHTON, M. Molecular epidemiology of recurrent clinical mastitis due to Streptococcus uberis: evidence of both an environmental source and recurring infection with the same strain. **J. Dairy Sci**, v. 97, n. 1, p. 285–90, 2014.

AKKOU, M.; BOUCHIAT, C.; ANTRI, K.; BES, M.; TRISTAN, A.; DAUWALDER, O.; MARTINS-SIMOES, P.; RASIGADE, J. P.; ETIENNE, J.; VANDENESCH, F.; RAMDANI-BOUGUESSA, N.; LAURENT, F. New host shift from human to cows within Staphylococcus aureus involved in bovine mastitis and nasal carriage of animal's caretakers. **Vet. Microbiol** v.223, p.173–180, 2018.

ALBUQUERQUE, P.; RIBEIRO, N.; ALMEIDA, A.; PANSCHIN, I.; PORFIRIO, A.; VALES, M.; DINIZ, F.; MADEIRA, H. Application of a Dot Blot Hybridization Platform to Assess Streptococcus uberis Population Structure in Dairy Herds. **Front. Microbiol**. v.8, p.1–11. 2017.

ALMEIDA, A.; ALBUQUERQUE, P.; ARAUJO, R.; RIBEIRO, N.; TAVARES, F. Detection and discrimination of common bovine mastitis-causing streptococci. **Vet. Microbiol**., v.164(3-4), p.370–377, 2013.

ALMEIDA, R. A.; DEGO, O. K.; HEADRICK, S. I.; LEWIS, M. J.; OLIVER, S. P. Role of Streptococcus uberis adhesion molecule in the pathogenesis of Streptococcus uberis mastitis. **Vet. Microbiol**, v.179(3-4), p.332–335, 2015.

ALMEIDA, R.A.; LUTHER, D.A.; PARK, H.M.; OLIVER, S.P. Identification, isolation, and partial characterization of a novel Streptococcus uberis adhesion molecule (SUAM). **Vet. Microbiol**. v.115, p.183–191, 2006.

ARTURSSON, K.; SÖDERLUND, R.; LIU, L.; MONECKE, S.; SCHELIN, J. Genotyping of Staphylococcus aureus in bovine mastitis and correlation to phenotypic characteristics. **Vet. Microbiol**, v.193, p.156–161, 2016.

BANNERMAN, T. L.; HANCOCK, G. A.; TENOVER, F. C.; MILLER, J. M. Pulsed-Field Gel Electrophoresis as a Replacement for Bacteriophage Typing of Staphylococcus aureus. **J Clin Micr**, v. 33, n. 3, p. 551–555, 1995.

BERRIDGE, B. R.; BERCOVIER, H.; FRELIER, P. F. Streptococcus agalactiae and Streptococcus difficile 16S-23S intergenic rDNA: Genetic homogeneity and species-specific PCR. **Vet Micr**, v. 78, n. 2, p. 165–173, 2001.

BOONYAYATRA, S.; THARAVICHITKUL, P.; OLIVER, S.P. Virulence-associated genes and molecular typing of Streptococcus uberis associated with bovine mastitis in northern Thailand. **Turk. J. Vet. Anim. Sci.** v.42, p.73-81, 2018.

BRADLEY, A. Bovine mastitis: an evolving disease. **Vet J.** v.164(2), p.116-128, 2002

CARIDADE, C.M.R.; MARCAL, A.R.S.; ALBUQUERQUE, P.; MENDES, M.V.; TAVARES, F. Automatic Analysis of Dot Blot Images. **Intell. Autom. Soft Comput.** v.21, p.607–622. 2015.

CASTAÑÓN –SÁNCHEZ, C.A. Patogenia molecular de Staphylococcus aureus **Evid Med Invest Salud** v.5 (3), p.79-84, 2012.

CERVANTES-GARCÍA, E.; GARCÍA-GONZÁLEZ, R.; MARÍA SALAZAR-SCHETTINO, P. General characteristics of Staphylococcus aureus **Rev Latinoam Patol Clin Med Lab**, v.61, p.28-40, 2014.

CHEN, X.; DEGO, O. K.; ALMEIDA, R. A.; FULLER, T. E.; LUTHER, D. A.; OLIVER, S. P. Deletion of sua gene reduces the ability of Streptococcus uberis to adhere to and internalize into bovine mammary epithelial cells. **Vet. Microbiol**, v.147(3-4), p.426–434, 2011.

COSGROVE, S. E.; QI, Y.; KAYE, K. S.; HARBARTH, S.; KARCHMER, A. W.; CARMELI, Y. The impact of methicillin resistance in Staphylococcus aureus bacteremia on patient outcomes: mortality, length of stay, and hospital charges. **Infect Control Hosp Epidemiol**, 26(2), 166–174, 2005.

COSTA, G.M; BARROS, R.A.; CUSTÓDIO, D.A.C.; PEREIRA, U.P.; FIGUEIREDO, D.J.; SILVA, N. Antimicrobial resistance in Staphylococcus aureus isolated from mastitis in dairy herds from the state of Minas Gerais, Brazil **Arq. Inst. Biol**., v.80, n.3, p. 297-302, 2013.

CREMONESI, P.; CASTIGLIONI, B.; MALFERRARI, G.; BIUNNO, I.; VIMERCATI, C.; MORONI, P.; MORANDI, S.; LUZZANA, M. Technical note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. **J Dairy Sci**, v. 89, n. 1, p. 163–9, 2006.

CREMONESI, P.; ZOTTOLA, T.; LOCATELLI, C.; POLLERA, C.; CASTIGLIONI, B.; SCACCABAROZZI, L.; MORONI, P. Identification of virulence factors in 16S-23S rRNA intergenic spacer genotyped Staphylococcus aureus isolated from water buffaloes and small ruminants. **J Dairy Sci**, v. 96, n. 12, p. 7666–74, 2013.

FIELD, T.R.; WARD, P.N.; PEDERSEN, L.H.; LEIGH, J.A. The hyaluronic acid capsule of Streptococcus uberis is not required for the development of infection and clinical mastitis. **Infect. Immun.** v.71, p.132–9. 2003.

FONTAINE, C.E.; PEREZ-CASAL, J.; XI-MING, S.; SHELFORD, J.; WILSON, P.J.; POTTER, A.A. Immunisation of dairy cattle with recombinant Streptococcus uberis GapC or a chimeric CAMP antigen confers protection against heterologous bacterial challenge. **Vaccine** v.20, p.2278–2286, 2002.

FOURNIER, C.; KUHNERT, P.; FREY, J.; MISEREZ, R.; KIRCHHOFER, M.; KAUFMANN, T.; STEINER, A.; GRABER, H. U. Bovine Staphylococcus aureus: Association of virulence genes, genotypes and clinical outcome. **Res Vet Sci**, v. 85, n. 3, p. 439–448, 2008.

FUDA, C. C.; FISHER, J. F.; MOBASHERY, S. Beta-lactam resistance in Staphylococcus aureus: the adaptive resistance of a plastic genome. **Cell Mol Life Sci**, v.62(22), p.2617–2633, 2005.

GAO, J.; BARKEMA, H.W.; ZHANG, L.; LIU, G.; DENG, Z.; CAI, L.; SHAN, R.; ZHANG, S.; ZOU, J.; KASTELIC, J.P.; HAN, B. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. **J Dairy Sci**. v.100(6), p.4797-4806, 2017.

GARCÍA-MARTÍNEZ, J.; MARTÍNEZ-MURCIA, A.; ANTÓN, A. I.; RODRÍGUEZ-VALERA, F. Comparison of the small 16S to 23S intergenic spacer region (ISR) of the rRNA operons of some Escherichia coli strains of the ECOR collection and E. coli K-12. **J Bacteriol**, v. 178, n. 21, p. 6374–6377, 1996.

GRABER, H.U.; NASKOVA, J.; STUDER, E.; KAUFMANN, T.; KIRCHHOFER, M.; BRECHBÜHL, M.; SCHAEREN, W.; STEINER, A.; FOURNIER, C. Mastitis-related subtypes of bovine Staphylococcus aureus are characterized by different clinical properties. **J Dairy Sci** v.92, 2009.

GRUET, P.; MAINCENT, P.; BERTHELOT, X.; KALTSATOS, V. Bovine mastitis and intramammary drug delivery: review and perspectives. **Adv Drug Deliv Rev**. v.50, p.245-259, 2001

GUO, Q.; YU, Y.; ZHU, Y.L.; ZHAO, X.Q.; LIU, Z.G.; ZHANG, Y.Y.; LI, G.L.; WEI, J.H.; WU, Y.M.; WAN, K.L. Rapid Detection of Rifampin-resistant Clinical Isolates of Mycobacterium tuberculosis by Reverse Dot Blot Hybridization. **Biomed. Environ. Sci**. v.28, p.25–35. 2015.

CUNHA, R.P.L.; MOLINA, L.R.; CARVALHO, A.U.; FACURY FILHO, E.J.; FERREIRA, P.M.; GENTILINI, M.B. Subclinical mastitis and the relationship between somatic cell count with number of lactations, production and chemical composition of the milk. **Arq. Bras. Med. Vet. Zootec.** v.60, n.1, p.19-24, 2008.

DOGAN, B.; BOOR, K. J. Short communication: growth characteristics of Streptococcus uberis in UHT-treated milk. **J. Dairy Sci.** v.87(4), p.813–815, 2004.

DUARTE RS, MIRANDA OP, BELLEI BC, BRITO MA, TEIXEIRA LM. Phenotypic and molecular characteristics of Streptococcus agalactiae isolates recovered from milk of dairy cows in Brazil. **J Clin Microbiol**. v.42, p.4214-4222, 2004

DUPIEUX, C.; CAMUS, C.; LINA, G.; VANDENESCH, F.; LAURENT, F.; RASIGADE, J.P. Does β-toxin production contribute to the cytotoxicity of hypervirulent Staphylococcus aureus? **J Infect Dis**. v.211(5), p.846-847, 2015.

EL-SAYED, A.; AWAD, W.; ABDOU, N.E.; CASTAÑEDA VÁZQUEZ, H. Molecular biological tools applied for identification of mastitis causing pathogens. **Int. J. Vet. Sci. Med**. v.5, p.89–97. 2017.

ERICSSON UNNERSTAD, H.; LINDBERG, A.; PERSSON WALLER, K.; EKMAN, T.; ARTURSSON, K.; NILSSON-OST, N.; BENGTSSON B. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. **Vet Microbiol.** v.137(1-2), p.90-97, 2009.

FERNANDES, C.J.; FERNANDES, L.A.; COLLIGNON, P. Cefoxitin resistance as a surrogate marker for the detection of methicillin-resistant Staphylococcus aureus. **J. Antimicrob. Chemother**. v.55, p.506–510, 2005.

FERREIRA, L. M.; NADER FILHO, A.; OLIVEIRA, E. de; ZAFALON, L. F.; SOUZA, V. de. Phenotypic and genotypic variabilities of Staphylococcus aureus strains isolated from bovine subclinical mastitis. **Ciência Rural**, v. 36, n. 4, p. 1228–1234, 2006.

FESSIA, A.S.; DIESER, S.A.; RASPANTI, C.G.; ODIERNO, L.M. Genotyping and study of adherence-related genes of Streptococcus uberis isolates from bovine mastitis. **Microb. Pathog.** v.130, p.295–301. 2019.

FEßLER, A.; SCOTT, C.; KADLEC, K.; EHRICHT, R.; MONECKE, S.; SCHWARZ, S. Characterization of methicillin-resistant Staphylococcus aureus ST398 from cases of bovine mastitis. **J Antim Chem**, v. 65, n. 4, p. 619–625, 1 abr. 2010.

GURJAR, A.; GIOIA, G.; SCHUKKEN, Y.; WELCOME, F.; ZADOKS, R.; MORONI, P. Molecular diagnostics applied to mastitis problems on dairy farms. **Vet Clin Food Anim**, v.28(3), p.565–576, 2012.

HARAN, K. P.; GODDEN, S. M.; BOXRUD, D.; JAWAHIR, S.; BENDER, J. B.; SREEVATSAN, S. Prevalence and characterization of Staphylococcus aureus, including methicillin-resistant Staphylococcus aureus, isolated from bulk tank milk from Minnesota dairy farms. **J Clin Microbiol**, v. 50, n. 3, p. 688–695, 2012.

HASSAN, A. A.; KHAN, I. U.; ABDULMAWJOOD, A.; LÄMMLER, C. Inter- and intraspecies variations of the 16S-23S rDNA intergenic spacer region of various streptococcal species. **Syst App Microb**, v. 26, n. 1, p. 97–103, 2003.

HOGAN, J.; SMITH, K.L. Managing environmental mastitis. **Vet Clin North Am Food Anim Pract**. v.28, p.217-224, 2012

JENSEN, M. A.; WEBSTER, J. A.; STRAUS, N. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. **Appl Environ Microbiol**, v. 59, n. 4, p. 945–52, abr. 1993.

JIANG, M.; BABIUK, L.A; POTTER, A.A. Cloning, sequencing and expression of the CAMP factor gene ofStreptococcus uberis **Microb. Pathog**, v.20, p.297-307, 1996.

JOHNSEN, L.B.; POULSEN, K.; KILIAN, M.; PETERSEN, P. Purification and cloning of a streptokinase from Streptococcus uberis. **Infect. Immun**. v.67, p.1072–107, 1999.

KEEFE, G. Update on control of Staphylococcus aureus and Streptococcus agalactiae for management of mastitis. **Vet Clin North Am Food Anim Pract**. v.28, p.203-216, 2012

KERRO DEGO, O.; ALMEIDA, R. A.; SAXTON, A. M.; ABDI, R. D.; ENSERMU, D. B.; OLIVER, S. P. Bovine intramammary infection associated immunogenic surface proteins of Streptococcus uberis. **Microb Pathog**, v.115, p.304–311, 2018.

KHORAMROOZ, S. S.; MANSOURI, F.; MARASHIFARD, M.; MALEK HOSSEINI, S. A.; AKBARIAN CHENARESTANE-OLIA, F.; GANAVEHEI, B.; GHARIBPOUR, F.; SHAHBAZI, A.; MIRZAII, M.; DARBAN-SAROKHALIL, D. Detection of biofilm related genes, classical enterotoxin genes and agr typing among Staphylococcus aureus isolated from bovine with subclinical mastitis in southwest of Iran. **Microb Pathog**, v.97, p.45–51, 2016.

KIRK, J.H.; MCCOWAN, B.; ATWILL, E.R.; GLENN, K.S.; HIGGINBOTHAM, G.E.; COLLAR, C.A.; CASTILLO, A.; REED, B.A.; PETERSON, N.G.; CULLOR, J.S. Association of minimum inhibitory concentration cluster patterns with dairy management practices for environmental bacteria isolated from bulk tank milk. **J Dairy Sci** v.88, Issue 10, 2005.

LANG, P.; LEFÉBURE, T.; WANG, W.; ZADOKS, R.N.; SCHUKKEN, Y.; STANHOPE, M.J. Gene content differences across strains of Streptococcus uberis identified using oligonucleotide microarray comparative genomic hybridization. **Infect Genet Evol**.v.9(2), p.179-188, 2009

LEIGH, J.A. Streptococcus uberis: a permanent barrier to the control of bovine mastitis? **Vet J**, v.157(3), p.225–238, 1999.

MAGALHÃES, V. D.; FERREIRA, J. C.; BARELLI, C.; DARINI, A. L. C. Pulsed field gel electrophoresis use in bacteriology – a technical review. **Revista Instituto Adolfo Lutz**, v. 64, n. 2, p. 155–161, 2005.

MAHMOOD, T.; YANG, P.C. Western blot: technique, theory, and trouble shooting. **N Am J Med Sci**, v.4(9), p.429–434, 2012.

MAKOVEC, J.A.; RUEGG, P.L. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. **J Dairy Sci**. v.86(11), p.3466-3472, 2003.

MCDOUGALL, S. Prevalence of clinical mastitis in 38 Waikato dairy herds in early lactation. **N Z Vet J.** v.47(4), p.143-149, 1999.

MENDONCA, E.C.L.; MARQUES, V.F.; MELO, D.A.; ALENCAR, T.A.; COELHO, I.S.; COELHO, S.M.O.; SOUZA, M.M.S. Phenogenotypical characterization of antimicrobial resistance in Staphylococcus spp. isolated from bovine mastitis. **Pesq. Vet. Bras**. v.32, n. 9, p.859-864, 2012.

MENG, J.; GU, Q.; LIN, S.; PENG, B.; LIU, L.; TIAN, Y.; LI, L. Dot-Blot Hybridization for Detection of Five Cucurbit Viruses by Digoxigenin-Labelled cDNA Probes. **Agric. Sci. China** v.6, p.1450–1455. 2007.

MONISTERO, V.; GRABER, H.; POLLERA, C.; CREMONESI, P.; CASTIGLIONI, B.; BOTTINI, E.; CEBALLOS-MARQUEZ, A.; LASSO-ROJAS, L.; KROEMKER, V.; WENTE, N.; PETZER, I.M.; SANTISTEBAN, C.; RUNYAN, J.; VEIGA DOS SANTOS, M.; ALVES, B.G; PICCININI, R.; BRONZO, V.; ABBASSI, M.; SAID, M.; MORONI, P. Staphylococcus aureus Isolates from Bovine Mastitis in Eight Countries: Genotypes, Detection of Genes Encoding Different Toxins and Other Virulence Genes. **Toxins (Basel)**. 10, 247, 2018.

MOSHYNSKY, I.; JIANG, M.; FONTAINE, M.C.; PEREZ-CASAL, J.; BABIUK, L.A.; POTTER, A.A. Characterization of a bovine lactoferrin binding protein of Streptococcus uberis. **Microb. Pathog**. v.35, p.203–215, 2003.

OGUNNIYI, A. D.; MAHDI, L. K.; TRAPPETTI, C.; VERHOEVEN, N.; MERMANS, D. VAN DER HOEK, M. B.; PLUMPTRE, C. D.; PATON, J. C. Identification of genes that contribute to the pathogenesis of invasive pneumococcal disease by in vivo transcriptomic analysis. **Infect Immun**, v.80(9), p.3268–3278, 2012.

OLIVEIRA, C.S.; HOGEVEEN, H.; BOTELHO, A.M.; MAIA, P.V.; COELHO, S.G.; HADDAD, J.P. Cow-specific risk factors for clinical mastitis in Brazilian dairy cattle. **Prev Vet Med**. v.121(3-4), p.297-305, 2015.

OLIVEIRA, L.; LANGONI, H.; HULLAND, C.; RUEGG, P.L. Minimum inhibitory concentrations of Staphylococcus aureus recovered from clinical and subclinical cases of bovine mastitis. **J Dairy Sci.** v.95(4), p.1913-1920, 2012.

OLIVER, S.P.; ALMEIDA, R.A.; GILLESPIE, B.E.; HEADRICK, S.J.; DOWLEN, H.H.; JOHNSON, D.L.; LAMAR, K.C.; CHESTER, S.T.; MOSELEY, W.M. Extended Ceftiofur Therapy for Treatment of Experimentally-Induced Streptococcus uberis Mastitis in Lactating Dairy Cattle. **J. Dairy Sci.** 87, 3322–3329, 2004.

OLIVER, S.P.; MURINDA, S.E. Antimicrobial resistance of mastitis pathogens. **Vet Clin North Am Food Anim Pract**. v.28(2), p.165-185, 2012.

PERRIG, M.S.; AMBROGGIO, M.B.; BUZZOLA, F.R.; MARCIPAR, I.S.; CALVINHO, L.F.; VEAUTE, C.M.; BARBAGELATA, M.S. Genotyping and study of the pauA and sua genes of Streptococcus uberis isolates from bovine mastitis. **Rev. Argent. Microbiol**. v.47, p.282–294. 2015.

PHILPOT, W.N.; NICKERSON, S.C. **Winning the Fight Against Mastitis** Westfalia Surge, Incorporated, 2000, 192 pages

PHUEKTES, P.; MANSELL, P.D.; DYSON, R.S.; HOOPER, N.D.; DICK, J.S.; BROWNING, G.F. Molecular Epidemiology of Streptococcus uberis Isolates from Dairy Cows with Mastitis. **J. Clin. Microbiol.** v.39, p.1460–1466. 2001.

PICCININI, R.; BORROMEO, V.; ZECCONI, A. Relationship between Staphylococcus aureus gene pattern and dairy herd mastitis. **Vet. Microbiol**., v.145, p.100–105, 2010.

POL, M.; RUEGG, P. L. Relationship between antimicrobial drug usage and antimicrobial susceptibility of gram-positive mastitis pathogens. **J Dairy Sci** v.90(1), p.262–273, 2007.

RABELLO, R.F.; SOUZA, C.R.V.M.; DUARTE, R.S.; LOPES, R.M.M.; TEIXEIRA, L.M.; CASTRO, A C.D. Characterization of Staphylococcus aureus isolates recovered from bovine mastitis in Rio de Janeiro, Brazil. **J. Dairy Sci**. v.88, p.3211–9, 2005.

REINOSO, E.B.; LASAGNO, M.C.; DIESER, S.A.; ODIERNO, L.M. Distribution of virulence-associated genes in Streptococcus uberis isolated from bovine mastitis. **FEMS Microbiol. Lett.** 318:183–188. 2011.

ROBERSON, J.R. Treatment of Clinical Mastitis. **Vet. Clin. North Am. Food Anim. Pract.** v.28, p.271–288. 2012.

ROSEY, E.L.; LINCOLN, R.A.; WARD, P.N.; YANCEY, R.J.; LEIGH, J.A. PauA: a novel plasminogen activator from Streptococcus uberis. **FEMS Microbiol. Lett.** 178:27–33. 1999.

ROYSTER, E.; WAGNER, S. Treatment of Mastitis in Cattle. **Vet. Clin. North Am. - Food Anim. Pract**. v.31, p.17–46. 2015.

SAINI, V.; MCCLURE, J. T.; LÉGER, D.; DUFOUR, S.; SHELDON, A. G.; SCHOLL, D. T.; BARKEMA, H. W. Antimicrobial use on Canadian dairy farms. **J Dairy Sci**, v.95(3), p.1209–1221, 2012.

SANTOS, A. L. dos; SANTOS, D. O.; FREITAS, C. C. de; FERREIRA, B. L. A.; AFONSO, I. F.; RODRIGUES, C. R.; CASTRO, H. C. Staphylococcus aureus: visiting a strain of clinical importance. **J. Bras. Patol. Med. Lab**., v. 43, p. 413–423, 2007.

SMITH, A. J.; WARD, P. N.; FIELD, T. R.; JONES, C. L.; LINCOLN, R. A.; LEIGH, J. A. MtuA, a lipoprotein receptor antigen from Streptococcus uberis, is responsible for acquisition of manganese during growth in milk and is essential for infection of the lactating bovine mammary gland. **Infect. Immun.** v.71(9), p.4842–4849, 2003.

SMITH, A.J.; KITT, A.J.; WARD, P.N.; LEIGH, J.A. Isolation and characterization of a mutant strain of Streptococcus uberis, which fails to utilize a plasmin derived beta-casein peptide for the acquisition of methionine. **J. Appl. Microbiol.** v.93, p.631–9. 2002.

SOMMERHÄUSER, J.; KLOPPERT, B.; WOLTER, W.; ZSCHÖCK, M.; SOBIRAJ, A.; FAILING, K. The epidemiology of Staphylococcus aureus infections from subclinical mastitis in dairy cows during a control programme. **Vet. Microbiol**, v.96, p.91–102, 2003.

STEENEVELD, W.; HOGEVEEN, H.; BARKEMA, H.W.; VAN DEN BROEK, J.; HUIRNE, R.B.M. The influence of cow factors on the incidence of clinical mastitis in dairy cows. **J. Dairy Sci.** v.91, p1391–1402, 2008.

STEVENS, M.; PIEPERS, S.; DE VLIEGHER, S. Mastitis prevention and control practices and mastitis treatment strategies associated with the consumption of (critically important) antimicrobials on dairy herds in Flanders, Belgium. **J Dairy Sci**. v.99(4), p.2896-2903, 2016

STOWELL, R.R.; INGLIS, S. **Sand for bedding**. Dairy Housing and Equipment Systems, 2000. NRAES 129.

SWENSON, J.M., BRASSO, W.B., FERRARO, M.J., HARDY, D.J., KNAPP, C.C., LONSWAY, D., MCALLISTER, S., RELLER, L.B., SADER, H.S., SHORTRIDGE, D., SKOV, R., WEINSTEIN, M.P., ZIMMER, B.L., PATEL, J.B. Correlation of cefoxitin MICs with the presence of mecA in Staphylococcus spp. **J. Clin. Microbiol**. 47, 1902–5. 2009.

TOMAZI, T. **Etiological and molecular profile of pathogens causing clinical mastitis, and antimicrobial use in dairy herds.** 2017. 187p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

TOMAZI, T.; DE SOUZA FILHO, A.F.; HEINEMANN, M.B.; SANTOS, M.V. Molecular characterization and antimicrobial susceptibility pattern of Streptococcus agalactiae isolated from clinical mastitis in dairy cattle. **PLoS One** 13, e0199561, 2018.

VASUDEVAN, P.; NAIR, M.K.; ANNAMALAI, T.; VENKITANARAYANAN, K.S. Phenotypic and genotypic characterization of bovine mastitis isolates of Staphylococcus aureus for biofilm formation. **Vet Microbiol.** v.92(1-2), p.179-185, 2003

VIGUIER, C.; ARORA, S.; GILMARTIN, N.; WELBECK, K.; O'KENNEDY, R. Mastitis detection: current trends and future perspectives. **Trends Biotechnol**. v.27(8), p.486-493, 2009

WARD, P. N.; FIELD, T. R.; DITCHAM, W. G.; MAGUIN, E.; LEIGH, J. A. Identification and disruption of two discrete loci encoding hyaluronic acid capsule biosynthesis genes hasA, hasB, and hasC in Streptococcus uberis. **Infect Immun**, v.69(1), p.392–399, 2001.

WARD, P. N.; LEIGH, J. A. Characterization of Paub, a novel broad-spectrum plasminogen activator from Streptococcus uberis **J Bacteriol**, v.184 (1), p.119-125, 2002.

WOESE, C. R.; KANDLER, O.; WHEELIS, M. L. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. In: National Academy of Sciences of the United States of America, **Proceedings…**1990.

YANG, F.; WANG, Q.; WANG, X.; WANG, L.; LI, X.; LUO, J.; ZHANG, S.; LI, H. Genetic characterization of antimicrobial resistance in Staphylococcus aureus isolated from bovine mastitis cases in Northwest China. **J. Integr. Agric**. v.15, p.2842–2847, 2016.

ZADOKS, R. N.; SCHUKKEN, Y. H. Use of molecular epidemiology in veterinary practice. **Vet Clin North Am Food Anim Pract**, v. 22, n. 1, p. 229–261, 2006.

ZADOKS, R.N.; GILLESPIE, B.E.; BARKEMA, H.W.; SAMPIMON, O.C.; OLIVER, S.P.; SCHUKKEN, Y.H. Clinical, epidemiological and molecular characteristics of Streptococcus uberis infections in dairy herds. **Epidemiol. Infect.** 130:S0950268802008221. 2003.

ZANETTE, E.; SCAPIN, D.; ROSSI, E.M. Suscetibilidade antimicrobiana de Staphylococcus aureus isolados de amostras de leite de bovinos com suspeita de mastite. **Unoesc Ciência - ACBS** v.1, p.65–70, 2010.

ZECCONI, A.; SCALI, F. Staphylococcus aureus virulence factors in evasion from innate immune defenses in human and animal diseases. **Immunol. Lett.** v.150, p.12–22. 2013.

ZURITA, J.; MEJÍA, C.; GUZMAN-BLANCO, M. Diagnosis and susceptibility testing of methicillin-resistant Staphylococcus aureus in Latin America. **Brazilian J. Infect. Dis.** v.14, p.97–107, 2010.