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Review Article
Livestock Diseases

Insights into *Staphylococcus aureus* pathogenesis and genetic diversity in bovine mastitis[1]

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ABSTRACT.- Vazquez H.C., Moliva M., Cerioli M.F., Vazquez M.A.C., Castañeda E.S., Farías E.C., Souza M.M.S. & Reinoso E. 2025. **Insights into *Staphylococcus aureus* pathogenesis and genetic diversity in bovine mastitis.** *Pesquisa Veterinária Brasileira 45:e07485, 2025.* Instituto de Biotecnologia Ambiental y Salud, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Río Cuarto, Ruta 8 Km 601, Córdoba, 5800, Argentina. E-mail: ereinoso@exa.unrc.edu.ar

Bovine mastitis is the most common infectious disease in dairy herds and causes important economic losses. Various pathogens can cause the disease, with *Staphylococcus aureus* being one of the most significant. *S. aureus* can cause clinical, subclinical, and chronic forms of the disease due to a variety of virulence factors, including protein A, coagulase, toxins, and adhesins. The presence of methicillin-resistant *S. aureus* strains complicates treatment protocols and raises concerns about antimicrobial resistance. The genetic characterization of these strains, utilizing techniques such as molecular typing by pulsed-field gel electrophoresis (PFGE), reveals the existence of different genetic and virulence profiles, as well as the geographical variations in the distribution of these genotypes. The role of next-generation sequencing (NGS) technologies, functional genomics, and multi-omics approaches in the genetic research of bovine mastitis are highlighted, providing a complete understanding of its genetic basis and opening opportunities for targeted interventions and improved disease control strategies in the dairy industry. This review highlights the importance of understanding the genetic diversity and pathogenic mechanisms of *S. aureus* in bovine mastitis to develop effective disease management strategies and reduce economic losses in the dairy sector globally.

INDEX TERMS: Infectious disease, dairy herds, genetic characterization, *Staphylococcus aureus.*

RESUMO.- [***Insights* sobre a patogênese de *Staphylococcus aureus* e diversidade genética na mastite bovina.**] A mastite bovina é a doença infecciosa mais comum em rebanhos leiteiros e causa importantes perdas econômicas. Diversos patógenos podem causar a doença, sendo *Staphylococcus aureus* um dos mais significativos. *S. aureus* pode causar formas clínicas, subclínicas e crônicas da doença devido a uma variedade de fatores de virulência, incluindo proteína A, coagulase, toxinas e adesinas. A presença de cepas de *S. aureus* resistentes à meticilina complica os protocolos de tratamento e levanta preocupações sobre a resistência antimicrobiana. A caracterização genética dessas cepas, utilizando técnicas como a tipagem molecular por eletroforese em gel de campo pulsado (PFGE), revela a existência de diferentes perfis genéticos e de virulência, bem como as variações geográficas na distribuição desses genótipos. Destaca-se o papel das tecnologias de sequenciamento de nova geração (NGS), da genômica funcional e das abordagens multi-ômicas na pesquisa genética da mastite bovina, proporcionando uma compreensão mais completa de sua base genética e abrindo oportunidades para intervenções direcionadas e melhores estratégias de controle da doença na indústria de laticínios. Esta revisão destaca a importância de compreender a diversidade genética e os mecanismos patogênicos de *S. aureus* na mastite bovina para desenvolver estratégias eficazes de manejo da doença e reduzir as perdas econômicas no setor de laticínios globalmente.

TERMOS DE INDEXAÇÃO: Doença infecciosa, rebanhos leiteiros, caracterização genética, *Staphylococcus aureus.*

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INTRODUCTION

Bovine mastitis remains a major challenge in the dairy industry and causes considerable economic losses worldwide. Different pathogens can cause the disease, and *Staphylococcus aureus* is the most prevalent contagious pathogen in dairy herds around the world. *S. aureus* is a Gram-positive bacteria that can be found on the skin and mucous membranes of animals and humans.

When *S. aureus* penetrates the cow's udder, usually during the milking process, it can cause an infection that triggers an inflammatory response in the mammary tissue. This leads to several characteristic symptoms, such as swelling, redness, warmth, and tenderness in the affected mammary gland. The milk produced by the infected cow may contain pus, resulting in changes in its appearance, such as a yellowish or greenish color, and may have an unpleasant odor.

Mastitis due to *S. aureus* can occur in acute or chronic form. In acute mastitis, symptoms are generally more severe and may include fever in the affected cow. In chronic cases, the infection can persist for long periods, with less obvious symptoms but with reduced milk production and lower quality.

Treatment of *S. aureus* mastitis usually involves the use of specific antibiotics. However, *S. aureus* can be resistant to certain antibiotics, making it difficult to treat. Additionally, this bacterium can form biofilms on the udder, which can protect it from antibiotics and make eradication difficult.

Prevention of *S. aureus* mastitis is essential and may include proper management practices such as hygiene during milking, maintaining clean and dry facilities, and using appropriate milking systems to prevent contamination. Control of *S. aureus* mastitis is important for both the health and well-being of cows and the production of quality milk in the dairy industry. A comprehensive understanding of the genetic diversity and pathogenic mechanisms of *S. aureus* is imperative to design effective disease control strategies. In this context, the present review attempts to provide insights into the pathogenesis and genetic diversity of *S. aureus* in bovine mastitis. A systematic exploration of the literature was conducted to outline the current state of knowledge in this domain. The methodologies covered the analysis of all the articles considered and the synthesis of pertinent information. Inclusion criteria were established to select relevant original articles and reviews, which included the following: articles published between 1990 and 2023, original research articles, review papers, and clinical studies, studies focusing on the pathogenesis, genetic diversity, and alternative treatments of *S. aureus* in bovine mastitis, articles published in English and Spanish, and peer-reviewed articles with robust methodologies and clear findings. Through this review, we aim to highlight the importance of understanding the mechanisms of genetic diversity and pathogenic mechanisms of *S. aureus* in bovine mastitis, with a view to formulating targeted interventions and strengthening disease control measures within the dairy industry.

BOVINE MASTITIS

Mastitis is the most common infectious disease in dairy herds around the world and is considered the disease that causes the greatest economic losses to the producer and the dairy industry (Hogeveen et al. 2011, Hadrich et al. 2018). Losses occur because of a significant decline in milk production, the need to discard milk due to treatment, and the excessive costs associated with antibiotics and veterinary services. Additionally, these factors contribute to a decrease in the overall quality and value of the milk.

The term "mastitis" originates from the Greek word "mastos," meaning mammary gland, and the suffix "itis," denoting inflammation. It refers to an inflammatory condition of the mammary gland tissues triggered by the invasion of microorganisms. Mastitis is characterized by damage to the glandular epithelium, leading to clinical or subclinical inflammation. The extent of pathological changes can vary, ranging from localized to generalized, depending on the severity of the damage (Garcia 2004).

The main causes of mastitis in cattle are bacteria. The occurrence and intensity of a case of mastitis depends on factors that are associated with the infected animal, the pathogenic bacterium, and the environment. The environment is determined by the handling conditions used in the dairy. Factors such as teat skin lesions, inadequate udder disinfection, and improper use of milking machines, among others, facilitate the entry of pathogens into the udder, leading to intramammary infection. It is important to consider the source and ways of transmission of the disease. Mastitis-causing microorganisms can be found in various environments, such as fecal matter, bedding, and skin. Ensuring the overall cleanliness of cows and their housing and implementing proper handling procedures (particularly during milking) are effective measures for controlling the spread of mastitis (Cheng & Han 2020).

Bovine mastitis can be classified into clinical and subclinical depending on the presence of signs of disease. Subclinical mastitis is not easily visible and cannot be detected without specialized tests. Although almost all affected quarters may appear normal, Garcia (2004) note a decrease in milk production and an increase in the number of somatic cells. Therefore, its detection is achieved by isolating the pathogenic agent and determining a high number of somatic cells, which may include macrophages, neutrophils, epithelial cells, and other cell types. Subclinical mastitis is considered the most important for several reasons, it is 15 to 40 times more common than clinical mastitis and usually precedes the clinical form, therefore, to control the clinical form, one must start by controlling the subclinical one, which is long-lasting and difficult to detect. The bacteria associated with this type of mastitis are *Staphylococcus aureus*, non-*aureus Staphylococcus* (NAS), *Streptococcus agalactiae* and *Streptococcus uberis*.

Clinical mastitis is a visible condition, and it is characterized by noticeable abnormalities in the milk. The affected quarter may exhibit heat, inflammation, and sensitivity (Kibebew 2017). Systemic symptoms often accompany clinical mastitis, including fever and loss of appetite, and in severe cases, it can lead to the death of the animal, known as hyperacute clinical mastitis. In addition, milk production decreases, and the milk may appear like blood serum. This type of mastitis is frequently caused by one of the major pathogens, staphylococci, streptococci, and coliforms.

Mastitis pathogens

Many microbial agents can come in contact with the udder and can enter the mammary gland through the teat canal. New infections can occur at any stage, either during lactation

or during the dry period. However, early lactating cows are susceptible to new infections due to stress conditions and immune suppression associated with the postpartum period (Petersson-Wolfe & Currin 2012).

More than eighty causative agents of mastitis have been identified, including species of bacteria, fungi, mycoplasmas, and algae (Cobirka et al. 2020). However, bacteria cause most of the infections (Fig.1). These mastitis-causing organisms have been classified based on bacterial origin into contagious, environmental and opportunistic pathogens and pathogens that cause mastitis less frequently (Langoni et al. 2011).

Contagious microorganisms are present on the udder or teat surface of cows. They are transmitted from infected quarters to uninfected quarters and from animal to animal during the milking process. The main contagious microorganisms are *Staphylococcus aureus, Streptococcus agalactiae, Corynebacterium bovis* and *Mycoplasma* species, being *S. aureus* the most frequently isolated contagious species (Barkema et al. 2009).

Environmental pathogens are those whose primary reservoir is the environment surrounding the animals. These organisms represent a heterogeneous group of bacterial genera and species, such as *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Streptococcus bovis*, *Enterococcus faecium*, *Enterococcus faecalis*, and coliform bacteria (*Escherichia coli, Klebsiella pneumoniae* and *Enterobacter aerogenes*) (Schroeder 2012). Among environmental bacteria, streptococci and enterococci are important causative agents of intramammary infection. They are the main pathogens responsible for high somatic cell counts in bulk milk tanks in properly managed herd dairies (Carrillo-Casas & Miranda-Morales 2012, Reinoso 2017). Mastitis caused by environmental organisms is opportunistic in nature and occurs when the host's immune system is depressed or when proper sanitation and hygiene are not practiced during or after milking (Schukken et al. 2003).

Opportunistic pathogens responsible for bovine mastitis include *Pseudomonas* spp., yeasts, *Prototheca spp*., *Serratia marcescens*, and *Nocardia* spp. These pathogens originate from the cow's environment, and their main mode of transmission is improper handling practices. Examples of such practices include wet litter, unsanitary grounds, udders wet with milk, inadequate udder and teat preparation before milking, housing systems that facilitate teat lesions, and exposing uninfected quarters to pathogens. These environmental conditions can arise at any point during a cow's lifespan. Infections caused by opportunistic pathogens typically occur sporadically, but outbreaks can arise within herds or entire regions, often due to hygiene or treatment issues.

In addition, coagulase-negative staphylococcal species (CoNS) have become the most commonly isolated bacteria from cows with mastitis and are recognized as emerging pathogens in mastitis cases.

S. aureus is one of dairy cattle's most prevalent major mastitis pathogens, causing significant economic losses globally. Although hygienic milking practices and effective dairy management systems have reduced its incidence, intramammary infections by this pathogen remain a major challenge for dairy farms, with a high prevalence (Vaughn et al. 2020).

S. aureus is a Gram-positive, catalase- and coagulase-positive, non-spore-forming, oxidase-negative, non-motile, cluster-forming, facultative anaerobe bacterium belonging to the family Micrococcaceae (Vaughn et al. 2020).

Moreover, *S. aureus* is not only a pathogen of animals but also a human pathogen. The identification of *S. aureus* as a human pathogen was reported by Ogston in 1881, after the discovery of the microorganisms by Robert Koch in 1878. In the following decades, it was considered the most important agent in nosocomial infections since it can produce from a simple abscess to fatal sepsis (Kim et al. 2014).

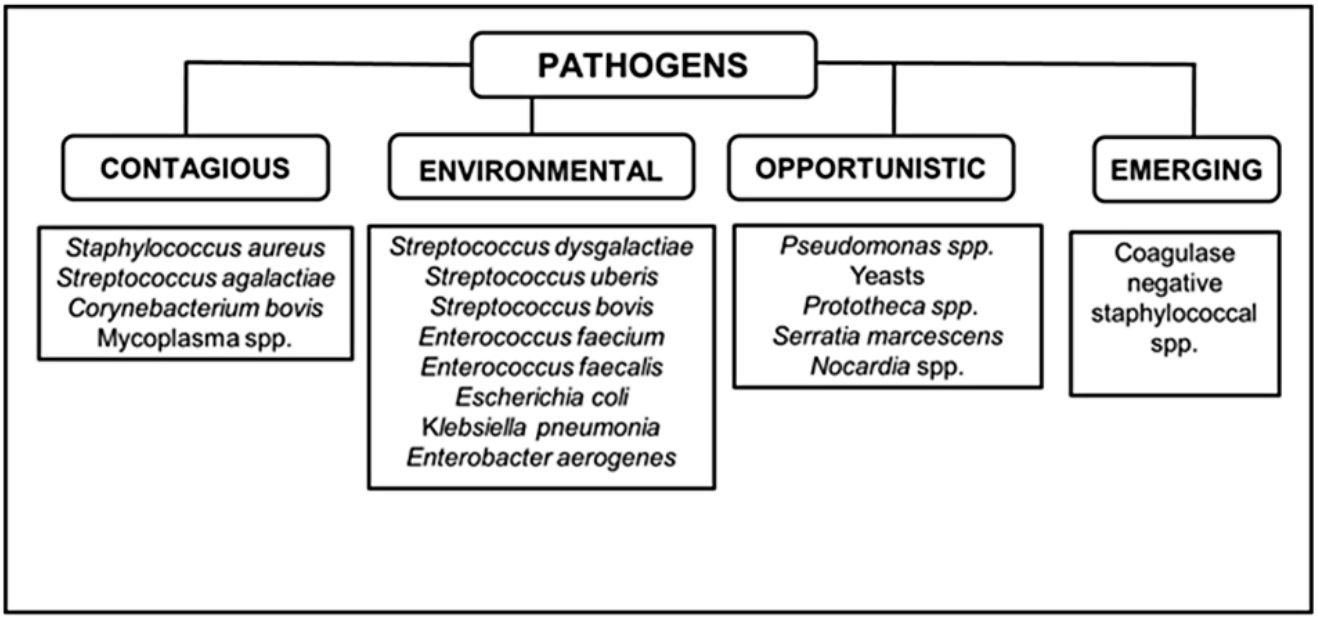

Fig.1. Principal bovine mastitis pathogens.

This highly contagious pathogen is frequently isolated in bovine intramammary infections worldwide, causing clinical, subclinical, and chronic mastitis and resulting in significant economic losses in dairy farms (Castañeda Vazquez et al. 2014, Reinoso 2017). The primary reservoir for this pathogen is the infected quarter, with transmission occurring frequently during the milking process.

Furthermore, *S. aureus* is part of the normal flora found on mammals and birds' skin and mucous membranes. It commonly colonizes the human colon, with the nose being the main site of colonization. Humans serve as a natural reservoir of *S. aureus*, and asymptomatic colonization is more prevalent than infection. The pathogenicity of *S. aureus* is attributed to the expression of virulence factors, which can be either surface-associated or secreted into the surrounding medium (Camussone & Calvinho 2013).

The *S. aureus* genome consists of a single circular chromosome of approximately 2.8 million base pairs (bp), with a GC percentage of 32-33% (Sivakumar et al. 2023). The first *S. aureus* genomes were sequenced in 2001 (Kuroda et al. 2001). The core genes, which comprise approximately 75% of the genome, contain necessary genes for cell survival and genes involved in bacterial metabolism, DNA and RNA synthesis, and replication (Stefani et al. 2012). These core genes are highly conserved, with a similarity of over 97%. Another piece of the *S. aureus* genome consists of variable core genes, which include more than 700 different genes. These genes encode surface or structural proteins that interact with the host. They are distributed throughout the genome and comprise 10-12%. The third component of the *S. aureus* genome is the mobile genetic elements (MGEs), which are genes introduced by horizontal gene transfer, as phage genes, antimicrobial resistance, and different virulence genes. This component constitutes approximately 10-20% of its genome. It is characterized by DNA fragments that can replicate independently or have specific mechanisms to insert themselves into chromosomes or plasmids that replicate (Malachowa & DeLeo 2010). The main transfer system for mobile genetic elements (MGEs) in *S. aureus* is transduction, followed by conjugation; transformation is much less common. Most MGEs can only be transferred to their genetic line or a restricted number of lines (Stefani et al. 2012, Lindsay et al. 2014). Accessory genes play an important role in the pathogenesis of *S. aureus* intramammary infections (Magro et al. 2017).

Virulence factors of *Staphylococcus aureus*

Different virulence factors have been described in *S. aureus* (Cheung et al. 2021). It is reported that these factors include 20 immune evasion molecules (such as protein A, coagulase, hemolysins and leucocidins, factors related to suppressing innate immunity), 15 microbial surface components recognizing adhesive matrix molecules associated with tissue adhesion (such as clumping factor A *clfA*, intercellular adhesion genes *icaA* and *icaD*), and 25 different toxins (such as enterotoxins SEA to SEQ, toxic shock syndrome toxin-1 TSST-1, exfoliative toxins Eta, Etb) among others. Table 1 summarizes the main virulence factors of *S. aureus*, detailing their functions and associated genes, providing a clear and concise overview of how each factor contributes to the pathogenesis of bovine mastitis.

Protein A is encoded by the *spa* gene, which has a conserved and a variable region. The X polymorphic region consists of a variable number of 24 base pair repeats and is located upstream of the coding region from the C-terminus of the cell wall. The region's diversity originated from spontaneous deletion and duplication of the repeat units and point mutations. The protein A domain, encoded by the X region, serves to extend the N-terminal immunoglobulin IgG-binding portion through the cell wall, preventing phagocytosis and complement fixation. The 24-bp repeats vary in number among different *S. aureus* strains and are commonly used as a molecular tool to study genetic variability (El-Sayed et al. 2006, Bhati et al. 2016).

Coagulase protein is another important virulence factor in *S. aureus.* The enzyme is a prothrombin activator, offering protection against phagocytosis. Like the *spaA* gene, the *coa* gene has a repeat polymorphic region that can be used to differentiate strains. The *coa* gene variable region comprises short 81 base pair repeat sequences that are variable in number and sequence (Chmagh & Al-Abbas 2019).

S. aureus strains can produce capsular polysaccharides *in vivo* or under defined culture conditions. The capsule is a virulence factor that has been demonstrated in different animal models since it inhibits phagocytosis. It is reported in the literature that capsule types 5 and 8 are the most common. However, a high percentage of *S. aureus* is encapsulated (Kuipers et al. 2016). A variable prevalence of the capsule type has been found in bovine isolates from different geographic regions of the world (Salimena et al. 2016).

Adhesins are considered the most important virulence factors in the early phases of infection since they facilitate the adhesion to different host cell types. Among them, fibronectin, laminin, elastin, osteoporin, sialoproteins and collagen stand out. Adhesins are called microbial surface-recognizing components of adhesive molecules on the host cell (termed MSCRAMMs), which are cell-wall attached proteins with structural traits like an N-terminal folded domain associated with ligand binding and a wall-spanning region followed by a sorting signal placed at the C-terminal that anchor the protein to the cell wall (Foster et al. 2014). Each of these MSCRAMMs is characterized as having a specificity for a single host protein. For example, the ability of *S. aureus* to bind fibrinogen and fibrin is due to the presence of the *clfA* gene that codes for the fibrinogen receptor. On the other hand, the *cna* gene encodes an adhesin that binds to collagen in the host cell. In addition to its role in adhesion, it also participates in immune evasion during human infection (Campos et al. 2022).

S. aureus is also an important pathogen due, in part, to the production of exotoxins, which are considered superantigens capable of eliciting an increased immunological response in the host by activating host T cells (Hu et al. 2021). Various exotoxins can contribute to the development of bovine mastitis (Campos et al. 2022). These exotoxins include alpha-toxin (α-toxin), beta-toxin (β-toxin), gamma-toxin (γ-toxin), and delta-toxin (δ-toxin). Alpha-toxin is known for its cytotoxic effects, damaging host cell membranes and contributing to tissue damage. These exotoxins can exacerbate inflammation and tissue injury in the udder during mastitis infections.

Furthermore, staphylococcal enterotoxins produced by *S. aureus* can also cause bovine mastitis. Enterotoxins are heat stable and not destroyed during pasteurization, so they are a potential biohazard (Hennekinne et al. 2012). While

their primary role is in causing food poisoning in humans, in the context of bovine mastitis, they can contribute to the inflammatory response in the udder and exacerbate the severity of mastitis infections. Enterotoxin-encoding genes are in mobile genetic elements, such as pathogenicity islands, phages, and plasmids (Otto 2014). Overall, the production of exotoxins and enterotoxins by *S. aureus* can contribute to the pathogenesis of bovine mastitis by promoting inflammation and tissue damage and exacerbating the host immune response.

In addition, the expression of extracellular proteins is subject to the coordinated regulation of several *loci*. The first to be discovered and most characterized is the *agr* regulator, which involves five genes (*agrA, agrB, agrC, agrD,* and *hld*). The *agr* system acts as a positive regulator of secretory proteins such as α, β and δ hemolysins, protease, DNAse, staphylokinase and toxic shock syndrome toxin. In contrast, it represses the transcription of protein A and coagulase genes and other wall-associated proteins (Wang & Muir 2016). The *sar locus* is required for *agr* expression, providing an additional level of regulation of the virulence factor in response to different signals.

Biofilm is considered a virulence factor in *S. aureus,* and its formation begins with the action of adhesive molecules, which help the union of *S. aureus* to the epithelial cells of the mammary gland. Then, the attached *S. aureus* strains multiply and accumulate by the involvement of the bacterial extracellular matrix, the polysaccharide intercellular adhesion molecule produced by *icaADBC* operon, which is considered the most virulent factor associated with biofilm production (Boonyayatra et al. 2014). Cytolytic toxins have also been reported as necessary in biofilm development (Huseby et al. 2010). Alpha-toxin is involved in cell communications, and biofilm-associated protein (Bap), associated with biofilm formation, has a role in perseverance intracellular and antibacterial resistance (Valle et al. 2012). Furthermore, regulation of *agr* is also involved in biofilm development. These traits that are involved in biofilm formation are closely related to persistence in the host.

Methicillin-resistant *Staphylococcus aureus* strains

The use of antibiotics in veterinary medicine is often employed for preventive measures, enhanced feed efficiency, and growth promotion, especially in developing countries. This extensive use of antimicrobials has led to the emergence of antimicrobial-resistant pathogens (Caneschi et al. 2023). The rise and dissemination of multidrug-resistant zoonotic pathogens have sparked growing concerns within both public and scientific communities regarding the widespread application of antimicrobial agents.

Antibiotic-resistant bacteria pose a significant challenge as they do not respond to conventional antibiotic treatments, thereby complicating the course of disease management. In

Table 1. Virulence factors of *Staphylococcus aureus*

Virulence factor	Description	Associated gene	Function
Immune evasion			
Proteo A	Immune evasion molecule, prevents phagocytosis and complement fixation	*spa*	Conserved and variable region, extends IgG-binding domain through the cell wall
Coagulase	Prothrombin activator, protection against phagocytosis	*coa*	81 bp variable repeat region, strain differentiation
Capsule	Inhibits phagocytosis, capsule types 5 and 8 are common	-	Protection in animal models, geographical variability
Adhesins			
Clumping factor A (ClfA)	Adhesion to fibrinogen	*clfA*	Binds to fibrin and fibrinogen
Intercellular adhesion genes (IcaA, IcaD)	Tissue adhesion	*icaA, icaD*	Facilitate adhesion to host cells
Collagen-binding adhesin	Binds to collagen, immune evasion	*cna*	Adhesion to collagen, immune evasion during human infection
Toxins			
Alpha-toxin (α-toxin)	Cytotoxic toxin, damages host cell membranes	-	Contributes to tissue damage
Beta-toxin (β-toxin)	Exotoxin involved in mastitis	-	Exacerbates inflammation
Gamma-toxin (γ-toxin)	Exotoxin involved in mastitis	-	Exacerbates inflammation
Delta-toxin (δ-toxin)	Exotoxin involved in mastitis	-	Exacerbates inflammation
Enterotoxins (SEA-SEQ, TSST-1, Eta, Etb)	Heat-stable toxins, cause inflammatory response	-	Potential biohazard, contribute to mastitis
Extracellular regulation			
agr system	Regulator of secretory proteins and virulence factors	*agrA, agrB, agrC, agrD, hld*	Regulates hemolysins and wall-associated proteins
Biofilm formation			
Polysaccharide intercellular adhesion	Formation of extracellular matrix in biofilm	*icaADBC*	Biofilm production, antibacterial resistance
Biofilm-associated protein (Bap)	Biofilm formation, cell communication	*bap*	Intracellular persistence, antibacterial resistance
Cytolytic toxins	Involved in biofilm development	-	Facilitate biofilm formation
agr regulation	Involved in biofilm development	-	Host persistence

the context of *S. aureus* strains, antibiotic resistance further complicates treatment protocols for infections. The resistance of *S. aureus* to antimicrobial agents, notably penicillin, became apparent shortly after the introduction of penicillin around 1945. Most *S. aureus* populations developed resistance by producing β-lactamase, an enzyme that hydrolyzes penicillin, encoded by the *blaZ* gene.

In response to the rise in penicillin resistance, methicillin, an antibiotic impervious to β-lactamase hydrolysis, was introduced into human medicine in the late 1950s. However, methicillin-resistant *S. aureus* (MRSA) strains were reported shortly after its introduction. MRSA is mediated by the *mecA* gene, which encodes another penicillin-binding protein, PBP2A, with low affinity for β-lactam antibiotics and is part of a large mobile genetic element known as the staphylococcal cassette chromosome *mec* (SCCmec). MRSA strains are typically resistant to multiple drugs (Algammal et al. 2020).

S. aureus strains can acquire the staphylococcal cassette chromosome (SCCmec), generating MRSA strains. The SCCmec can be transferred horizontally, carrying the resistance gene *mecA* that codes for penicillin-binding protein (PBP2a) with low affinity for β-lactam antibiotics, providing resistance to β-lactams commonly used for mastitis treatment (Markey & Leonard 2023). MRSA arose in the community during the 1960s. Subsequently, MRSA was reported as a pathogenic agent causing bovine mastitis in dairy cattle in Europe. Since then, livestock-associated MRSA (LA-MRSA) strains have been described (Kadlec et al. 2019). Most LA-MRSA isolates are reported as multi-resistant to antibiotics and lack toxins such as PVL and enterotoxins (Khanal et al. 2022).

Notably, MRSA has emerged as a concerning issue in veterinary medicine, representing a new dimension of zoonotic diseases. Initially described as a hospital-based cause of infection, MRSA has garnered attention as a significant concern in the broader veterinary context (Vanderhaeghen et al. 2012). The implications of MRSA in veterinary settings highlight the complex interplay between antibiotic usage, resistance development, and the potential for zoonotic transmission, underscoring the need for comprehensive strategies to address this evolving challenge.

Otherwise, previous studies (Mendonça et al. 2012, Soares et al. 2012, 2021, Silva et al. 2013, Melo et al. 2014) have reported several phenotypic methicillin-resistant *Staphylococcus* spp. isolates that are not correlated with the presence of the *mec*A gene. Further investigation allowed Melo et al. (2020) to report the discovery of a *mec*A gene variant in bovine samples containing mutations in the annealing region that prevent detection of the gene with the primers described so far. A two-set study was conducted to confirm this hypothesis. Firstly, original primers based on the nucleotide sequences of the *S. aureus mec*A gene (HE681097) were tested in bovine, human and equine strains. Those primers failed to amplify the whole *mec*A gene segment in bovine strains. The impairment of *mec*A gene detection in bovine strains sheds light on the specificity of bovine samples. Next, a second-step primer set was based on a sequence of *Staphylococcus sciuri mec*A gene (AY820253) and only yielded *mec*A gene segments for bovine strains. The multiple alignments of *mec*A gene sequences from bovine, human, and equine origins revealed that bovine ones presented punctual but significant differences leading to the observed impairment of *mec*A gene detection in bovine strains, probably due to some selective pressure in the dairy environment (Melo et al. 2014). The increase in selection pressure can indeed promote the spread of resistance genes and the emergence of specific mutations, making it challenging to accurately analyze resistance in dairy production environments. It is important to note that many intramammary pharmaceuticals containing cloxacillin are ineffective against methicillin-resistant strains, which is often overlooked as a selection criterion (Fischer-Tenhagen et al. 2023). Considering the impact of MRSA on human health, it is crucial to assess the risks of using this class of antimicrobials in animal production without precise indication.

Molecular typing of *Staphylococcus aureus* strains

Molecular typing is essential to understanding the evolution of pathogens and their genetic relationships; thus, a greater understanding is achieved during epidemiological investigations (El-Sayed et al. 2017, Vázquez et al. 2018, García et al. 2018).

A variety of molecular techniques, with different degrees of discrimination, are used to type *S. aureus*, including A-surface protein typing (*spa* typing), multiple *locus* sequence typing (MLST), plasmid profile analysis, restriction fragment length polymorphism (RFLP) analysis (MLVA), whole genome DNA sequence analysis and pulsed-field electrophoresis (PFGE) (Sabat et al. 2013, Dendani et al. 2022). Among the techniques, PFGE is considered the gold standard for having a high discriminatory power, with the capacity to produce different profiles, easy to interpret and reproduce (Zadoks & Schukken 2006, Reinoso 2020). The analysis of *S. aureus* isolates using PFGE is an essential tool to establish the clonal origin of bacterial isolates, helping to establish epidemiological relationships between strains of the same species isolated from one or different herds. Knowledge of a certain genetic profile makes it possible to understand the distribution of strains with infective capacity, allowing the identification of virulence factors associated with mastitis infection and helping develop more effective treatments (Ote et al. 2011).

Table 2 compares various techniques for the genetic characterization of *S. aureus*, outlining their principles, advantages, limitations, and applications. Each technique provides unique insights into the genetic diversity and epidemiology of *S. aureus*, contributing to a comprehensive understanding of its pathogenesis and aiding in the development of effective control measures.

A significant development in DNA technology occurred in the early 1990s when Williams et al. (1990) and Welsh & McClelland (1990) simultaneously developed a strategy based on polymerase chain reaction (PCR) by using profiles of DNA fragments amplified using a given primer (Reinoso 2020).

A first advance in molecular typing was the analysis of variable tandem repeat (VNTR) sequences at different virulence *loci*. The number of repeat units at the same *locus* always varies from strain to strain and can be detected by PCR. Amplification of repeated regions of different genes, such as coagulase (*coa*), protein A (*spaA*), fibrinogen receptor (*cflA*) and collagen adhesion (*cna*), have been used for reliable and accurate typing. *spaA* gene typing is especially important for the rapid typing of MRSA isolates, as it offers higher resolution than *coa* gene typing (Shopsin et al. 1999).

spa typing is a fast and cheap method widely used for characterizing MRSA. *spa* typing emerges as a cost-effective

and accessible technique and allows its use in geographically distant laboratories, taking advantage of the various types of *spa* that prevail in various regions (Krawczyk & Kur 2018).

Although typing methods are not 100% resolving, it should be noted that bacterial strains will undergo mutation events as they propagate in different environments. So, it is important to recognize that there is no typing technique that allows definitive differentiation (an exception to whole genome sequencing) because strains could be continually mutating. However, it is of fundamental importance to establish the criteria for the use of these methods so that the results obtained are reproducible and precise.

Genetic variation of *Staphylococcus aureus*

Virulence factors of *S. aureus* strains that cause bovine mastitis are well-known, although the reasons why different genotypes with variable virulence factors can cause infection remain unclear. Molecular typing techniques have been used in epidemiological investigations worldwide, and the results have shown that disseminated identical or related clones are responsible for intramammary infections within and among cattle in specific geographical areas. Table 3 summarizes studies on the genetic variation of *S. aureus* in bovine mastitis. Marques et al. (2013), when analyzing strains using PFGE, did not detect a predominant profile. Likewise, the presence or absence of virulence genes could not be associated with the PFGE profiles, confirming the marked diversity of circulating clones in the evaluated dairy herds.

Castañeda-Vázquez et al. (2020) analyzed 335 cows from 27 stables in 10 municipalities in the state of Jalisco, observing a genetic variation of 14.9%. The analyzed strains were grouped into profiles with 95% or more genetic similarity, resulting in 12 PFGE profiles.

In a study conducted by Dieser et al. (2017) in Argentina, genetic profiles of 43 *S. aureus* strains were determined using PFGE. Chromosomal DNA digestion resulted in eight to 15 macrorestriction fragments ranging from 48.5 to 436.5 Kb. Two distinct PFGE profiles (A and B) were identified, with Profile A representing 76.7% (33/43) of the *S. aureus* strains and only one identified as subtype Aa. Profile B was found in 23.2% (10/43) of the *S. aureus* strains, and among the seven analyzed strains, four subtypes (Ba, Bb, Bc, and Bd) were distinguished. Profile A was recovered from 80% of the analyzed farms. Furthermore, the study revealed a high number of virulence profiles, indicating that strains with different virulence profiles may be capable of causing mastitis without a predominant profile being detected.

PFGE results obtained by Vaughn et al. (2020) showed the presence of 16 PFGE types throughout the farms, of which three types were the most frequently isolated. This study did not find an association between type, genotypic, and phenotypic virulence factors.

Recent advances in understanding *S. aureus* genetic variation have focused on studying clonal complexes. It has been reported that *S. aureus* clones causing bovine mastitis belong to complexes CC151, CC97, CC133, CC479, and CC771 (Zadoks et al. 2011, Schlotter et al. 2012). Hoekstra et al. (2020) showed that CC479 was strongly associated with clinical mastitis. A limited number of *S. aureus* complexes were responsible for bovine mastitis, and the complex type influences the clinical outcome of the disease. Additionally, the study identified specific genes associated with clinical mastitis.

Monistero et al. (2018) evaluated the genetic lineages of 120 *S. aureus* isolates from eight countries using RS-PCR and examined 26 virulence factors. Novel genotypes associated with South African strains were detected, while new variants of existing genotypes were identified in other countries. Specific genotypic patterns were found for each country, demonstrating a wide variety of genotypes and confirming

Table 2. Comparison of genetic characterization techniques for *Staphylococcus aureus*

Technique	Principle	Advantages	Limitations	Applications
PFGE	Separation of large DNA fragments by pulsed field gel electrophoresis	High discriminatory power, reproducible, and easy to interpret	Labor-intensive, time-consuming, and requires specialized equipment	Establishing clonal relationships, epidemiological investigations
NGS	Sequencing of entire genomes	Provides comprehensive genetic information, high resolution	Expensive, requires bioinformatics expertise	Whole genome analysis, detection of genetic mutations, antibiotic resistance profiling
Multi-omics	Integration of genomics, transcriptomics, proteomics, and metabolomics	Holistic understanding of pathogen biology, identifies functional pathways	Complex data analysis, high cost, and resource-intensive	Understanding pathogen physiology, identifying virulence factors, and developing targeted interventions
spa Typing	PCR amplification of the X region of the *spa* gene	Fast, cost-effective, good for MRSA typing, geographically adaptable	Lower resolution compared to whole genome sequencing	Rapid typing of MRSA, epidemiological tracking
MLST	Sequencing of multiple housekeeping genes	High reproducibility, good for evolutionary studies	Moderate discriminatory power, more expensive than spa typing	Phylogenetic studies, tracking evolutionary relationships
Plasmid profile analysis	Characterization of plasmid content in strains	Helps in understanding plasmid-mediated antibiotic resistance	Limited to plasmid-carrying strains, lower resolution	Studying horizontal gene transfer, antibiotic resistance mechanisms
RFLP Analysis (MLVA)	Restriction digestion followed by fragment length analysis	Moderate discriminatory power, useful for various *loci*	Time-consuming, less discriminatory than PFGE or NGS	Typing based on virulence *loci*, epidemiological studies

PFGE = pulsed field gel electrophoresis, NGS = next-generation sequencing, MLST = multiple locus sequence typing, RFLP = restriction fragment length polymorphism, MLVA = , MRSA = methicillin-resistant *Staphylococcus aureus*.

the genetic diversity associated with the geographical origin of the isolates.

Leijon et al. (2021) compared the sequence types of *S. aureus* collected from cases of bovine clinical mastitis in Sweden from 2002 to 2003 with sequence types of a set of strains isolated from 2013 to 2018 using core genome multi-*locus* sequence typing (cgMLST). The study showed that the frequent sequence types recovered from 2002 to 2003 belonged to the clonal complexes CC97, CC133, and CC151. Furthermore, these clonal complexes were also detected among the isolates from 2013 to 2018. However, there was a population change from one complex to another (CC133 to CC97). Additionally, CC151 was detected over time.

Furthermore, *S. aureus* exhibits some host-specific features, suggesting that the bacterium displays distinct characteristics or adaptations depending on the host species it infects. Genomic and epidemiological evidence indicates that *S. aureus* has undergone multiple interspecies transfers throughout its evolutionary timeline (Matuszewska et al. 2020). This implies that *S. aureus* may have evolved specific mechanisms or traits to successfully colonize and interact with different hosts. These host-specific features could include variations in virulence factors, immune evasion strategies, or even changes in genetic or phenotypic traits that enable *S. aureus* to thrive within specific host environments (Howden et al. 2023). Different studies have shown that certain clonal lineages of *S. aureus* are exclusively or more frequently associated with a certain host species (Richardson et al. 2018, Howden et al. 2023, Lima et al. 2023).

Emerging trends in genetic research on bovine mastitis

In recent years, significant progress has been made in genetic research related to bovine mastitis, providing novel perspectives and tools for comprehending and addressing this widespread ailment in dairy herds. These emerging trends contribute to a more nuanced understanding of the genetic foundations of mastitis, opening avenues for innovative disease control and prevention strategies.

Next-generation sequencing (NGS) technologies. One of the most impactful advancements is the widespread adoption of NGS technologies. These methodologies enable the rapid and cost-effective sequencing of entire bacterial genomes, allowing for a detailed exploration of *S. aureus* strains associated with bovine mastitis. NGS facilitates a comprehensive analysis of the genetic landscape, revealing subtle variations and enabling a more precise classification of strains (Wilkes 2023).

Functional genomics studies. Advancements in functional genomics have deepened our understanding of the specific genes and pathways contributing to *S. aureus* virulence in the context of bovine mastitis. Researchers now employ techniques such as CRISPR-Cas9 technology to manipulate bacterial genomes, allowing for targeted investigations into the role of individual genes in the pathogenicity of *S. aureus* (Liu et al. 2017).

Integration of multi-omics approaches. The integration of multi-omics approaches, combining genomics with transcriptomics, proteomics, and metabolomics, provides a holistic view of host-pathogen interactions during bovine mastitis. This systems biology approach offers insights into dynamic changes in gene expression, protein production, and metabolite profiles, unraveling the complex molecular mechanisms underlying the disease (Naserkheil et al. 2022).

Machine learning and bioinformatics. Bioinformatics and machine learning algorithms are increasingly vital in managing the vast datasets generated by genomic studies. These tools assist in identifying patterns, predicting virulence factors, and assessing antimicrobial resistance profiles. Integrating machine learning into genetic research enhances our ability to predict and mitigate the impact of specific *S. aureus* strains on bovine mastitis outcomes (Esener et al. 2021).

Global collaborative genomic studies. With the advent of global collaborative initiatives, researchers can now pool large datasets from diverse geographic regions. This facilitates a more comprehensive analysis of the global genetic diversity of *S. aureus* associated with bovine mastitis, considering regional variations, and allowing for the identification of commonalities and unique characteristics.

Table 3. Summary of genetic variation studies of *Staphylococcus aureus* in bovine mastitis

Study	Location	Sample size	Typing method	Major findings
Marques et al. (2013)	Fluminense South, Rio Claro, Piraí, Paracambi, Seropédica, Brazil	38 isolates	PFGE	High genetic diversity, no predominant profile, diverse virulence genes
Castañeda Vazquez et al. (2020)	Jalisco, Mexico	32 isolates	PFGE	14.9% genetic variation, 12 PFGE profiles
Dieser et al. (2017)	Argentina	43 isolates	PFGE	2 PFGE profiles (A and B), high virulence diversity
Vaughn et al. (2020)	Tennessee, USA	111 isolates	PFGE	16 PFGE types, no association with virulence factors
Hoekstra et al. (2020)	11 countries	125 isolates from CM 151 from SCM	Clonal Complex Analysis	CC479 associated with clinical mastitis
Monistero et al. (2018)	8 countries	120 isolates	RS-PCR	Country-specific genotypes, wide genetic diversity
Leijon et al. (2021)	Sweden	225 and 229 isolates collected 2002-2003, 2013-2018	cgMLST	Clonal complexes CC97, CC133, CC151 prevalent over time

CM = clinical mastitis, SCM = subclinical mastitis, PFGE = pulsed field gel electrophoresis, RS-PCR = RNA template-specific polymerase chain reaction, cgMLST = core genome multi-*locus* sequence typing.

CONCLUSIONS

Bovine mastitis remains a significant challenge for the global dairy industry. Controlling mastitis involves implementing measures to ensure overall cleanliness in cows and their housing, emphasizing proper handling procedures during milking, and addressing factors like teat skin lesions and udder disinfection. *Staphylococcus aureus*, a Gram-positive bacterium, is a prevalent mastitis pathogen with significant economic implications. It possesses various virulence factors, including protein A, coagulase, toxins, and adhesins, contributing to its ability to cause clinical, subclinical, and chronic mastitis. The emergence of methicillin-resistant *S. aureus* (MRSA) strains adds complexity to treatment protocols and raises concerns about antimicrobial resistance.

Various molecular typing techniques, such as pulsed-field electrophoresis (PFGE), have been employed to characterize the genetic profiles of *S. aureus* strains. Studies from different regions have identified multiple PFGE profiles, indicating the presence of distinct genetic clusters within *S. aureus* populations associated with bovine mastitis. Furthermore, these studies have demonstrated the presence of different virulence profiles among *S. aureus* strains causing mastitis, suggesting the existence of multiple pathogenic mechanisms involved in the development of the disease. The absence of a predominant virulence profile emphasizes the complexity of the pathogenesis and highlights the ability of different strains with diverse virulence factors to cause mastitis in cows. Moreover, geographic variations in genotypes have been observed, indicating the influence of geographical factors on the genetic diversity of *S. aureus* strains causing mastitis. Different countries and regions exhibit specific genotypic patterns, suggesting the presence of local strains and highlighting the impact of geographical origin on the genetic makeup of these strains.

Genetic research on bovine mastitis has witnessed notable advancements, with next-generation sequencing (NGS) technologies, functional genomics, multi-omics approaches, machine learning, and global collaborative studies playing crucial roles. These trends contribute to a more comprehensive understanding of the genetic basis of mastitis, paving the way for targeted interventions and improved disease control strategies in the dairy industry.

As the genetic basis of this disease's complexities continues to unravel, the dairy industry will benefit from targeted interventions, ultimately reducing economic losses and improving dairy herds' overall health.

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