VERÓNICA KAREN CASTRO PÉREZ

DETECTION OF VIRULENCE AND ANTIMICROBIAL RESISTANCE GENES IN *Staphylococcus aureus* ISOLATED FROM BOVINE MASTITIS IN BRAZIL

LAVRAS – MG

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Ciências Veterinárias, área de concentração em Ciências Veterinárias, para obtenção do título de Mestre.

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VERÓNICA KAREN CASTRO PÉREZ

DETECÇÃO DE GENES DE VIRULÊNCIA E RESISTÊNCIA AOS ANTIMICROBIANOS EM *Staphylococcus aureus* ISOLADOS DE MASTITE BOVINA NO BRASIL

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APROVADA em 22 de março de 2019.
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LAVRAS – MG
2019

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A minha irmã Jeanette, meu avô Pedro e minhas tias Angie e Roxana, pelo amor incondicional.
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“A mais bela coragem é a confiança que devemos ter na capacidade de nosso esforço”.

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“Así que no temas, porque yo estoy contigo; no te angusties, porque yo soy tu Dios. Te fortaleceré y te ayudaré; te sostendré con mi diestra victoriosa”.

Isaías 41.10

RESUMO

Staphylococcus aureus pode expressar diferentes fatores de virulência e resistência a vários agentes antimicrobianos em casos de mastite bovina. O presente estudo teve como objetivo avaliar os fatores de virulência e os mecanismos genéticos de resistência à drogas em 400 isolados de *S. aureus* de mastite bovina no Brasil, bem como verificar a associação entre essas características, ano de isolamento e origem geográfico. A identificação dos genes de virulência e resistência foi realizada por PCR *singleplex* e *multiplex*. A identificação fenotípica de formação de exopolissacarídeos (biofilme) foi realizado no caldo Triptona de Soja com Vermelho Congo e sacarose e incubados a 37 °C por 48 horas. Como resultado, 83,5% dos isolados foram produtores de biofilme. Os genes de resistência *icaAD* foram detectados em 98,5% isolados. Os genes *luk* (leucocidina Panton-Valentine), *seb* (enterotoxina estafilocócica B), *sec* (enterotoxina estafilocócica C), *sed* (enterotoxina estafilocócica D), *tst* (toxina 1 da síndrome do choque tóxico) foram observados em 3,5%, 0,5%, 1%, 0,25% e 0,74% dos isolados, respectivamente. Os genes das hemolisinas foram observados em 82,85% (*hla* + *hlb* +), 16,5% (*hla* +), 0,75% (*hlb* +). O gene *blaZ*, associado à resistência à penicilina, foi detectado em 82,03% dos isolados, enquanto os genes *tetK* de resistência à tetraciclina e *aac(6') -Ie–aph(2')-Ia* de resistência à aminoglicosídeos foi exibido em 33,87% e 45.15% dos isolados, respectivamente. O gene *mepA* associado a resistência a fluoroquinolonas foi detectado pela primeira vez em todos os isolados. Os genes de resistência identificados com menor frequência foram *tetM* (3,22%), *tetL* (1,61%), *ermA* (14,29%), *ermB* (14,29%), *ermC* (33,3%), *ermT* (9,52%), *ermY* (4,76%), *msrA* (9,52). %), *mphC* (9,52%). Concluí-se que houve uma alta frequência de *S. aureus* carregando genes de virulência para biofilme e hemolisina. Além disso, foi encontrada uma grande variedade de genes de resistência que conferem resistência a todas as classes de antimicrobianos utilizados em animais e população humana. Esses resultados mostram o potencial patogênico de *S. aureus* isolados de mastite bovina para causar doenças tanto em humanos quanto em animais.

Palavras-chave: Staphylococci. Infecção intramamária. Biofilme. *icaAD. blaZ. mepA.*

ABSTRACT

Staphylococcus aureus can present many mechanisms in order to remain in mammary gland. The present study aimed to evaluate virulence factors and genetic mechanisms of drug resistance in 400 *S. aureus* strains isolated from bovine mastitis in Brazil, as well as to assess the association between these characteristics, year of isolation and geographic origin of the strains. Singleplex and multiplex PCR was used to identify virulence factors and drug resistance encoding genes. Detection of biofilm-forming was carried out using Congo red Tryptic Soy Broth assay. As a result, 83.5% isolates were biofilm-forming and 98.5% strains exhibited the biofilm gene *icaAD*. Virulence genes *luk* (Panton–Valentine Leukocidin), *seb* (Staphylococcal Enterotoxin B), *sec* (Staphylococcal Enterotoxin C), *sed* (Staphylococcal Enterotoxin D), *tst* (Toxic shock syndrome toxin 1) were observed in 3.5%, 0.5%, 1%, 0.25% and 0.74% of the strains, respectively. Hemolysin genes were observed in 82.85% ($hla^{+}hlb^{+}$), 16.5% (hla^{+}) and 0.75% (hlb^{+}) isolates. The gene *blaZ*, associated with penicillin resistance, was detected in 82.03% isolates, whereas tetracycline resistance gene *tetK* and aminoglycoside gene *aac(6')-Ie–aph(2')-Ia* were observed in 33.87% and 45.15%, respectively. Fluoroquinolone resistance gene *mepA* was detected for the first time in all fluoroquinolone resistance *S. aureus* isolates. Resistance genes *tetM* (3.22%), *tetL* (1.61%), *ermA* (14.29%), *ermB* (14.29%), *ermC* (33.3%), *ermT* (9.52%), *ermY* (4.76%), *msrA* (9.52%) and *mphC* (9.52%) were detected in low frequency among the isolates. Our results showed a high frequency of *S. aureus* carrying mainly biofilm and hemolysin genes. Moreover, a wide variety of antimicrobial resistance genes that confers resistance to all classes of antimicrobial agents used in animals and human population were observed. These results highlight the pathogenic potential of *S. aureus* from cattle to cause severe disease in both humans and animals.

Keywords: Staphylococci. Intramammary infection. Biofilm. *icaAD*. *blaZ*. *mepA*.

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PRIMEIRA PARTE

1. GENERAL INTRODUCTION

Bovine mastitis is considered one of the most prevalent disease with the greatest impact on dairy cattle production worldwide, since it reduces the quantity and quality of milk produced (KEEFE, 2012; HOGEVEEN; VAN, 2017). *Staphylococcus aureus* is considered the main agent involved in bovine mastitis (ROLLIN et al., 2015; BOBBO et al., 2017). The ability to cause infections are related to the expression of various virulence factors, structures, products or mechanisms (KOT et al., 2016; MELLO et al., 2016; MONISTERO et al., 2018), frequently acquired by mobile genetic elements (LOWY, 1998) that facilitates adhesion and colonization in the mammary glandular epithelium resulting in persistence in the tissue's host. Although this problem affects directly animals, it is also of concern for human health since strains carrying these virulence genes can reach human population by variety of routes.

Antimicrobial resistance is also a biggest concern in animal and public health. It is known that genetic modifications by mutation and selection or by gene exchange between bacteria occurs as a naturally phenomenon over time (BISWAS et al., 2008). Nonetheless, a selection of drug resistance isolates can be accelerated due to incorrect use of drugs. Besides, it has been set that low concentrations of antimicrobial agents in animals can allow for enrichment and selection of bacteria carrying multidrug-resistance plasmids, causing, maintenance, multiplication and spread of these genes between bacteria (TER KUILE et al., 2016). The presence of antimicrobial resistance genes (ARGs) often located on mobile genetic elements, allows easily transmission between different hosts including humans, animals, and environment.

Hence, from the animal and public health point of view, it is essential to define which microorganisms are involved in the etiology of bovine mastitis and their potential to cause severe infections.

Thus, in the first paper will be presented a review of the role of virulence and antimicrobial resistance genes in bovine mastitis, the relationship between them and its risk to human health.

In the second paper, will be presented a research paper about the identification of the principal virulence and antimicrobial resistance genes in *S. aureus* isolated from bovine

mastitis in Brazil, as well as to verify the association of among these characteristics, the year of isolation and geographic origin of the strains.

2. OBJECTIVES

The objectives of this study are:

2.1 GENERAL OBJETIVE

i. Characterize *S. aureus* isolated from bovine mastitis.

2.2 SPECIFIC OBJECTIVES

i. Define the virulence profile of *S. aureus* isolated from bovine mastitis.
ii. Define genetic determinants of antimicrobial resistance in *S. aureus* isolated from bovine mastitis.
iii. Verify association between virulence factors and drug resistance.

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SEGUNDA PARTE

ARTIGO 1 - REVIEW PAPER

RELATIONSHIP BETWEEN VIRULENCE AND ANTIMICROBIAL RESISTANCE IN *Staphylococcus aureus* FROM BOVINE MASTITIS

Article drafted in accordance with the standards for submission in the Journal of Global Antimicrobial Resistance

Relationship between virulence and antimicrobial resistance in *Staphylococcus aureus* from bovine mastitis

Highlights

- Implication of the expression of virulence factors for bovine mastitis and human health.
- Presence of AMR in *S. aureus* from bovine mastitis and its importance in humans.
- Relationship between β-lactams and the expression of virulence factors.

ABSTRACT

Staphylococcus aureus can present many mechanisms of virulence and antimicrobial resistance in mammary gland infection. The pathogenicity of *S. aureus* infection is attributed to a wide array of virulence determinants rather than to any single one. In addition, different mechanisms of antimicrobial resistance play an important role in the permanence of the bacteria in the host. The possibility of exchange resistance genes among different bacteria is a serious concern in livestock husbandry, as well as in the treatment of other staphylococci human infections. Thus, the aim of this review is to summarize the literature on the role of virulence and antimicrobial resistance genes in bovine mastitis, the relationship between them and its risk to human health.

Keywords: *Staphylococci*, Intramammary Infection, Bovine, Virulence Factors, Resistance Mechanism, Public Health.

1. Introduction

Bovine mastitis is one of the most prevalent disease that affects the world dairy production because it decreases quantity and quality of milk produced [1]. The interaction between host, environment and infectious agents results in mastitis disease [2]. *Staphylococcus aureus* is the main agent involved in bovine mastitis [3, 4]. It is important to know which virulence factors, structures, products or mechanisms are produced and how it facilitates adhesion and colonization of the microorganism in the mammary glandular epithelium resulting in persistence, success in its installation and maintenance in the tissue's host. Although this problem affects directly animals, it is also of concern for human health since strains carrying these virulence genes can reach human population by variety of routes, being foodstuffs one of these.

Antimicrobial resistance is also a biggest concern in animal and public health. It is known that genetic modifications by mutation and selection or by gene exchange between bacteria occurs as a naturally phenomenon over time [5]. Nonetheless, a selection of drug resistance isolates can be accelerated due to incorrect use of drugs. It has been set that low concentrations of antimicrobial agents in animals can allow for enrichment and selection of bacteria carrying multidrug-resistance plasmids, causing, maintenance, multiplication and spread of these genes between bacteria [6]. In addition, the presence of antimicrobial resistance genes (ARGs) often located on mobile genetic elements, allows easily transmission between different hosts including humans, animals, and environment.

In this context, the present study aims to review the main virulence factors and determinants of drug resistance in *S. aureus* isolated from bovine mastitis, focusing mainly on the association between these characteristics.

2. Bovine mastitis

Mastitis is defined as an inflammatory process in the mammary gland that can result from trauma, injury, chemical irritation or microbial infection in the udder [7]. Most cases of mastitis are caused by microbial invasion in the mammary gland, mainly by bacteria, however viruses, yeasts and algae may also be involved [8]. In general, mastitis can be classified as clinical / acute or subclinical / subacute, latter frequently leading to development of chronic mastitis [9]. Clinical mastitis is the one with obvious signs such as inflammation of at least one quarter, change in the appearance of the milk due to the inflammatory response because of infection, udder edema, lumps, temperature increase, hardening and pain in the mammary gland [10]. On the other hand, in subclinical mastitis, although the infection is present, there are no visible signs or variation of the characteristics of the milk [11].

Depending on the type of microorganisms causing infection, mastitis can also be classified as contagious or environmental. The contagious microorganisms are those that are disseminated at the time of milking through the infected quarters, mainly by the hands of the milker [12]. Contagious mastitis is usually caused by *Staphylococcus aureus*, *Streptococcus agalactiae*, coagulase negative *Staphylococcus* (CoNS), *Mycoplasma* spp. and *Corynebacterium bovis*. On the other hand, environmental microorganisms are those that are not adapted to the mammary gland, acting as opportunist pathogens. This type of mastitis is typically caused by ubiquitous bacteria, found in feces, water, contaminated fomites, soil,

milking equipment, and outside of contaminated quarters or udder, which, via the teat cistern, reach the udder causing infection and often leading to the development of clinical or subclinical diseases [12, 13]. The most frequent environmental microorganisms involved in mastitis are *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Proteus* spp., *Pseudomonas* spp., *Streptococcus uberis*, *Streptococcus dysgalactiae*, yeasts, algae and fungi [14].

3. Bovine mastitis caused by *S. aureus*

S. aureus is a Gram-positive cocci, catalase and coagulase-positive, facultative anaerobe, with capsule, immobile and not sporulated [15]. Because of capacity of contagiousness and the ability to induce long lasting chronic infections, *S. aureus* is considered one of the major pathogens associated with endemic mastitis all over the world [16-18]. Although different countries have been implemented mastitis prevention programs, the prevalence of *S. aureus* in cows still remains [19-21].

Intramammary infection begins when *S. aureus* passes through the teat canal, interacts with the mammary tissue cells, multiplies in milk and disseminate in the cisterns and throughout the duct system [22]. The release of secreted bacterial products acts as microbe-associated molecular patterns (MAMPs) which contributes to the detection of bacteria by the immune system in the mammary gland [23, 24]. The inflammatory response associated with mastitis results in a decrease in milk production and quality of milk [25]**.** In addition, mastitis also results in an increase of whey proteins, serum albumin, immunoglobulin, chloride, sodium, pH, free fatty acids the milk**.** Also, in the reduction of the synthesis of components of milk, such as lactose, fat, non-fat solids and casein [26].

Costs associated with mastitis include milk production losses, pharmaceuticals, veterinary services [27, 28]. The importance of *S. aureus* in milk and other dairy products is because its capacity to produce various toxins [29]. Another important fact is the antimicrobial residues in food, due to the extensive use of drugs as treatment and control of diseases, that can cause sensitization of normal individuals and development of antimicrobial resistant strains [30]. The presence of genes in *S. aureus* that initially where though to be restricted only to animals, in recent years have been also identified in humans, this highlight the necessity to characterize isolates from bovine mastitis.

4. Importance of virulence factors of *S. aureus* to bovine mastitis and in public health

S. aureus presents multiple virulence factors, some of them related to the severity of intramammary infection [31]. There is a complex network of transcriptional regulatory factors that control the expression of genes that encode virulence factors of *S. aureus* [32]. Thus, it was observed that during *in vitro* culture of *S. aureus* the virulence factors associated with the bacterial surface are firstly expressed in the logarithmic phase of growth, while the secretion factors are released in the post logarithmic phase. This biphasic expression of virulence factors could fulfill the function of organizing the infection process [33]. Initially, surface adhesins would recognize the structures of the host, facilitating colonization and later multiplication of the microorganism and secretion of toxins (α, β and γ hemolysins, leukotoxins, enterotoxins) and enzymes (serine proteases cysteine, proteases, lipases) [34]. However, it has been postulated that, in order to reach intracellular persistence, *S. aureus* must avoid the immune and inflammatory response of the host, for which it would negatively regulate the expression of virulence factors [35]. This sophisticated regulatory network would be the key in the pathogenesis of infection by *S. aureus* that leads chronicity of the disease and at the same time, allows adaptation of microorganism to microenvironment changes during the course of the infection and its survival [35]. In the following lines, will be described main virulence genes that affect cattle and have implications for human health.

4.1 Enterotoxins

S. aureus can express many enterotoxin genes. Among Staphylococcal enterotoxins (SEs) and staphylococcal enterotoxin-like proteins genes, *sea, sec, sed, see, seg, seh, sei, selj, selk, sell, selm, seln, selo, selp, selq* and *selu* have been detected in *S. aureus* isolated from raw milk samples in earlier studies [22, 36].

Several studies have shown that most *S. aureus* strains isolated from bovine mastitis harbor one or more enterotoxin genes [37]. Thus, the frequency of genes in dairy herds are variable, some reports mention that *S. aureus* isolates have at least one gene encoding superantigen toxin [38]. In contrast, other studies showed that enterotoxin genes were absent or seldom detected in *S. aureus* isolated from cows with mastitis [22, 39, 40]. Nonetheless, recently studies have reported the impact of toxins in the mammary gland. For example, it was observed that effect of staphylococcal enterotoxin H *in vitro*, induced bovine mammary epithelial cells (bMECs) apoptosis. The stimulation of lymphocyte proliferation decreased the viability of bMECs and induced the cells to undergo apoptosis in a time-dependent manner

[41]. Another study that analyzed the biological characteristics and potential pathogenic activity of enterotoxin C, observed that super antigenic activity, induces proinflammatory cytokine release and inflammation responses, and subsequently induces mammary tissue damage [42].

From public health point of view, *S. aureus* can produce a wide variety of enterotoxins, however 95% of cases of food poisoning are caused by the group consisting of the exotoxin's *sea, seb, sec, sed* and *see* [29]. SEs keep their biological and immunological activities even following pasteurization, food processing and exposure to gastrointestinal proteases [43]. The fact of detecting SEs in cow´s milk represents not only a problem for the dairy husbandry but also a high risk to public health due to consumption of unpasteurized milk and products derived from milk.

4.2 Toxic shock syndrome toxin

Intoxication in humans is most commonly determined by toxic shock syndrome toxin 1 (TSST-1). TSST- 1 can disturb the host immune response by causing a non-specific polyclonal activation of immune cells [44]. This toxin presents numerous immunomodulating effects such as induce the release of interleukin 1 and the tumor necrosis factor from monocytes [45]. It has been observed that when bovine T-lymphocytes are experimentally exposed, TSST-1 can act as a superantigen for bovine immune cells, and thus potentially contributes to the mammary pathology associated with *S. aureus* infections [45]. A study in 120 *S. aureus* isolates from bovine mastitis, that compares the molecular-epidemiologic profiles of strains from different countries around the world, founded that 37% of Argentinian, 23% of German, 16% of Tunisian and 6% of Italian isolates carried *tst* gene [46]. Besides, it has been reported that co-production of TSST-1 and SEs by *S. aureus* may contribute to a more severe inflammatory reaction [47].

In humans, toxic shock syndrome by TSST-1 is a relatively rare condition, however it is difficult to obtain accurate estimates of the incidence of *S. aureus* intoxications because most cases are not reported [48]. This syndrome is characterized by causing fever, hypotension, congestion in various organs and lethal shock [49], stimulates the non-specific proliferation of T cells and induces production of IL-1, IFN-ɣ and TNF-α [50]. Although pasteurization kills *S. aureus*, heat-stable TSST-1 can retain their biological activity [43]. Strong resistance to pepsin and trypsin digestion has also been observed, even after treatment

TSST-1 continuous keeping significant super antigenic and lethal shock activities [51]. Due to the importance of this toxin in bovine and public health, is necessary an efficient screening to detect prevalence of enterotoxin in *S. aureus* strains isolated from bovine milk and derivates.

4.3 Hemolysin

Hemolysins are considered important virulence factors of *S. aureus* that contribute to bacterial invasion and escape from the host immune response [52]. *S. aureus* mainly produce α and β hemolysins, encoded by *hla* and *hlb* genes, respectively [53, 54]. The most prominent cytotoxin produced by *S. aureus* is α -hemolysin present in a wide range of host cells. Its pathogenicity depends on its hemolytic, dermonecrotic and neurotoxic effects [44, 55]. However, some studies consider that β-hemolysin is produced by most *S. aureus* isolates from strains isolated from bovine mastitis and chronic skin infections in humans [53, 56]. Despite of not causing cell lysis, β-hemolysin is a highly active sphingomyelinase against bovine erythrocytes [57, 58]. The destruction of sphingomyelin increases the permeability of the plasma membrane with progressive loss of the negative electrical charge of the cell surface, allowing easy adherence of the bacterial cell [56]. It was detected a high percentage of *S. aureus* strains harboring *hla* and *hlb* gene isolated from bovine mastitis [59]. In fact, interaction between α and β hemolysin increased both the adherence to bovine mammary epithelial cells and the proliferation of *S. aureus* [56]. Also, a capacity of these toxins to be stable in high temperatures [60], makes this an important fact for public health because of consumption of products derived from milk represents a high risk to humans.

4.4 Leukotoxins

Leukotoxins belong to a family of pore-forming toxins and are responsible for the destruction of phagocytic cells, such as monocytes and polymorphonuclear cells. The staphylococcal Panton-Valentine Leukocidin (PVL) is encoded by *LukS-PV* and *LukF-PV* genes and the more recently described LukM/FPV(P83) is encoded by *LukF-PV83/LukM* [61, 62]. Toxic effect depends on the synergistic action of both class S (slow elution)-related and class F (fast elution)- related proteins on polymorphonuclear cells and monocytes [63]. *S. aureus* can acquire two phages encoded leukocidins, PVL and a bicomponent leucocidin LukMF´. However, while the PVL genes is associated to human strains, LukMF´ genes are associated with animal strains, especially with isolates from bovine mastitis [64, 65].

The main role of PVL is associated with necrosis of skin and soft tissues and its presence has been reported worldwide [66]. On the other hand, LukMF is highly expressed and is the most potent toxin killing bovine neutrophils [67]. The presence of *LukF-PV83/LukM* can lead to reduction of host defense and facilitate more rapid colonization of the bovine udder by pathogens, and its presence determine whether *S. aureus* behaves as an obligatory or accidental pathogen for the host organism [64]. Considering this, *LukF-PV83/LukM* positive strains could be regarded as obligatory pathogens to cattle, which also implied that they might easily spread and persist in herds. On the other hand, *LukF-P83/LukM* negative strains could be accidental strains in cattle, possibly being transferred from other species such as humans or rodents into cows [64].

4.5 Biofilm forming

The ability of *Staphylococci* to form biofilms is one of the virulence factors that facilitate the adherence and colonization of these pathogens to the mammary gland epithelium, contributing to the evasion of the immunological defenses and for the recurrent or persistent infections, and thereby avoiding its eradication [68]. Biofilm is an exopolysaccharide, a slime matrix around multiple layers of cells. *Staphylococcus* biofilm formation mechanisms involve the participation of many kinds of proteins, and genes [69]. Firstly, the bacteria adhere to a surface mediated by a capsular antigen polysaccharide/adhesin (PS/A). Then, multiply to form a multilayered biofilm, which is associated with production of polysaccharide intercellular adhesin (PIA). Synthesis of PIA and PS/A in staphylococcal species, is mediated by the intercellular adhesion operon (*ica*) formed by the *icaA*, *icaB*, *icaC* and *icaD* genes and a regulatory gene, *icaR*, that encodes the proteins ICAA, ICAB, ICAC and ICAD [70, 71]. The presence of the *ica* locus in all the mastitis *S. aureus* isolates confirms its potential role as a virulence factor in the pathogenesis of mastitis in ruminants. However, *ica*-independent biofilm formation by *S. aureus* has also been reported, although in a small percentage of clinical isolates [72], suggesting potential that surface proteins such as Aap and Bap and secretory proteins can replace the function of PIA during biofilm development by *ica*-deficient strains of *S. aureus* [73, 74].

5. Impact of antimicrobial resistance to bovine mastitis and in public health

Antimicrobial drugs have been used for many years in animals. A study estimates that between 2010 to 2030, global consumption of antimicrobials in livestock production will increase by two thirds, and that it will double Brazil, Russia, India, China, and South Africa

[75].Due to the emergence of multiresistant strains reports in humans and animals, it becomes critical to develop new drugs that allows to control multiresistance. In the following lines are described the mechanism of antimicrobial resistance of principal drugs frequently used in dairy herds.

5.1 Resistance to β -lactams

After the introduction of penicillin as treatment, a methicillin-resistant *Staphylococcus aureus* (MRSA) isolate was reported in England. Since then, *S. aureus* has become an important pathogen involved in antimicrobial resistance. Resistance to penicillin is caused by the production of penicillinases (β-lactamase), this enzyme inactivates the antibiotic through hydrolysis of the peptide bond in the β-lactam ring in antibiotics such as penicillin G, carboxypenicillins and ureidopenicillins [76]. The mechanism of resistance is encoded by *blaZ* gene, which typically resides on a large transposon on a plasmid. In the absence of penicillin, β-lactamase is expressed at low level [76, 77]. A study in *S. aureus* isolates from bovine mastitis in Brazil, detected that 84% isolates harbored *blaZ* gene [78]. This high prevalence of *blaZ* gene in *S. aureus* isolates from bovine mastitis has also been reported in different countries [79, 80].

Resistance to methicillin is conferred by the production of the penicillin-binding protein (PBP2a) encoded by *mecA* gene, which has a low affinity for β-lactam [81, 82]. Methicillin-resistant *Staphylococcus aureus* (MRSA) appears when methicillin-susceptible *S. aureus* (MSSA) exogenously acquires a staphylococcal cassette chromosome *mec* (SCC*mec*), transmissible among staphylococcal species as a mobile element [83]. SCC*mec* typing is one of the most important molecular tools for understanding the epidemiology and clonal relation of MRSA isolates, because SCC*mec* is a vehicle for drug resistance genes [84]. Recent evidence suggests that Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) may be present in people who are in close contact with animals [85], representing a high risk of zoonoses.

On the other hand, it was determined that there are others chromosomally determined factors that cause resistance, for example the operon *femAB*, which acts as a regulatory gene. The cooperation between *femA* and *mecA* genes is essential for the expression of methicillin resistance in *S. aureus* [86].

In addition, a new divergent *mecA* homolog (*mecC*) was described in a novel SCCmec designated as type XI [87]. An epidemiological study by García-Álvarez, Holden [88] on bovine mastitis in England described an isolate of *S. aureus* phenotypically MRSA (resistant to oxacillin and cefoxitin), but did not contain the *mecA* gene or the PBP2a protein. Genetic study of this strain revealed the presence of *mecC* gene. This gene shared 69% homology with the *mecA* gene and encoded for production of a protein that had 63% amino acid homology with PBP2a. The proteins encoded by *mecC,* have less affinity for oxacillin than cefoxitin, which would explain the difficulties on the detection by phenotypic methods [89, 90]. In addition, a retrospective study carried out in Denmark and United Kingdom identified *mecC* in 65 strains, isolated from cattle and humans. The majority of MRSA *mecC* isolates belong to the CC130 clonal complex and less frequently to the ST42518 type sequence [88]. Both genetic lineages of MRSA are usually from animals, suggesting a zoonotic origin of the *mecC* gene, probably from ruminants and that subsequently spread to humans. The MRSA *mecC* strains predominantly cause skin and soft tissue infections, but they have also been described as causative agents of bone infections [91], nosocomial pneumonia [88] and bacteremia [92]. Likewise, these strains produce a variety of infections in various species of domestic animals and livestock, they have been mainly described as a cause of mastitis in dairy cows [93]. MRSA strains from animals are not only important from the point of view of animal health and economic perspective, but also because can act as a zoonotic reservoir, enter the food chain and cause antimicrobial resistance in humans.

5.2 Resistance to tetracyclines

Tetracyclines are broad-spectrum antimicrobials that have been widely used in human and veterinary medicine. The main resistance mechanisms against tetracycline in *S. aureus* are efflux pump, that is a result of the acquisition of *tetK* or *tetL* by mobile *tet* genes, and the ribosomal protection, that is conferred by the *tetM* and *tetO* genes [94]. It is also mention by Trzcinski, Cooper [95] that both, efflux and ribosomal protection are inducible in *S. aureus* by subinhibitory concentrations of tetracycline. Moreover, it is discussed that in animals staphylococci, resistance to tetracyclines is often mediated by the genes *tetK* and *tetL* [94]. A study in *S. aureus* isolated from bovine mastitis found that 90% *S. aureus* isolates from bovine mastitis, harbored at least one of the *tet* genes, being the most prevalent *tetK*. Whereas, *tetL*, *tetM* and *tetO* genes were found in 8·8%, 2·2% and 1·1% isolates, respectively [78].

It was reported a presence of *tetK*, *tetL* and *tetM* genes in bovine and swine nares, highlighting their importance in public health because these genes are on mobile genetic element, as small plasmids or conjugative transposons and can spread and cause treatment failure both in veterinary and human medicine [96]. Furthermore, is also to mention that tetracycline resistant strains are more frequently isolated from farmers and veterinarians than from people without contact with livestock especially pigs [97].

5.3 Resistance to macrolides, lincosamides and streptogramins

Antimicrobials such as macrolides, lincosamide and streptogramin (MLS_B) are widely used in the treatment of staphylococcal infections. Main genes associated with MLS_B resistance are *erm*, *msr*, *mph*, *vat* and *lnu*. The *erm* genes that have been detected in staphylococci of animal origin, being *ermA* and *ermB* genes associated with transposons. The *ermA* gene has been identified in *S. aureus*, mostly MRSA and the *ermB* gene has been detected in LA-MRSA, both genes from cattle [98]. It is interesting to mention that plasmids that harbor gene *ermC* are commonly located on small plasmids and usually not carry additional resistance genes, while *ermT* is often found on large multi resistance plasmids [99]. These genes have been identified in *S. aureus* (including MRSA) from cattle [98, 99]. The *msrA* gene confers resistance to macrolides and streptogramin type B [100], whereas the gene *mphC* only confers resistance to macrolides [101, 102]. Interesting, *mphC* often occurs linked to *msrA* but when *mphC* phosphotransferase is alone low-level resistance to macrolides is observed [103]. On the other hand, the *lnuA* gene that confers resistance to lincosamides is often located on small plasmids and has been identified in *S. aureus* including MRSA from dairy cattle [104]. Genes *vatA*, *vatB* or *vatC* confers inactivation to streptogramin A antibiotics in staphylococci [105].

MLS_B, especially macrolides, are frequently used as treatment in bovine mastitis because its excellent diffusion into the mammary gland, have long half-life effect, low protein binding, lipid solubility and high intracellular concentration. *S. aureus* can harbor different genes that brings resistance to MLS_B, isolates carrying more than one resistance gene has been reported [80]. This fact suggests that one or several new resistance mechanisms for macrolides may be widespread among *S. aureus* isolates.

5.4 Resistance to aminoglycosides

Although aminoglycosides are widely used for mastitis treatment [106], the number of studies carried out on genotypic resistance are very limited compared to phenotypic resistance studies in *S. aureus* isolated from animals [102, 107]. Resistance to aminoglycosides is based on several inactivating enzymes, which differ in their specific substrate spectra. For example, the gene *aacA-aphD* widely distributed in staphylococci of animal origin, including *S. aureus* isolated from bovine mastitis, is located on transposon Tn4001 and confers resistance to gentamicin, kanamycin, tobramycin and when over-expressed to amikacin [94, 98]. A recent study in bovine clinical mastitis was detected *aacA-aphD* gene in 23% of *S. aureus* isolates [80]. In contrast, a study in *S. aureus* from bovine mastitis in Turkey, detected that the most prevalent gene was *aph (3')-IIIa* [108]. Thus, difference of aminoglycosides resistance genes can be attributed to the difference among *S. aureus* isolates from different geographical regions.

Therefore, is essential to determine resistance to aminoglycosides in *S. aureus*, in order to know its prevalence that will allows us to control the misuse and overuse of antimicrobial drugs in dairy cattle and so avoid multiresistant strains.

5.5 Resistance to fluoroquinolones

In general, two important mechanisms cause fluoroquinolone resistance in *S. aureus*. The first one is attributed to mutations occurring in the quinolone-resistance determining region (QRDR) of *GrlA*/ *GrlB* (topoisomerase IV, encoded by genes *grlA*/*grlB*) and *GyrA*/*GyrB* (DNA gyrase, encoded by genes *gyrA*/ *gyrB*), which decrease the affinity of the drug [109]. Nonetheless, fluoroquinolone resistance can also be mediated by drug efflux, a mechanism that is less well characterized. Several efflux pumps have been described in *S. aureus*, including the chromosomally encoded *norA, norB, norC, mdeA, mepA, sepA* and *sdrM*, as well as the plasmid-encoded *qacA/B, qacG, qacH, qacJ* and *smr* [110]. In contrast to the data on human *S. aureus* strains, very little is known about the genetic basis of fluoroquinolone resistance in animal staphylococci. It has been reported resistance rates to fluoroquinolones, tetracyclines, and macrolides and the corresponding genes in *S. aureus* from poultry [111]. A recent study in China, detected a high frequency of genes *norA, gyrA, grlA* in *S. aureus* isolated from bovine mastitis [112]. However, more studies necessary in order to know the fluoroquinolone resistance profile.

6. Association between virulence and antimicrobial resistance

In recent years, it has been described different mechanism of antimicrobial agents modulating staphylococcal virulence factors. It is known that β-lactams are widely used in human and animals. Studies showed that low levels of these drugs can stimulate biofilm formation, increasing adhesion protein expression by releasing extracellular DNA (eDNA) and modifying the extracellular matrix composition, this was prominently noted in strains MRSA than MSSA [113, 114]. The same results were observed for low levels of clindamycin, which modify eDNA release and autolysis rate by increasing the expression of adhesion factors and secreted proteins, resulting in a more compact and stable biofilm [115].

In a study was reported an increase of α-toxin gene expression due to β -lactams and fluoroquinolones exposure. It was observed that MRSA strains have more α-toxin production when treated with β-lactams than in MSSA [116, 117]. In contrast, the use of clindamycin and erythromycin drugs abolished α-toxin expression [116]. Studies indicate that protein synthesis inhibitors, especially clindamycin and linezolid, prevent the translation but not transcription of α-toxin [117, 118].

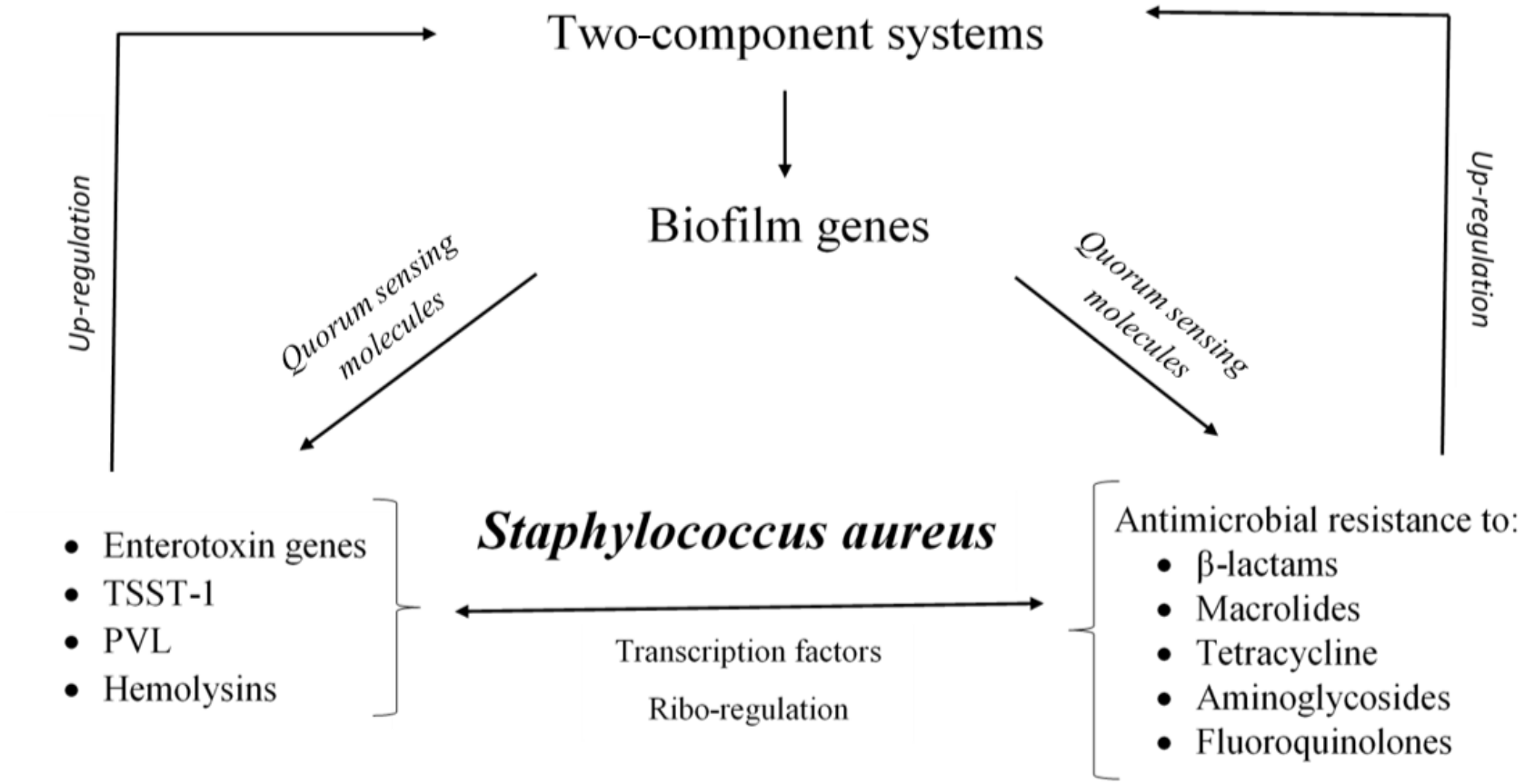
The increase of PVL expression was also seen when that *S. aureus* were cultured with β -lactams. Similar effects have been seen in TSST-1 expression where the use of β -lactams treatment increased its expression and decreased expression after protein synthesis inhibitory antibiotic treatment. [117, 119]. Nonetheless, it was noticed that clindamycin and gentamicin drugs suppressed TSST-1 production, reducing it by up to 95% and 75%, respectively [120]. It could be because clindamycin act mainly by blocking ribosomal function and suppressing the protein synthesis of virulence factors as well as the synthesis of regulators of virulence expression [121].

The enterotoxin gene regulation during therapeutic simulations was studied in the hollow-fiber model. This study evaluated *sec4*, *sek*, *seq*, and *sel2* genes expression during treatment with clindamycin, linezolid, minocycline, trimethoprim-sulfamethoxazole, or vancomycin. As a result, both clindamycin and linezolid increased enterotoxin expression [118].

As shown, the use of antimicrobial drugs can influence the expression of virulence genes. It was also observed that regulation of virulence genes influences expression of antimicrobial resistance genes and vice versa. The gene expression can be indirectly or

indirectly influence by a host of environmental factors [122]. *S. aureus* presents two quorum sensing systems that acts on biofilm genes and can control the expression of toxins, virulence factor and antimicrobial resistance genes, likewise these genes can up-regulate (Figure 1). It has been study that for anaerobic respiration in *S. aureus* two-component system SrrAB is necessary. SrrAB down-regulate the regulatory RNA *agr*-RNAIII, which contribute in the excretion of the virulence factors: serine protease and α-hemolysin. Also, it has been observed an increase of extracellular polysaccharide by the increase expression of *ica* operon [123]. Moreover, regulation of antimicrobial resistance through transcription factors has been studied in MRSA. A studied showed that regulatory proteins YycH and YycI reduce vancomycin

Figure 1. Relationship between virulence and antimicrobial resistance genes in *Staphylococcus aureus*



susceptibility in *S. aureus*, particularly in strains with intermediate level resistance to vancomycin [124].

7. Final considerations and future perspectives

The high prevalence of bovine mastitis by *S. aureus* makes important to understand different mechanism of virulence and antimicrobial resistance. However, despite the importance, very few studies are carried out. The use of techniques that allows to understand the relationship between virulence and resistance genes are necessary. For example, the use of high-throughput sequencing techniques, can allows to seek connections between resistance genes and virulence factors. An example of this can be the transposon insertion site sequencing (TnSeq) experiments that is a useful and often unbiased tool to study the link between antimicrobial resistance genes and fitness.

In brief, further studies are needed for a more robust understanding of what drives the link between resistance and virulence facts, and so understand how it influences in the pathogenesis of the bacteria, allowing released of multiple virulence factors or expression of antimicrobial resistance genes.

Declarations

8. Conflicts of interest: None

9. Funding: None

10. Ethical Approval: Not required

11. Competing Interests: The authors declare that there are no conflicts of interest.

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TERCEIRA PARTE

ARTIGO 2 - RESEARCH PAPER

DETECTION OF VIRULENCE AND ANTIMICROBIAL RESISTANCE GENES IN *Staphylococcus aureus* ISOLATED FROM BOVINE MASTITIS IN BRAZIL

Article drafted in accordance with the standards for submission in the Journal of Veterinary Microbiology

Detection of virulence and antimicrobial resistance genes in *Staphylococcus aureus* isolated from bovine mastitis in Brazil

Highlights

• High prevalence of biofilm and hemolysin genes.

• First detection of *mepA* in *S. aureus* from bovine mastitis.

•Detection of high prevalence of β-Lactams resistance gene *blaZ*.

ABSTRACT

Staphylococcus aureus can present many mechanisms in order to remain in mammary gland. The present study aimed to evaluate virulence factors and genetic mechanisms of drug resistance in 400 *S. aureus* strains isolated from bovine mastitis in Brazil, as well as to assess the association between these characteristics and the year of isolation and geographic origin of the strains. Singleplex and multiplex PCR were used to identify virulence factors and drug resistance encoding genes. Detection of biofilm-forming was carried out using Congo red Tryptic Soy Broth assay. As a result, 83.5% isolates were biofilm-forming and 98.5% strains exhibited the biofilm gene *icaAD*. Virulence factors genes *luk*, *seb*, *sec*, *sed* and *tst* were observed in 3.5%, 0.5%, 1%, 0.25% and 0.74% of the strains, respectively. Hemolysin genes were observed in 82.85% (hla^+hlb^+), 16.5% (hla^+) and 0.75% (hlb^+) isolates, while enterotoxin genes *sea* and *see* were not detected. The gene *blaZ*, associated with penicillin resistance, was detected in 82.03% isolates, whereas tetracycline resistance gene *tetK* and aminoglycoside gene *aac(6') -Ie–aph(2')-Ia* were observed in 33.87% and 45.15% of the isolates, respectively. Fluoroquinolone resistance gene *mepA* was detected for the first time in all fluoroquinolone resistance *S. aureus* isolates. Resistance genes *tetM* (3.22%), *tetL* (1.61%), *ermA* (14.29%), *ermB* (14.29%), *ermC* (33.3%), *ermT* (9.52%), *ermY* (4.76%), *msrA* (9.52%) and *mphC* (9.52%) were detected in low frequency among the isolates. Our results

showed a high frequency of *S. aureus* isolated from bovine mastitis in Brazil carrying mainly biofilm and hemolysin genes. Moreover, a wide variety of antimicrobial resistance genes that confers resistance to all classes of antimicrobial agents used in animals and human population were observed. These results highlight the pathogenic potential of *S. aureus* from cattle to cause severe disease in both humans and animals.

Keywords: Staphylococci, intramammary infection, biofilm, *icaAD*, *blaZ*, *mepA*.

1. INTRODUCTION

Bovine mastitis is one of the most common disease that affects the world dairy production, being responsible for decreasing quantity and quality of milk produced (Hogeveen and Van, 2017; Keefe, 2012). *Staphylococcus aureus* is one of the main pathogens isolated from bovine mastitis and causes significant production and economic losses (Bobbo et al., 2017; Rollin et al., 2015) in different parts of the worlds where dairy farming is expressive. In Brazil several studies have showed the importance of *S. aureus* in the epidemiology of mastitis in cattle (Brito et al., 1999; Costa et al., 2013; Mello et al., 2016; Silva et al., 2014).

The *S. aureus* ability to cause infections and the severity the diseases are related to the expression of various virulence factors, structures, products or mechanisms (Kot et al., 2016; Mello et al., 2016; Monistero et al., 2018), frequently acquired by mobile genetic elements (Lowy, 1998). It is well documented that *S. aureus* strains from mastitis can produce a wide variety of extracellular toxins, such as enterotoxin, encoded by *sea*, *seb*, *sec*, *sed* and *see* genes, toxic shock syndrome toxin 1 (*tst*), Panton–Valentine Leukocidin (PVL) (*luk*), α and β hemolysin (*hla* and *hlb*) (Iandolo, 1989; Kot et al., 2016), among others. The ability produce toxins by *S. aureus* strains from animal origin causes harm not only to the animals but also to public health, since some of these products, in addition to favoring the infection, are also thermostable and remain active even after thermal treatments used in milk (Asao et al., 2003; Sabini et al., 2001; Singh et al., 2014).

Furthermore, the production of extracellular polymeric substances (EPS), among which highlights exopolysaccharide (slime), appears to play a crucial role in the infection, adhesion and colonization of the microorganism in the mammary glandular epithelium, promoting not only the formation of biofilm and its extracellular persistence, but also ensuring success in its installation and maintenance in the host tissues (Coelho et al., 2011; Saei, 2012). In fact, biofilm production in *S. aureus* strains isolated from mastitis, which is usually associated with the presence of *icaA* and *icaD* genes (Vasudevan et al., 2003), may be associated with

antimicrobial resistance (Cucarella et al., 2004). The mechanisms responsible for drug resistance include the physical and chemical diffusion barrier formed by the exopolysaccharide matrix, which make difficult the penetration of antimicrobials, besides creating microenvironments that antagonize the antibiotic (Costerton et al., 1999; Marques et al., 2017).

Antimicrobial resistance is a major problem in animal and public health lately. Increase of drug resistance has been reported in Brazilian dairy farms and worldwide (Medeiros et al., 2011; Mehli et al., 2017; Nobrega et al., 2018) among *S. aureus* strains. Staphylococci of animal origin can harbor a wide variety of resistance genes that confer resistance to almost all classes of antimicrobial agents approved for use in animals. Antimicrobial resistance genes (ARG) commonly reported in *Staphylococcus* isolated from cattle are mainly *mecA* and *blaZ* (β-lactams resistance), *tetK*, *tetL* and *tetM*, (tetracycline resistance), *ermA*, *ermB*, *ermC*, *ermT*, *ermY*, *msrA*, *mphC* [macrolide, lincosamide, streptogramin B (MLSB) and macrolide phosphotransferase resistance], *aac(6')-Ie–aph(2')-Ia* [aminoglycoside modifying enzyme (AME)], *mepA* (fluoroquinolone resistance, efflux pumps) and *grlA/grlB* and *gyrA/gyrB* (fluoroquinolone resistance, mutation in topoisomerase IV and DNA gyrase) . Additionally, it has been set that carriers of ARG among staphylococci from animals, such as plasmid and transposon, play a key role in the transmission of resistance, because they facilitate the exchange of resistance genes with staphylococci from human origin and with other Gram-positive bacteria. This is extremely important because it can increase the risk of antimicrobial resistance transmission from animals to humans (Juhász-Kaszanyitzky et al., 2007).

From the animal and public health point of view, it is essential to define which microorganisms are involved in the etiology of bovine mastitis and their potential to cause severe infections, in order to adopt appropriate hygienic measures and a rigorous monitoring program. Therefore, the aims of this study were to evaluate virulence factors and genetic

mechanisms of drug resistance in *S. aureus* isolated from bovine mastitis in Brazil, as well as to verify the association of among these characteristics and the year of isolation and geographic origin of the strains.

2. MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 400 *S. aureus* strains isolated from cows with mastitis were used in the present study. These were representative strains selected from the Collection of Microorganisms of Agribusiness Interest from Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Gado de Leite (Brazilian Agricultural Research Corporation – Dairy Cattle), selected between 1994 and 2016 isolated from different Brazilian states. The distribution of the isolates per year and state are shown in the Figure 1. The determination of the antimicrobial susceptibility profile (cefoxitin, oxacillin, ampicillin, enrofloxacin, ciprofloxacin, cephalothin, ceftiofur, amoxicillin + clavulanic acid, erythromycin, neomycin, gentamicin, tetracycline, sulfamethoxazole + trimethoprim, penicillin-novobiocin, ampicillin-colistin) of the strains was previously performed by Abreu (2016) and is shown in the Supplemental Table S1.

Strains were reactivated by incubation on Brain Heart Infusion (BHI) agar (Difco, USA) at 37° C for 24 hours in aerobic conditions. Bacterial mass was inoculated in phosphate buffered saline (PBS) (0.01 M pH 7.4) for DNA extraction and in BHI broth (Difco, USA) + 20% glycerol and stored at -80 °C to preserve the isolate.

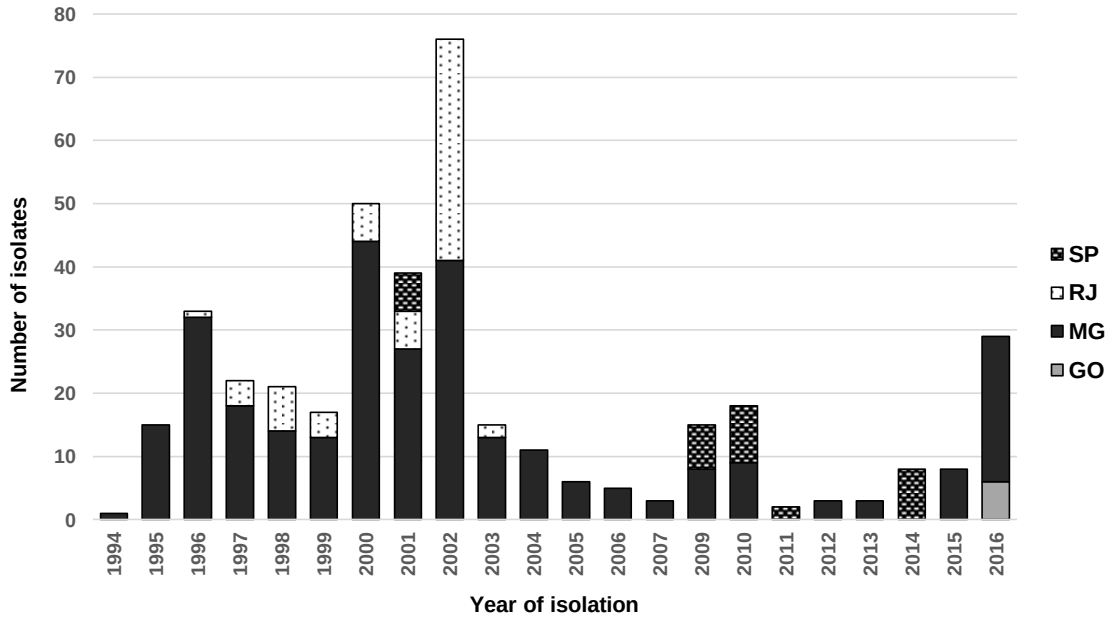

Figure 1. Distribution of *Staphylococcus aureus* isolated from bovine mastitis in Brazil, according to year of isolation and state.

Isolation of DNA

The extraction of the genomic DNA was performed according to Pitcher et al. (1989). The quantity and quality of the extracted DNA was assessed by spectrophotometry using NanoVue™ Spectrophotometer (GE Healthcare, USA) according to described by Russell and Sambrook (2001). DNA samples were kept at −20°C until the analysis.

Identification of S. aureus

All strains were confirmed as *S. aureus* by amplification of the conserved thermonuclease gene (*nuc*) using the primers described in Table 1. The PCR conditions used were as described by Cremonesi et al. (2005), with some modifications in the cycle (annealing for 30 s and extension at 72 °C for 30 s).

Phenotypic detection of biofilm-forming

For the phenotype identification of biofilm-forming strains, 4 colonies of each strain were inoculated in trypticase broth (TSB) (Difco, USA) supplemented with Red Congo (0.8 g / L) and sucrose (36 g / L) and incubated for 48 hours at 37 °C, as described by Lee et al. (2016).

S. aureus ATCC 51651 and *Staphylococcus chromogenes*, isolated from bovine mastitis (Custódio, 2019), were used as positive and negative controls in all assays, respectively.

Detection of virulence genes

Detection of *icaAD* gene was carried out according to previously described by Sun et al. (2003). Identification of the enterotoxin genes *sea*, *seb*, *sec*, *sed* and *see* was performed as described by Mehrotra et al. (2000), with minor modifications (2.5 mM $MgCl_2$). The presence of hemolysin genes *hla* and *hlb* was investigated by multiplex PCR according to Jarraud et al. (2002).

Multiplex PCR for detection of toxic shock syndrome toxin (TSST-1) and *femA* was performed as described by Mehrotra et al. (2000), using the following thermal cycle: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, ending with a final extension at 72 °C for 7 min.

The identification of the Panton-Valentine Leukocidin (PVL) gene was performed by singleplex PCR according to Lina et al. (1999), using following thermal cycle: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min, ending with a final extension at 72 °C for 7 min.

All reagents of the PCR mix without DNA was routinely used in each assay, as negative control. Positive controls and primers used are described in Table 1.

Table 1. Virulence genes investigated in *Staphylococcus aureus* isolated from bovine mastitis in this study.

Target	Gene	Primer sequence (5' to 3')	Annealing temperature (°C)	Amplicon size (bp)[1]	Positive control (ATCC)[2]	Reference
Thermostable Nuclease (*S. aureus* species-specific)	*nuc*	F AGT TCA GCA AAT GCA TCA CA R TAG CCA AGC CTT GAC GAA CT	56	400	25923	Cremonesi et al. 2005
Staphylococcal Enterotoxin A	*sea*	F GGTTATCAATGTGCGGGTGG R CGGCACTTTTTTCTCTTCGG	57	102	13565	Mehrotra et al., 2000
Staphylococcal Enterotoxin B	*seb*	F GTATGGTGGTGTAACTGAGC R CCAAATAGTGACGAGTTAGG	57	164	14458	Mehrotra et al., 2000
Staphylococcal Enterotoxin C	*sec*	F AGATGAAGTAGTTGATGTGTATGG R CACACTTTTAGAATCAACCG	57	451	19095	Mehrotra et al., 2000
Staphylococcal Enterotoxin D	*sed*	F CCAATAATAGGAGAAAATAAAAG R ATTGGTATTTTTTTTCGTTC	57	278	23235	Mehrotra et al., 2000
Staphylococcal Enterotoxin E	*see*	F AGGTTTTTTCACAGGTCATCC R CTTTTTTTTCTTCGGTCAATC	57	209	27644	Mehrotra et al., 2000
Resistance to methicillin	*femA*	F AAAAAAGCACATAACAAGCG R GATAAAGAAGAAACCAGCAG	57	132	25923	Mehrotra et al., 2000
Toxic shock syndrome toxin 1	*tst*	F ACCCCTGTTCCCTTATCATC R TTTTCAGTATTTGTAACGCC	57	326	33586	Mehrotra et al., 2000
Panton–Valentine Leukocidin (PVL)	*luk*	F ATCATTAGGTAAAATGTCTGGACATGATCCA R GCATCAASTGTATTGGATAGCAAAAGC	62	433	25923	Lina et al. 1999b
α-Hemolysin	*hla*	F CTGATTACTATCCAAGAAATTCGATTG R CTTTCCAGCCTACTTTTTTATCAGT	53	209	8096	Jarraud et al. 2002
β -Hemolysin	*hlb*	F GTGCACTTACTGACAATAGTGC R GTTGATGAGTAGCTACCTTCAGT	53	309	13565	Jarraud et al. 2002
Biofilm	*icaAD*	F CCTAACTAACGAAAGGTAGG R TTAGCGTTGGGTATTCCCTC	58	1266	51651	Sun et al.2009

[1]Base pairs (bp); [2]American Type Culture Collection (ATCC).

Detection of antimicrobial resistance genes

Strains described as resistant, according to the phenotype showed previously (Abreu, 2016) were screened for the presence of the following genes. Detection of gene *blaZ* (β -lactams) and tetracycline genes *tetK*, *tetM*, *tetL* was carried out according to Schnellmann et al. (2006) and Aarestrup et al. (2000), respectively. The PCR thermal cycle was: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing temperature according to each gene described in Table 2, and extension at 72 °C for 1 min, ending with a final extension at 72 °C for 10 min.

Macrolide resistance genes *ermA*, *erm B* and *ermC*, was performed according Sutcliffe et al. (1996). The thermal cycle for genes *ermA* and *ermC* was: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 52 °C for 45 seconds, and extension at 72 °C for 2 min, ending with a final extension at 72 °C for 7 min. For gene *ermB*, initial denaturation at 93 °C for 3 min, followed by 35 cycles of denaturation at 93 °C for 1 min, annealing at 51 °C for 1 min, and extension at 72 °C for 1 min, ending with a final extension at 72 °C for 5 min. Macrolide/lincosamide/streptogramin B (MLSB) genes *ermT* and *ermY*, was performed as described by Gómez-Sanz et al. (2010). The PCR conditions were: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing temperature according to each gene described in Table 2, and extension at 72 °C for 1 min, ending with a final extension at 72 °C for 5 min. Macrolide resistance gene *msrA*, was carried out according to Lina et al. (1999). The PCR thermal cycle was: initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min 30 seconds, ending with a final extension at 72 °C for 7 min. Macrolide resistance gene *mphC* was performed was mentioned by Schnellmann et al. (2006). PCR thermal conditions was: initial denaturation at

94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 1 min, ending with a final extension at 72 °C for 5 min. The aminoglycoside resistance gene *aac(6') -Ie–aph(2')-Ia* was performed according to Vakulenko et al. (2003). Fluoroquinolone resistance genes *mepA, grlA and gyrA* was developed as described by Couto et al. (2008) and Pan et al. (2002). Only strains resistant to a given class were tested for the corresponding resistance gene. Multidrug resistance was defined as resistance to three or more antimicrobial groups (Magiorakos et al., 2012). The antimicrobial groups were defined according to Clinical and Laboratory Standards Institute (CLSI) M100 manual (28th ed.). PCR singleplex was developed for each gene. All reagents of the PCR mix without DNA were routinely used in each assay, as negative control. Positive controls and primers used are described in Table 2.

Table 2. Antimicrobial resistance genes investigated in resistant *Staphylococcus aureus* isolated from bovine mastitis in this study.

Target	Gene	Primer sequence (5' to 3')	Annealing temperature (°C)	Amplicon size (bp)[1]	Positive control[2]	Reference
β-Lactams resistance	*blaZ*	F CAGTTCACATGCCAAAGAG R TACACTCTTGGCGGTTTC	45	772	#60	Schnellmann et al. 2006
Macrolide resistance - rRNA erm methylase	*erm(A)*	F TCTAAAAAGCATGTAAAAGAA R CTTCGATAGTTTATTAATATTAG	52	645	#75, #76 #78	Sutcliffe et al. 1996
Macrolide resistance - rRNA erm methylase	*erm(B)*	F GAAAAGTACTCAACCAAATA R AGTAACGGTACTTAAATTGTTTA	51	639	#76, #60	Sutcliffe et al. 1996
Macrolide resistance - rRNA erm methylase	*erm(C)*	F TCAAAACATAATATAGATAAA R GCTAATATTGTTTAAATCGTCAAT	52	642	#184, #398	Sutcliffe et al. 1996
Macrolide/lincosamide/streptogramin B (MLSB) resistance	*erm(T)*	F CCGCCATTGAAATAGATCCT R TTCTGTAGCTGTGCTTTCAAAAA	50	200	#161	Gómez-Sanz et al. 2010
Macrolide resistance - rRNA erm methylase	*erm(Y)*	F AGGCCCCTTTTAAAGACGAAGGCA R GGCGCGATTGTTCATTTTAAGGCCC	59	320	#60	Gómez-Sanz et al. 2010
Macrolide resistance - Efflux pump	*msr(A)*	F GGCACAATAAGAGTGTTTAAAGG R AAGTTATATCATGAATAGATTGTCCTGTT	50	940	#60, #352	Lina et al. 1999a
Macrolide resistance - macrolide phosphotransferase	*mph(C)*	F ATGACTCGACATAATGAAAT R CTACTCTTTCATACCTAACTC	45	900	#60, #352	Schnellmann et al. 2006
Tetracycline resistance (efflux pump)	*tet(L)*	F CATTTGGTCTTATTGGATCG R ATTACACTTCCGATTTCGG	49	456	#240	Aarestrup et al. 2000
Tetracycline resistance (efflux pump)	*tet(K)*	F TTAGGTGAAGGGTTAGGTCC R GCAAACTCATTCCAGAAGCA	56	697	#184, #82	Aarestrup et al. 2000
Tetracycline resistance (ribossomal protection)	*tet(M)*	F GTTAAATAGTGTTCTTGGAG R CTAAGATATGGCTCTAACAA	55	657	#75, #78	Aarestrup et al. 2000
Aminoglycosides resistance - aminoglycoside-modifying enzyme	*aac(6') -Ie–aph(2')-Ia*	F CAGAGCCTTGGGAAGATGAAG R CCTCGTGTAATTCATGTTCTGGC	55	348	#137, #386	Vakulenko et al. 2003

(AME)						
Fluoroquinolone resistance (efflux pump)	*mepA*	F ATGTTGCTGCTGCTCTGTTC R TCAACTGTCAAACGATCACG	53	718	ATCC[3] 33591	Couto et al. 2008

[1]Base pairs (bp); [2]# strains used as positive controls were from the collection of Laboratório de Bacteriologia, Departamento de Medicina Veterinária, Universidade Federal de Lavras; [3]American Type Culture Collection (ATCC).

Agarose gel electrophoresis for PCR products

Visualization of the amplified products of all PCR reactions was performed in 1.0% agarose gel in tris-borate-EDTA buffer (TBE) (89 mM Tris Base, 89 mM boric acid and 2 mM EDTA pH 8.0) and stained with ethidium bromide (0.5 mg / mL). Following electrophoresis, the gels were visualized under ultraviolet light and photographed (L-PIX EX, Loccus Biotechnology, Brazil). The molecular weight marker 100 bp DNA ladder (KASVI, Brazil) was used in each electrophoresis.

Statistical analyzes

Prevalence was obtained in cross tabulations and expressed in percentage. All associations between the variables were carried out by univariate analysis using chi-square or Fisher's exact tests, $P < 0.05$ was considered significant (Sampaio, 2002). All statistical analyzes were performed using GraphPad Prism 5.0 (GraphPad Software, USA).

3. RESULTS

All strains were confirmed by PCR as *S. aureus.*

Prevalence of biofilm-forming ability and biofilm associated genes

Prevalence of positive isolates for biofilm-forming was 83.5% (334/400), while 16.5% (66/400) of the isolates maintained red color in the medium and were considered negative. PCR analysis for detection of the *icaAD* biofilm gene revealed that 98.5% (394/400) isolates harbored *icaAD* gene. Interestingly, 83.25% (333/400) of the isolates that phenotypically were biofilm-forming also exhibited *icaAD* gene, however 15.25% (61/394) did not produce biofilm but harbored these genes. A significant association between phenotype and genotype for biofilm was observed ($P < 0.001$) (-3). Moreover, it was also observed an association between biofilm-forming ability and year of isolation ($P < 0.05$) in the tested *S. aureus* strains (Table 3). The odds of the strain being a biofilm producer in the phenotypic test increased over the evaluated years. The years were grouped based on the cumulative distribution in

percentiles according to the number of isolates (25%, 50% and 75%) in the following periods 1994-1998, 1999-2001, 2002-2004 and 2005-2016.

Table 3. Association between biofilm-forming and the year of isolation and presence of *icaAD* gene among *Staphylococcus aureus* strains isolated from bovine mastitis in Brazil.

Variable	Biofilm-forming[1]		P-value[2]	Odds Ratio (95% CI)[3]
	Positive	Negative		
Year			*0.0244*	
1994 to 1998	84/92	8/92		Base category
1999 to 2001	91/106	15/106		1.73 (0.70 – 4.30)
2002 to 2004	77/102	25/102		3.41 (1.45 – 8.01)
2005 to 2016	82/100	18/100		2.30 (0.95 – 5.60)
***icaAD* gene**			*0.0005*	
Positive	333/394	61/394		Base category
Negative	1/6	5/6		27.30 (3.13 – 237.71)

[1]Phenotype; [2]Chi-square test or Fisher's exact test; [3]Confidence Interval

Prevalence of virulence genes

The Table 4 summarize the prevalence of the virulence genes investigated among the *S aureus* strains isolated from bovine mastitis. Among the genes associated with virulence, the most common in the studied population were those coding for α and β hemolysins.

In order to analyze frequency of virulence genes according to the year of isolation, previous categorization (1994-1998, 1999-2001, 2002-2004 and 2005-2016) was used for the distribution of the isolates per year and the strains were also classified according to the number of virulence genes found as follows ≤ 1, 2, 3 and ≥ 4 genes. Most of the strains showed at least 3 virulence genes tested [77.0% (308/400)], being *icaAD* and *hla* the genes were the most prevalent among the tested isolates. Percentual distribution of *S. aureus* strains according to the number of virulence genes exhibited and the year of isolation is shown in the Figure 2. As a result, no pattern was observed for the presence of virulence genes over the

years among the isolates tested, although the percentage of strains that exhibited 3 virulence genes increased over the analyzed period.

Table 4. Prevalence of virulence factors genes *in Staphylococcus aureus* strains isolated from bovine mastitis in Brazil.

Gene	N° of isolates	%
Enterotoxins		
sea^{+}	0/400	0
seb^{+}	2/400	0.5
sec^{+}	4/400	1.0
sed^{+}	1/400	0.25
see^{+}	None	0
Toxic shock syndrome toxin – 1 (TSST)		
tst^{+}	3/400	0.74
Hemolysin		
hla^{+}*hlb*$^{-}$	66/400	16.5
hla^{-}*hlb*$^{+}$	3/400	0.75
hla^{+}*hlb*$^{+}$	329/400	82.85
hla^{-}*hlb*$^{-}$	2/400	0.5
Panton–Valentine Leukocidin (PVL)		
luk^{+}	14/400	3.5
Biofilm		
icaAD^{+}	394/400	98.5

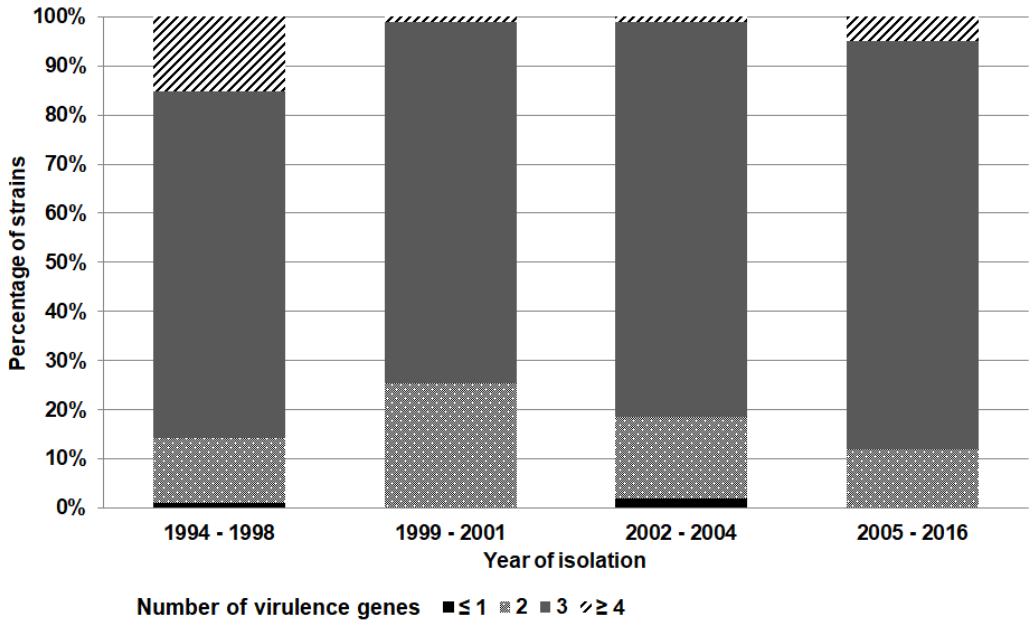

Figure 2. Percentual distribution of *Staphylococcus aureus* isolated from bovine mastitis in Brazil, according to the number of virulence genes exhibited (*luk, hla, hlb, sea, seb, sec. sed, see, tst and icaAD*) and the year of isolation.

Prevalence of antimicrobial resistance genes

The prevalence of ARG among resistant *S. aureus* isolated from bovine mastitis in Brazil are showed in Table 5. The genes most frequently found among resistant *S. aureus* isolates (only strains resistant to a given class were tested for the corresponding resistance gene) were *mepA* (fluoroquinolone resistance), *blaZ* (β -lactam resistance), *aac(6')-Ie–aph(2')-Ia* (aminoglycosides resistance), *tetK* (tetracycline resistance) and *ermC* (macrolides resistance) (Table 5).

Table 5. Prevalence of antimicrobial resistant genes (ARG) among resistant *Staphylococcus aureus* isolates from bovine mastitis in Brazil.

Antimicrobial class	Gene	Number of resistant strains[1]	Number of strains showing ARG[2] (%)
Penicillin	*blaZ*	217	178 (82.03)
Tetracyclines	*tetK*	62	21 (33.87)
	tetL	62	1 (1.61)
	tetM	62	2 (3.22)
Macrolides	*ermA*	21	3 (14.29)
	ermB	21	3 (14.29)
	ermC	21	7 (33.30)
	ermT	21	2 (9.52)
	ermY	21	1 (4.76)
	msrA	21	2 (9.52)
	mphC	21	2 (9.52)
Aminoglycosides	*aac(6')-Ie–aph(2')-Ia*	13	6 (45.15)
Quinolones	*mepA*	7	7 (100)

[1]Strains described as resistance by Abreu (2016); [2]ARG -antimicrobial resistance genes.

Association between virulence and antimicrobial resistance

Combinations for the presence of virulence genes and ARG were observed, albeit not very common [2.5% (10/400)] (Table 6). Furthermore, evaluation of the distribution of the number of virulence genes according to the antimicrobial resistance profile revealed that most of the resistant strains exhibited at least 3 virulence genes (Figure 3). In addition, it was observed a significant association between biofilm-forming and resistance to penicillin (P = 0.002), having the penicillin resistance strains 2.44 (95% confidence interval; 1.38 - 4.34) times more chance to produce biofilm, compared to susceptible strains.

Table 6. Virulence factors and antimicrobial resistance genes (ARG) in *Staphylococcus aureus* isolates from bovine mastitis in Brazil.

Genes	N° of isolates
hla^{+}*hlb*$^{+}$*luk*$^{+}$*icaAD*$^{+}$*blaZ*$^{+}$	5
hlb^{+}*seb*$^{+}$*icaAD*$^{+}$*blaZ*$^{+}$	2
hla^{+}*luk*$^{+}$*icaAD*$^{+}$*tetK*$^{+}$	1
hla^{+}*hlb*$^{+}$*luk*$^{+}$*icaAD*$^{+}$*ermB*$^{+}$	1
hla^{+}*hlb*$^{+}$*luk*$^{+}$*icaAD*$^{+}$*tetK*$^{+}$*blaZ*$^{+}$	1

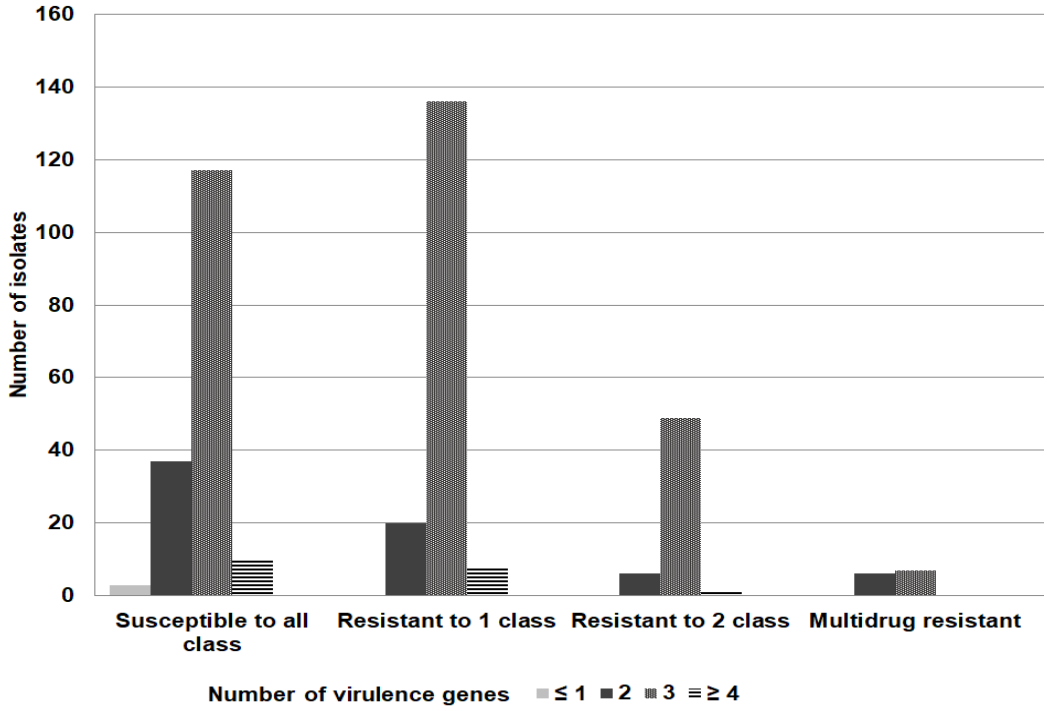

Figure 3. Distribution of *Staphylococcus aureus* isolated from bovine mastitis in Brazil, according to the number of virulence genes exhibited (*luk, hla, hlb, sea, seb, sec, sed, see, tst and icaAD*) and the antimicrobial resistance profile.

4. DISCUSSION

A further understanding of the potential for damage of *S. aureus* isolates from cattle is of great importance for animal and human health, since this agent is considered one of the main pathogens causing food poisoning (Cretenet et al., 2011) and main agent responsible for the contagious mastitis worldwide (Hogeveen and Van, 2017). In the present study, we investigated some of the major virulence factors and genetic mechanisms of antimicrobial

resistance in *S. aureus* strains isolated from bovine mastitis and found that Brazilian staphylococci of animal origin have a great potential to cause severe infections.

Biofilm formation and maturation is mediated by synthesis of polysaccharide intercellular adhesin (PIA), also known as polymeric N-acetyl-glucosamine (PNAG), which is encoded by the *icaADBC* operon (Foster et al., 2014). There are several methods for the identification of biofilm-forming bacterial strains. In this study, the strains were tested using the Congo red TSB broth supplemented with sucrose as proposed by Lee et al. (2016) and investigated for the presence of *icaAD* gene, as this gene is commonly detected in biofilm-forming *S. aureus* strains isolated from bovine mastitis. Moreover, also the coexpression of *icaA* and *icaD* appears to lead a significant increase in enzymatic activity, being related to phenotypic expression of the capsular polysaccharide (Felipe et al., 2017; Foster et al., 2014; Gerke et al., 1998).

In the present study, a high frequency (83.25%) of biofilm-forming strains that also harbored the *icaAD* gene was observed. High frequency of *icaA* and *icaD* genes in *S. aureus* isolates from bovine mastitis were also reported in similar studies (Castelani et al., 2015; Li et al., 2012; Vasudevan et al., 2003). As expected, a positive association was observed between phenotype and genotype for ability to form biofilm. Thus, these results reveal that *icaAD* gene may be crucial biofilm associated genes since this gene was present in biofilm-positive strains. Although in this study was detected a presence of *icaAD* gene in non-biofilm-forming strains (15.25%), this could be because *ica* expression can be regulated by multiple accessory regulators (Li et al., 2012). Besides, in this study was observed, in low frequency, the expression of biofilm by strains that did not carry *icaAD* gene, the reason could be because the existence of other *ica*-independent biofilm formation mechanisms (Cucarella et al., 2004; Figueiredo et al., 2017; Mootz et al., 2015). According to Cucarella et al. (2004), biofilm production by microorganisms isolated from mastitis is associated with antimicrobial

resistance, since presence of exopolysaccharide matrix precludes the antimicrobial penetration. For this reason, association between the biofilm-forming ability and the antimicrobial susceptibility profile (Abreu, 2016) was investigated, being observed that penicillin resistant strains had 2.44 (95% CI: 1.38-4.34) times more chance to produce biofilm compared to susceptible strains. Indeed, β-lactams, especially penicillin, are widely used to intramammary treatment of bovine mastitis and the strains of the studied population exhibited a high level of resistance to this drug. Furthermore, it was also observed a significant association between biofilm production and year of isolation (P = 0.02), which could be partially explained considering the acceleration of the process of natural selection of antimicrobial resistance due the extensive use of these drugs in animal production.

In addition to issues related to drug resistance, *S. aureus* is also a pathogen of great importance in public health due to its ability to produce a wide variety of enterotoxins, frequently involved in foodborne diseases outbreaks (Cretenet et al., 2011). The majority (95%) of food poisoning cases are caused by exotoxins *sea, seb, sec, sed* and *see* (Hennekinne et al., 2010), however, in this study, the presence of enterotoxins genes was observed in low frequency among the *S. aureus* strains isolates from bovine mastitis (Table 4). Similarly, other studies conducted in Turkey and Brazil also found low frequency or absence of *sec* gene in *S. aureus* isolated from cattle (Boynukara et al., 2008; Rall et al., 2014). In contrast, Rall et al. (2008) in Brazil, detected a prevalence of 20.5% for this gene, similar frequencies of 15.5% and 16.1% were also observed by Akineden et al. (2001) and Cremonesi et al. (2005), in Germany and Italy respectively in *S. aureus* from bovine milk. Regard to the *seb* and *sed* genes, the results observed in the present study are corroborated by other findings, since frequency of these genes seems to appear low in *Staphylococcus* spp. or were not observed (Wang et al., 2009, Ruaro et al., 2013, Liu et al., 2014). Likewise, in present study *sea* neither *see* genes were detected, similar to the results found by Yang et al. (2012) that did not observe

any *sea* positive *S. aureus* (n=39) strain among isolates from bovine clinical mastitis in China. However, the enterotoxin gene *sea* was the most frequent detected in Brazil (41%) and Turkey (23.6%) in *S. aureus* isolated from bovine milk, respectively (Boynukara et al., 2008; Rall et al., 2008).

Another important virulence factor related to the production of toxins, the *tst* gene, which encodes toxic shock syndrome toxin-1, was detected a low frequency (0.74%), as well as observed in China (2.6%) and Poland (2.4%) for *S. aureus* isolates from bovine mastitis (Kot et al., 2016; Yang et al., 2012). Nonetheless, it is interesting to note that, two *tst* positive strains identified also exhibited the *sec* gene. A comparable relationship between presence of these two genes has been reported in the literature (Fitzgerald et al., 2000; Stephan et al., 2001). The presence of *tst* and *sec*, has been identified as part of the bovine *S. aureus* pathogenicity island SAPIbov (Haenni et al., 2010). Both toxins can exhibit various biological activities and act as superantigens for cells of the bovine immune system, contributing to the pathological mechanisms of bovine mastitis (Yokomizo et al., 1995), especially in peracute mastitis (Zschöck et al., 2004). Moreover, considering that Staphylococcal enterotoxins (SEs) and TSST-1 can keep their biological and immunological activities even following pasteurization (Asao et al., 2003), the detection of strains able to produce both toxins in cow milk samples represents not only an issue for the dairy husbandry but also a threat to public health.

Likewise, *luk* gene was exhibited by few isolates, which was expected since other studies, PVL gene was rarely detected in bovine isolates (Fluit, 2012; Shrivastava et al., 2018). Interestingly, 93% (13/14) of PVL-positive strains also harbored $hla^{+}hlb^{+}icaAD^{+}$ genes. Despite the low prevalence among the studied *S. aureus* isolates, it is worth noting that PVL-positive strains in bovine milk poses a potential public health risk to the community due to its characteristic of pore-forming toxins and its association with necrosis of skin and soft tissues.

Differently from that observed for genes encoding for SEs, TSST-1 and PVL, our findings for the presence of hemolysin genes (*hla* and *hlb*) revealed a large proportion (82.85%) of the tested isolates positive for both genes, which has also been reported by others (Silva et al., 2005; Yang et al., 2012). In this sense, the community risk related to the detection of hemolysin genes in *S. aureus* from mastitis are even higher compared to the other toxins investigated in the present study, considering the expressive number of isolates carrying these genes and that the hemolysins, especially *hlb*, have relative stability against inactivation in high temperatures (thermostable below 90 °C for 30 minutes) (Singh et al., 2014). Moreover, the interaction between α and β hemolysins increase both, the adherence to bovine mammary epithelial cells and the proliferation of *S. aureus* (Cifrian et al., 1996). Only two isolates did not harbor α and β hemolysin genes, probably because carried other hemolysin gene or none (Aarestrup et al., 1999).

Considering that there is a strong association between virulence and antimicrobial resistance genes, whether for the importance in public and animal health or because of common mechanisms of dissemination and co-selection, this study was analyzed the distribution of ARG among *S. aureus* isolated from bovine mastitis in Brazil. The *blaZ* gene, screened among β-lactams resistant strains (ampicillin, amoxicillin+clavulanic acid, penicillin-novobiocin, ceftiofur), was the main genetic mechanism of resistance observed for this antimicrobial class, in contrast to *mecA* gene, which was previously tested in all isolates of the present study that were all negative (Abreu, 2016). In fact, resistance to β-lactams are principally conferred by *mecA* and *blaZ* genes, and the importance of detecting *mecA* gene in *S. aureus* isolated from bovine mastitis is because its presence implies resistance to almost all β-lactams agents. Furthermore, detecting *blaZ* and, especially, *mecA* is also important because it will help to determine strategies for treatment, control and prevention of dissemination of resistance between animals and humans (Becker et al., 2013; Wielders et al., 2001). High

prevalence of *blaZ* among *S. aureus* isolated from animal origin has also been reported by studies conducted in China and Brazil (Marques et al., 2017; Qu et al., 2019). Additionally, in the present study for an expressive proportion of the isolates [39/217 (18%)] neither *mecA* nor *blaZ* were detected, thereby further studies are need in order to identify the genetic determinate of resistance for theses isolates that were phenotypically β-lactams resistant. Recently *mecC* gene has also emerged as an important mechanism of resistance to this group among *S. aureus* from mastitis (García-Álvarez et al., 2011).

For tetracycline resistant *S. aureus* (tetracycline) the principal gene found was *tetK*, followed by *tetM* and *tetL*, as well as observed in a study conducted by Martini et al. (2017) in *S. aureus* isolated from bovine mastitis in Brazil. The detection of these genes in *S. aureus* from animal origin has a critical importance in public health, since these genes are in mobile genetic elements, as small plasmids or conjugative transposons, which help to spread several resistance genes and consequently can lead treatment failure in both veterinary and human medicine (Huys et al., 2005). In this context, resistance to macrolides (such as erythromycin) and lincosamide (such as lincomycin and clindamycin), antimicrobials widely used in the treatment of staphylococcal infections, are also predominant among staphylococci (Chang et al., 1995; Sanchez et al., 1993). The investigation of macrolides, lincosamide and streptogramin B (MLS_B) resistance genes (*erm*, *msrA* and *mphC*) revealed that most of the isolates resistant to macrolides (erythromycin) carried the gene *ermC*, as well as observed by LI et al. (2015) that found *ermC* as the most prevalent resistance gene in *S. aureus* isolates. Likewise, the low frequency of *ermA* and *ermB* genes observed in the present study has also been reported elsewhere (Qu et al., 2019), showing that these genes are not commonly detected in *S. aureus* from bovine mastitis. The other genes related to macrolides resistance (*ermT*, *msrA* and *mphC*) were found in a lower frequency, suggesting a possible less importance of these mechanisms in the resistance to this class in *S. aureus* from animal origin.

It has been mentioned that *mphC* often occurs linked to *msrA* and that the presence of *mphC* gene alone confers low-level resistance to macrolides (Lüthje and Schwarz, 2006). In the present study, was found two isolates harboring *msrA* and *mphC* and one of them associated to *ermY, blaZ, ermB* genes.

Although aminoglycosides are widely used in Brazil for mastitis treatment (Martins et al., 2016), few studies are focused on the identification of mechanism of resistance against this class.

In this study, among aminoglycoside resistance genes, *aac(6')-Ie–aph(2')-Ia* (also known as *aacA-aphD*) was detected in 45.15% of *S. aureus* isolates. Similar studies have reported a high prevalence of *aac(6')-Ie–aph(2')-Ia* in *S. aureus* isolated from mastitis (Goni et al., 2004; Qu et al., 2019; Schnellmann et al., 2006; Wendlandt et al., 2013). In contrast, a study conducted in *S. aureus* strains from bovine mastitis in Turkey detected the gene *aph (3')-IIIa* as the most prevalent (Turutoglu et al., 2009). Thus, difference of aminoglycosides resistance genes can be attributed to the difference among *S. aureus* isolates from different geographical regions.

For fluroquinolones, in general, two important mechanisms are responsible for resistance in *S. aureus*. The first one is attributed to mutations occurring in the quinolone-resistance determining region (QRDR) of *grlA* / *grlB* (topoisomerase IV) and *gyrA* / *gyrB* (DNA gyrase), which decrease the affinity of the drug (Ng et al., 1996; Takahata et al., 1996). Nonetheless, fluoroquinolone resistance can also be mediated by drug efflux, a mechanism that is less well characterized. Several efflux pumps have been described in *S. aureus*, including *norA, norB, norC, mdeA, mepA, sepA* and *sdrM* genes (Poole, 2007). In this study, it was identified by the first-time fluoroquinolone resistance gene *mepA* in all isolates of *S. aureus* from bovine mastitis that were phenotypically resistance or intermediate susceptible to ciprofloxacin or enrofloxacin. Four isolates also carried *blaZ* and *mepA* genes. The

importance of the detection of the *mepA* gene among bovine mastitis *S. aureus* goes beyond resistance to fluoroquinolones, as this gene also confers resistance to a wide range of compounds, including various dyes and biocides (Correia et al., 2017; Kaatz et al., 2005), such as iodine, quaternary ammonium and chlorhexidine, widely used in cows post dipping. A recent study in China, detected a high frequency of genes *norA, gyrA, grlA* in *S. aureus* isolated from bovine mastitis (LI et al., 2015). However more studies are necessary in order to know the actual profile of fluoroquinolone resistance among *S. aureus* from animal origin.

5. CONCLUSIONS

Our results showed that *S. aureus* strains isolated from bovine mastitis in Brazil carried mainly biofilm and hemolysin genes, whereas, virulence genes associated with enterotoxins, PVL and TSST-1 were less frequently observed. Moreover, a wide variety of resistance genes that confer resistance to almost all classes of antimicrobial agents approved for use in animals and in human population were found. Together these data point to the great pathogenic potential of staphylococcal infections caused by *S. aureus* of animal origin.

6. ACKNOWLEDGMENTS

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7. CONFLICT OF INTEREST STATEMENT

None to declare.

8. APPENDIX

Table 7. Supplemental Table S1. Antimicrobial susceptibility profile of *Staphylococcus aureus* isolated from bovine mastitis from Minas Gerais, Rio de Janeiro, São Paulo and Goiás from 1994 to 2016. Source: ABREU (2016).

OXA	AMP	CFL	CFO	CTF	AMC	PNM	AMC45	ENO	CIP	ERI	NEO	GEN	TET	SUT	N° of isolates
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	165
S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	145
S	R	S	S	S	S	S	S	S	S	S	S	S	R	S	36
S	R	S	S	S	S	S	S	S	S	S	S	S	I	S	7
S	S	S	S	S	S	S	S	S	S	I	S	S	S	S	5
S	R	S	S	S	S	S	S	S	S	R	S	S	S	S	4
S	S	S	S	S	S	S	S	S	S	S	S	S	I	S	3
S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	3
S	R	S	S	S	S	I	S	S	S	S	S	S	S	S	3
S	R	S	S	S	S	S	S	S	S	R	R	S	R	S	3
S	R	S	S	S	S	S	S	S	S	R	S	S	R	S	3
S	S	S	S	S	S	S	S	I	S	S	S	S	S	S	2
S	R	S	S	S	S	S	S	S	S	S	I	R	R	S	2
S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	1
S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	1
S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	1
S	R	S	S	S	S	S	S	S	I	S	S	S	S	S	1
S	R	S	S	S	S	S	S	S	R	S	S	S	S	S	1
S	R	S	S	S	S	S	S	S	S	S	S	S	S	I	1
S	R	S	S	S	S	S	S	S	S	S	I	S	I	S	1
S	R	S	S	S	S	S	S	I	S	S	S	S	R	S	1

S	R	S	S	S	S	I	S	S	S	S	S	S	R	S	1
S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	1
S	S	S	S	S	S	S	S	S	S	R	S	S	R	S	1
S	R	S	S	S	S	S	S	S	S	S	I	S	S	S	1
S	R	S	S	S	S	S	S	S	S	S	I	R	S	S	1
S	R	S	S	S	S	S	S	S	S	I	S	S	I	S	1
S	R	S	S	S	S	S	S	S	S	I	R	S	S	S	1
S	R	S	S	S	S	S	S	I	S	R	S	S	S	S	1
S	R	S	S	S	R	S	S	S	I	I	I	S	S	S	1
S	S	S	S	S	S	S	S	S	S	S	I	R	S	S	1
S	S	S	S	S	S	S	S	S	S	I	I	R	S	S	1

S: susceptibility, I: intermediate, R: resistance

CFL, cephalothin; CFO, cefoxitin; OXA, oxacillin; AMC: amoxicillin–clavulanic acid; AMP, ampicillin; ENO, enrofloxacin, CIP, ciprofloxacin; CTF, ceftiofur; AMC45, ampicillin-colistin; ERI, erythromycin; NEO, neomycin; GEN, gentamicin; TET, tetracycline; SUT, sulfamethoxazole–trimethoprim.

9. REFERENCES

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