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Pathogenesis, Diagnosis, Control, and Prevention of Bovine Staphylococcal Mastitis

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Abstract

Bovine mastitis is the single most costly disease usually caused by Bacteria. The genus *Staphylococcus* is major bacteria that cause mastitis in dairy cattle. Staphylococci that cause bovine mastitis are commonly divided into two major groups such as 1) *Staphylococcus aureus* and 2) non-aureus staphylococci (NAS). *Staphylococcus aureus* causes clinical and subclinical mastitis in dairy cows. Accurate diagnosis of *Staphylococcus* species can be made by Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF), 16S RNA gene sequencing, and polymerase chain reaction (PCR). In well-managed dairy farms that fully applied mastitis control measures, the incidence of *S. aureus* mastitis significantly reduced. However, staphylococcal mastitis is still major problem in most farms due to variation in management and presence of some species of non-aureus staphylococci in the environment. There is no effective vaccine that prevent staphylococcal mastitis. Treatment with antibiotics is increasingly less effective and increases development of antimicrobial resistant bacteria. Sustainable non-antibiotic staphylococcal mastitis prevention measures such as vaccines, probiotics, good herd health management and other improved methods are required. To develop an innovative control tool detailed understanding of staphylococcal virulence factors, pathogenesis, and host immunological responses is critically important. This chapter discusses the pathogenesis, host responses and current control and prevention methods.

Keywords: bovine mastitis, intramammary infection, *Staphylococcus aureus*, non-aureus staphylococci, pathogenesis, innate immunity, adaptive immunity, virulence factor

1. Introduction

A healthy mammary gland is the fundamental source of monetary gain in the dairy industry. The understanding of mastitis and developing control and prevention measures at the farm level is of paramount importance. Improved knowledge on the control and prevention of mastitis will help to improve practices that decrease the occurrence of mastitis and thereby improve diminished revenue due to production losses.

Mastitis is most frequently caused by bacteria. The genus *Staphylococcus* is among the major bacteria that cause mastitis in dairy cattle. The most common staphylococci that cause mastitis include *Staphylococcus aureus* and non-aureus staphylococci, such as *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans* and *S. xylosus* [1–3]. The name *Staphylococcus* was derived from the Greek word “staphyle” which means “a cluster of grapes.” *Staphylococcus* is Gram-positive, catalase-positive, non-motile, non-spore-forming, facultative anaerobic cocci that grow in aggregating grape-like morphological clusters. The first line of mammary gland defense is physical barriers, which prevent the entry of mammary pathogens into the mammary gland. For example, the teat sphincter closes the teat opening. It acts as the mammary gland’s first line of defense against invading infectious agents lingering in the environment (manure, milking machines, soil, or bedding).

Most commonly, bacteria enter via teat opening into the teat canal and multiply rapidly and subsequently produce toxins and other enzymes, inducing an inflammatory reaction.

Staphylococci that cause mastitis are divided into two main groups: (1) *Staphylococcus aureus* and (2) coagulase-negative *Staphylococcus* species (CNS), also frequently referred to as non-aureus *Staphylococcus* species (NAS). *Staphylococcus aureus* is typically found in the infected mammary gland and is one of the major contagious mastitis pathogens on dairies. Over one-third of all dairy cows have a staphylococcal infection due to one of these above-mentioned groups [1]. Clinically mastitis can be classified as a subclinical or clinical case. Clinical mastitis is characterized by local visible inflammatory changes in milk and udder tissue. Clinical mastitis can be acute, peracute, subacute, or chronic. Per acute mastitis is manifested by a rapid onset of severe inflammation, pain, and systemic symptoms resulting in a severely sick cow within a short time. Acute mastitis is a very rapid inflammatory response characterized by systemic clinical signs, including fever, anorexia, shock, and local inflammatory changes in the mammary gland and milk. Subacute mastitis is the most frequently seen form of clinical mastitis characterized by few local signs of mild inflammation in the udder and visible changes in milk, such as small clots.

Chronic mastitis is a long-term recurring, persistent case of mastitis that may show few symptoms of mastitis between repeated occasional flare-ups of the disease where signs are visible and can continue over several months. Chronic mastitis often leads to irreversible damage to the udder from the repeated occurrences of the inflammation, and often these cows are culled. Unlike clinical mastitis, subclinical mastitis does not manifest visible inflammatory changes in milk, such as flakes, clots, or discoloration of milk or mammary gland tissue. Diagnosis of subclinical mastitis can be made by somatic cell count (SCC) or California Mastitis Test (CMT). With the stringent application of current mastitis control measures, the incidence of staphylococcal mastitis can be reduced but not fully controlled yet. Treatment of staphylococcal mastitis with antibiotics is ineffective, and increased use of antimicrobials on dairy farms leads to the development of antimicrobial-resistant *Staphylococcus aureus* and non-aureus *Staphylococcus* species. Therefore, the development of one or more alternative non-antibiotic sustainable control measures such as an effective vaccine, phage therapy, probiotics, antimicrobial peptides, and use of CRISPR-Cas antibacterial system coupled with good dairy herd health management, use of teat sealants, and good quality nutrition (balanced ration for dairy cows), are required.

Despite strong efforts in the past several years to control staphylococcal mastitis, it still remains to be one of the major mastitis pathogens for dairy cows worldwide. The persistent staphylococcal infection of udder tissue cells over an extended time

as small colony variant (SCV) [4–6] hiding from the host immune system and antimicrobial drugs treatment might be responsible for the difficulty in curing staphylococcal mastitis.

Detailed understanding of staphylococcal virulence factors and pathogenesis of staphylococcal intramammary infections (IMI) in the dairy cow is necessary to develop an effective vaccine. In addition, the knowledge of the innate and adaptive immune responses during the early stages of host-pathogen interactions that may limit the progress of infection to mastitis is also important for the proper design of an innovative vaccine against staphylococcal mastitis. Understanding the pathogenesis of staphylococcal mastitis and its effects on the host immune system is critically important to develop effective vaccines to prevent the establishment of IMI, clinical disease, and subsequent production losses.

2. Staphylococcal virulence factors

The severity and duration of staphylococcal mastitis are partially due to the wide range of bacterial virulence factors. These virulence factors are produced at differing quantities depending on the stage of infection and host immune response [2]. *Staphylococcus* bacteria exhibit virulence factors, which include but are not limited to biofilm, surface proteins, several secreted toxins (exotoxins, membrane-impairing toxins), adaptability (mutations), and increasing resistance to antibiotics [3].

Staphylococcal virulence factors can be divided into intrinsic and acquired classes. Intrinsic factors refer to virulence factors that are an integral part of the bacterium or secreted from a bacterium, including biofilm, surface proteins, and secreted toxins. Intrinsic virulence factors may be considered as the bacteria's innate abilities. Acquired virulence factors refer to the procurement and adaptability of additional defenses, namely antibiotic resistance genes through horizontal gene transfer, discussed later in the chapter in great detail. Acquired virulence factors through genetic variation are obtained in four ways. These include (1) transformation—bacterium takes up a piece of free-floating DNA, (2) transduction—DNA is transferred from one bacterium to another through a virus/bacteriophage, (3) conjugation—DNA is exchanged between bacteria through a pilus/tube-like structure, and (4) mutation—DNA is spontaneously changed during bacterial replication.

A multitude of factors attributes to the ability of staphylococcal bacterial virulence and antibiotic resistance. This section focuses on specific virulence factors of *Staphylococcus aureus* and non-aureus staphylococci (NAS) attributable to antibiotic resistance and other defenses. Approximately 95% of *Staphylococcus* isolates from bovine mastitis is *S. aureus* and about 15% of non-aureus staphylococci have been linked to bovine mastitis, of which *S. epidermidis*, *S. xylosus*, *S. chromogenes*, *S. simulans*, and *S. haemolyticus* are predominant isolates from bovine milk [7].

2.1 Intrinsic virulence factors

2.1.1 Biofilm formation

Biofilm is an important virulence factor, creating an impenetrable layer via the structure produced. The biofilm is composed of exopolysaccharides, creating a slime-like defensive matrix. The biofilm matrix allows the bacteria to become walled off from the host immune defenses [8]. Biofilm overall promotes the attachment and colonization of staphylococci on the mammary gland epithelium and inner

mammary tissue [8]. Additionally, biofilms cannot be engulfed by individual macrophages due to their large mass, impeding the efficiency of host defense cells [9].

The initial attachment of the biofilm complex is via a capsular antigen: polysaccharide/adhesin (PS/A). Following the initial attachment is the multiplication and maturation of cell layers, resulting in the entire conglomerate biofilm. After biofilm formation is finalized, the subsequent production of polysaccharide intercellular adhesin (PIA) begins [10] which represents a factor of the staphylococcal biofilm matrix responsible for protecting against bovine innate immune defenses [11]. Along with PIA production, the bacteria are also able to detach and disperse, furthering the spread of infection in a mechanism known as metastasis [12]. Backtracking a few steps, post attachment of staphylococci to host epithelium, proteases (enzymes that break down proteins and peptides) play a role in transitioning from adhesion to invasion by cleaving host proteins [13]. These adhesion and invasion factors create a deep-seated, persistent infection that even intramammary antibiotics cannot reach.

The formation of different biofilms has also been associated with additional slime production, thought to increase bacterial adhesion and colonization. Slime is an additional extracapsular layer of the biofilm but is not found on all biofilms [14]. The biofilm/slime partnership depends on the bacterial strain. A study from Poland found that most *S. aureus* isolates (80%) producing slime could also form a strong biofilm. However, for non-aureus staphylococci, 87% with and 84.2% without slime production exhibited the ability to produce strong biofilm [15]. Strong biofilm is characterized by the ability of staphylococcal isolates to colonize and form large, distinct biofilms completely covering a stainless-steel surface under laboratory conditions [14]. Further research is needed to understand why some biofilm produces additional slime and others do not and its implications on the strength of defense mechanisms.

The production of PIA and PS/A in staphylococcal species is mediated by the intercellular adhesion operon (*ica*). The *ica* is formed by the *icaA*, *icaB*, *icaC*, and *icaD* genes in addition to a regulatory gene, *icaR*, which encodes the proteins IcaA, IcaB, IcaC and IcaD [10]. Although the role of the *icaB* gene is not fully understood, studies have shown that the coexpression of *icaA* and *icaD* facilitate the production of slime while *icaC* serves as a polysaccharide receptor [16]. The presence of these specific genes controlling the intercellular adhesion operon function also depends on the specific strain of bacteria. For example, the aforementioned study performed in Poland demonstrated the presence of the *icaA* gene was only determined in NAS (24.1%). In comparison, the *icaD* gene was found in both NAS (21.4%) and *S. aureus* (100%) [15]. Understanding the phenotypic and genotypic requirements for biofilm formation is essential in developing effective treatment against staphylococcal mastitis.

The intercellular adhesion operon (*ica*) is present in almost all *S. aureus* isolates from bovine mastitis; however, *in some cases, S. aureus biofilm formation has also been reported without this operon*. The lack of *ica* indicates other surface proteins can replace the function of PIA and PS/A synthesis. Staphylococcal pathogens have several mechanisms to generate biofilm and do not require one specific gene [10]. Furthermore, the biofilm complex matrix structure varies with staphylococcal species and within the environment where the bacterial species reside [9]. These complications are another barrier illustrating why antibiotics are ineffective in overcoming staphylococcal mastitis.

2.1.2 Surface proteins

Surface proteins increase bacterial colonization of the host tissue and inhibit the ability of phagocytes, a host immune defense, to engulf the bacteria. Therefore,

surface proteins, such as protein A can form “immunological disguises” for the invasive *S. aureus* bacteria to utilize [3].

Staphylococcal protein A (SpA) is a surface protein anchored to the cell wall of the *S. aureus*. Protein A is a virulence factor providing aid in decreasing host adaptive immune defenses via several pathways. First, SpA binds the Fc γ domains of the IgG antibody and defends the *S. aureus* from being identified and cleared out of the host. Second, SpA binds to the Fab domain of IgM, triggering cross-links between B-cell receptors. This cross-linking between B-cell receptors alters host adaptive immune responses by programming B lymphocyte death as daughter cells arise from parent cells [17]. Thus, the host adaptive immune system has been crippled using SpA.

Coagulases are polypeptides tightly bound to *S. aureus* bacterial surfaces that react with prothrombin in the blood to form staphylothrombin. The formation of staphylothrombin enables the conversion of fibrinogen, a plasma protein, to fibrin. Fibrin catalyzes blood clots, which protect the bacteria from phagocytosis and other host immune defenses [18]. Along with blood coagulation, coagulase influences fibrinogen-binding proteins’ production, facilitating further cell clumping. Coagulation and cell clumping in parallel protect the bacteria from host immune defenses due to the fibrin coat acting as a shield [19]. Coagulase is one of many adhesion proteins involved in staphylococcal virulence.

As stated previously, surface proteins also play a role in biofilm formation, especially when *ica* is absent. The adhesion proteins include SSP-1 and SSP-2 (Staphylococcal Surface Proteins 1 and 2), AtlE adhesin (autolysin E), aae adhesin (autolysin/adhesin Aae), and the fibrinogen-binding protein. Staphylococcal adhesin proteins and coagulase belong to a group of proteins called Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM). MSCRAMM also includes fibronectin-binding proteins A and B (fnbA and fnbB), fibrinogen-binding proteins also called clumping factors A and B (clfA and clfB), cell wall components (type 5 and 8 Capsules), collagen-binding proteins (can), and fibrinogen-binding proteins (fib). All MSCRAMM proteins facilitate adhesion by binding to various extracellular proteins and surfaces [15]. The MSCRAMM group of adhesion proteins enhances the binding to the intramammary epithelial cell lining to initiate a strong initialization of biofilm formation.

Biofilm-associated protein (Bap) plays a critical role in biofilm formation. A study by Shukla et al. in 2017 illustrated that the Bap gene (*bap*) was significantly more likely to be found in non-aureus staphylococci than *S. aureus*. Another study performed by Shukla et al. [12] indicated that the Bap gene (*bap*) was more likely (56.6%) to be found in *Staphylococcus* strains isolated from subclinical mastitis cases compared to clinical cases. With many different structures, functions, and mechanisms, Bap is another surface protein playing an important role in virulence.

Invasins are enzymatic proteins associated with the initial pathogenic attachment and invasion into eukaryotic host cells via outer membrane transporters. The proteins locally damage host cells to facilitate the immediate growth and spread of *S. aureus* through the help of leukocidins, kinases, and hyaluronidase. Each of these secreted bacterial enzymes function differently to diminish host immune defenses and help further spread the bacteria.

Leukocidins destroy leukocytes by breaking down the cell membrane and cytoplasmic granules, which contain enzymes used to combat foreign pathogens.

Kinases break down fibrin and clots formed by the host to prevent an isolated infection through phosphorylation.

Hyaluronidase aids in bacterial spread by hydrolyzing hyaluronic acid, a polysaccharide present in connective tissue [3].

After invading the host cells through the outer membrane transporters, the outer membrane of invasins releases the aforementioned bacterial enzymes, toxins, and proteases to damage local cells. Invasins also utilize an adhesion mechanism to remain on the surface of host cells [20]. The cell damage usually only occurs in and around the site of bacterial growth and may not lead to mass cell death, unlike other virulence factors. Generally, invasins tend to be broad in function compared to other virulent proteins like exotoxins. However, some invasins, such as staphylococcal leukocidins, have a relatively specific cytopathic effect [3]. Surface proteins are important virulence factors for evading host immune systems and sustain habitation on epithelial surfaces.

2.1.3 Exotoxins

Exotoxins are another group of enzymatic proteins characterized by the characteristic manner of inducing harm to host cells. Type I exotoxins signal host cell membranes, type II damage host cell membranes, and type III enter host cells and directly alter the cell [21]. All these exotoxin types are secreted by virulent bacteria, with the secreted toxin portrayed as a major determinant of virulence. As stated previously, exotoxins tend to be more specific in function in comparison to invasins. However, some may still play a role in initial cell invasion, initiating damage in many ways [3]. *S. aureus* exotoxins typically damage host tissue by entering the host cells and catalyzing covalent modifications [22]. The harmful covalent modification caused by a type III exotoxin occurs in several ways. Almost all *S. aureus* strains associated with bovine mastitis secrete exotoxins in the form of enzymes and cytotoxins encompassing: hemolysins, nucleases, proteases, lipases, hyaluronidase, and collagenase, to name a few. These enzymes and cytotoxins convert local host tissue into nutrients required for bacterial growth [23]. The exotoxins that cause damage and create the sustainable existence of pathogenic bacteria in the host are important for establishing a proliferating colony.

As previously mentioned, leukocidins, a type of pore-forming cytotoxin, target and destroy essential bovine immune cells. The target immune cells, leukocytes, are also called polymorphonuclear (PMN) cells, consisting of neutrophils, basophils, and eosinophils. Different leukocidin forms have also been revealed in bovine mastitis, such as lukS/lukF (γ -hemolysin), lukD/lukE, and especially lukM/lukF-PV(P83) [13]. By disarming the host immune defenses, these leukocidins contribute to rapid colonization of the intramammary tissue [10].

Enterotoxins Some strains of *S. aureus* also secrete another type of exotoxin known as staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH, and SEI) [24]. They are characterized as type I toxins because the group of proteins does not directly enter cells. Instead, these toxins bind to extracellular surface receptors and trigger a cascade of specific responses inside the cell. These enterotoxins act as superantigens which are potent immune system stimulators by stimulating T-cells and a large aggregation of pro-inflammatory cytokines [13]. Mammary tissue damage caused by excessive proinflammatory cytokine release is commonly seen in clinical mastitis cases (Table 1).

Membrane-impairing toxins are a group of toxins that specifically lyse eukaryotic cell membranes and interfere with immune system components. Toxins start causing tissue damage after the penetration and multiplication of *S. aureus* at the site of infection, the teat canal. These toxins also include some hemolysins, leukotoxins, and leukocidins and can be considered a subdivision of type II exotoxins. They are also referred to as pore-forming toxins. The insertion of a transmembrane pore causes the breakdown of the cell membrane of a host cell. The pore in the outer membrane results in the disruption of selective ion transfer across the membrane, leading to the deterioration of the membrane itself [3].

Level	Prevalence	Source
Quarter	1.6–53.3%	[25–27]
Cow	2.9–41.9%	[26, 28–31]
Bulk tank/herd/farm	7.4–54.1%	[32–35]

Table 1.
Staphylococcal enterotoxins (contained one or more enterotoxin genes) prevalence at udder quarter, cow, and farm levels.

Staphylococcal α and β toxins also referred to as α and β hemolysins, are a type of membrane-impairing toxins expressing varying effects based on concentration levels. They can inhibit leukocyte chemotaxis, which decreases the inflammatory response at lower concentrations and can cause necrosis and tissue damage at higher concentrations. Therefore, it can be concluded that subclinical cases may be associated with lower concentrations, and clinical cases may be associated with higher concentrations of α and β toxins. A study from Brazil found that β toxins were present in 69% of hemolytic isolates, suggesting that beta-toxin may contribute to the virulence and pathogenesis of mastitis [8]. In addition, the α - and β -toxins aid in bacterial invasion and escape from the immune response by increasing the attachment of toxins to bovine mammary epithelial cells and the expansion of the *S. aureus* infection [8]. The amplification of infection leads to the persistence of staphylococcal bacterial growth and colony formation in the mammary gland, developing into chronic infections [10].

2.2 Acquired virulence factors

2.2.1 Antimicrobial resistance

Antimicrobial resistance is a continuously emerging challenge when treating staphylococcal mastitis. Antibiotic resistance results from both innate and acquired virulence factors leading to the evolution of staphylococcal bacteria. Antibiotic resistance itself can also be divided into intrinsic and acquired resistance. One of the most potent intrinsic antibiotic resistance is biofilm formation. As mentioned previously, the ability to form biofilm in all strains induces a deep-seated infection resistant to antibiotics. There are several reasons for this association as outlined by Raza et al. [9], which include (1) the exopolysaccharide make-up, preventing the initial physical antibiotic penetration, absorption, and enhance binding to the antibiotics themselves due to their negative charge; (2) the deeply embedded bacteria in the biofilm are not fast-growing and are smaller in size resulting in increased difficulty for the antibiotics to target these hidden pathogens; (3) biofilm contains enzymes that inactivate the antibiotics that have successfully reached the surface and (4) bacteria residing in the biofilm exhibit cell membranes more likely to block antibiotic molecules. The increased blockage of antibiotic molecules occurs considering most antibiotics are inactivated by reactive oxidants like hypochlorite and H_2O_2 present around biofilm. These reactive agents are released when phagocytes generate a respiratory burst, often caused by the aforementioned surface proteins. Overall, biofilm provides an ideal environment for antibiotic resistance through its specialized structure and function [9].

Antibiotic resistance in all staphylococcal mastitis strains is related to the pathogenic genotype and expression of genes. One of the most important adaptive mechanisms in the acquisition of antibiotic resistance is horizontal gene transfer. The diversity of staphylococcal mastitis strains and their developing virulence, resistance, and transmission is partially due to the exchange of genetic material via

transformation, transduction, conjugation, and mutation, all of which have been previously defined. Horizontal gene transfer is also known as lateral gene transfer, an adaptation allowing *S. aureus* and non-aureus staphylococci strains to transfer DNA to different genomes.

The development of bacterial strains with increased resilience can be anticipated due to horizontal gene transfer of the aforementioned genes responsible for potent biofilm. Mobile genetic elements (MGE) further capture, accumulate, and spread these emerging virulence and resistance genes to more strains resulting in broad antibiotic resistance. The specific resistance genes are difficult to target as a result of their involvement in different stages of mastitis development and infection. Additionally, the presence of certain resistance genes, such as the superantigen genes, varies by region due to differences in strains, management, and antibiotic use. Studies also showed that different combinations of genes most likely influence the ability of a strain to induce a persistent infection [36].

Several studies reported a worldwide increase in resistance to β -lactam antibiotics in both *S. aureus* and non-aureus staphylococci. β -lactam antibiotics include, but are not limited to, penicillin, ampicillin, oxacillin, and methicillin [37]. Besides β -lactam antibiotics, there are several other classes of antibiotics *Staphylococcus* species have developed resistance to as highlighted by Pérez et al. [10]. For *S. aureus* strains, a study has shown that cows infected with more common *S. aureus* genotypes experience shorter durations of inflammation compared to cows infected with less common genotypes. This suggests that less common genotypes contain genes enabling more virulence factors and longer-lasting infections. In addition, these less common *S. aureus* strains are more likely to possess certain genes such as the pyrogenic toxin superantigens (PTSAg), enterotoxin-encoding genes (*sed* and *sej*), and a penicillin resistance gene (*blaZ*) [38].

Although resistance in both *S. aureus* and non-aureus staphylococci are known, overall, non-aureus staphylococcus species are more resistant to antibiotics than *S. aureus* strains. A study in Korea analyzing over 300 non-aureus staphylococci isolates found that *S. chromogenes* was the predominant species, but *S. epidermidis* was concluded to have the highest antibiotic resistance. This may be due to the specificity of the *S. chromogenes* pathogen and its tendency to cause chronic infections. Moreover, *S. epidermidis* persistently produced biofilms and carried the *mecA* gene, responsible for methicillin resistance. However, in the same study, the *mecA* gene was not found in most other methicillin resistance non-aureus staphylococci isolates, suggesting that other genes or factors also influence antibiotic resistance. Methicillin-resistant staphylococci (including MRSA) are especially important, being found to transmit from animal to human, for example, the *S. epidermidis* isolates with identical genotypes found in milk samples and samples from milkers' skin in Denmark (Table 2) [37].

2.3 Conclusion

To develop an effective vaccine, (a) understanding of virulence and pathogenesis of staphylococcal intramammary infections (IMI) in the dairy cow and (b) the knowledge of the innate and adaptive immune system during early stages of host-pathogen interactions potentially limiting the progress of infection to mastitis are required. Considerable advances have been made in molecular microbiology and bacteriology research as more knowledge is accumulated about the ability of biofilm to create an impenetrable infection, the mechanisms in which embedded surface proteins and secreted toxins damage host immune defenses, and the process employed by resistance genes transfer among bacterial strains.

Level	Prevalence	Source
Quarter	0–3.89%	[39, 40]
Cow	0.3–6.3%	[29, 39, 41]
Bulk tank/herd/farm	3.8–4.4%	[41–46]

Table 2.
Methicillin-resistant Staphylococcus aureus (MRSA) prevalence at udder quarter, cow, and farm levels.

Staphylococcal virulence factors that are an integral part of bacterial cell surface are good targets for vaccine development because these virulence factors for vaccine development need to be exposed to the host immune system. The induced antibody must have access to the epitopes that induced its production to target the bacterium for antibody-mediated killing. Therefore, it is critically important to target bacterial cell surface virulence factors or proteins expressed during the early stages of staphylococcal-host interactions for vaccine development to effectively control mammary gland colonization by staphylococci.

3. Host immune response

As previously mentioned, staphylococcal mastitis is a very prevalent and economically devastating disease in the dairy industry. There are several members of NAS, with the major isolates from bovine IMI including *S. chromogenes*, *S. haemolyticus*, *S. epidermidis*, *S. simulans*, *S. hyicus*, and few less prevalent species [47]. The virulence factors of staphylococci and their effect on mammary tissue have been well studied over the past 50 years, although pathogenic mechanisms of some species during IMI have yet to be thoroughly examined and defined. Understanding the pathogenesis of staphylococcal mastitis and its effects on the host immune system is critically important to develop effective vaccines to prevent the establishment of IMI, clinical disease, and subsequent production losses. The genus *Staphylococcus* includes numerous species and strains within species (serovars) capable of causing mastitis and halting treatment efforts in dairy operations. Different species and strains can be classified based on various genetic and phenotypic characteristics [48]. Strains such as the small colony variants (SCV) of *S. aureus* have adapted to form better biofilms and succeed at internalization into host cells, resulting in the protection of the SCV strain from the host adaptive immune responses [48]. *S. aureus* and non-aureus staphylococci cause bovine mastitis through various pathogenic mechanisms, including evading host immune responses.

3.1 The immune system

A dairy cow’s immune response against staphylococcal IMI includes innate and adaptive immunity. While both are extremely vital in preventing, expelling, and protecting against foreign antigens, the two branches have unique mechanisms. Innate immunity has a specialized ability to quickly identify microorganisms and provide a rapid defense to halt initial IMI before it develops into mastitis. The adaptive immune response can specifically identify and memorize the antigen to prevent future severe infection. Understanding how dairy cattle’ innate and adaptive immune responses work together is valuable to develop effective vaccines or immunotherapy to increase overall resistance to invading pathogens.

3.1.1 Innate immunity

Innate immunity is a non-specific immune response that utilizes a cascade of cells and cytokines powered by molecules, such as chemoattractants, to target and destroy invading pathogens. The first line of physical defense is skin and mucous membranes. Once the first line of protection has been crossed, the innate immune response, or second line of defense, will be induced. The most common innate immune responses are phagocytic cells (neutrophils and macrophages), inflammation, and complement system activation.

Once the *S. aureus* or NAS induces tissue damage and provokes the secretions of chemoattractants, an inflammatory response is activated. During an inflammatory response, chemoattractants are released in response to invading antigens and attract the recruited groups of neutrophils to the site of infection. Neutrophils are phagocytic white blood cells patrolling the bloodstream for a signal that activates and recruits them to the site of inflammation in the body. Staphylococcal lipoteichoic acid (LTA), or the macroamphiphilic molecule of the Gram-positive bacteria, can induce a stronger secretion of chemoattractants similar to lipopolysaccharide (LPS) of Gram-negative bacteria [49]. Once the response is activated, the chemoattractants attract polymorphonuclear cells (PMNs) and monocytes to the site of infection and subsequent inflammation. The neutrophils recognize when to halt their migration by interpreting the chemical gradient levels as they travel through the bloodstream. Once a high level of chemoattractants is met, the cells slow down their movement through blood circulation by binding to their endothelial ligands at the target area of inflammation.

Additionally, other cells such as lymphocytes residing in the tissues which are produced in the bone marrow are recruited to the site of inflammation following neutrophils to promote phagocytosis. If phagocytosis of the *S. aureus* by phagocytic cells (neutrophils or macrophages) failed to destroy bacteria. In that case, the innate immune system uses the natural killer cells (NK) to kill phagocytic cells and release the bacteria from them to allow for the second round of phagocytosis [50]. Murphy et al. [51] found differences among the *S. aureus* strains and their ability to survive killings by neutrophils. If bacteria survive past the first line of the innate immune response, the second line of innate defense will be induced against the invading *S. aureus* depending on the strain. The phagocytic cells play a larger role in achieving bacteria cell death in the second line of defense.

Murphy et al. [51] found that secretion of cytokines and chemokines by the innate immune system significantly differed with the strain of *Staphylococcus*. Murphy et al. [51] measured different quantities of IL-6 and IL-8 chemoattractants in response to different strains. They found that IL-8 is a key chemokine in neutrophil recruitment, and the release of IL-8 by the innate immune system is crucial in the neutrophils' ability to respond rapidly [51].

It was shown that while all strains resulted in IL-6 and IL-8, the *S. aureus* strain known as CC97 produced significantly more IL-6 than the other strains, especially when compared to the strain CC151. This study also set out to determine if this difference in chemoattractant production could potentially positively affect the migratory response of the PMNs and monocytes to the site of inflammation. Murphy et al. [51] showed a significantly greater chemotaxis response in the systems that were exposed to those strains that also produced significantly greater IL-6 and IL-8 chemoattractants. Response to invading pathogens is important, considering any delay in response can cause the innate immune system to fail, so the phagocytosis step of rapid response is important in providing support.

As previously mentioned, there are coagulase-positive *S. aureus* and non-aureus staphylococci, also called CNS. The CNSs are increasingly becoming more common causes of IMI [52]. The most common *Staphylococcus* isolates from cases of mastitis

in dairy cattle include *Staphylococcus aureus* and CNS (*S. chromogenes*, *S. haemolyticus*, *S. epidermidis*, *S. simulans*, and *S. hyicus*) [47]. For example, *S. haemolyticus* can induce macrophage apoptosis by utilizing cytotoxins [53]. Apoptosis of the macrophages makes the innate immune system vulnerable to CNS invasion and dissemination from the site of infection.

3.1.2 Adaptive immunity

The second and possibly the most important branch of immunity is adaptive or acquired immunity. The humoral line of immunity is commonly associated with the antibody-mediated response. The adaptive mechanism requires an invading antigen phagocytosed by antigen-presenting cells, broken down into small peptides, and loaded on major histocompatibility molecule II (MHC-II). The broken-down peptides are then transported to the cell surface and presented to naïve T cells patrolling the body. The naïve T cells recognize a foreign peptide bond to MHC-II through its T cell receptor (TCR) and become activated T helper cells: Th1, Th2, Th17, Tfh, Treg, or cytotoxic T cells. Depending on which T helper cell is induced by the antigen, an effector immune response (antibodies or activated killer cells) will be delivered specifically to the invading antigen. Adaptive immunity is often stronger than innate immunity; however, there is a much longer delay in response before the invading antigen is specifically targeted and removed from the body. Without adaptive immunity or lack of adaptive immune response within the animal immune system hindering the growth and proliferation of antigens, the same pathogen previously seen by the immune system would constantly be responsible for the eradication of entire animals.

Just like the innate immune system uses T-cells and B-cells to eradicate invading pathogens, the adaptive immune system also utilizes natural killer cells to fight off infection. For the body to exploit the adaptive immune system, it must have already been previously exposed to the pathogen. The adaptive immune system's ability to activate natural killer (NK) cells and utilize them in a way that allows them to recall antigen-specific memories of particular pathogens. Familiar recognition of pathogens responsible for infecting the body in the past is one of the key defense mechanisms, including NK cells, to get rid of infecting pathogens. Only after the induction of the innate immune system through phagocytic cells with the proper presentation of bacterial antigens by antigen-presenting cells, the adaptive immune response would be triggered against the invading *S. aureus* and non-aureus staphylococci [54]. Since induction and activation of adaptive immunity require antigens uptake and presentation by antigen-presenting cells and the requirement of prior memory of exposure to mount a quick and robust response for exposure to a new pathogen, the adaptive immune response takes 5–7 days to be effective.

Once the *Staphylococcal* bacterium enters the body, it replicates and starts releasing antigens, such as staphylococcal superantigen analogous to SSL5 and SSL10. First, the neutrophils, specifically, are recruited by the innate immune system [55]. Although these staphylococcal superantigens can work as a protective measure for the *S. aureus* bacteria, they also will serve as a source of alarm to B and T cells. The antigens from invading bacteria will attach to the cell's outer layer, allowing the natural killer (NK) cells to recognize and terminate the invading bacterium. To protect the body from future infections by the same pathogen, the adaptive immune system generates a percentage of its helper T-cells to serve as memory T cells [53]. If the pathogen is in the intracellular area of infected cells, the adaptive immune response will induce the helper T-cells to become cytotoxic T cells meant to identify and kill infected cells. If antigens or pathogens are in the extracellular area, the helper T cells activate B-cells to secrete antibodies specific to those particular

antigens. After the B-cells secrete antibodies or immunoglobulins, the antibodies will bind to the foreign antigens that induced their production. Furthermore, the immunoglobulins bind to the foreign antigens that induced their initial production and block their ability to bind to receptors on host cells [56]. Blocking the foreign antigens or bacteria can reduce numbers and stop bacterial cell communication and replication [55].

The two main branches of the dairy cow immune system, adaptive and innate, are essential in protecting against infectious agents. The innate immune system rapidly responds with an effector mechanism composed of neutrophils' rapid recruitment to the site of infection and inflammation. The adaptive immune system works as the second line of defense, and while often delayed, can recruit lymphocytes to respond to foreign antigens specifically. Understanding the collaboration of the dairy cow's innate and adaptive immunity is valuable in preventing infections by enhancing adaptive immunity with effective vaccines.

3.2 Pathogenesis of staphylococcal IMI

The dairy cow is exposed to several pathogens daily; the role of innate and adaptive immunity is to remove these pathogens before the infection is established and progresses to disease or further to persistent or chronic infection. Both *S. aureus* and non-aureus staphylococci infections are difficult to clear in dairy cows due to the harbored resistance to the animal's defenses and antibiotic treatment.

3.2.1 Entry, adhesion, and evasion

Almost all mammary pathogens enter the udder via teat opening (orifice), except in rare cases where the udder gets infected secondarily via systemic infection (e.g., *Mycoplasma bovis* mastitis). The teat orifice is closed by a layer of the teat sphincter muscle, also known as the Rosette of Furstenberg, located directly above the streak canal. It is made up of loose folds of a membrane that smooth out as milk accumulates in the udder. It aids in preventing milk leakage between milkings and serves as a physical barrier for entry of mastitis pathogens through the teat orifice. Within the streak canal, keratin is produced by the teat duct epithelium to serve as a physical barrier against the entrance of mastitis pathogens. Keratin also has an antibacterial effect consequential of different bacteriostatic fatty acids such as myristic, lauric, palmitoleic, and linoleic acids. Fibrous proteins also make up keratin specifically implied to bind and destroy the cell wall of the pathogens. Damage to keratin by incorrect intramammary infusion or by faulty milking machine systems increases the potential for teat canal colonization by bacteria [8, 22, 23]. Upon entry through the teat opening, *S. aureus* adheres to the host's cells and invades into surrounding tissues to overcome being flushed out by the milk [48]. *Staphylococcus aureus* has several virulence factors, including surface proteins, fibronectin-binding proteins (FnBP), fibrinogen binding proteins (FgBP), collagen-binding proteins (cna), clumping factors, and biofilm, all of which will allow it to adhere to these epithelial cells and evade the immune system [48].

Staphylococcus aureus uses the staphylococcal Fn-binding proteins, FnBPs, on the bacteria to connect to the fibronectin α -5B1 integrin on the host cellular surface [57]. This FnBP expression is extremely important for cellular adhesion but may vary across *Staphylococcus* strains. For example, methicillin-resistant *S. aureus* strains require an additional FnBR 11 to promote cell invasion [57]. After several cellular interactions between FnBPs, Fn, and α -5B1 integrins have taken place between the bacteria and host cell, the *S. aureus* will deploy the actin cytoskeleton and enter the cell's fluid membrane [57]. The coagulase-negative staphylococci can also utilize adhesion capabilities, such as laminin-binding proteins, to adhere to host cells and tissues [58].

After adhering to the desired host cell, the *S. aureus* and non-aureus staphylococci, or CNS, secrete several exotoxins and enzymes that will assist in invading, penetrating, and destroying cells and tissues [48]. For instance, the exotoxin hemolysin and exoenzymes are responsible for breaking down the epithelial tissue in the ducts and alveoli of the mammary glands, contributing to milk production losses during mastitis in dairy in cattle [48].

As mentioned prior, the phagocytic cells of the innate immune system will engulf and destroy *S. aureus* and non-aureus staphylococci. However, *S. aureus* and CNS have several evasion strategies. Two evasion proteins utilized by many strains of staphylococci are the extracellular fibrinogen-binding proteins (Efb) and the leukotoxin subunit (LukM) [59]. The Efb protein can mask the surface of the *S. aureus* with a capsule-like shield to avoid binding of antibodies to *S. aureus* and subsequent removal by phagocytic neutrophils. The LukM binding subunit is capable of eliminating the leukocytes by interacting with its target receptor on the surface of the neutrophils [59]. *Staphylococcus epidermidis* employs its exopolysaccharide PIA to form a biofilm that hinders phagocytosis by phagocytic cells of the innate immune system [60]. This biofilm formation will protect and preserve the bacterium until conditions are more ideal for the bacterium to thrive.

In conclusion, the dairy cow's immune system plays a major role in protecting the animal from prevalent pathogens, such as persistent agents causing staphylococcal infections. The pathogenesis of staphylococcal infections depends on several virulence factors that allow them to overcome the host's immune system. Understanding the detailed pathogenesis of staphylococcal IMI and the host's innate and adaptive immune responses against IMI is the key to improving mastitis control by vaccine or immunotherapy.

4. Clinical manifestation

Mastitis causes physical, chemical, and microbial changes in the milk due to pathological alterations in the mammary gland tissue [61]. The most common or cardinal signs of mastitis or signs of inflammation of the udder are redness, heat, pain, swelling, and altered or reduced milk production of the mammary glands. Staphylococci follow the same pattern of infection, and the consequential inflammatory signs are displayed locally in milk and udder tissue or systemically in the infected animal. Depending on the symptoms as well as the duration of infection caused by the infecting bacteria, mastitis can be classified as clinical or subclinical.

4.1 Clinical mastitis

The incidence of clinical mastitis (CM) is estimated to range between 16 and 48% of cases and the prevalence of subclinical mastitis (SCM) is reported to be 20–80% globally [62]. Clinical mastitis can be detected on the farm based on the physical clinical symptoms expressed in either the cow's milk or udder. If clinical mastitis progresses beyond local inflammation of the mammary gland to systemic involvement, as in the case of acute or peracute mastitis, infected animals will express systemic signs. The secondary systemic signs may include increased body temperature, elevated pulse, and respiratory rates, loss of appetite, and dehydration [63].

4.2 Subclinical mastitis

In comparison, subclinical mastitis does not present physical symptoms as seen in clinical cases. In most herds, subclinical mastitis incidence is 15–40 times higher

than clinical mastitis [64]. *Staphylococcus aureus* is a major cause of bovine mastitis; however, it is mainly associated with subclinical infection. Mastitis caused by *S. aureus* is extremely persistent and reoccurs easily, becoming resistant to conventional antimicrobial treatments through selective pressure. Therefore, the only way to avoid the risk of transmission to the entire herd is to remove or cull the infected cows [65]. The diagnosis of subclinical mastitis is challenging due to the absence of visible manifestations. Subclinical staphylococcal mastitis does not result in physical changes in the milk and/or udder. The milk will appear normal without clots or flakes; however, subclinical mastitis decreases milk quality and quantity.

5. Diagnosis of mastitis

The methods of detecting causative agents of bovine mastitis have been intensively developed and improved over the years. The traditional gold standard methods are somatic cell count (SCC) and milk bacteriological culture, which are still predominantly used worldwide today. For subclinical mastitis, on-farm screening tests are used, such as the California mastitis test (CMT) [66]. The CMT test is conducted by mixing the test reagent (CMT reagent) with an equal volume of milk [67]. The reagent breaks the cell membranes and releases DNA from the nuclei of the somatic cells in the milk, forming a gel. The reaction is then visually scored as 0, Trace, 1, 2, or 3, depending on the gel that forms. The formation of more viscous gel indicates the presence of a higher somatic cell count [67]. Thus, the CMT is an ideal test for farmers to have on hand to quickly, easily, and accurately identify questionable cases of mastitis, or narrow down specific quarters of cows, causing an increase in the composite SCC. While these methods are quick and on-farm accessible, they require skilled personnel, and false positive or negative results are still possible.

The most efficient approach to detect clinical mastitis is during the pre-milking stripping process, also known as the “Strip Cup Test”, which allows milk screening for abnormalities. The strip cup test is the method commonly used for mastitis detection on the farm. In this practice, the milker visually examines the foremilk for clinical signs of mastitis mentioned above, such as blood, flakes, clots, or watery milk (change in color) [68]. Similarly, udder tissue can be examined for visible abnormalities, namely swelling, redness, and pain. Additional factors to consider are a significant reduction in individual milk quality and milk yield [64].

Somatic cell count is the most common way to detect changes in milk composition and quality. SCC is widely used, and a reliable indicator of udder health. Crucial monitoring of milk somatic cell count in a herd may allow dairy farm herds-men to track and identify the sources of disease. Somatic cells are mainly white blood cells, including granulocytes (neutrophils, eosinophils, and basophils) and monocytes, macrophages, and lymphocytes. A small fraction of milk-producing epithelial cells are also included in the somatic cells count [69]. Since leukocytes in the udder increase as the number of infecting pathogens increases, SCC indicates the degree of mastitis in an individual cow or the herd, depending on the test being conducted [70–74]. Higher numbers of somatic cells are detected due to the mammary epithelial cells initiating defense mechanisms against invading pathogens.

Infection with *Staphylococcus aureus* or CNS induces leukocytes and epithelial cells to produce chemoattractants, cytokines, and acute-phase proteins that attract neutrophils to the site of the infection [69]. Neutrophils engulf, phagocytize, and destroy the pathogen via oxygen and protease-dependent mechanisms, which result in the release of enzymes, such as N-acetyl- β -D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH). N-acetyl- β -D-glucosaminidase (NAGase) is an enzyme released into milk during inflammation and acts as an early mastitis

indicator. Lactate dehydrogenase (LDH) is a widely distributed enzyme in cells of various living systems for carbohydrate metabolism. LDH found in dairy milk originates from somatic cells, leukocytes, and other invading microorganisms. As a result, milk production decreases, milk pH changes, and conductivity increases [69].

5.1 Mastitis detection at individual cow level

Individual cow composite milk SCC above 200,000 cells/mL of milk is considered an indication of subclinical mastitis. The legal limit for milk SCC in the USA is 750,000 cells/mL for bulk tank milk. However, milk premium decreases as SCC increases and milk quality parallelly decreases [63]. Dairy producers receive higher premiums, or higher prices, for their milk with SCC < 250,000 cells/mL of milk, along with minimal cases of mastitis and minimal use of antibiotics on their farm.

Dairy farmers and dairy associations frequently use SCC to determine and monitor milk quality [63]. The most common method for monitoring mastitis in the dairy herd is the SCC of bulk tank milk samples at Dairy Herd Improvement (DHI) labs. The DHI organization is known to have a service many farmers take advantage in which monthly composite samples are taken from all the individual cows in the milking herd to test for SCC. The results are returned on time, allowing the farmers to take swift action against high SCC cows or those with subclinical cases of mastitis.

On-farm bacteriological culture may also help a producer decide to utilize a specific antibiotic or not to treat a cow at all [75–77]. Cultures that show no bacterial growth usually require no treatment because these cows are self-cured or cleared off infection due to the immune system has already cleared the bacterial infection. On-farm culture is designed for quick mastitis treatment decisions [75–77]. Producers can identify the difference between Gram-negative bacterial pathogens that are usually cleared or unresponsive to treatment. Most Gram-positive bacterial pathogens respond effectively to antibiotic treatment, although some are not susceptible to antibiotics.

5.2 Mastitis detection at farm level

Bulk tank samples are also vital in the continuance of quality milk and low somatic cell count monitoring in a herd. The Wisconsin Mastitis Test (WMT) is a well-known lab testing method that is a rapid screening test for mastitis-causing bacteria in bulk milk samples. The test is based on an increase in leukocytes that is followed by an increase in viscosity when the detergent reagent is mixed with the milk sample. In both tests (WMT and CMT), the same reagent is used, a 3% sodium lauryl sulfate solution. In a CMT, the resultant reaction is qualitatively estimated, while in WMT the test result reaction is measured quantitatively (mm) [78–80]. These tests provide practical and inexpensive methods to detect subclinical mastitis in the dairy herd.

Another test used to determine mastitis infection is the pH levels in the milk. The amount of sodium and chloride ions increases in mastitic milk due to the damaged epithelial cells and weakened milk-blood barrier [63]. The potassium levels decrease, with all these changes leading to a fluctuation in electroconductivity (EC) of milk and subsequently increased pH of milk. These parameters are widely used to identify mastitis and infection rates in the herd [63]. The electrical conductivity of milk can be determined by using a handheld (portable) electrical conductivity meter (milk checker or digital mastitis detector). The measurement of EC of milk is expressed in the unit of milk siemens/cm [78–80]. While the EC method is not very common, it is low-cost and provides easily recordable information in dairy herds with automatic

milking systems. The EC sensors are becoming increasingly used as automatic milking systems are more widely adopted into previously traditional parlor herds.

The high frequency of false negatives by culture-based methods encouraged the development of molecular diagnostic tests. These include polymerase chain reaction (PCR) and MALDI-TOF with high test sensitivity, specificity, and detection of growth-inhibited and non-viable bacteria [63]. The only aspect the MALDI-TOF MS lacks is the catalog of pathogens not as commonly seen or causing severe disease. With time and use, the inventory will grow, and thus with it, the sensitivity to detect specific mastitis-causing pathogens.

In conclusion, early detection of mastitis enables to limit the spread of infection within a herd. Infected cows that are not detected or do not receive correct treatments may potentially develop chronic, long-term infections that lower production and spread infections further throughout the herd. It only takes a few infected animals to lower milk quality by increasing the bulk milk SCC. Diagnosis tools such as somatic cell count, CMT, and others are crucial to a farm’s mastitis control program (Tables 3 and 4).

Level	Prevalence	Source
Quarter	5.7–46.2%	[44, 45, 81, 82]
Cow	4–41.7%	[26, 29, 82–84]
Bulk tank/herd/farm	47.2–97.6%	[41, 42, 46, 85]

Table 3.
Staphylococcus aureus mastitis prevalence at udder quarter, cow, and farm levels.

Level	Prevalence	Source
Quarter	8.4–31%	[37, 81, 86–88]
Cow	7.4–34.4%	[37, 83, 87, 89]
Bulk tank/herd/farm	16–56.8%	[81, 90–92]

Table 4.
CNS prevalence at udder quarter, cow, and farm levels.

6. Staphylococcal mastitis control and prevention

Typically, staphylococcal mastitis has been treated in the past by antibiotics via intramammary infusion and parenteral injection. However, the efficacy of antibiotics has become increasingly limited. Staphylococcal mastitis can spread easily via milkers’ hands, pre- and post-dips, flies, or other vectors and fomites with the potential to come into direct contact with the teat end. Due to the lack of treatment, any spreading of the infection from cow to cow will ultimately be detrimental to the milk production of all affected animals (Tables 3 and 4).

6.1 Treatment

6.1.1 Therapeutic antibiotics

While there are presently no successful antibiotics available offering to mitigate the effects of staphylococcal mastitis, others have been proven slightly beneficial compared to the alternative absence of treatment. However, the cost may not

outweigh the benefit of regained milk production if the animal remains infected with the staphylococcal pathogen. For example, one currently marketable drug for prophylactic use as an antibiotic mastitis treatment in staphylococcal mastitis is mupirocin. This topical antimicrobial is particularly effective in patients that are known carriers of *Staphylococcus aureus*; however, the treatment is not feasible in a dairy environment. Systemic antibiotics, such as ampicillin, have been deemed less effective than mupirocin and cannot clear the animal of the staphylococcal pathogen.

Once clinical mastitis has been diagnosed, most times farmers do not speciate using a diagnostic test due to increased cost. Instead, most herdsmen choose to use a common treatment, such as Spectramast or Pirsue. Spectramast, ceftiofur hydrochloride, offers a much broader spectrum of treatment for clinical and subclinical mastitis cases. However, even with extended eight-day treatment, there is only mitigation of the severity of the infection and no clearance of staphylococcal bacteria responsible for the infection. A high rate of recurrent infection is also seen in staphylococcal infected quarters and the pathogen is often spread to other quarters and more importantly other cows sharing the environment. When evaluating the effectiveness of ceftiofur as an antibiotic, no significant difference has been found between animals treated for clinical or subclinical mastitis in regards to visual severity or SCC [93–95]. However, extended treatments are effective in only reducing elevated SCC in both clinical and subclinical infections in some studies [93–95]. Pirsue (pirlimycin hydrochloride), is a far more targeted antibiotic specifically developed for species of streptococcus and marketed to reduce the severity of staphylococcal mastitis cases but is not effective in clearing the animal of the pathogen leading to recurring infection and a chance of spread. At most, a 50% *S. aureus* cure rate is achieved by Pirsue treatments measured by lowering SCC and bacterial numbers [96]. Further research is needed to develop a more sustainable antimicrobial for the treatment of staphylococcal mastitis. The efficacious antibiotic must also parallel economic and applicable practicality to be deemed a viable option for the treatment of staphylococcal clinical and subclinical mastitis.

6.1.2 Prophylactic antibiotics

Prophylactic use of antibiotics is defined as the use of prescribed antibiotics before the onset of the infection. This is also commonly called dry cow therapy, which is the long-acting intramammary antibiotic treatment of cows at the end of their lactation period, directly after their last milking. The dry period typically ranges from 50 to 70 days. The infusion of dry cow antibiotic may follow by an intramammary infusion of teat sealant. There are two types of dry cow therapy: blanket treatment (BDT) and selective treatment (SDT). Blanket dry cow therapy (BDT) is the intramammary antibiotic treatment of all dry cows in all actively milking quarters, whereas selective dry cow therapy (SDT) is the intramammary antibiotic infusion into quarters with high SCC.

Dry cow therapy utilizes the dry period as a means for treating deep-seated infections or prevention of new pathogens from colonizing the mammary gland directly during dry period. Some antibiotics used for dry cow therapy include Spectramast and Pirsue. Similarly, teat sealants such as Orbeseal, Lock Out, and U-Seal can be used at dry off. There are many different options for dry cow therapy and teat sealants, as well as dry cow therapy intramammary antibiotic injections as well as combinations within. Spectramast is a broad-spectrum intramammary antibiotic injection created ideally for the treatment of bacterial mastitis. Pirsue is another intramammary antibiotic injection; however, it is used to treat mastitis associated with staphylococcal infections in particular. Orbeseal,

Lock Out, and U-seal is all non-antibiotic teat sealants. Teat sealants are used to prevent any risk of infections, working by sealing the ends of the teat, preventing any environmental microorganisms from entering the mammary gland, and causing subsequent mastitis. While a reduction in both SCC and CMT score can be achieved utilizing selective dry cow therapy and existing IMI can be reduced by up to 78%. *S. aureus* exhibits a persistent degree of resistance to selective dry cow therapy control and prevention methods [97, 98]. Both teat sealants and antibiotic treatments, if used, are vital measures utilized to prevent mastitis while the cow is in her dry period.

6.2 Hygienic control measures

There are two different control plans dairy farmers use to prevent mastitis. One is known as the 5-point mastitis control plan, which has been around for nearly 50 years. The 5-point plan includes (1) recording and treatment of all clinical cases, (2) disinfecting the teat ends post-milking, (3) using prescribed dry-cow treatment when drying off, (4) culling any chronically infected cows, and (5) performing regular maintenance on the milk machines. The origin of the 5-point plan comes from the National Institute for Research in Dairying (NIRD). The objective of this plan was to prevent new infections through management control efforts. The 5-point plan achieved a significant reduction in the incidence of contagious mastitis pathogens but has a very limited effect on environmental mastitis pathogens. So to control environmental mastitis pathogens the National Mastitis Council (NMC) later developed 10-point plan.

The 10-point mastitis control plan includes (1) establishing udder health goals, (2) maintaining a clean, dry, and comfortable environment, (3) establishing clean and safe milking procedures, (4) frequent maintenance of milking equipment and machines, (5) excellent record keeping, (6) safe and healthy management of clinical mastitis during lactation, (7) effective and proper dry cow management, (8) maintaining biosecurity within the farm for contagious pathogens, (9) proper management of udder health status, and (10) a frequent review of the mastitis control program. The 10-point protocol originated from the American Veterinary Medical Association and National Milk Producers Federation. This plan did not come about until after the 1990s, however, the 10-point plan is the most up-to-date management protocol used today in the industry. Moreover, creating and maintaining efficient biosecurity guidelines on the farm can lead to improved cow health, milk quality, and overall milk production by reducing opportunities for the spreading of pathogens across the facility or between animals.

6.3 Vaccines against staphylococcal mastitis

There are two commercial vaccines for *Staphylococcus aureus* mastitis on the market, Lysigin® (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) in the United States and Startvac® (Hipra S.A, Girona, Spain) in Europe and some other countries [99]. None of these vaccines confer protection under field trials as well as under controlled experimental studies [89, 100–102]. Several field trials and controlled experimental studies have been conducted testing the efficacy of Lysigin® and Startvac®. Results of some studies showed a reduced incidence, severity, and duration of mastitis in vaccinated cows compared to non-vaccinated control cows [89, 102, 103] whereas other studies did not find the difference between vaccinated and non-vaccinated control cows [99, 104]. None of these bacterin-based vaccines prevents new *S. aureus* IMI [89, 100–102].

The Startvac® (Hipra, Girona, Spain) is the commercially available polyvalent vaccine that contains *E. coli* J5 and *S. aureus* strain SP140 [105]. In a field trial, Freick et al. [99] compared the efficacy of Startvac® with Bestvac® (IDT, Dessau-Rosslau, Germany) another herd-specific autologous vaccine in a dairy herd with a high prevalence of *S. aureus*, and found that the herd prevalence of *S. aureus* mastitis was lower in the Startvac® and Bestvac® vaccinated cows compared to the control cows. However, there were no other differences in terms of improvement of udder health. These authors [99] concluded that vaccination with Startvac® and Bestvac® did not improve udder health. In another field efficacy study on Startvac® in the UK, Bradley et al. [102] found that Startvac® vaccinated cows had clinical mastitis with reduced severity and higher milk production compared to non-vaccinated control cows [102].

Similarly, Schukken et al. [89] evaluated effect of Startvac® on the development of new IMI and the duration of infections caused by *S. aureus* and CNS. These authors [89] found that vaccinated cows had decreased incidence rate and a shorter duration of *S. aureus* and CNS mastitis. Piepers et al. [103], also tested the efficacy of Startvac® through vaccination and subsequent challenge with a heterologous killed *S. aureus* strain and found that the inflammatory response in the vaccinated cows was less severe compared to the control cows. These authors [103] suggested that Startvac® elicited a strong Th2 immune response against *S. aureus* in vaccinated cows and was more effective at clearing bacteria compared to the control cows. Contrary to these observations, Landin et al. [104], evaluated the effects of Startvac® on milk production, udder health, and survival on two Swedish dairy herds with *S. aureus* mastitis problems and found no significant differences between the Startvac® vaccinated and non-vaccinated control cows on the health parameters they evaluated.

An experimental *S. aureus* vaccine made up of a combination of plasmids encoding fibronectin-binding motifs of fibronectin-binding protein (FnBP) and clumping factor A (ClfA), and plasmid encoding bovine granulocyte-macrophage-colony stimulatory factor, was used as a vaccine with a subsequent challenge with bacteria to test its protective effects [106]. These authors [106] found that their experimental vaccine-induced immune responses in the heifers were partially protective upon experimental challenge [106]. Another controlled experimental vaccine efficacy study was conducted on the slime associated antigenic complex (SAAC) which is an extracellular component of *Staphylococcus aureus*, as a vaccine antigen in which one group of cows were vaccinated with a vaccine containing a low amount of SAAC and another group with a high amount of SAAC and the unvaccinated group served as a control [107]. Upon intramammary infusion (challenge) with *S. aureus*, no difference in the occurrence of mastitis among all three groups despite the fact that the vaccine with high SAAC content induced higher production of antibodies compared to the vaccine with a low amount of SAAC [107]. Similarly, Pellegrino et al. [108], vaccinated dairy cows with an avirulent mutant strain of *S. aureus* and subsequently challenged with *S. aureus* 20 days after the second vaccination which resulted in no significant differences in the number of somatic cell count (SCC) or number of bacteria shedding through milk despite increased IgG antibody titer in the vaccinated cows compared to the control cows.

7. Discussion

Mastitis, an inflammation of the mammary glands is one of the most challenging diseases to control due to its multifactorial causes [109]. *Staphylococcus* species are

considered the most frequent and important cause of both clinical and subclinical mastitis in dairy cattle [109, 110]. The clinical course of the infection and contagious nature of the disease depends on virulence factors of the staphylococcal strain involved in causing the disease [111, 112]. Staphylococcal species employ many virulence factors to evade the host immune system, resist antibiotics, and eventually damage the mammary gland cells [113, 114]. Identifying the roles of major staphylococcal virulence factors is important to understand the epidemiology and control measures of mastitis.

Staphylococcal virulence factors can be categorized as intrinsic (an integral part of the bacteria) and extrinsic (acquired) factors [115]. Intrinsic virulence factors are mostly chromosomally encoded and are integral parts of the bacteria, whereas extrinsic virulence factors are acquired from mobile genetic elements, such as plasmids, or obtained through transformation, conjugation and transduction. Staphylococcal intrinsic virulence factors include biofilm, surface proteins, coagulases, biofilm-associated protein (Bap), invasins (leukocidins, kinases, and hyaluronidase), toxins (exotoxins and endotoxins/enterotoxins), membrane-impairing toxins, and staphylococcal α and β toxins [116, 117].

Staphylococcal biofilm is one of the major virulence factors that help the bacteria to become resistant to host immune defense and antibiotics [14]. Most staphylococcal surface proteins play a key role in evading host immune systems and adhesion to host cell surfaces, whereas others hydrolyze host cells, leading to cell death [118]. Surface proteins, such as protein A, help the bacteria elude host adaptive immune defenses via a variety of ways. Coagulases allow the conversion of fibrinogen to fibrin, and then fibrin catalyzes blood clots, protecting the bacteria from phagocytosis [119].

Staphylococcal exotoxins such as enterotoxins are considered as superantigens as they cause severe host immune reaction toxic shock syndrome. Other exotoxins such as α - and β -hemolysins and exfoliative toxins help the bacteria turn host cellular components into nutrients that the bacteria utilize to grow [120].

Infection of the mammary gland occurs when the udder host defense mechanism is not able to contain the virulent *Staphylococcus* spp. The host usually employs innate and adaptive defense mechanisms to fight the invading bacteria. Innate immunity is the first line of defense, which can quickly identify pathogenic microorganisms and provide a rapid defense to stop IMI infection before it advances into mastitis. The host cell uses various mechanisms such as lactoferrin, complement, and phagocytic cells to clear the invading pathogen [49, 121].

The adaptive immune response also called acquired immunity, is most commonly associated with antibody-mediated and cell-mediated responses [51]. The adaptive mechanism requires an invading antigen phagocytosed by antigen-presenting cells and presented to major histocompatibility molecule II (MHC-II). The adaptive response eliminates virulent staphylococcal species or stops their growth through antibody responses and/or cell-mediated immune responses [50].

Mastitis can be classified into two categories: subclinical or clinical. Clinical manifestations are vital, yet feasible for the milker to detect based on visible signs of mastitis either locally in milk or systemically in the body. More problematic mastitis cases are subclinical mastitis due to the evading nature of staphylococcal pathogens. Subclinical mastitis exhibits an elevated SCC and decreased milk production and requires diagnostic tools such as the CMT and electrical conductivity test to detect. Clinical and subclinical mastitis are costly to the industry, with strains varying by region, milking practices, and season making it nearly impossible to control the long-lasting effects of staphylococcal mastitis.

Early detection of mastitis is vital to prevent clinical cases from progressing further and poor quality milk entering the bulk tank. Most importantly, the detection of subclinical cases can be performed at a quarter, cow, or herd level to ensure high quantity and quality milk production. Diagnostic tools such as CMT, WMT, on-farm culturing, and electroconductivity are used [122]. The PCR and MALDI-TOF have been crucial for identifying causative bacteria at the species level to treat individual infections appropriately. The MALDI-TOF database is continuously growing, but currently, the lack of CNS speciation is problematic in identifying particular species under this category [123].

Once staphylococcal mastitis has been detected and the cow and quarter have been identified, diagnostic methods may further help identify at least the genus of the pathogen for appropriate treatment choice. Currently, there are only two widely used intramammary infusion antibiotics in the industry: Spectramast and Pirsue. While Pirsue is marketed to reduce staphylococcal mastitis severity, this antibiotic lacks the aspect of prevention [96]. The 10-point control programs and implementation of procedures, such as selective dry cow therapy, reducing staphylococcal infections via the environment, and cow to cow spread is possible. The prevalence of *S. aureus* and CNS in bulk tank milk persists at upwards of 97% and 56%, respectively, making staphylococcal mastitis an egregious issue. Without proper prevention methods, such as an efficacious vaccine, staphylococcal mastitis will continue to be a major problem for the dairy industry and the most costly disease of dairy cattle.

Based on current knowledge, both innate and balanced (humoral and cellular) adaptive immunity is required to control staphylococcal mastitis. Therefore, an intensive evaluation of bacterial cell surface-exposed staphylococcal virulence factors expressed during the early stages of host-bacterial interactions is required for vaccine development to identify immunogenic antigens.

8. Conclusion

Mastitis remains the most common and costly disease of dairy cows to date. Reduction in milk yield resulting from mammary tissue damage constitutes the major portion of the total cost of mastitis. Though several bacteria cause mastitis, *S. aureus* is considered one of the most common pathogens. Staphylococcal mastitis is extremely contagious and very challenging to control as it usually causes subclinical mastitis lacking any visible changes in milk and the mammary gland. *S. aureus* can invade the intracellular area evading the host immune system and bactericidal or bacteriostatic effects of common antibiotics used to treat mastitis by hiding within phagocytic and other non-phagocytic cells. This suggests effective management of staphylococcal mastitis using antibiotics alone is not effective and sustainable. Controlling staphylococcal mastitis is challenging for dairy farmers since all farmers are not equally applying mastitis control measures. However, control of *S. aureus* mastitis is important for the success of the farm. Although several efforts are underway to develop a vaccine, there is no effective vaccine against *Staphylococcus aureus* mastitis. Thus, with the current rise in antimicrobial resistance and poor staphylococcal mastitis treatment outcomes, it is important to focus on developing innovative sustainable tools to control staphylococcal mastitis such as an effective vaccine, probiotics, phage therapy, and others coupled with improved herd health management and good nutrition.

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