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Mastitis in Dairy Cattle, Sheep and Goats

Edited by Oudessa Kerro Dego

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Meet the editor

Dr. Kerro Dego is a veterinary microbiologist with training in veterinary medicine, microbiology, and anatomic pathology. Dr. Kerro Dego is an assistant professor of dairy health in the department of animal science, the University of Tennessee, Institute of Agriculture, Knoxville, Tennessee. He received his D.V.M. (1997), M.S. (2002), and Ph.D. (2008) degrees in Veterinary Medicine, Animal Pathology and Veterinary Microbiology from College of Veterinary Medicine, Addis Ababa University, Ethiopia; College of Veterinary Medicine, Utrecht University, the Netherlands and Western College of Veterinary Medicine, University of Saskatchewan, Canada respectively. He did his Postdoctoral training in microbial pathogenesis (2009 - 2015) in the Department of Animal Science, the University of Tennessee, Institute of Agriculture, Knoxville, Tennessee. Dr. Kerro Dego's research focuses on the prevention and control of infectious diseases of farm animals, particularly mastitis, improving dairy food safety, and mitigation of antimicrobial resistance. Dr. Kerro Dego has extensive experience in studying the pathogenesis of bacterial infections, identification of virulence factors, and vaccine development and efficacy testing against major bacterial mastitis pathogens. Dr. Kerro Dego conducted numerous controlled experimental and field vaccine efficacy studies, vaccination, and evaluation of immunological responses in several species of animals, including rodents (mice) and large animals (bovine and ovine).

Contents

Preface

Mastitis, an inflammation of the mammary glands, is the most costly disease in dairy farming, mainly caused by a broad range of bacteria categorized into contagious and environmental bacteria. Contagious bacteria live in the infected mammary glands and spread from infected glands to health glands during milking by milkers' hands or milking machine liners or towels. Environmental bacteria live in the environment of dairy cows, such as soil, feces, bedding materials, and spread from these sources to the mammary glands at any time of the year. Control of contagious bacteria focuses on improving hygiene during milking and treating infected animals or culling chronically infected animals that do not respond to treatment. The control of environmental bacteria is difficult because it is difficult to get rid of bacteria from the environment. However, cleaning manure and keeping animals in dry housing reduce teat contamination and infection. This book is a concise summary of mastitis in dairy cattle, sheep, and goats, which mainly focuses on etiological agents, epidemiology, pathogenesis, clinical manifestation, pathological and histopathological changes, diagnosis, prevention, and control measures. The book serves as a textbook on mastitis in dairy cattle, sheep, and goats for dairy veterinarians, veterinary students, animal science students, dairy technicians, animal health professionals. Researchers from various countries contributed to this book. The book contains the latest information on mastitis in dairy cattle, sheep, and goats and antimicrobial usage to prevent and control mastitis. Based on the reader's feedback, *Mastitis in Dairy Cattle, Sheep and Goats* can be considered to become a book series with a certain time interval between each published volume.

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Section 1

Bovine Mastitis

Epidemiology of Bovine Mastitis and Its Diagnosis, Prevention, and Control

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Abstract

Mastitis is an inflammation of mammary glands that is prevalent in dairy bovines. It causes a significant proportion of economic losses to the dairy farmers in India. Cattle and buffalo farming contribute significantly to the economy of the state. Various infectious agents such as bacteria, fungi, and algae may cause mastitis. Hence, it is essential to understand the etiological agents and predisposing factors that lead to mastitis in susceptible bovine populations in Madhya Pradesh state so that appropriate prevention and control strategies can be implemented. In this chapter, epidemiology, diagnosis, prevention, and control measures of mastitis in general and in India, the state of Madhya Pradesh, in particular, will be presented.

Keywords: mastitis, cattle, buffalo, bovine, Madhya Pradesh, epidemiology, diagnosis, prevention, control

1. Introduction

Mastitis, an inflammation of the mammary gland, is a very common disease in bovines. Among all the pathogens, bacteria are commonly implicated as the cause of mastitis [1]. Mastitis is characterized by inflammatory changes in milk and udder tissue. Mastitis is prevalent worldwide in dairy farming, causing heavy economic losses to the dairy industry. In those countries having well-developed dairy industry, morbidity of mastitis in dairy cows is 40% [2]. Affected bovines may lose their total milk production. Infectious agents like bacteria residing on the udder tissue or from the environment can enter through the teat canal. Milk from bovines with mastitis is unfit for human consumption because some mastitis-causing bacteria are zoonotic and can cause human infection [3]. Once the susceptible dairy bovine develops mastitis, it loses its milk production capacity significantly. Milk from a cow with mastitis is discarded due to inferior milk quality. Dairy animal owners have to bear extra costs for treating and maintaining such infected animals [4]. Clinically, there are two forms of mastitis: clinical form and subclinical form. The clinical form is characterized by local visible inflammatory changes in milk and udder tissue with or without systemic clinical signs, whereas subclinical form does not manifest clinical signs of mastitis but increased somatic cell counts with the presence of the causative agent. The diagnosis of the subclinical form of mastitis requires cow side test such as California mastitis test (CMT) or various laboratory tests including somatic cell

count (SCC) and milk bacteriological culture. Early diagnosis of subclinical form of mastitis is very much essential for successful treatment and control of infection [5]. Generally, high milk-producing cows are more suffered from mastitis than low milk producers [6]. This chapter highlights the epidemiology of mastitis and available diagnostic methods, prevention, and control measures with major focus on India in general and the state of Madhya Pradesh in particular.

1.1 The dairy sector and bovine population in the state of Madhya Pradesh, India

Madhya Pradesh is the second largest state in India with an area of 3,08,000 sq. km. The state is the part of the peninsular plateau of the country situated in the north-central part. It is one of the landlocked states in India (**Figure 1**).

It has three major seasons; summer, monsoon, and winter. The temperature of the entire state during summer (March–June) ranges above 29.4°C. Monsoon starts mid-June, and between June and September, the state gets the majority of its share of rainfall. In the winter season (November–February), the temperature remains low in the northern parts of the state compared to the southern parts. The state has over 70 million (7 crores) human population. The majority of the population of Madhya Pradesh (75%) resides in the villages, and most of them have income from agriculture. Tribal population accounts for 20% of the total population of Madhya Pradesh [7]. Livestock rearing provides them extra income and food security. Madhya Pradesh state Livestock and Poultry development corporation envision to increase the income levels of farmers involved in animal husbandry, particularly women by adopting a series of measures including a) increase in milk production, b) protect farmers from economic losses, and c) educate farmers about better management practices [8]. Madhya Pradesh state cooperative dairy federation limited has set up three-tier structures for dairy cooperatives. Primary village cooperatives (I tier), regional milk unions (II tier), and the apex federation (III tier) work in tandem for smooth functioning at field and plant operation levels and also for marketing the
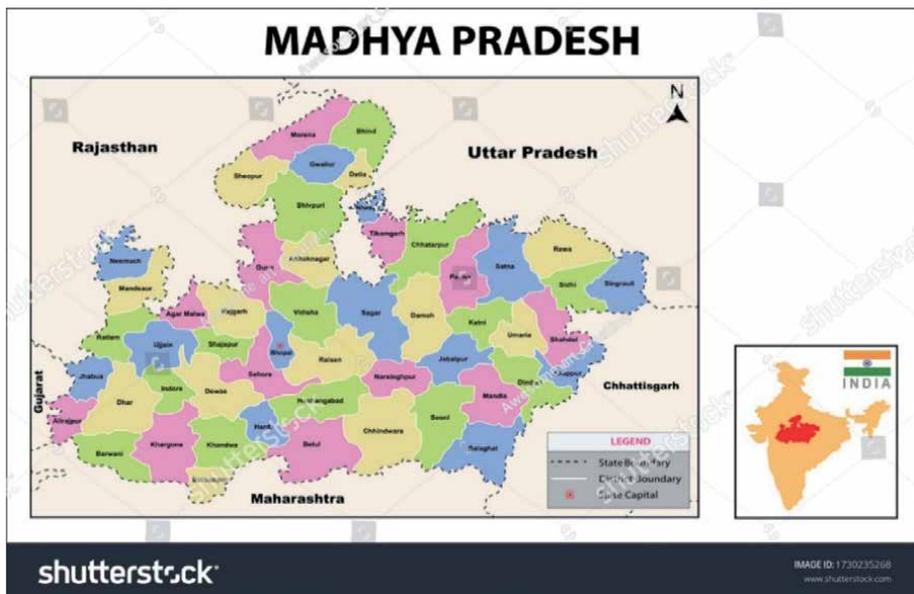
Figure 1.
Madhya Pradesh state in India.

Animal	Livestock population (in million)				Change in percent
	India (2019)		Madhya Pradesh		
	Total	**Female**	**2012**	**2019**	
Cattle	192.49	145.12	19.6	18.7	−4.42
Buffalo	109.85	100.57	8.2	10.3	25.88

Table 1.
Bovine population in India and Madhya Pradesh.

branded products (Sanchi and alike). During 2018–2019, there were 517 milk routes and 4698 functional dairy cooperative societies. A total of U.S. $ 148.71 million (Indian ₹ 1096.92 crores) has been paid to milk producers [9]. The total bovine population in 2019 increased by 1% to that of the total bovine population recorded in the previous census. According to 20th livestock census (provisional) statistics, the total bovine population in India stands at 302.79 (Cattle, Buffalo, Mithun, and Yak) million in 2019 [10]. However, in the state of Madhya Pradesh, only cattle and buffalo are reared. The cattle and buffalo population of the country and the state of Madhya Pradesh from the above-mentioned census is presented in **Table 1**.

Mastitis is an economically important infectious disease of cattle and buffalo in the state. It is essential to know its epidemiology, diagnosis, prevention, and control measures in India in general and in in the state of Madhya Pradesh in particular.

1.2 Institutes working on mastitis in the state of Madhya Pradesh

Madhya Pradesh state at present is having three constituent veterinary colleges covering the western (Dr. Ambedkar Nagar-Mhow) and north-central and north-eastern region (Jabalpur and Rewa) of the state (**Figure 2**) under the auspices of Nanaji Deshmukh Veterinary Science University, Jabalpur. The laboratories there are well equipped for the diagnosis of cases of mastitis. Besides, the state also boasts of a vibrant veterinary service where disease diagnostic laboratories are the main-stay to avert any serious issues about infectious diseases.
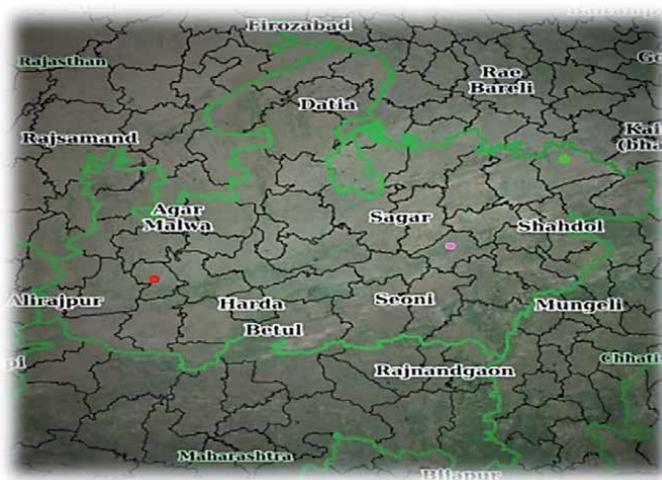
Figure 2.
Locations of institutes working for diagnosis and prevention and control of mastitis in the state of Madhya Pradesh (red dot—Dr. Ambedkar Nagar-Mhow, pink dot—Jabalpur, green dot—Rewa).

Work published on mastitis from the western and north-central regions from the state of Madhya Pradesh is available. However, there are very few published reports presently available from the north-eastern region of the state. Although recently a study was conducted for the assessment of subclinical mastitis in bovines in and around Rewa district [11], in this study, a 31.4% prevalence rate for subclinical mastitis in cattle was reported. It is essential to improve awareness of agriculture and livestock owners in general and dairy animal owners in particular about prevalent bovine diseases including mastitis in the state of Madhya Pradesh to minimize production losses. So, information and toolkits regarding mastitis, its diagnosis, prevention, and control measures are being distributed.

2. Economic impact due to mastitis

Among the two forms of mastitis, the clinical mastitis is readily diagnosed by clinical manifestations (**Figure 3**) and affected animals can be treated but the subclinical mastitis is asymptomatic and often diagnosed late, after days or weeks of infection. In Asia, incidence rates of clinical and subclinical mastitis ranged from 1-8% and 55–60%, respectively. The dairy industry in India in general and in the state of Madhya Pradesh, in particular, contributes to the economy by uplifting people from poverty and also helps in earning a regular income. Mastitis is responsible for nearly 70% of milk loss. Economic losses due to mastitis in India were estimated to be around U.S. $ 971.39 million (Indian ₹ 7165 crore) [12].

Figure 3.
Cow affected with clinical form of mastitis showing visible udder swelling.

3. Epidemiology of mastitis in India and Madhya Pradesh

3.1 Clinical and subclinical mastitis

Clinical mastitis is diagnosed readily by visible clinical signs and changes in the milk. In one of the studies conducted in India, in buffaloes from rural and urban dairy farmers, there was 18.74% prevalence of clinical mastitis [13]. In one of the other studies, 4.77% incidence of clinical mastitis was reported in the state of Madhya Pradesh [14]. Out of 260 cases of bovines in the Jammu region of the

country, 30 cases (11.54%) were positive for clinical mastitis. Prevalence of clinical mastitis in bovines ranged from 4.77 to 18.74% [13–15].

A higher level of incidence of subclinical mastitis (47.79%) was reported in the state of Madhya Pradesh [14]. In subclinical mastitis, there are no visible changes in milk or udder appearances in the affected bovines. Incidence of subclinical mastitis ranged between 19.2 and 83% in cows of Punjab state in India [16]. Recently, 40% of the overall incidence of subclinical mastitis was recorded in buffaloes in the state of Madhya Pradesh [17]. However, in lactating cows, the overall occurrence of subclinical mastitis was 27.81% [18]. In a recent study carried out in 2020, the reported overall prevalence of subclinical mastitis in cows was 31.55%. The prevalence of subclinical mastitis was highest in 5–7 years of age group (38.50%). The prevalence percentages of subclinical mastitis in organized (scientifically reared animals with adequate floor space availability) and unorganized dairy farms (animals reared in open space by livestock owners) were 29.82 and 41.66%, respectively [19]. In one of the other studies conducted in India, in buffaloes, there was 32.9% prevalence of subclinical mastitis [13].

When compared to clinical mastitis, subclinical mastitis is 15 to 40 times more prevalent [13, 20]. The prevalence of subclinical mastitis in dairy herds varied from 5 to 75% [21].

Subclinical mastitis results in greater economic losses to the farmers rearing dairy cattle and buffalo. No visible clinical signs are noticed in subclinically affected animals. In India, about 70–80% of economic losses have been attributed to subclinical mastitis alone. It occurs worldwide and also has adverse impacts on animal health and the quality of milk produced. Besides culling chronically infected animals from herds, decreased fecundity in affected animals and cost of treatment for mastitis lead to major economic losses. Subclinically infected cattle and buffalo can be a source of infection to other susceptible populations in the herd [22].

The prevalence of subclinical mastitis in bovines in India ranged from 9.88 to 86.87% [23]. In Madhya Pradesh, the prevalence of subclinical mastitis was reported highest in Jersey Cross (86.87%) than Holstein Friesian (75%), Malvi (57.35%), Sahiwal (75%), and Gir (80%) [24]. Among the various bacterial causative agents, staphylococci and streptococci were reported to be the most common pathogens of mastitis in India [25].

3.2 Etiology of mastitis

Mastitis is caused by physical, chemical, and biological agents. Generally, bacterial infections are the main causes of mastitis. Among many of the different microorganisms isolated from cases of bovine mastitis, the most common are Staphylococci (*Staphylococcus aureus*), Streptococci (*Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis*), and members of the family *Enterobacteriaceae* (*Escherichia coli, Klebsiella pneumoniae*) and other less common causative agents are *Pseudomonas aeruginosa, Mycoplasma* species, *Mycobacterium* species, *Nocardia asteroides*, *Candida* species, *Cryptococcus* species, and *Aspergillus* species. Rarely viruses are implicated in producing mastitis in bovines. The mastitis may be acute, per acute, subacute, chronic, and subclinical. Entry of the pathogen *via* teat canal into the mammary gland is characterized by increased leucocyte count in the milk [26].

Staphylococci, streptococci, *Escherichia coli, Pseudomonas* species, *Corynebacterium* species, *Mycoplasma* species, *Streptococcus agalactiae, Streptococcus dysgalactiae*, and *Mycobacterium tuberculosis* are isolated from buffalo suffering from mastitis. Among all the pathogens of bovine mastitis, staphylococci, streptococci, micrococci, *Corynebacterium* species, and *Escherichia coli* were isolated in Madhya

Pradesh. Listeria organisms were isolated from raw milk. Incidence of strepto-cocci (32.53%), micrococci (5.74%), *Corynebacterium* (1.91%), and *Escherichia coli* (0.95%) was reported in cases of bovine mastitis. However, recently, 17.19% mastitis cases in buffalo were due to infection with *Escherichia coli* [27–31].

Depending on the mode of transmission of mastitis pathogens from their natural habitat to the mammary glands, there are contagious and environmental mastitis pathogens. Contagious mastitis pathogens exist on the udder or teat surface of infected cows. These are the primary source of infection from where they are transmitted during milking to uninfected cows. Major contagious bacterial mastitis pathogens are coagulase-positive *Staphylococcus aureus*, *Streptococcus agalactiae,* and *Mycoplasma bovis. Corynebacterium bovis* is the less common contagious mastitis pathogen [26]. *Staphylococcus aureus,* a bacterial species, is most commonly isolated from cases of bovine mastitis [32]. The reported prevalence of *Staphylococcus aureus* was 58.85% in Madhya Pradesh. *Staphylococcus aureus* is known to acquire antimicrobial resistance very quickly. Methicillin resistance *Staphylococcus aureus* (MRSA) is also a causative agent of mastitis in dairy cattle. Detection of MRSA has serious public health significance. In dairy cattle, the reported prevalence of MRSA mastitis was 16.47% in the Jabalpur region of Madhya Pradesh [33].

Environmental mastitis pathogens are in the environment of dairy cows and they can transfer to the mammary glands at any time. Major environmental mastitis pathogens include environmental streptococci (*Streptococcus uberis* and *Streptococcus dysgalactiae*) and coliform bacteria (*Escherichia coli*, *Klebsiella* species, *Enterobacter* species, and *Citrobacter* species). Some of them are opportunistic pathogens and infect mainly the immunocompromised host [26, 34]. Coliform bacteria (*Escherichia coli*) do not normally live on the skin or in the udder. These organisms can enter the teat canal when the animal comes in contact with a contaminated environment. Contaminated bedding materials, soil, manure, and organic matter in the environment can be a source of *Escherichia coli* that can lead to environmental mastitis. This kind of environmental mastitis was reported from several countries. *Escherichia coli* are one of the most frequently isolated bacteria from clinical infections. In severe, naturally occurring clinical cases of mastitis due to *Escherichia coli* there can be necrosis of the mammary epithelium [35].

4. Mastitis risk factors

Mastitis risk factors include managemental factors and cow factors. In management, the most common measures that can be used to avoid mastitis at the farm level are regular floor cleaning, use of appropriate milking techniques, and udder washing before milking and pre- and post-milking teat dipping in antiseptic solutions. Culling is hardly practiced in the country and also in the state of Madhya Pradesh. Regular screening of bovines for mastitis is not practiced at the larger scale as it needs to be in the state. Generally, dairy owners approach to the testing facilities after the cases of mastitis in bovines are clinically visible. Cow factors include the stage of lactation, breed, history of mastitis, and parity. Even if udder defense mechanisms are there, microbial infection overpowers at times and causes mastitis. Additionally, at times due to inadequate livestock management and husbandry practices such as unhygienic maintenance of livestock, inadequate floor space available to the animals, improper ventilation, and faulty milking techniques used by milkman also contribute as predisposing factors for mastitis. Physical injury to teat skin, teat canal, and mammary cistern are also important predisposing factors for entry of microbial pathogens in the udder to cause mastitis [36].

5. Diagnosis

Rapid cow side tests that are used to diagnose mastitis are highly required to implement prevention and control strategies. Some of the tests used in the diagnosis of clinical and subclinical mastitis are described below.

5.1 Cow side test—strip cup test

The physical appearance of milk is checked by a test named strip cup test. Strip cup or strip plate-based visual examination is routinely used for detection of clinical mastitis in individual and herd animals. The quality of the foremilk is examined visually after squirting few stripes of milk on the strip cup for gross examination of blood, flakes, clots, wateriness.

5.2 California mastitis test (CMT)

California mastitis test (CMT) is a simple rapid screening test, based on the estimation of the number of somatic cells in the milk sample. The somatic cell population consists of 75% leucocytes and 25% epithelial cells. The rise in the leucocytes indicates mastitis. The CMT reagent is mixed with the milk samples and the reagent causes lysis of somatic cells and release of DNA that form a gel. The CMT test result is qualitatively estimated (**Figure 4**). The average SCC of 2,00,000 cells/mL is considered as normal milk. For bulk tank milk >2,00,000 cells/mL of milk shows the presence of mastitis with a significant loss in milk production; for composite milk, from all the four quarters of a cow >2,00,000 cell/mL is considered mastitis whereas for milk from a single quarter of a cow >1,00,000 cells/mL is considered mastitis milk [37].

5.3 Milk bacteriological culturing and identification

For isolation and identification of bacteria-causing mastitis, the milk samples for bacteriological examination are first centrifuged and the resulting sediment is streaked on ordinary, selective, or differential media and incubated aerobically. Attempts should be made to isolate mycotic and anaerobic organisms. Milk sample

Figure 4.
California mastitis test paddle showing gel formation.

is streaked on sheep blood agar (0.05–1% aesculin), which supports the growth of most of the pathogenic bacteria-causing mastitis. The growth of bacteria can be further confirmed by primary and secondary biochemical tests and also by molecular detection methods.

After isolation of the bacterial pathogen, further identification is carried out using phenotypic and genotypic methods. Isolation and identification of the bacterial pathogen from cases of bovine mastitis are of paramount importance for testing effective antibacterial drugs against the bacterial isolate. Phenotypic characterization of bacterial pathogen includes: a) evaluation of bacterial morphology and growth characteristics, b) testing the ability of bacteria to metabolize substrates (biochemical tests), and c) testing of the antimicrobial sensitivity. Many of the commercial bacterial identification testing kits are developed using these phenotypic traits. These tests are easy to perform, readily available in the markets and cost effective. However, growing mastitis causing bacteria or fungi in the laboratory requires time and manpower. The major limitation for using tests based on phenotypic characterization is the variable expression of phenotypic characteristics by bacteria, even if the bacterial isolates are from the same species. This led to difficulty in identifying the bacteria correctly. However, culture systems at the farm site are increasingly being used for the diagnosis of mastitis [38].

Staphylococci—Colonies of *Staphylococcus aureus* (gram-positive cocci, generally appearing as irregularly arranged clusters) are round, shiny, golden yellow. These colonies are surrounded by a double zone of hemolysis on blood agar. Baired Parker agar and Mannitol salt agar are the selective growth media used to grow this species. Coagulase test (slide agglutination test and tube agglutination test) gives positive result in case there is a pathogenic strain of staphylococci. Streptococci (gram-positive cocci, generally arranged irregularly in chains) produce small translucent colonies on blood gar (with alpha, beta, and gamma hemolysis) [26, 37].

Streptococci-Edward's medium is a selective as well as an indicator medium for haemolysis and aesculin hydrolysis. Darkening of colonies shows hydrolysis of aesculin. *Streptococcus uberis and Enterococcus faecalis* hydrolyze aesculin. *Streptococcus agalactiae* and *Streptococcus dysgalactiae* are hydrolysis negative. Only *Enterococcus* grows on MacConkey agar and produces red pinpoint colonies. Christie-Atkins-Munch-Peterson (CAMP) test is used to identify hemolytic streptococci [26, 37].

Coliform bacteria—*Escherichia coli*, *Klebsiella pneumoniae,* and *Enterobacter aerogenes* are the most common species of bacteria isolated from milk samples collected from bovines suffering due to mastitis. MacConkey agar is used to grow coliform bacteria. *Escherichia coli* gives a metallic sheen appearance on Eosin Methylene Blue (EMB) agar. Colonies of *Escherichia coli* are generally non-mucoid. *Klebsiella pneumoniae* (non-motile) and *Enterobacter aerogenes* colonies are generally mucoid [26, 37].

5.4 Species and strain determination for a bacterium isolated from a case of mastitis

Nowadays, species and strain determination for a bacterium are carried out using a technique based on matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). It is a reliable, easy to use, and cost-effective technique. This technique can have 100% sensitivity and specificity for identifying the infectious agent from the cases of mastitis [39–42]. The major limitation of MALDI-TOF MS is, it can only have existing bacterial protein profiles for any specific interpretation. The technology is not available to common laboratories that have mastitis diagnosis facilities in the state of Madhya Pradesh. Genotypic methods use nucleic acid for the identification of species as well as strain typing [43]. Polymerase chain reaction

Molecular method	Species level	Strain level	Reference
AFLP	✓		[44]
RFLP		✓	[45]
MLVA		✓	[46]
Ribotyping	✓		[47]
tDNAiSLP	✓	✓	[48, 49]
PFGE		✓	[50]
DNA Sequencing	✓	✓	[51]

AFLP, Amplified fragment length polymorphism; RFLP, Restriction fragment length polymorphism; MLVA, Multiple locus variable-number tandem repeat analysis; tDNAiSLP, Transfer DNA intergenic spacer length polymorphism analysis; PFGE, Pulsed field gel electrophoresis.

Table 2.
Molecular methods for identification of infectious agents of mastitis.

(PCR) is highly sensitive and the most commonly used molecular method to amplify target nucleic acid of a specific infectious agent causing mastitis. Virulent strains of organisms and those organisms that are not grown in culturing are also identified by using these molecular diagnostic methods (**Table 2**).

5.5 Electrical conductivity test

An increase in conductivity of milk from bovines suffering from mastitis is due to an increase in the salt concentration, which can be measured by an electrical conductivity test. Mastitis led to changes in ion concentrations [52], which impacts on the electrical conductivity of milk. Electrical conductivity can be measured [53] and the electrical conductivity rises with the increase in the concentration of sodium chloride in milk. Therefore, the measurement of electrical conductivity is used as a simple physical method to diagnose mastitis. An electrical conductivity meter is used to determine the electrical conductivity (EC). The EC of milk is expressed in the unit of milliSiemens (mS). This test has the following advantages: 1) One-time marginal investment is enough, 2) no special training is needed, and 3) easy to do and results are readily available.

5.6 Somatic cell count (SCC)

An increase in somatic cell count (SCC) in milk samples from bovines suffering from mastitis is measured by various tests such as CMT, white slide test, direct microscopic count, catalase test and anti-trypsin test, Brabant mastitis test (BMT), and Wisconsin mastitis test (WMT). Because of inflammation, the composition of milk in the animal suffering from mastitis is changed from normal to abnormal [54]. Somatic cell count is used to diagnose subclinical mastitis. The SCC includes direct microscopic somatic cell count (DMSCC), the bulk milk somatic cell count (BMSCC), and individual cow somatic cell count (ICSCC). The BMSCC is the universally accepted screening test for mastitis. In DMSCC, milk sample is smeared on a clean glass slide in the area of 1 cm^2 and stained with 1% methylene blue to examine 60 fields under the microscope for the count. The average number of cells in the fields is multiplied by the multiplication factor of the microscope to obtain the number of cells/ml of milk sample. Electronic somatic cell counters are used for DMSCC. A count of less than 1,00,000 signifies normal udder [1, 55, 56].

5.7 Chlorine test

An increase in chloride concentration of milk is an indicator of mastitis and it can be detected chemically. The presence of an increased quantity of chlorides in mastitic milk forms the basis of the test whereas normal milk contains about 0.07% chlorides. In mastitis, there is decreased amount of lactose and an increased amount of sodium chloride to maintain the normal milk osmotic pressure; hence during inflammation, there is an increase in the chloride content (> 0.14 percent) [57].

5.8 N-acetyl-B-D-glucosaminidase (NAGASE) test

This is a enzyme-based test that measure cell-associated enzyme N-acetyl-B-D-glucosaminidase in the milk. The highest level of the enzyme indicates a high cell count. It is a simple, effective, and most reliable test for the detection of subclinical mastitis. The readings for normal milk with 0.5×10^4 cells/ml and for mastitis milk with 1.5×10^4 cells/ml are 0.0053 and 0.034 moles/min/ml, respectively [58].

5.9 Indicators

Indicators of inflammation used to diagnose mastitis are well documented [59]. Recently, acute phase proteins were compared for diagnosis of subclinical mastitis in cross-bred cows in India [60].

5.10 Serological tests

Serological tests such as dot blot and enzyme-linked immunosorbent assays were used to detect antibodies to *Listeria* species antigens in the milk [61].

6. Mastitis prevention and control strategies

Five-point core plan for mastitis prevention and control include the following—a) disinfection of teats, b) adherence to hygienic practices in the milking procedures, c) removal of cows with chronic mastitis, d) dry cow therapy with antibiotics, and e) treatment of clinical mastitis [62, 63]. Additionally, in cow herds the measures used in prevention and control of mastitis are as follows—a) reduce introduction of new infections, b) shorten the duration of existing infections, c) maintenance of the normal udder health [64], d) dry and clean storehouse for fodder, feeders, and mangers, e) supply of palatable water, f) disinfection of milking area, g) shoe dipping disinfecting solutions for visitors to check any entry of the pathogen, h) check spread of infection by sprinkling lime powder [65], and i) keeping dairy animals stress free that encourage the development of healthy immune system [66].

10 point mastitis control program in cow herds recommended by National Mastitis Council is described in brief as follows: 1) establishment of goals for udder health and review those to prioritize changes in management to achieve set goals, 2) maintenance of a clean and comfortable environment by bedding managment, keeping areas clean and dry, ensuring proper ventilation and provision of feed soon after milking so that animals remain in standing position, 3) adopting proper milking procedures by washing and drying teats, cleaning of udder with single-use clothes, examining foremilk and palpating glands to facilitate early detection of clinical cases, maintaining clean hands or wear gloves during the process of milking, 4) proper maintenance and use of milking equipment by routinely servicing it

and thoroughly washing and sanitizing, 5) good record keeping of incidence and prevalence of clinical and subclinical mastitis in cow herd, individual examinations, and treatments, 6) appropriate management of clinical mastitis during lactation by selecting appropriate therapeutic regimen and avoid treatment of cases suffering from resistant microbial and non-responsive agents, 7) effective dry cow management by drying cows off abruptly and careful administration of all quarters of all cows with suitable antibiotic, 8) maintenance of biosecurity for contagious pathogens by assessing test reports of cows (SCC, CMT) and also obtaining aseptically collected milk cultures from suspect cows before purchasing are must. Newly purchased cows must be kept and milked separately to ensure the absence of intramammary infection. Cows with a persistently high SCC (greater than 3,00,000) are segregated and observed for response to the treatment. Marketing of chronically infected cows with *Staphylococcus aureus* and antimicrobial-resistant microbial agents (Mycoplasma, Nocardia, Pseudomonas, *Arcanobacterium pyogenes*) that are unresponsive to treatment is carried out, and 9) regular monitoring of udder health status by enrolling cows for SCC program and monitoring rates and distributions of cows with high SCC. These cows with high SCC and cows with clinical infection are used for cultural examination. Inflammation is monitored by cow-side CMT. Reports from the regulatory agencies and marketing organizations are used for monitoring udder health for the herd, and 10) periodic review of the mastitis control program is evaluated by representatives from veterinary, industry and extension fields.

Bedding materials are the primary source of environmental mastitis. Hence, reducing the bacterial count in bedding generally decreases the risk of environmental mastitis. Teat cleaning (which includes wet cleaning followed by manual drying)/pre-milking teat dipping in antiseptic solution is important to reduce bacterial counts on the skin. In cows kept indoors, it reduces the incidence of new intramammary infection [67]. Teat dipping after milking dairy animals results in a reduction in the rate of new infection significantly. It is most effective against contagious mastitis pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*. During the dry period, administration of, broad-spectrum antibiotics in each quarter of the udder at the last milking of lactation reduces the incidence of new intramammary infections. Dry cow therapy is the best way to cure chronic and subclinical mastitis. Effective and timely treatment of active cases is helpful to prevent new cases. Antibacterial therapy through the intramammary route is not always successful for treatment against mastitis. Particular antibiotics must be selected based on nature of the pathogen, results of antibiogram assay, and drug characteristics [68–70].

Farmer's awareness about a) what causes mastitis and b) how to prevent it, is essential to minimize economic losses not only in the state of Madhya Pradesh but also in India [71, 72]. Feeding micronutrients such as selenium and vitamin E are also very important to boost the immune response of the animals. Besides, recommending improvements in managemental practices, there must be proper attention made to provide better nutrition to animals for increasing immunity and reducing stress, and also encouraging farmers to participate in various awareness programs [73]. Knowledge of risk factors and characteristics of pathogens causing mastitis are essential to control the disease at farm level [74–77].

7. Summary

Mastitis affects the bovine population in India, it is also of major health concern to the bovine population of the state of Madhya Pradesh. Madhya Pradesh ranks fifth in milk production in India. Activities related to milk production and sale

fetch many livelihoods in the state. Bovine cases of clinical and subclinical mastitis caused by various pathogens are reported from the state and have economic implications for the dairy animal owners in terms of direct and indirect losses. There are various mastitis diagnostic tests routinely carried out by the institutes in the state for early and timely diagnosis of mastitis. It is aiding the authorities involved in livestock health for the better treatment of mastitis. Farmer awareness campaigns are undergoing but more impetus is needed to spread the word among them for better implementation of mastitis prevention and control strategies in bovines recommended by the National Mastitis Council.

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Chapter 2

Bovine Mastitis in Ethiopia

Tadele Tolosa Fulasa and Feyissa Begna Deressa

Abstract

Ethiopia is located in tropical region and livestock production represents a major national resource and forms an integral part of the Agricultural production system and livelihood of the society. Dairy farming being one of the agricultural production in Ethiopia, is practiced mainly as an extensive type of management system, which involves smallholder farmers in rural areas and semi-intensive and intensive managements in per urban and urban areas. Despite a large number of milking cows, there is low milk production because of many factors, including low genetic potential of indigenous breeds, extensive and poor husbandry practices, and widespread livestock diseases. Among the dairy cows' diseases, mastitis is prevalent in the dairy production system incurring high economic losses and social burden. Several reports on mastitis in Ethiopia are present but are scattered. We focused on reviewing articles published in indexed journals reporting bovine mastitis to summarize its common etiologies, prevalence, and risk factors in Ethiopia. The common pathogens reported from different parts of Ethiopia are *Staphylococcus aureus (Staph. aureus)*, non-aureus staphylococci, *Streptococcus* spp. (*Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*), coliforms (*E. coli, Klebsiella pneumonae*), *Trueperella pyogenes* and *Mannheimia haemolytica (M. haemolytica), Pseudomonas aeruginosa (P. aeroginosa)*, *Enterobater aerogenes*, *Bacillus* species, *Micrococcus* species. *Staphylococcus aureus* and *E. coli* are the most common isolates from clinical mastitis (CM). *Staphylococcus aureus* is also the most frequently isolated pathogen from sub-clinical mastitis (SCM). Sub-clinical mastitis which usually ranges from 25.4% to 73.3%, is highly prevalent than the clinical cases of mastitis which ranges from 3.2% to 26.5%. Several mastitis risk factors were reported. These were breed of animals, parity number, stage of lactation, presence of teat/udder lesion and hygiene measure of the farms. Thus, it is essential to plan and implement control measures including maintenance of good dairy farm environment, udder and milking hygiene at farm level; regular monitoring of udder health with special attention to exotic, crossbred and lactating cows and culling of older cows. Isolation, characterization and conducting antibacterial sensitivity test should be integral part of mastitis control strategy for effective control of the mastitis causing pathogens.

Keywords: Bovine mastitis, Ethiopia, prevalence, risk factor

1. Introduction

The Ethiopian economy is highly dependent on agriculture, which involves crop and livestock production in the highland areas and mainly livestock production in the lowland areas. The livestock subsector plays a vital role as source of food, income, services and foreign exchange to the Ethiopian economy, and contributes 16.5% and 45% of the total and agricultural GDP, respectively [1, 2]. It also accounts for 12–15% of the

total export earnings, second in order of importance [3]. Despite the huge number of livestock in the country, the performance of the sector is poor considering its potential. Dairy farming is an important sub-sector of livestock production, and serves as a source of food and income to many resource poor communities in the country.

In Ethiopia, dairy farming is mainly under the extensive type of management system, which involves smallholder farmers in rural areas. Currently, there are also emerging semi-intensive and intensive dairy production systems, practiced by farmers who have good access to the markets. However, the dairy production is being challenged by major constraints such as low genetic potential of indigenous cattle breed, diseases, inadequate feed and water and poor advancement in dairy development technologies. The problem of diseases is becoming very important with the importation of exotic breeds into the country for improved genetics and milk production. Among the diseases, mastitis is known to be prevalent in different dairy production systems in the country, incurring high economic losses.

Mastitis, an inflammation of the mammary gland, is usually a consequence of a bacterial intramammary infection (IMI) [4, 5]. Its can be presented with visible or invisible inflammatory responses of the udder. Mastitis with visible symptoms is called clinical mastitis, whereas mastitis without visible symptoms is called sub-clinical mastitis (SCM) [6].

Clinical mastitis is recognized by the presence of abnormal milk or udder characterized by discoloration, clots or swelling of the infected quarter [7, 8]. Cows with acute clinical mastitis may show generalized symptoms such as fever, loss of appetite, reduced mobility due to pain in the swollen udder, and systemic shock. Mastitis also threatens animal welfare [9, 10] due to pain, higher mean rectal temperature, increased heart rate, and respiratory rate caused by clinical mastitis. Severe cases of mastitis can even result in the death of the infected animal [9]. Furthermore, discarding milk from lactating animal suffering from mastitis results in substantial food losses, which causes nutritional shortage to the children and nursing women resulting in nutritional deficiency diseases.

Subclinical mastitis refers to inflammation of the mammary gland in the absence of visible symptoms, which can develop into clinical mastitis and vice versa. This type of mastitis causes an invisible reduction in milk production [11, 12], changes in milk quality [13], and composition [12]. Severe or chronic inflammation can result in loss of quarter (s) or teats. Cows with blind quarters produce less [14] and are more likely to be prematurely culled than healthy herd mates [14]. In Ethiopia, different reports showed that the prevalence of mastitis is different in different parts of the country and different breeds [15–18]. These reports also indicated that a number of factors influences bovine mastitis at individual animal and farm level. Therefore, this book chapter aims to provide summarized information on the etiology, prevalence, associated risk factors, and control measures for bovine mastitis in Ethiopia.

2. Etiology

A variety of microorganisms have been isolated from the milk of a cow with mastitis [5]. Mastitis-causing pathogens can be grouped into Gram-positive or Gram-negative based on their Gram-staining characteristics or major or minor pathogens based on potential damage they cause to the host or contagious or environmental based on their mode of transmission [19].

Contagious mastitis pathogens are in the infected mammary gland of the host, and mainly spread from infected to uninfected udders during milking [19]. The cow's environment is the main source of infection for environmental mastitis causing pathogens. Their number can be high in soil, manure, bedding, or contaminated water [19].

Staphylococci, Streptococci and coliforms are the most common causes of bovine mastitis [3]. *Staphylococci* are Gram-positive, catalase-positive cocci and are categorized into *Staph. aureus* and non-aureus staphylococci. *Streptococcus agalactiae*, *Strep. dysgalactiae* and *Strep. uberis* are the most common streptococcal species that cause bovine mastitis [5]. All the three are Gram-positive and catalase-negative major pathogens causing mastitis. *Streptococcus agalactiae* and *Strep. uberis* are known as a typical contagious and environmental mastitis pathogen, respectively, whereas *Strep. dysgalactiae* is likely to spread from cow to cow than from the environment to the cow.

Escherichia coli and *Klebsiella* spp. belong to the coliform group and are the most common Gram-negative pathogens that cause bovine mastitis. Both are major environmental mastitis causing pathogens.

The pathogens distribution of clinical and subclinical mastitis has been studied in several countries. For example, *Strep. uberis* was the most frequently isolated pathogen from clinical mastitis cases of British and Flemish herds of Belgium [20, 21], whereas *Staph. aureus* was the most frequently isolated pathogen causing clinical mastitis in Canada and Ireland [22, 23]. Non-aureus staphylococci (*Staph. chromogenes*, *Staph. epidermidis*, *Staph. haemolyticus*, *Staph. simulans*, and *Staph. xylosus*) were the most common cause of subclinical mastitis cases in the UK and Flanders part of Belgium [20, 24, 25]. Non-aureus staphylococci were also reported to be the most common isolates from subclinical cases of mastitis in Uganda [26].

Numerous organisms have been reported associated with mastitis in Ethiopia. These include *Staphylococcus* spp. (*Staph. aureus*, *non-aureus* staphylococci), *Streptococcus* spp. (*Strep. agalactiae, Strep. dysgalactiae, Strep. uberis*), Coliforms (*E. coli, Klebsiella pneumonae and other Klebsiella species*), *Trueperella* and *M. haemolytica*, *P. aeruginosa*, *Enterobater aerogenes*, *Bacillus* species, *Micrococcus* species and others (**Table 1**). Among all the pathogens of bovine mastitis, *Staph. aureus* is recognized as the most common causative agent of bovine mastitis in Ethiopia [18, 27–36]. The authors also reported that the most common contagious pathogens are *Staph. aureus* and *Strep. agalactiae* indicating that their presence in high prevalence could be due to lack of effective udder hygiene and poor milkers' hygiene practice during

Isolates	Study areas						
	Addis Ababa[a]		Selale[b]	Asella[c]		Mekele[d]	
	CM	SCM	SCM	CM	SCM	CM	SCM
Staphylococcus aureus	21	50	43	29	20	11	43
Non-aureus staphylococci	10	20	24	8	24	4	10
Streptococcus agalactiae	18	2	13	15	11	11	6
Streptococcus dysgalactiae	3	5	2	7	7	11	1
Streptococcus uberis	5	5	10	3	4	0	3
Escherichia coli	23	0	1	14	3	57	19
Klebsiella spp.	0	2	0	0	0	7	9
Others	21	16	7	24	31	0	9

a = *Addis Ababa capital city of Ethiopia.*
b = *Capital town of North Shewa zone Oromia state.*
c = *Capital town of Easter Arsi Zone of Oromia state.*
d = *Capital city of Tigray state.*

Table 1.
Pathogen distribution (in %) of clinical mastitis (CM) and subclinical mastitis (SCM) samples collected from different Ethiopian regions.

Figure 1.
Map showing study areas conducted on bovine mastitis in Ethiopia.

milking. The most common environmental mastitis pathogens were coliform bacteria [37]. Transmission of environmental mastitis pathogens may occur at any time including during milking and between milkings since they are in the environments of dairy cows. e.g., *Trueperella pyogenes* [38] and *Bacillus* species as summarized under the heading of others **Table 1** were also isolated [18].

The pathogen distribution of clinical mastitis (CM) and subclinical mastitis (SCM) has also been studied in different Regions of Ethiopian (**Table 1**; **Figure 1**). *Escherichia coli* was the most common pathogen causing CM in farms in and around Addis Ababa [39] and Mekele [37] whereas *Staph. aureus* was the most common pathogen causing CM in farms in and around Addis Ababa and Asella [18]. *Staphylococcus aureus* was the most frequently isolated pathogen from SCM samples in farms in and around Addis Ababa [39], Selale [17] and Mekele [37]. *Non*-aureus staphylococci species were more prevalent in the SCM samples in farms in and around Asella and Selale compared to the prevalence of *non*-aureus staphylococci species in the other regions (**Table 1**) [18].

3. Prevalence of mastitis in Ethiopia

Dairy farms in Ethiopia are not registered, and therefore, information on the exact number and distribution of dairy farms is lacking. However, reports indicated that the number of farms are increasing yearly although it does not commensurate with human population growth in the country [40]. The number of herds, which are indicated below, is retrieved from prevalence studies carried out in different Regions. Most studied farms are similar in average herd size, milk production and farming practices. In addition, most farms are hand-milked, and cows are managed under zero-grazing conditions. However, the differences observed in prevalence of mastitis between studies might be explained by the differences between individual farm management, environment, and breed of the animals.

3.1 Clinical mastitis

Clinical mastitis in cows has been reported with varying degree of occurrence in Ethiopia (**Table 2**) [17, 18, 37, 41–43]. In Ethiopia, the prevalence of clinical mastitis ranged from 3.2% to 26.5% at the cow level and from 0.9% to 14.9% at the quarter level

Region	Herd	Cows			Quarters			Reference (s)
	n	n	% CM	% SCM	n	% CM	% SCM	
Sodo and Awassa	20	307	15	25.4	1133	7.3	11.4	[15]
Addis Ababa	51	363	6.6	46.6	1452	2.7	26.7	[41]
Selale	109	500	3.2	29.4	2000	0.9	13.2	[17]
Asella	42	223	26.5	38.1	892	14.9	30.4	[18]
Adama	95	206	6.3	41.7	794	2.4	22.2	[42]
Mekelle	13	305	3.6	33.8	1220	14.3	11.9	[37]
Jimma	42	176	11.4	61.9	704	9.8	46.3	[43]
Assella		66	12.1	54.5		10	32	[44]
Holota		107	22.4	48.6		10	34.8	[45]
Bishoftu Town		262		40.1			16.1	[46]
Eastern Harrarghe Zone		384	12.5	51.8		10.7	46.4	[47]
Hawassa		201	5	25.4		2.1	8.2	[48]
Wolayita Sodo		349	2.6	26.9				[49]
Haramaya		384	6.77	56.25		6.38	22.66	[50]
Holleta		90	7.8	73.3		5.59	75.3	[51]
Ambo		302	9.9	32.8		9.3	32.8	[52]
Meta analysis		39	8.3	37				[53]

Table 2.
The prevalence of clinical mastitis (CM) and subclinical mastitis (SCM) from different studies conducted in Ethiopian.

(**Table 2**). The lowest prevalence of clinical mastitis was reported in farms in Selale [17] while the highest was in farms in and around Holota [45]. The variability in prevalence of clinical mastitis among different studies conducted at different areas of the country might be attributed to differences in management practices, environmental conditions in the study areas and other factors. Unlike other countries, no longitudinal studies have been performed on clinical mastitis in Ethiopia [21, 22, 54]. Consequently, the incidence and average duration of clinical mastitis cases are unknown.

3.2 Subclinical mastitis

Subclinical mastitis is considered as the most economically important type of mastitis because of long term effects of chronic infections. The prevalence of subclinical mastitis (SCM) ranged from 25.4% to 73.3% at the cow level and from 8.2% to 75.3% at the quarter level (**Table 2**). The lowest quarter level prevalence of SCM was observed in Southern Ethiopia (Awasa) [48] and the highest quarter level prevalence of SCM were observed in West Shewa of Ethiopia (Holota) [51]. The variation of findings among studies might be attributed to differences in management and environmental conditions in the different study areas as well as cow breed variations in susceptibility to mastitis. Nevertheless, it can be concluded that comparison to other countries, the prevalence of subclinical (or clinical or both) mastitis is high in Ethiopia (**Table 2**). Moreover, different scholars have reported varying ranges of clinical and subclinical cases of mastitis (**Table 2**; **Figure 1**) [27–36].

4. Risk factors

Mastitis is a multifactorial disease. Identification of risk factors and characteristics associated with the likelihood of the disease, can lead to better control of mastitis. Different herd, cow, and quarter characteristics associated with pathogen-specific IMI or subclinical mastitis has been identified [27–35, 54–56]. However, as management largely differs between regions, not all risk factors associations can be generalized, leaving the need for implementation of region- or even herd-specific control plans.

In Africa, researchers from Zimbabwe (pure breed Friesian, Jersey and Red Dane and their crosses compared with Mashona indigenous breed) and Rwanda (pure-breed Friesian, Jersey and their crosses and local breed Ankole and Sahiwal) reported that farms with pure and cross-breed herds had higher odds to mastitis compared with the indigenous breed [57, 58]. It is also reported that farms which use pre-milking teat dipping in antiseptic solution (0.5% iodine) had lower odds to mastitis compared with farms not using pre-milking teat dipping [57].

Risk factors	Outcome	Level	Reference
Age (cow) ↑	CM↑	2 (Middle, old)	[41]
Age (cow) ↑	↓	2 (≤4 years, >4 years)	[59]
Parity↑	↑	3 (1, 2, 3)	[41]
Lactation stage ↑	↑	2 (Mid, late)	[41]
Body score↓	↑	3 (Good, fair, poor)	[41]
Leaking milk↑	↑	2 (Yes, no)	[41]
Previous udder problem	↑	2 (Yes, no)	[41]
Heifers purchased in the last year (herd) ↑	↓	2 (Yes, no)	[59]
Teat injury (quarter)	↑	2 (No, yes)	[59]
Age (cow) ↑	SCM↑	2 (Middle, old)	[41]
Parity↑	↑	3 (1, 2, 3)	[41]
Lactation stage↑	↑	2 (Mid, late)	[41]
Breed		2 (Cross, Zebu)	[16]
Lactation stage↑	↑	2(>210 DIM vs. <121 DIM)	[16]
Parity↑	↑	3 (1or2, 3–5, >5)	[16]
Lactation stage↑	↑	3 (Beginning, mid, end)	[17]
Breed		2 (Cross, Arsi)	[18]
Parity↑	↑	7 (1–7)	[18]
hygiene↓	↑	2 (poor Vs good)	[18]
Lactation stage↑	↑	3 (< 90 DIM, >90–180 DIM, >180 DIM)	[43]
Body score↓	↑	3 (Good, fair, poor)	[41]
Lesion on teat/udder	↑	2 (Yes, no)	[17]

Table 3.
Reports on association of different risk factors with the prevalence of mastitis (SCM, CM), data from different scholarly articles from Ethiopia.

In Ethiopia, several scholars have reported the significant association of breed, physiological state, parity, stage of lactation and presence of lesion on udder/teat skin with the prevalence of mastitis (**Table 3**).

Higher prevalence of the disease was reported on exotic or cross breed (Holstein-Friesian) than local indigenous zebu [15, 18]. This indicates higher yielding cows are more likely suffering from the disease. The findings reported from various parts of Ethiopia have also indicated parity of the dairy cows is a risk factor of mastitis. Increased prevalence of mastitis with increasing parity number was reported by many authors [15, 17, 18]. The significant difference in prevalence of mastitis at different lactation stages was also reported. Cows in early lactation stage are more likely affected with mastitis than mid lactation [15]. Difference in prevalence of mastitis was also observed among cows with different body conditions. Cows with poor body condition are more likely affected with mastitis than cows with good body condition [41, 43]. The presence of predisposing factors such as teat and/or udder lesions and tick bites have a significant influence on the prevalence of mastitis. Cows with teat and/or udder lesions and tick bites were more affected by mastitis than without these factors (**Table 3**) [15, 17, 18].

5. Diagnosis

Monitoring udder health performance is impossible without reliable and affordable diagnostic methods [60]. Diagnosing udder health problems needs to distinguish between Intramammary infections (IMI), clinical mastitis, and subclinical mastitis which requires laboratory facility [60].

The diagnosis starts with physical clinical examination which involves palpation of the mammary gland and visual inspection of milk. In CM cases, the infected quarter may manifest inflammatory changes such as hot, red, swollen and painful. Inflammatory changes in milk include change of color such as bloody or watery milk, change in consistency which can be viscous, watery and/or clots, and change in smell of the milk [18].

Since milk seems normal in SCM, diagnosis is based on additional testing of milk samples such as direct somatic cell count (SCC) or indirect estimation of somatic cells in milk by California Mastitis Test (CMT) or other quick cow side tests. The SCC can be measured at the quarter, cow and herd-level. The CMT qualitatively estimates the number of somatic cells in milk secretions and is performed by mixing 2 mL of milk sample with a 2 mL of the CMT detergent which dissolves cell walls and releases DNA. The more cells in milk the more DNA is released, the thicker the mixture would be indicating the presence of high SCC. Hence, SCC can be scored based on the degree of thickening or gel formation. The test is cheap, applicable on farm but comes with inter-operator variation and has a low sensitivity [60, 61]. According to European Union countries, bulk milk SCC gives an indication of the presence of SCM in the herd's when it counts above 400,000 cells/mL, >200,000 cells/mL SCC for individual cow of composite milk and individual quarter SCC (>50,000 cells/mL) [62].

Other tests which indicates the presence SCM in the milk are measuring N-acetyl-β-D glucosaminidase (NAGase), lactate dehydrogenase (LDH) and electric conductivity of milk but are less frequently used compared to SCC [63]. These tests indicate the association between the activities of NAGase and LDH and SCC with respect to udder health status. The stronger the relationship between NAGase and LDH activity and SCC indicates the presence of mastitis. On the other hand, in both SCM and CM, intramammary infections can be detected by bacteriological culture or PCR test on milk samples. Bacteriological culturing of milk can be done

from bulk milk, individual quarter or cow. This gives a clue to evaluating udder health and mastitis control at the herd, cow or quarter level when it is performed combined with SCC.

6. Control measures

Mastitis control includes treatment of existing IMI and prevention of new IMI [64]. In the 1960s, the Five-Point Plan was initiated in the UK which includes (1) early detection and treatment of clinical cases, (2) blanket dry cow therapy, (3) post milking teat disinfection (4) identification and culling of chronically infected cows and (5) the routine maintenance of the milking machine [65–67]. In Ethiopia, early detection and treatment of clinical cases is most applicaple in majority of rural farms. In urban and periurban farms, application of the Five-Point control/prevention plan are possible. Yet, application of the other points in the rural farms are not possible. For example, tubes for dry cow therapy are not availability on the local markets and almost all farms in rural Ethiopia are not using milking machine.

Implementation of the five-point control/prevention plan was mainly successful in controlling contagious mastitis pathogens but less effective against environmental mastitis pathogens [20]. Therefore, an extended 10-point mastitis control plan was designed by the National Mastitis Council (NMC, a global organization for mastitis control and milk quality). This program includes preventive measure against environmental mastitis pathogens such as maintenance of a clean, dry, comfortable environment. Yet, not all 10-points mastitis control plan are applicable on rural farms in Ethiopia. Some of the 10-point mastitis control plan can be customized in Ethiopian by increasing the awareness of the farmers. For example, establishing goals for udder heath, maintaining a clean, dry, comfortable environment, good record keeping, management of clinical mastitis during lactation, and maintenance of biosecurity can be adopt and most big farms are applying them.

7. Conclusion

The prevalence of bovine clinical mastitis ranged from 3.2% to 26.5% at the cow level and from 0.9% to 14.9% at the quarter level while subclinical mastitis ranged from 25.4% to 73.3% at the cow level and from 8.2% to 75.3% at the quarter leve in Ethiopia. This indicates that the subclinical mastitis incurs more economic losses to the farmers and the nutritional supply of the community. Both contagious and environmental pathogens of mastitis are commonly isolated from dairy cows at different corners of the country. *Staph*. *aureus* and *Escherichia coli* are the most common isolates from clinical cases of mastitis, whereas *Staphylococcus aureus* is the most frequently isolated pathogen from Subclinical mastitis. Staphylococci other than *Staph*. *aureus* are also more prevalent in the SCM in some parts of the country. Several risk factor for mastitiss such as breed, parity number, stage of lactation, teat/udder lesion, tick infestation, and hygienic measures of the farms have been identified in Ethiopian dairy farms. Some of these factors such as hygiene, tick control are modifiable at farm level whereas most are beyond the control of the farmers. Example, the farmer does not have an influence on the parity or stage of lactation of his cows. To this end, there is a need to identify management practices that have an effect on mastitis and that are really relevant in the field by establishing national guideline in Ethiopia. The essential control plans can be adoppted from mastitis control plans designed by the National Mastitis Council (NMC) and implement with local modifications. The most important control measures against bovine

mastitis to be mentioned are good dairy environmental hygeign practice, udder, and milking hygiene at farm level; regular monitoring of udder health with special attention to exotic, crossbreed, and lactating cows.

Conflict of interest

The authors declare that they have no conflict of interest.

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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 Fcγ 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 B-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 herdsmen 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 suh 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 her 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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Etiology of Bovine Mastitis

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Abstract

Mastitis in dairy animals is the primary concern of dairy farmers, which is the most common disease that causes huge economic losses in the dairy industry. The economic losses due to mastitis are from a reduction in milk yield, condemnation of milk with antibiotic residues, veterinary treatment costs, and death. In addition, some mastitis pathogens also cause serious human diseases associated with the contamination of milk or milk products with bacteria or their toxins. Bovine mastitis is mainly caused by a wide range of environmental and contagious bacterial mastitis pathogens. Contagious pathogens are those whose main reservoir is the infected udder. Contagious pathogens mainly spread among animals during milking process whereas environmental pathogens spread from environment to udder at any time. The source of the environmental pathogens is the surrounding environment of an animal. The major contagious pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Mycoplasma* spp. and the minor contagious pathogens include *Corynebacterium bovis* and others. Major environmental pathogens include coliform bacteria (*Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp.), environmental streptococci (*Strep. dysgalactiae, Strep. uberis*). This chapter covers detailed review of published data on contagious and environmental pathogens responsible for bovine mastitis.

Keywords: Bovine mastitis, Etiology of mastitis, Microorganism, Contagious pathogen, Environmental pathogen

1. Introduction

Mastitis is an inflammation of the mammary gland caused by microorganisms or trauma. Its purpose is to eliminate or neutralize infectious agents or repair injury and set the stage for healing and restoring normal functioning [1]. Inflammation can be caused by many types of injuries, including infectious agents and their toxins, chemical irritation, and physical trauma [2, 3]. In dairy cows, mastitis is most often caused by microorganisms, usually bacteria that enter the udder and multiply in the milk and gland tissues, producing toxins and other virulence factors that cause direct damage to the gland tissue [4]. Mastitis is one of the main diseases of dairy animals (e.g., cattle, buffalo, sheep, goats and camels). It causes several

problems including reduction in milk production, affect quality of milk to be processed and milk and dairy products quality as well as a huge financial loss for the dairy industry [5]. Mastitis affects the physical and chemical properties, bacteriological load, and other milk qualities. In the milk of infected animals, pathogens and their toxins may present. So, the disease is also very important from the consumer's health risk point of view [6]. The presence of heat-resistant pathogenic spores and toxins in commercially available raw milk poses a serious threat to consumer's health and wellbeing [7–9].

Mastitis can be caused by a single pathogen or combination of two pathogens. According to the US National Mastitis Council Guidelines for diagnosis of mastitis, isolation of more than three pathogens in a milk sample is considered contamination. About 137 microbes have been isolated from milk [4]. Environmental microorganisms that can cause mastitis include *Strep. uberis, Strep. agalactiae, Trueperella pyogenes, Enterobacter aerogenes, Klebsiella* spp., *E. coli,* some yeast, and fungi [10]. In herds that lack an effective mastitis control program, infectious agents such as *Staphylococcus aureus* and *Streptococcus agalactiae* are generally considered to be the main organisms causing mastitis [11]. The incidence rates of these pathogens were significantly reduced with strict adoption of mastitis control programs in countries with well-established dairy farming systems. However, in well managed dairy farms with strict application of mastitis control programs environmental pathogens are of more concern in well-established dairies [12, 13]. Prior to the implementation of mastitis control strategies such as 5-point mastitis control program and later on 10-point mastitis control program by National Mastitis council, contagious mastitis pathogens were considered as the main causative agents of mastitis in dairy cows, even in developed countries [14–16]. The epidemiological field study of mastitis concluded that agents such as *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* account for over 75% of mastitis cases, and *Staphylococcus aureus* is the most prevalent, resistant and challenging candidate among them [8, 15, 17]. The bacterial entry into mammary glands leads to bacteria interaction with the mammary epithelial cells, resulting in local inflammatory signs and deteriorated milk quality. Environmental microorganisms can accidentally enter the udder during intramammary injection [18]. Moreover, contagious intramammary infection can be transmitted by milker's hands, cleaning towels, flies, and milking machines [19, 20]. *Streptococcus dysgalactiae, Strep. uberis, Klebsiella* and *E. coli* are the most common environmental pathogens, gaining access to udder at any time including during milking process. Clinical mastitis manifest symptoms such as udder/quarter swelling, abnormal milk quality and quantity, and anorexia [21–23].

2. Contagious mastitis pathogens

2.1 Major pathogens

2.1.1 Staphylococcus aureus

Staphylococcus aureus is major pathogen causing infectious mastitis in dairy cows, with prevalence of 43–74% [24]. It is a gram-positive, catalase and coagulase-positive, non-spore-forming, oxidase-negative, immobile, and facultative anaerobic bacteria [25]. *Staphylococcus aureus* is the most common mastitis pathogen [26]. While it is possible to reduce the incidence of *S. aureus* mastitis through hygienic milking and proper management systems, it remains a major challenge for dairy farms with a prevalence rate higher than 60% [8, 27]. The incidence of *S. aureus* mastitis differs due to changes in hygienic milking practices and general differences in the management of infectious

mastitis on farm [20, 28]. Optimal milking parlor hygiene can considerably decrease the incidence of new *S. aureus* mastitis in the herd but cannot exclude existing cases in the herd [29]. Based on early observations by Neave et al. [29], numerous studies have reported that treatments can decrease the number of new cases of mastitis [12] but cannot eliminate persistent infections in the herd. In the United States, the occurrence of clinical and subclinical *S. aureus* mastitis is 10–45% and 15–75%, respectively [30]. Its virulence is due to its ability of producing wide array of virulence factors that enhance its pathogenicity and persistence in epithelial linings of udder. These virulence factors contribute to microbial attachment, colonization, longer persistence and escaping the immune response. Such abilities make *S. aureus* one of the most important challenging pathogen for animal and human health [31, 32]. *Staphylococcus aureus* isolated from udder infections in ruminants are found producing a layer of slime around them, which enables them to resist host immune system and antibiotics [8]. This slime layer also helps in adherence and colonization of pathogen in udder glandular cells [33]. *Staphylococcus aureus* virulence factors and pathogenicity associated mechanisms such as resistance to phagocytosis, adherence and biofilm formation enable it to cause persistent and chronic infections [34].

Staphylococcus aureus has numerous virulence factors, that can be divided into two categories. These include non-secretory factors which are surface restricted structural component that acts as virulence factors, and secretory factors that are produced by bacterial cells, and act on a variety of target sites in the host. Both secretory and non-secretory factors enable this pathogen to evade host's defenses and colonize the udder [35–37]. Microbial membrane proteins, including fibrinogen-binding protein, collagen-binding protein, penicillin-binding protein, elastin-binding protein, and lipoteichoic acid can act as non-secretory virulence factors [36, 38, 39]. Cell wall binding factors such as lipoprotein, peptidoglycans, protein A, phthalic acid, protease, and β-lactamase can act as secretory virulence factors [39, 40]. Other virulence factors related with the cell surface include exopolysaccharides, biofilms, and capsules [37, 41, 42]. Overall, *Staphylococcus aureus* has more than 13 secreted proteins and 24 surface proteins involved in immune evasion [43], as well as about 15–26 proteins involved in biofilm formation [44]. The most familiar secretory virulence factors are toxins, including non-enteric exfoliative toxins, staphylococcal enterotoxins, leucocidin, toxic shock syndrome toxin 1, and hemolysins (α, β, δ, and γ) [45]. Likewise, enzymes like staphylokinase, coagulase, phosphatase, DNase, phospholipase, lipase, and hyaluronidase are also virulence factors of *Staphylococcus aureus* [7, 46, 47].

2.1.2 *Streptococcus agalactiae*

Streptococcus agalactiae is the contagious mastitis pathogen and the infected mammary gland acts as reservoir of the bacterium in the herd. Transmission of the bacterium is mainly through milking equipment, milker's hands, and regular towels [48]. Developed dairy sectors have overcome this challenge by optimal managemental and biosecurity practices but *Streptococcus agalactiae* is still an important cause of intramammary infections (IMI) around the globe [16, 49–51]. A study from dairy farms in Colombia indicated a higher prevalence of *Streptococcus agalactiae* induced IMI in cattle ranging from 28–35% [52]. Moreover, *Streptococcus agalactiae* reemergence has also been reported in Northern Europe [53]. Non-dairy sources (e.g., humans) have been reported to be the main cause of reintroduction of this pathogen into dairy herds [54].

Capsular polysaccharide is the most important virulence factor of *Streptococcus agalactiae* [55], which protects bacteria from phagocytosis by macrophages and subsequent depletion [55]. An additional virulence factor for *S. agalactiae* is the

surface protein, which provides resistance to proteases. Emaneini et al. [55] discovered that 89% of cattle mastitis causing *Streptococcus agalactiae* isolates possess gene encoding (*rib*). *Streptococcus agalactiae* is extremely contagious but responds well to antibiotic treatment, allowing its removal from the herd with effective mastitis control programs [56]. As a result of standard managemental practices, *Streptococcus agalactiae* mastitis has been significantly reduced and is now rare in developed dairy systems [57].

2.1.3 Mycoplasma *spp.*

Mycoplasma is a highly contagious microorganism, but not to the same extent as *Streptococcus agalactiae* or *Staphylococcus aureus*. However, *Mycoplasma* damages the secretory tissue and causes abscess and lymph node fibrosis as well as gland fibrosis [4, 16]. Animals of any age and at any time of lactation are sensitive to *Mycoplasma* infection. Those in the early stages of lactation are susceptible to *Mycoplasma* infection and may be isolated from asymptomatic high producing animals. Mycoplasmosis is usually associated with the appearance of mastitis, the appearance of new animals, previous respiratory or joint diseases, and herds of cattle that have not responded to antibiotic treatment [18, 58]. *Mycoplasma* infection is suspected if there is at least one recurrence of mastitis, asymptomatic disease, and no response to treatment [59].

The species detection in *Mycoplasma* mastitis is usually carried out by PCR with defined endpoints for *Mycoplasma bovis*, *Mycoplasma bovigenitelium*, *Mycoplasma californicum*, and *Mycoplasma alkalescens.* Laboratory monitoring of dairy herds showed the presence of *Mycoplasma* spp. in at least one cow of the herd [60]. Herd-level study of 463 Northwest Dairy Association miking herds reported that 93 herds were positive for *Mycoplasma* mastitis. Cattle in milk were noted more prone to *Mycoplasma* infection. Moreover, *Mycoplasma* infection was noted indirectly related to herd size [61].

Mycoplasma mastitis is less common than other bacterial mastitis, but it can cause severe mammary infections and has unique epidemiology and risk factors [58, 61]. It can usually be distinguished from mastitis caused by staphylococci and streptococci because it is (1) highly infectious, (2) infects more than one quarter, (3) causes significant milk yield loss, (4) is often resistant to antibiotic treatment, and (5) can become purulent. In some cases, affected cows may appear normal and not show obvious clinical signs. Since *Mycoplasma* mastitis is considered incurable, culling remains the most commonly recommended control measure [58, 62].

2.2 Minor contagious pathogens

2.2.1 Mannheimia *spp.*

Mastitis, caused by *Mannheimia* (formerly known as *Pasteurella*) *haemolytica* and *Pasteurella multocida*, is common in sheep and manifests itself as peracute gangrenous, but less commonly in goats and cattle [63, 64].

2.2.2 Corynebacterium bovis

Corynebacterium bovis (C. bovis) is a common infectious agent, most associated with asymptomatic infections. However, in 7% of cases, the bacteria were isolated from cows with clinical mastitis [16]. From the herds where pathogens that cause infectious mastitis were controlled, it accounted for higher number of clinical cases. There is a continuing discussion about the importance of *Corynebacterium bovis* infection for udder health and milk production [16, 19, 21]. Studies have shown

that this bacterium has tendency for the teat canal. This characteristic is associated with lipids requirements for its growth (probably inside the keratin plug). It could be possible that *C. bovis* occlusion of the streak canal may cause competition with other ascending bacterial infections for nutrients, thus decreasing the IMI [15, 16]. Moreover, the small increase in SCC linked with *C. bovis* infection may increase the ability of the udder quarter to show response against new intramammary infections. A higher SCC than normal is caused by infection with a minor mastitis pathogens in the udder and increases the udder's resistance to invasion by other contagious pathogens [1, 65].

In herds with endemic *C. bovis* mastitis, the infection rate was noted lowest in comparison to major pathogens infected herds [15, 66]. Intramammary *C. bovis* infections are mostly associated with clinical manifestations but generally have a reasonable increase in somatic cells count. Milk in such infections is usually thicker than normal and milk loss is usually undetectable [16, 22, 23, 67].

3. Environmental pathogens

In modern dairy systems, environmental mastitis is the most common and costly challenge [59]. Environmental mastitic pathogens include various bacteria such as coliform (e.g., *Escherichia coli, Klebsiella* spp.*, Enterobacter* spp., etc.*),* environmental streptococci (e.g., *Streptococcus uberis*, *Streptococcus agalactiae, etc.*) [15]. In addition, farm floor, pasture and cattle manure are the main sources of environmental mastitis pathogens, especially *E. coli* and *Streptococcus uberis* [68]. Major environmental pathogens causing severe damage to bovine udder include *Streptococcus uberis, Streptococcus dysgalactiae*, coliforms, and non-aureus *staphylococci* [69]. Mixed IMI of major and environmental mastitis pathogens frequently cause severe, persistent and non-responsive mastitis, with a significant increase in somatic cell count and obvious clinical manifestations [59].

Due to emerging concern of increasing antibiotic resistance, preventive strategies for controlling environmental mastitis pathogens are needed [47, 70]. Control of significant risk factors, pasture management, optimal managemental and feeding practices is a prime goal of such strategies. There are preventive mastitis vaccines in the market that are reported to reduce the infection, but unfortunately, none of them provided promising results [53]. Understanding the transmission pathways, better diagnostic tools and implementation of mastitis control program in efficient way can lead to drastically lessen the mastitis burden in dairy industry [71–73].

3.1 Major environmental pathogens

3.1.1 Environmental streptococci

Environmental streptococcal species are considered as one of the significant pathogens that cause clinical and subclinical mastitis in dairy herds. Among these, *Streptococcus uberis* is the most common mastitis pathogen that damages the bovine udder. Mastitis control measures have minimal effect on the incidence of mastitis, caused by environmental *Streptococcus* species, coliforms and some non-aureus staphylococci [74]. Dairy environment is the key risk factor that leads to the development of mastitis particularly due to *S. uberis, S. dysgalactiae (Streptococcus dysgalactiae subsp. dysgalactiae).* Other members of *Streptococcus* species that cause mild bovine mastitis are *Streptococcus sanguis, Streptococcus salivarius* and *Streptococcus parauberis* [75].

3.1.2 Escherichia coli

Mastitis is caused by multiple bacterial etiologies, where *E. coli* is known as one of the most significant causes of clinical mastitis in dairy animals, typically occurred in high producing cows as wells as cows in the early lactation period with low somatic cell counts [76]. *Escherichia coli* (*E. coli*) is a gram-negative environmental pathogen and is positive for catalase test and negative for coagulase test [77, 78]. Many animals are the carriers, but cattle are the main carriers of *E. coli*. Pathogenic strains of *E. coli* can be differentiated from the strains of normal flora on the basis of the presence of virulence factors such as adhesin proteins, antibiotic resistance, and biofilm production [79, 80]. There are distinctive *CITED2* (Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2), *SLC40A1* (Solute Carrier Family 40 Member 1), and *LGR4* (Leucine Rich Repeat Containing G Protein-Coupled Receptor 4) genes specific to *E. coli* isolated from the bovine mastitis [81]. Moreover, *E. coli* isolates from bovine mastitis cases contain a variety of serogroups [82]. It has been reported that multiplication of *E. coli* occurs in mammary secretions without its adherence to mammary glands epithelium. A study on mastitis epidemiology has revealed that the severity of *E. coli* mastitis is mainly linked with cow factors, as well as strain characteristics [83]. *E. coli* is the udder pathogen causing mastitis in dairy animals, and its endotoxin is potential health threat at consumer end [84]. Its long persistence and associated virulence factors are more often a point of concern in the dairy farm environment [85]. Toll-like receptor-4 has major role in the pathogenesis of *E. coli* in mastitis [86]. Cephalosporins and non-steriodal anti-inflammatory drugs are commonly recommended for the treatment of *E. coli* mastitis, to which microbe has evolved the resistive character [84, 87]. The chronic nature of *E. coli* mastitis deteriorates the milk quality without notice of handlers [22]. The prevalence of subclinical mastitis in different districts of province Punjab was reported to be 32% with *E. coli* as second most common isolate from samples with incidence rate of 16.18% [88]. The *E. coli* isolation rate from subclinically infected cows was 13% with subclinical mastitis 36% [89]. 25% mastitis prevalence with *E. coli* isolation rate of 18.47% in dairy buffaloes was reported by [90].

3.1.3 Nocardia *spp.*

Mastitis caused by *Nocardia* spp. is rare in cattle and presents as mastitis with extensive granulomatous udder lesions. Nocardia is gram-positive, aerobic bacteria with filamentous branches [91]. Nocardia is an ever-present environmental saprophyte with more than 30 identified species [92].

3.1.4 Bacillus *spp.*

Bacillus cereus and *Bacillus subtilis* are saprophytes and they are the only pathogens that can cause mastitis. These are responsible for acute hemorrhagic mastitis in cattle [15, 16, 93]. *Bacillus cereus* cases are usually linked with teat injury or surgical infection. Mastitis can also occur in cattle during calving and is linked with brewing grains mixed with *Bacillus cereus* spores. Several strains of the *Bacillus* species are non-pathogenic, and the isolated strains from clinically healthy bovine teat change rapidly over time [91].

3.1.5 Klebsiella *species*

Mastitis caused by *Klebsiella pneumoniae* can be severe as it responds poorly to commonly used mastitis treatment protocols and rapid progression to toxic

shock, resulting in death [94, 95]. *Klebsiella pneumoniae* is still a challenge to dairy animals and causes udder infections even after the advancement in control of mastitis [96, 97]. Mastitis caused by *K. pneumoniae* tends to be prolonged and severe because of its low sensitivity to antibiotic treatment and can lead to animal death if left untreated. *Klebsiella* species cause more losses to the dairy industry than *E. coli* in terms of mastitis [96].

3.1.6 Pseudomonas aeruginosa

Pseudomonas aeruginosa is one of the causative agents of bovine mastitis [98, 99]. Most strains of *Pseudomonas aeruginosa* have a type III secretion system that can induce an increase in the number of somatic cells count in the mastitic milk. In addition, most *Pseudomonas aeruginosa* strains can form biofilms, reducing the effectiveness of antibiotics [100].

3.1.7 Other Pseudomonas species

Pseudomonas species are potential environmental pathogens, frequently associated with wet bedding and water used in milking parlor [98, 100]. Trauma to teat ends due to improper milking increases the chances of *Pseudomonas aeruginosa* infections. *P. aeruginosa* is commonly isolated from mastitic animals and possesses different virulent factors like exo-enzyme, exotoxin A and protease that initiate an inflammatory response and cellular death [51, 101]. It can survive in different environmental conditions and infect susceptible cows through teat canal. Immuno-compromised cows, due to infectious diseases and nutritional deficiencies, are more susceptible to *P. aeruginosa* infection. This microorganism is reported as extremely resistant to commonly used antimicrobials [97]; therefore, adopting the hygienic practices, isolation, and culling of infected cows are the only available control measures [100].

3.2 Minor mastitis pathogens

Minor mastitis pathogens include a range of different environmental microorganisms including some non-aureus staphylococci and *Corynebacterium* species. Some non-aureus staphylococci are opportunistic environmental bacteria that normally reside on the nasal tissue, teats, and hands of milking personnel [102]. Non-aureus staphylococci are considered as the emerging mastitis-causing bacterial pathogens [19, 103, 104]. Non-aureus staphylococci exhibit less pathogenicity as compared to other principal mastitis-causing pathogens and infections, most of the time remain subclinical. However, persistent non-aureus staphylococci infection can lead to reduced milk production and milk quality, increased somatic cell count, and severe damage to the udder [105]. *Trueperella pyogenes* causes summer mastitis and low-grade mastitis in the cows, often being clinically well but with a very enlarged and painful quarter [106]. Despite the high-frequency isolation, non-aureus staphylococci are considered minor mastitis pathogens but still a significant challenge for dairy farmers [12, 107].

4. Other mastitis pathogens

Some members of *Enterococcus* species like *Enterococcus faecalis, Enterococcus saccharolyticus* and *Enterococcus faecium* cause bovine mastitis [75]. Moreover, *Aerococcus viridans* has also been reported as a causative agent of mastitis, but its potential role has not been elucidated yet [108].

5. Conclusion

Mastitis is the most common and economically important disease for dairy industry, regarding milk quality and quantity. Microorganisms enter the udder and multiply in the glandular parenchyma, producing toxins that cause direct harm. Bovine mastitis is caused by a wide range of environmental and contagious pathogens. Contagious pathogens are those whose main reservoir is infected udder of an animal. The major contagious agents include *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Mycoplasma* species. On the other hand, environmental mastitis is caused by pathogens such as *Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus uberis, Trueperella pyogenes, Enterobacter aerogenes, Klebsiella* species*,* some yeast, fungi and *Pseudomonas* species. Mammary gland infections caused by these pathogens are of short duration and have severe clinical presentation. Environmental pathogens are usually linked with unsanitary managemental practices, resulting in the clinical symptoms (udder/quarter swelling, abnormal milk quality and quantity, and anorexia). Due to emerging concern of increasing antibiotic resistance, preventive strategies for controlling mastitis pathogens are needed. Control of significant risk factors, pasture management, optimal sanitary and feeding practices is a prime goal of such strategies. There are some mastitis vaccines against specific bacterial pathogen in the market that are reported to reduce the challenge, but unfortunately, none of them has provided promising results against all mastitis pathogens. Understanding the transmission pathways, better diagnostic tools and implementation of mastitis control program in efficient way can lead to drastically lessen the mastitis burden in dairy industry.

Conflict of interest

Authors declare no conflict of interest.

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Section 2

Ovine and Caprine Mastitis

Mastitis in Small Ruminants

Christine T. Mwenge Kahinda

Abstract

Bacterial mastitis in small ruminants is a complex disease, with massive economic loss in dairy sheep/goat industry due to poor productivity. The current mastitis prevention strategy relies on culling of infected ewes or does and or the use of antimicrobial agents to eliminate the bacterial infection. This has a potential risk for developing antibiotic resistant bacteria, posing human health risk from consumption of raw sheep or goat dairy products. Existing experimental and licensed vaccines on the market are ineffective against reducing the risk of mastitis in herds or flocks. Raising the needs for development of improved vaccines against mastitis for use in sheep and goats. This review examines, current understanding of the pathological processes and immunological responses against bacterial mastitis, using *S. aureus* as an example. By highlighting the protective defense mechanism induced in the udder against *S. aureus* mastitis. Based on evidence from published studies on pathological process and protective immune response mechanism, the need for improved vaccines for prevention of mastitis in small ruminant is highlighted and the development of a vaccine capable of enhancing immune response mechanism, that reduce the establishment of intramammary infection through induction of local IgA, IgG2 and Th17 immune responses is proposed.

Keywords: Mastitis, *S. aureus*, *Pathogenesis*, pathophysiology, vaccination

1. Introduction

Mastitis is a complex disease that results in inflammation of the mammary glands due to infection or mechanical injury. Host, pathogen, and environmental predisposing factors play major role in the development of mastitis. Most cases of mastitis of small ruminant occur as a result of bacterial intramammary infection (IMI), which are generally a contagious infection resulting from mammary and cutaneous carriages of bacterial agents and or spreading of bacteria during the milking process. The inflammatory response induced by the host is aimed at removing the irritant and repairing damaged mammary gland tissues to ensure normal functioning of the udder. Inability of the host to control IMI leads to persistent inflammatory response (chronic mastitis) that leads to premature culling of the affected ewes [1], loss of udder function, reduced milk yield [2], and quality [3, 4] and occasionally death. In addition, reduced milk production also affects livestock productivity, as it results in lower growth rates of suckling lambs [1]. As such, mastitis of small ruminant has a significant economic impact in the livestock industry. Mastitis in small ruminants caused by bacterial intramammary infections presents in two forms i.e., subclinical mastitis or clinical mastitis as depicted in **Table 1**, with varying severity from acute infections that last for short period of time to chronic

Ruminant	Inflammatory Changes		
	Milk	**Clinical Manifestation**	**Udder**
Sheep	No visible abnormality; high bacterial count; reduced milk production; SCC > 500 x10^3 cells/mL; changes in milk composition	Subclinical	No visible signs of inflammation;
	Visible changes in milk e.g., may be blood tinged or yellow, may be thick, "lumpy", or very watery	Clinical	Visible abnomalities in the udder; udder may be firm, swollen etc. the gravity of the abnomality varies based on disease severity.
	May contains clots, flakes, or discolored secretion	Subacute (mildly clinical)	Swollen, red udders, hot and painful to the touch; hard sensitive udder;
	Reduced milk secretion, contains clots, flakes, or discolored secretion; appears watery	Acute (sudden onset of inflammation, can be fatal)	Swollen; hot; red and painful to touch
	Abnormal milk appearance; bloody fluid	Peracute (Severe inflammation, fatal or loss of affected udder)	Visibly abnormalities; swollen; cold; blue/black; may slough off; gangrenous
	May have no milk production; reduced yield and composition; contains purulent material (pus);	Chronic	Hard or lumpy; abscesses; may have scars; may be fibrotic; swollen teat; may contain a hard core of pus; asymmetrical appearance; enlarge or shrunken;
Goat	No visible abnormality; But laboratory test present with: high bacterial count; reduced milk production; reduced antioxidant content; changes in milk composition	Subclinical	No visible signs of inflammation;
	Visible abnormalities in the milk (varies based on severity); increase in whey proteins; increase in albumin concentration; a reduced lactose concentration and milk fat; increase electrical conductivity	Clinical mastitis	Visible abnormalities in the udder (varies based on severity)
	Contains clots, flakes, or discolored secretion;	Subacute (mildly clinical)	slightly swollen and tender
	Reduced milk secretion, contains clots, flakes, or discolored secretion; appears watery	Acute (sudden onset of inflammation, can be fatal)	Swollen, red udders, hot and painful to the touch
	Serum-like milk secretion. Milk may appear reddish and may contain gas	Peracute (Severe inflammation, fatal or loss of affected udder)	Swollen, discolored (reddish to purple/black), cold to touch; may be gangrenous
	Milk may contain flakes, purulent material (pus) and be discolored; Reduced yield and composition	Chronic (persistent infection, may be incurable and recurring)	Hard, fibrotic, abscesses shrunken or lumpy

Table 1.
The different forms of mastitis in small ruminant and plausible signs in relation to the severity of infection.

infection which are persistent and long term. Herein, we discuss mastitis in small ruminant, focusing on sheep and goat.

1.1 Sheep

The most common form of mastitis in sheep is subclinical mastitis, with a reported prevalence of 5–30% [5] and sometimes up to 50% [6]. Subclinical mastitis is difficult to identify, mainly due to lack of clinical signs. Subclinical mastitis can only be detected by milk bacteriological test or somatic cells count (SCC) (i.e., inflammatory cells and some epithelial cells) [7–10]. In sheep a SCC of >500 x 10^3 cells/mL of milk [7, 8] and or a positive California Mastitis Test (CMT) [9, 10] is considered subclinical mastitis. In most cases, ewes with subclinical mastitis appear healthy, but have decrease milk production [11, 12] and changes in the composition of milk due to the inflammation. Subclinical mastitis may affect lambs of infected ewes by causing them to have a poor growth rate, lower weaning weight and occasional death [11, 13]. As such, subclinical mastitis has significant financial implications for both dairy sheep flocks due to its impact on milk production and quality [11, 12] in meat-producing sheep flocks as it affects lambs growth rate and weaning weight [11, 13].

Subclinical mastitis due to bacterial IMI may progress to acute or chronic mastitis. Progression of subclinical mastitis to clinical mastitis can occur as a result of the following events. 1) Subclinical mastitis-causing bacteria can be transmitted from an infected under to an uninfected udder during milking as a result of poor hygiene practices by milkers whereby they can transmit the infecting bacteria from their hands or from using contaminated shared milking equipment and udder washcloths. These practices provide the bacteria access to the teat canal, where successful bacterial growth subsequently results in mastitis. 2) Nutritional deficiency adversely affects the animal host defense mechanism, and may promote disease progression to clinical mastitis. Katsafadou et al. [14], associated nutritional deficiencies with impaired leucocyte function or mammary defense. Here nutritional elements such as Selenium, Zinc, vitamin E and Vitamin A deficiencies have been linked to an increased risk of mastitis in ewes [14–16]. For example, Selenium and Vitamin E are important in maintaining neutrophil function [17], they are known to protect leucocytes against reactive oxygen species (ROS)-induced damage [14–16]. Zinc forms part of teat keratin and skin, it has been suggested that deficiency in Zinc and vitamin A negatively affects the integrity of the teat and epithelia [14–16]. This could allow the colonization of the teat by infecting bacteria, coupled with other deficiencies that result in the establishment of clinical mastitis.

Clinical mastitis usually occurs in less than 5% of mastitis cases in sheep [5, 10, 18]. However, clinical mastitis in sheep is often observed as sporadic cases or during occasional herd outbreaks [5, 10]. Clinical mastitis can transition from subacute to chronic with increasing disease severity. Clinical mastitis is easy to identify, it presents with clearly observable clinical signs and physical changes in the udder, such as blue discoloration of the udder. Udders with clinical mastitis are usually swollen and sometimes painful to the touch. sheep affected with clinical mastitis go off feed, are lethargic, and often refuse to allow their lambs to nurse, resulting in lower growth rates of suckling lambs. The appearance and composition of milk obtained from ewes affected by clinical mastitis is abnormal, it may be discolored, watery, may contain blood or serum, may be foul-smelling if it contains pus and has visible clots or flakes.

1.2 Goats

The prevalence of subclinical mastitis in goat is 5–45% [5, 19]; some authors suggest it's 15–40 times more prevalent than clinical mastitis [20]. As in other ruminants, subclinical mastitis in goat is difficult to identify by clinical signs. Subclinical mastitis in goat presents with high bacterial count in milk; reduced milk production and quality, as well as a high SCC. However, SCC are not reliable indicators of subclinical mastitis diagnosis in goat [21, 22]. Generally, healthy goats have a higher milk SCC compared to sheep, and other ruminants such as cows. In addition, the number of SCC in goat milk various based on stage of lactation, SCC has been reported to reach 3.6×10^6 cells/ mL at end of lactation [23]. Some have reported a SCC $\geq 10^6$ cells/mL as an indication of subclinical mastitis in goat; however, this set minimum is usually combined with a bacteriological test to confirm diagnosis. SCC alone is not used to diagnose subclinical mastitis in goats, as shown by Hussein et al. [21], SCC $\geq 10^6$ cells/mL threshold was unable to differentiate subclinical mastitic goat from healthy goats, thus confirming the use of bacteriological test as the most reliable indicator of subclinical mastitis in goats [21].

In goat subclinical mastitis usually precedes clinical mastitis, as its act as a source of infection for healthy animals [19]. Clinical mastitis presents with visible abnormalities in the udder and or milk that varies based on the severity of the infection, as mentioned in **Table 1**. Clinical mastitis in goats is also classified into four groups based on disease severity, i.e. subacute, acute, peracute and chronic. As mentioned in **Table 1**, above, this ranges from a mild infection to severe, presenting with pain, heat, swelling, redness, and reduced and abnormal secretion such as clots, flakes, or watery milk. May develop fever, depression, weakness, anorexia and may be fatal.

2. Important bacterial pathogens for vaccine development against mastitis in small ruminants

Several bacterial agents are associated with clinical or subclinical mastitis in small ruminants [5]. A very exhaustive list of gram-positive and gram-negative bacteria has been implicated in mastitis of sheep and goats. However, for the purpose of this review, the most implicated organism with potential for commercial vaccine development against mastitis of both sheep and goats will be discussed.

2.1 Sheep

Over 30 bacterial species have been isolated from sheep with mastitis [1, 24–26]. The most implicated organisms in sheep mastitis are *Staphylococcus aureus* [24, 27, 28]; *Mannheimia* spp. [29] *Streptococcus* spp. [30, 31]; and non-*aureus* staphylococci.

S. aureus is a zoonotic, Gram-positive bacteria that occurs as a mammalian commensal and opportunistic pathogen. *S. aureus* is the most common cause of mastitis in sheep and the major mastitis-causing agent isolated in 70% of clinical mastitis cases in dairy flocks [10, 24]. It is responsible for about 40% of mastitis cases in ewes suckling lambs and 80% mastitis cases in milking ewes [24, 27, 32]. Cases of mastitis due to *S. aureus* ranges from subclinical mastitis to severe gangrenous mastitis. It is important to note that *S. aureus* subclinical mastitis infections are extremely difficult to treat, cure and are highly contagious. As such, animals with *S. aureus* mastitis are culled or milked last to prevent spread of infection to other members of the herd or flock.

Other non-aureus staphylococci, are associated with subclinical intramammary infections [33], of these, *Staphylococcus epidermidis* is the most common species associated with ovine mastitis [34].

M. haemolytica is an aerobic, non-motile, bipolar, gram-negative rods, non-spore-forming opportunistic bacterium carried in the nasal and nasopharyngeal cavities of healthy animals. *M. haemolytica* is the most common cause of mastitis in meat and wool sheep producing systems [1, 29, 32]. *M. haemolytica* causes severe clinical mastitis, where the infected mammary glands are greatly enlarged, tense, blue-black, with watery secretion containing flakes and widespread gangrenous necrosis of the udder [29]. In some flocks *M. haemolytica* mastitis is more significant than *S. aureus* induced mastitis, due to its transmission by suckling lambs [29]. As a result, pneumonia may be observed in suckling lambs of ewes with *M. haemolytica* mastitis. Based on a 2008 research study by Arsenault *et al.*, [1], the prevalence of *M. haemolytica* clinical mastitis is similar to *S. aureus* mastitis in meat-producing flocks, thus making it a significant organism in the etiology of mastitis in sheep.

Streptococcus spp. are zoonotic, anaerobic, non-spore-forming, Gram-positive, catalase-negative, homofermentative, spherical or ovoid cocci that occur as single or in pairs or chains [23]. They are usually responsible for sporadic outbreaks of mastitis in sheep and goats [30, 31, 35, 36]. Mastitis due to *Streptococcus* spp. occurs at a rate of 23–31% in flocks [25, 37]. Mastitis caused by *Streptococcus* spp. are more frequent in machine-milked flocks [25, 38, 39] or as a result of poor hygiene during milking [40], suggesting that proper milking practices may reduce the incidence of mastitis due to streptococci. *S. agalactiae*, *S. uberis*, *S. dysagalactiae* and *S. equi subsp.zooepidemicus* are the most isolated *Streptococcus* spp. causing mastitis in dairy ruminants [41].

2.2 Goats

Several bacterial species have been isolated from goats with mastitis, for example. *Staphylococcus* spp.; *Streptococcus* spp.; *Bacillus* spp.; *Listeria* spp.; *Corynebacterium* spp.; *Pseudomonas* spp.; *Mycoplasma* spp.; *Mannheimia haemolytica; Clostridium perfringens* and *Escherichia coli* [5, 10, 22, 25, 41–45]. However, non-*aureus* staphylococci are the most prevalent causative agent of subclinical mastitis in goats [10, 22, 42, 46–48] and *Staphylococcus aureus* is considered to cause the highest pathogenicity in goat mastitis, along with minor prevalence of *Escherichia coli*, *Streptococcus agalactiae, Mannheimia haemolytica* [10, 22, 42, 45–49]. *S. aureus*-induced mastitis in goat ranges from subclinical to gangrenous mastitis, which is the most severe form of the disease [45, 47]. As with sheep mastitis, *S. aureus* mastitis are difficult to treat, infected goats act as reservoir and source of spread.

Non-aureus staphylococci are the most prevalent bacteria from goats with subclinical mastitis, with up to 100% incidence [10, 48, 50–52]. The most isolated bacteria from these species are *Staphylococcus chromogenes, Staphylococcus epidermidis, Staphylococcus simulans, Staphylococcus xylosus*, and *Staphylococcus caprae* [10, 52–54]. *S. chromogenes* and *S. epidemidis* are associated with higher milk SCC [53] compared to other non-*aureus* staphylococci, however, the increase in SCC is lower than in *S. aureus* mastitis [10]. *S. caprae* and *S. simulans* have been linked to persistent mastitis in goat [55]. These bacteria do not produce coagulase, an enzyme responsible for prothrombin activation leading to the coagulation of plasma [56, 57]. They are opportunistic bacteria with the ability to produce biofilms [58], which enables these bacteria to persist on milking equipment, serving as a source of spread to other animals in cases of poor hygiene and milking practices. Non-*aureus* staphylococci have been reported to carry a wide range of antimicrobial resistance genes, which allows for persistent infections [59].

For vaccine development against mastitis in sheep and goat, it is imperative that intension and efforts are made towards the development of a vaccine containing

bacteria that have the highest pathogenicity or prevalence and has potential to impact human safety due to their zoonotic nature such as; *S. aureus*.

3. Pathogenesis of bacterial intramammary infection in small ruminant, using *S. aureus* as an example

The pathogenesis of bacterial intramammary infections in small ruminants is very complex. It is dependent on the infecting bacteria, bacterial virulence factors, and the interaction of these virulence factors with the host immunological response in the udder. Of the various bacteria known to cause mastitis in sheep and goats, *S. aureus* is used in this paper as an example to describe the pathogenesis of intramammary infection in sheep and goats, with emphasis on pathological processes that are essential for vaccine development.

The pathogenesis of *S. aureus* mastitis is very complex. It is associated with various surface proteins and virulence factors that are differentially expressed at various phases of the infection. This process entails three key steps, that is adhesion, invasion and evasion. In brief, the first step in the pathogenesis process is adhesion to epithelial cells and extracellular matrix, which permits the bacteria to avoid being flushed out of the udder from milk flux pressure [60]. During this step *S. aureus* expresses several virulent factors involved in adhesion, such as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM), e.g. protein A, elastin-binding proteins, collagen-binding protein etc.; surface-associated capsule (which inhibits phagocytosis and promotes adhesion); peptidoglycan (which activates complement); teichoic acids (involved in adhesion and colonization, cell division and biofilm formation) [60–64]. Here, the formation of biofilm protects *S. aureus* from host immune response or antibiotics [65, 66]. In the second step of this process, *S. aureus* again expresses different virulence factors to establish infection by invasion into host cells and tissues. This step or phase entails penetration and destruction of mammary glands tissues by the bacteria and involves the expression of the following virulence factors: haemolysins (i.e., alpha, beta, gamma & delta, these lyse cells); leukocidin (damages polymorphonuclear leucocytes); panton-valentine leukocidin (a β-pore-forming toxins); enterotoxins (heat stable toxin); epidermolytic toxin (this is a serine protease that causes splitting of desmosomes or intercellular bridges in the stratum granulosum); toxic shock syndrome toxin (TSST-1, causes leakage of endothelial cells and penetration of mucosal barrier) [60–64]. Together, these virulence factors result in damage to the epithelium of the cistern, duct and alveoli and perpetuate the infection, eventually leading to the clinical signs observed during mastitis. Subsequent to these events or perhaps in unison, the final step in the pathogenetic process is an evasion of the host immune response. Here, *S. aureus* escapes the host immune response by producing the various virulence factors that helps it not only to evade but also modulate the host immune response in its favor. For example: enzymes such as coagulase (that activates a coagulase reacting factor (CRF), which is believed to coat bacteria with fibrin to prevent opsonisation and phagocytosis); staphylokinase (fibrinolysin); Hyaluronidase (hyaluronic acid, which facilitates the spread of *S. aureus* through tissues); deoxyribonuclease; lipase; phospholipases; proteases; and again other already expressed virulence factors that were released during the adhesion and invasion process, these are continuously differentially expressed as required to anchor the infection and avoid elimination of *S. aureus* by mammary gland immune responses. *S. aureus* virulence factors target the main cells involved in mammary immunity, such as neutrophils and macrophages by counteracting their actions [60].

3.1 Immune response and pathological process in the mammary gland

3.1.1 Teat and teat cistern

The teat canal is the first physical barrier that bacterial agents meet before they can spread into the mammary glands [67–70]; protection against bacterial agents is provided by bacteriostatic fatty acids that are present in the keratin plug in the teat canal [68–70]. As such, invading microbial organisms are trapped in the lining of the teat canal by these hydrophobic lipids. The trapped microbial organisms are flushed out together with teat canal epithelial cells during the first outflow of milk. In addition, ewes teat are known to close 20 to 30 minutes; however, total closure occurs two hours post milking [69], as part of the first line of defense against invading microbial organisms. For this reason, it is recommended to move ruminants to clean areas after milking and provide feed to avoid laying down and exposure to contaminating environmental microbial organisms until teats end close [69]. If bacteria gain access to the teat canal, the bacterial adherence property is used to establish infection. As mention previously, using *S. aureus* as an example, MSCRAMM and capsular proteins are differentially expressed to permits attachments of the bacteria to the epithelial tissues. This mechanism is not only employed by *S. aureus* but other mastitis causing bacteria such as, M. *haemolytica* [71], *Streptococcus* spp. [72]. Therefore, adherence of microbial agent to teat epithelial tissue permits them to invade or penetrate this protective barrier and migrate to the teat duct.

3.1.2 The teat duct

Once the bacteria reach the teat duct, a cascade of complex sequence of events determines the outcome of the immune response induced. Here, somatic cells/leukocytes present in milk and component of the innate immune response in the teat duct act as a defense against any invading bacteria that has managed to by-passed the physical barrier in the teat cistern. The milk leukocytes act as phagocyte and secrete an array of immune-related components in milk, such as cytokines (e.g. TNF-*α*, IFN-*γ*, GM-CSF, IL-8, and IL-12), chemokines, reactive oxygen species (ROS), and antimicrobial peptides (Lactoferrin, defensins, and cathelicidins) [73]. In addition, inducible lymphoid nodules (containing B and T lymphocytes, as well as immune cells that express major histocompatibility complex (MHC) class II) that are present in the teat duct act in synergy with viable milk leucocytes to get rid of the invading bacteria. Based on this, an array of multiple cells are involved in the immune response of the teat duct against invading bacteria, these includes neutrophils, macrophages, αβ T cells, γδ T cells, B cells and inducible lymphoid cells etc. [69, 73].

A plausible scenario for the sequence of events that occurs when a bacteria is invading through the teat duct could be summarized as follows. As the bacteria invade the teat duct to colonize and establish infection, it releases a mirage of virulent factors, amongst these pathogen-associated molecular patterns (PAMPS) such as peptidoglycan and lipoteichoic acids in the case of *S. aureus*. These are recognized by Toll-like receptor (TLR)-2 on the surface of epithelial cells lining the teat ducts [70, 74, 75]. TRL2 stimulation leads to the release of IL-8; CCL2 and CCL4 [69, 74, 76]. IL-8 is a potent chemo-attractant and activating factor for neutrophils. CCL2 and CCL4 are chemoattractant for monocytes and macrophages [69, 76]. *S. aureus* PAMPs can also be recognized through formylated peptide receptors, mannose-binding lectins (MBL), ficolins, and complement molecules [70], resulting in the activation of the complement system leading to ingestion and killing of *S. aureus*. In addition, B cells in milk and milk macrophages process antigens from invading

bacteria and present these antigens in association with MHC class I or II on their membranes to different T cells.

As part of the innate response, activated neutrophils and tissue macrophages migrate into mammary glands to eliminate the invading bacteria and initiate the inflammatory response. The outcome of these early events results in increased neutrophils in the milk and an elevation in SCC, seen in subclinical mastitis, under normal state the udder tissue and milk mainly contain macrophages and during infection neutrophils are dominant in the udder tissue and milk [70].

Neutrophils/PMNs (Polymorphonuclear neutrophils) are the first recruited cells at the site of infection and are known to form part of the earliest protective response against bacterial mastitis in ruminants [77]. Their primary function is to engulf, phagocytose, and destroy invading bacteria. This is done through two pathways, the oxygen-dependent (respiratory burst, which includes the production of hydroxyl and oxygen radicals) pathway and or the oxygen-independent (which uses peroxidases, hydrolytic enzymes) pathway. Neutrophils also modulate vascular permeability and release a variety of inflammatory mediators that coordinate both the innate and adaptive immune response [69, 74]. In addition, neutrophils contain bactericidal peptides such as defensins; myeloperoxidase; S100-A9 protein, elastase; cathepsin types B, D, and G; procathepsins within their intracellular granules, these peptides can kill a variety of mastitis pathogens [69, 78–80]. However, neutrophil release oxidants and proteases are non-specific, as such they may also contribute to host mammary epithelium damage, and e.g., hydroxyl radicals may damage mammary epithelium [81]. Neutrophils undergo apoptosis or programmed cell death after completing their task and are removed by macrophages [81].

Milk macrophages and recruited macrophages (blood monocytes that differentiate in mammary tissue) act as antigen-presenting cells (APCs), by processing and presenting antigens to CD4+ T cells in association with MHC class II [82]. These macrophages ingest and phagocytose the invading bacteria, destroying them with proteases and ROS. However, as with neutrophils, macrophages also contribute to mammary gland epithelial damage due to their non-specific killing with proteases [83]. Macrophages have been shown to be inefficient in killing some mastitis pathogen by promoting their multiplication intracellularly [83].

In healthy udders, the predominating lymphocytes are αβ T cells, with a CD3+ CD8+ phenotype, that act as cytotoxic or suppressor T cells [84]. γδ T cells mediate cytotoxicity in a none restricted manner, with variable involvement of MHC molecules and also play a role in antibacterial immunity through production of granulysin [73, 85] expression of CD95L and TNF-related apoptosis-inducing ligand, that engage with several death receptors on target cells [85]. In addition, activated γδ T cells stimulate other immune cells e.g., dendritic cell maturation, through the production of TNF-α [85], IFN-γ, and IL-17 [86]. In mastitis, these cells form part of the early response as is reported in a mouse experimental mastitis study, where infection with *S. aureus*, induced an early influx of γδ T cells producing IL-17 into the mammary glands [87]. IL-17 activates mammary epithelial cells and enhances neutrophil infiltration through an expression of CXCL1, CXCL2, and CXCL5 chemokines. This enhances host clearance of the invading bacteria [87, 88]. It has also been suggested that γδ T cells plays a role in repairing damaged mammary gland tissues during and after mastitis [89].

Whereas, αβ T cells recognize an antigen through membrane receptors, as such their specificity, diversity, and memory features are defined by the type of receptor they used to recognize antigens [73].

The efficiency of phagocytic killing culminating from events mentioned above determines the severity of the disease being established, i.e., the disease progression from subclinical mastitis to gangrenous mastitis.

Mastitis causing bacteria may by-pass the natural defense and innate immunity in the teat canal and establish infection in the intramammary area. Indeed, mastitis-causing organism, such as *S. aureus* secretes an array of virulence factors to facilitate invasion and deeper penetration into the mammary glands. *S. aureus* secretes cytolytic toxins such as α, β, γ, δ-haemolysins [90], phenol soluble modulins (PSMs) and bi-component leukocidins. These exert their role through pore-forming on host immune cell membrane, causing osmotic leakages of cell content, leading to lysis of neutrophils, monocytes, platelet, and erythrocytes. *S. aureus* also engages a wide range of virulence factors to restrain neutrophil activation, chemotaxis and phagocytosis and also target key host effector proteins that are released by host immune cells. For example, extracellular fibrinogen-binding (Efb) protein, coagulase (Coa), extracellular matrix-binding protein (Emp), extracellular adhesive protein (Eap), chemotaxis inhibitory protein (CHIPS) and staphylococcal complement inhibitor (SCIN) proteins. For example; Efb plays an immunosuppressive role by interfering with the complement system, it has been reported to significantly exacerbate *S. aureus* infections, impairs wound healing, and inhibit platelet aggregation and thrombus formation [91]. Through this mechanism, Efb facilitate *S. aureus* survival and persistent infection. CHIPS, is a potent inhibitor of neutrophil and monocyte chemotaxis towards C5a and formylated peptides [92]. Furthermore, macrophages also synthesize complements, such as component 3 (C3) in the mammary gland. These complements are involved in evoking and controlling the inflammatory process, bacterial opsonization and presentation, leukocytes recruitment and killing of microbial agents in the mammary glands [93, 94].

In a nutshell, *S. aureus* virulence factors promote the establishment of mastitis in the udder, through secretion of an array of virulence factors that facilitates adherence to mammary epithelium, invasion of mammary glands, and evasion of mounted host immune response mechanism against it by modulating counter-responses, e.g. *S. aureus* expresses T-cell superantigens such as, TSST-1, staphylococcal enterotoxin A, staphylococcal enterotoxin B, etc.) that bind to a specific subset of the variable Vβ chains of the T-cell receptor (TCR), leading to polyclonal proliferative responses and clonal deletion of T lymphocytes [95].

3.1.3 Alveoli

Mechanisms of the innate immune response previously described remain the same, but failure to resolve the induced inflammatory response lead to engagement of the adaptive immune response to eliminate the bacteria. *S. aureus* protective immune response mechanism entails both arms of the adaptive immune response, i.e. cell-mediated and humoral mediated arms of the immune response play a role in the clearance of bacterial mastitis. Numerous studies have shown the role of both T helper cells, cytotoxic T cells and B cell responses in clearance and resolving of bacterial infections. However, these cells have also been implicated in disease pathogenesis.

Using our previous scenario, once the bacteria reach the alveoli, the mammary epithelial cells lining acts as defense mechanism against the invading bacteria triggering a cascade of immunological responses directed against the invading bacteria's virulence factors. In addition, the magnitude of the induced immunological response determines the outcome of the inflammatory response (mastitis), i.e., the early expression of various inflammatory reaction modulators play a role in the severity of the subsequent inflammatory response e.g., IL-1, IL-8, IFN-γ, TNF-α, and G-CSF enhanced the activation of neutrophils, whereas IL-12, M-CSF, and GM-CSF stimulate enhanced activation of macrophages; IL-2 and IL-6 Stimulates

B cells differentiation, and so forth [96] and the type of effector T cell response induced is also related to the cytokines in its surrounding.

As the bacteria continue to proliferate in the alveoli, the mammary epithelial cells lining the alveoli have various role in protection against invading microbial organisms. For instance: 1) the mammary epithelial cells sense and recognizes microbial agents through pattern recognition receptors (PRRs), e.g., these cells are known to express TLR2 and TLR 4 [97, 98]. These cells recognize invading bacteria via the MyD88- dependent TLR (Toll-like receptor) signaling pathway [74, 79], 2) the mammary epithelial cells synthesize inflammatory mediators and antimicrobial peptides upon recognition of microbial organisms. According to various mastitis studies and reports, mammary epithelial cells produce cytokines such as, interleukin-8; chemokines such as, CCL5; β-defensins; haptoglobin; cathelicidin; lactoferrin; lysozyme, and serum amyloid A [42, 99–101]. The release of these inflammatory mediators activate local leukocytes that are normally present in the mammary gland tissue, such as macrophages, dendritic cells, and intra-epithelial lymphocytes, and also immune cells in milk and circulating immune cells, such as neutrophils (polymorphonuclear neutrophils (PMN)), and cytotoxic natural killer cells.

The adaptive immune response is initiated by activated macrophages; these produce cytokines and chemokines, antimicrobial peptides, such as, β-defensins and cathelicidins [102, 103]. In addition, macrophages process and present antigens through MHC class I or II mechanisms.

Macrophage present antigen through MHC class II to naïve circulating T helper cells. Upon activation, these cells subsequently differentiate into antigen-specific effector T helper cells based on the type of cytokine in the immediate surrounding, e.g., presence of IL-12 surrounding will induce polarization of CD4+ T helper 1 (Th1) cells, IL-4 will induce polarization of Th2 and a combination of IL-4, TGF-β, IL-22 induces Th9 polarization, whereas IL-1β, IL-6, TGF-β & or IL-23 combination induces polarization towards Th17 [104, 105] and so forth, thereby inducing specific local immune responses. The antigen-specific activated T-cell clonally expands into effector cells that produce specific cytokines which activate and induce polarization of other cells that participate in the immune response. These cells eventually differentiate into memory cells. Mastitis results in changes to cytokines concentration in the milk and udder; these changes are reported to differ based on the infecting bacteria. In *S. aureus* mastitis, protective immune responses that leads to eventual clearance of the bacteria, is facilitated by an initial increase in the numbers of activated Th17, Th1, Th2 cells associated with an increase in pro-inflammatory cytokines in the mammary gland, followed by an increase in Treg and anti-inflammatory cytokines IL-10 [88]. The study conducted in an *S. aureus* mastitis mice model showed that the frequency of these cells changed throughout the course of infection [88]. Whereby Th 17 cells producing IL-17 are increased in the early phase of a *S. aureus* mastitis infection, along with an increase in Th1 cells [88]. As previously mentioned, IL-17 enhances neutrophil infiltration facilitating bacterial clearance. IL-2, TNF-β and IFN-γ produced by Th1 cells promote the activation and proliferation of cytotoxic lymphocytes, natural killer cells, and macrophages. As the infection progress, the effector T helper cell response shifts to a Th 2 response, this is thought to limit the tissue damage due to the inflammatory response, Th2 secreted cytokines such as, IL-4 which regulate macrophage functions, and inflammatory cytokines were increased. IL-4 increase expression of IL-10 [106], which inhibits IL-17 expression [88, 107]. As part of the protective immune response against mastitis, Zhao et al. [88], reported that Treg cells and IL-10 tightly regulate the inflammatory response to mastitis, as observed after the peak of infection in mice *S. aureus* mastitis model.

Sometimes during infection, extracellular bacteria such as *S. aureus* avoid being targeted by the host response and persist by invading cells and multiplying within them, becoming an intracellular infection. In addition, phagocytosed *S. aureus* is able to replicate and multiply in phagocytic cells such as macrophages [108, 109]. Here, bacterial antigens are processed and presented on MHC class 1, where they are recognized for killing by antigen-specific cytotoxic T cells (CD8+), releasing *S. aureus* for the second round of opsonophagocytosis [110]. Activated antigen-specific CD8+ cytotoxic T-cells mainly produce IFN-γ and CD8+ suppressor T-cells produce regulatory IL-4. The severity of the disease outcome is dependent on the efficiency of phagocytic killing that occurs and this as already mentioned, is dependent on the early expression of the various inflammatory mediators [96]. Therefore, as the inflammatory response amplifies, with increasing migration of phagocytic cells and antigen-specific cytotoxic T cells to the site of infection, *S. aureus* has the ability to form abscess and release a wide variety of virulence factors such as haemolysins (alpha, beta, and delta) [60–63], T cells superantigens (enterotoxins, TSST-1) [60–63] and several others as mentioned in Section 3 above. These virulent factors enable the bacterium to evade detection by the immune system and inhibit the host immune response by destroying immune cells.

The humoral response plays an important role in the prevention and control of bacterial infections. Three different classes of immunoglobulins, i.e., IgG (subclass: IgG1, IgG2, IgG3), IgM, and IgA, play significant roles in mammary gland defense against bacterial pathogens. In sheep the predominant immunoglobulin G is IgG1, followed by IgG2 then IgG3, however this is dependent of the infecting organisms. IgG1 producing plasma cells are associated with a Th2 response whereas IgG2 producing cells are associated with a Th1 response. It has been suggested that immunoglobulins in colostrum and milk, are transported from blood into the mammary secretions as part of normal physiological process during colostrum and milk production or through leakage into the mammary gland during inflammation. For example, during normal physiological process, such as colostrum or milk production, blood derived IgG1 specific to intestinal antigens is trafficked into the mammary glands, blood derived IgG1 is produced by plasma cells derived from stimulated B lymphocytes of the Peyer's patches [111–114], and has no major role in intramammary infection. However, blood derived IgG2 leaks into the mammary glands during inflammation and is though to play a significant role in intramammary infection, as it is produced by plasma cells in the skin-associated lymphoid tissue and regional lymphoid tissues [111–114]. Blood derived IgG2 has specificity to bacterial antigens associated to skin infections [111–114]. In addition, during intramammary infection, antigen-activated plasma cells from regional lymphoid nodes present within the mammary glands produce IgA, IgM and IgG2 that are specific for antigens present in the mammary gland [111–114]. IgG1, IgG2 and IgM function by opsonising, invading bacterial pathogens and make them detectable by neutrophils and macrophages for opsonophagocytic destruction [115]. Phagocytosis by PMN is regarded as one of the most important defense mechanisms of the mammary gland. However, this defense mechanism can be hindered by toxins produced by *S. aureus* such as leukotoxin. IgA acts as a neutralizing antibody to protect the mammary gland against bacterial toxins [114]. In addition, IgA prevents the establishment of mastitis in the mammary gland through complement fixation, prevention of adhesion of pathogenic microbes to the endothelial lining by binding various adhesion receptors, and inhibition of bacterial metabolism by blocking enzymes [113], such as Staphyloccocus Enterotoxins. IgA also acts in bacterial agglutination, limiting bacterial dissemination and colonization [114, 116]. Immunoglobulin, specifically IgA, may play a very import role in protection and prevention of mastitis in small ruminants [113, 114].

4. Approach to new vaccine developments for the prevention of mastitis

Vaccination is a control strategy used to increase the adaptive immunity of the animal in order to prevent new infections. The purpose of using vaccines is to enhance immunity and reduce the reliance on the use of antimicrobial drugs (antibiotics), more so in the case of mastitis in sheep and goats, as the use of antibiotics in treatment may result in antimicrobial resistance, e.g., antibiotics resistance in *Staphylococcus* spp., such as methicillin resistant *Staphylococcus aureus*. This poses a human health risk, especially because most mastitis-causing bacteria are zoonotic, and some have been reported as cases in humans due to consumption of raw sheep and goat dairy product [117–121]. In addition, there are very few veterinary pharmaceutical products licensed for specific use in sheep and goats globally. Furthermore, the use of non-steroidal anti-inflammatory agents to allevi-ate clinical signs of mastitis and improve animal welfare [122], has no impact on milk quality. As such, alternatives strategies are needed to prevent mastitis in small ruminants. Several experimental vaccines against mastitis, based on formalin-inactivated whole cells, whole-cell lysate, polyvalent whole-cell Bacterin cultures of the vaccine strains or bacteria of interest, produced using old technologies, have been shown to play a role in mastitis prevention, by reducing the severity of clinical and subclinical mastitis, but does not reduce the incidence of the disease [123–126]. Although experimental vaccines against mastitis, based on formalin-inactivated whole cells, whole-cell lysate, polyvalent whole-cell Bacterin cultures of the vaccine strains [123–126], stimulates humoral immune responses, the levels of opsonizing antibodies in milk is poor or absent [127]. The lack of efficacy observed in conversional experimental vaccines may explain why mastitis vaccines for use in sheep and goats have not been developed further. Hence there are currently very few commercial vaccines licensed for use in sheep and goats. In addition, current vaccines on the market licensed against mastitis are mostly targeted at staphy-lococcal mastitis in bovine, there aren't many vaccines against mastitis targeting sheep and goats. Of the few vaccines against mastitis on the market, none of them are effective against mastitis but label claim indicate some effect. For example, Lysigin® (Boehringer Ingelheim) is the only vaccine against staphylococci in the US. While, Startvac (Hipra, spain) is the only vaccine licensed in Europe and few other coutries including Canada with label claim of some effect against *S. aureus, E. coli,* CNS. However, in controlled experimental studies their effects were none to very limited [127–130]. Another vaccine on the market is J5 vaccine from different manufacturer (zoetis, Boeringer, etc.) against *E. coli* mastitis. As with the other vaccines mentioned previously, this vaccine is also not very effective but claimed for some effect. Lastly, UBAC® (Hipra, Spain) with label claim against *S. uberis* mastitis is yet to be validated under field condition [130]. In comparison, only two licensed vaccine, Blue udder (Onderstepport biological products (OBP), South Africa), and Vimco ® (Hipra, Girona, Spain), targeting mastitis in sheep and goat are available on the market. As with the other mastitis vaccine, label claim of some effects against *S. aureus*, *M. heamolytica* and *S. aureus* respectively. Highlighting the need for the development of an efficacious mastitis vaccines for sheep and goat. In the past 10 years, a wealth of knowledge on the pathogenesis of disease and protective immune response mechanisms against bacterial mastitis has been gained in ruminants. This knowledge needs to be applied in the development of an effec-tive mastitis vaccine. Based on our current understanding of the immunological responses in the mammary gland of ewes against bacterial mastitis, as discussed above. The significant role played by antibody-mediated immune response, such as the importance of induction of locally produced antigen-specific IgA antibodies [131], and cell-mediated immune response geared towards a local Th17 response at

the onset of infection in preventing mastitis [87, 88, 132], supports the use of novel vaccines technologies in the improvement of already existing experimental vaccine. For example, already licensed vaccines for bacterial mastitis used in sheep and goats, could be improved in the following manner:

1. Inclusion of other prevalent mastitis-causing bacteria virulence factors such as toxins, surface proteins etc. In order to target more bacteria rather than focusing on one organism. For example, studies have shown that anti-leukotoxin antibodies have an important role in protection against mammary infection of ruminants. This was demonstrated through vaccination of ewes with partially purified leukotoxin and α-haemolysin, which conferred partial protection against an intramammary challenge with a mastitis-causing strain of *S. aureus* [133].

2. Use of delivery systems (formulation strategies and novel adjuvants) in order to stimulate the development of immunity towards a Th17 type response [132, 134] and stimulate local production of IgA and IgG2 responses [135]. In addition, to early recruitment of neutrophils. To induce Th17 responses in vaccines various adjuvants have been studied. For example; *S. pneumonia* whole cell antigen vaccine formulated in aluminum hydroxide enhanced the quality of antibodies and Th17 CD4+ T cell response [136]. In TB infections Cyclic dinucleotides (CDNs) adjuvanted vaccine has be shown to elicit a Th17 immune response correlating with enhanced protection against infections [137]. The bacterial components, muramyl dipeptide (MDP) a NOD2 ligand has been shown to induce Th17 response [138], lipopolysaccharide (LPS) a TLR4 ligand induces Th17 [139]. Therefore, prospective mastitis vaccine aiming on eliciting a Th17 response, maybe formulated in currently used adjuvants such as aluminum hydroxide gel in combinations with TRL ligands, such as TLR4 or TLR8/7 ligands; NOD2 ligands and CDNs. Alternatively, these ligands could also be formulated in combination with novel nanoemulsion oil and water adjuvants for the development of efficacious vaccine.

3. Exploring alternative vaccination routes, such as mucosal vaccine administration, in order to achieve the desired immune response, for example, in cow vaccination route have an impact on the subsequent immune response [132, 140, 141]. For example, studies, have shown that intramammary administration of antigens (e.g., inactivated *S. aureus*) in non-lactating ewe enhance the kinetics of neutrophil influx with no involvement of complement in the immunological response.

4. Use of newer technologies, such as biofilm matrix polysaccharides, have also been used to induce protective immune response against *S. aureus* mastitis in ewes [142]. Vaccines developed using this approach offers some degree of improved efficacy against *S. aureus* mammary infection and mastitis [143]. Mastitis Vaccines licensed for sheep such as, the Vimco ® vaccine based on biofilm-producing *Staphylococcus* has been shown to reduce the incidence of mastitis in sheep [144]. In addition, omics technologies could be harnessed to fully characterize immunological responses in mastitis and identify relevant vaccine candidates for more efficacious vaccine development against mastitis causing bacteria.

5. Conclusion

Lack of effective vaccines against mastitis in sheep and goat has long been attributed to lack of knowledge on the disease pathogenesis and protective immune

response mechanism required. In the past decades, a wealth of knowledge has been gained on the pathological processes leading to mastitis in sheep and goat caused by the most prevalent pathogenic bacteria, i.e. *Staphylococcus* spp. Using *Staphylococcus* spp. as an example, we now know that the pathological processes leading to sub-clinical and clinical mastitis depends on bacterial virulence factors and the induced host immune response. The pathogenesis of *S. aureus* mastitis entail three processes, i.e. adhesion, invasion and evasion. During these three processes *S. aureus* differentially expresses virulence factors that aids colonization of the host mammary glands. In addition, we now have a better understanding of which virulence factors target the main cells involved in mammary immunity and how their actions are counteracted by the bacteria. We have also gained more understanding of the immune response required to limit *S. aureus* infection. Although we do not fully know the mechanisms of the protective immune response in the mammary glands of ruminants and still do not know how to induce such a protective response. Our current knowledge, points to a local protective response that most likely entail early recruitment of neutrophils to control bacterial inversion and IgG2 antibodies isotypes, and to a potential role for IgA. In addition, a local cellular response geared towards a Th17 immunity plays a role in bacterial cleareance and neutrophil recruitment. This knowledge could be used to improve current conventional experimental vaccines against mastitis in small ruminants by employing immunostimulatory adjuvants or delivery systems capable of stimulating a local Th17 responses, by using TLR4, 7/8; NOD2 and CDNs ligands in adjuvant formulations.

Due to the lack of efficacy observed with conventional vaccines, research on the development of efficacious mastitis vaccine for small ruminant can be fast track by exploiting rapidly advancing omics technologies and developing immunological tools (reagents) for characterization of ruminant adaptive immune response in great detail. Reverse vaccinology approaches could be used to discover candidate vaccine antigens from mastitis causing bacteria. Omics technologies can also be applied to gain understanding on the protective adaptive immune response to mastitis infections by mapping relevant antigen through transcriptomics and proteomics, and characterizing antibody and T-cell repertoires through immuno-proteomics. Data generated from these approach may reveal correlates of protection to which vaccination strategies can be based.

Conflict of interest

The author declares no conflict of interest.

Notes/thanks/other declarations

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Antimicrobial Usage in Dairy Farms for the Prevention and Control of Mastitis

Antimicrobial Usage for the Management of Mastitis in the USA: Impacts on Antimicrobial Resistance and Potential Alternative Approaches

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Abstract

Mastitis is the most frequently diagnosed disease of dairy cattle responsible for the reduction in milk quantity and quality and major economic losses. Dairy farmers use antibiotics for the prevention and treatment of mastitis. Frequent antimicrobial usage (AMU) undeniably increased antimicrobial resistance (AMR) in bacteria from dairy farms. Antimicrobial-resistant bacteria (ARB) from dairy farms can spread to humans directly through contact with carrier animals or indirectly through the consumption of raw milk or undercooked meat from culled dairy cows. Indirect spread from dairy farms to humans can also be through dairy manure fertilized vegetables or run-off waters from dairy farms to the environment. The most frequently used antibiotics in dairy farms are medically important and high-priority classes of antibiotics. As a result, dairy farms are considered one of the potential reservoirs of ARB and antimicrobial resistance genes (ARGs). To mitigate the rise of ARB in dairy farms, reducing AMU by adopting one or more of alternative disease control methods such as good herd health management, selective dry-cow therapy, probiotics, and others is critically important. This chapter is a concise review of the effects of antimicrobials usage to control mastitis in dairy cattle farms and its potential impact on human health.

Keywords: antimicrobial resistance, bovine mastitis, intramammary infection, antimicrobials, mastitis, bovine, dairy cattle

1. Introduction

Since the discovery of antibiotics, microbes have continued to uncover new ways to survive and thrive in the presence of antibiotics [1]. In recent years, the emergence and spread of antimicrobial resistance (AMR) worldwide have increased at an alarming rate [2]. AMR has been detected almost as quickly as newer antibiotics were developed and used [3]. Mastitis, an inflammation of the mammary gland, mainly caused by bacteria, is the most frequent reason for antibiotic use in dairy cattle. Mastitis causes significant economic losses to the dairy industry directly through a reduction in milk yield and quality and indirectly by

increasing the cost of its management [4]. The indirect cost includes heavy use of antibiotics, which contributes to the occurrence of AMR. In addition, some AMR mastitis pathogens can pose public health threats through the consumption of milk and milk products [5].

The rise in AMR occurs mainly due to the imprudent use of antimicrobials which increasingly undermines the sustainable use of antimicrobials. Studies reported that the amount of antimicrobials used (AMU) to treat clinical and subclinical mastitis accounts for nearly twice the quantity of antibiotics used for all other health problems in dairy cows [6, 7]. The United States Department of Agriculture (USDA), National Animal Health Monitoring System (NAHMS) survey of 2013 reported a 24.8% clinical mastitis in all cows involved [8]. The majority (87.3%) of the cows with clinical mastitis were given antibiotic treatment. Nearly three-fourths of the farms (73%) used cephalosporins, 34.4% used first-generation cephalosporins (FGCs), and 38.6% of them used third-generation cephalosporins (TGCs). The NAHMS also reported that out of 21.4% of cows treated for mastitis, the primary treatments given were TGCs (50.7%), lincosamide (24.7%), and FGCs (15.2%). The same report showed that there are seven approved intramammary (IMM) antimicrobial products in the United States but no systemic products for treating clinical mastitis except limited extra-label usage of some products. While one approved IMM antimicrobial product is classified as a lincosamide (pirlimycin) and six IMM antimicrobial products are classified as beta-lactams. The beta-lactams that are used as IMM products include FGCs (cephapirin) and TGCs (ceftiofur), aminopenicillins (amoxicillin and hetacillin), penicillin G, and penicillinase-resistant penicillins (cloxacillin) [9].

Another most common AMU is for dry cow therapy (DCT). Dairy cows are susceptible to intramammary infection (IMI) during the early and late dry period [10–12]. To prevent IMI during the dry period, the National Mastitis Council (NMC) recommends IMM of long-acting IMM antibiotics, also known as dry cow therapy (DCT), as a prophylactic control measure for the management of mastitis. The DCT is routinely used at the end of lactation to cure existing subclinical mastitis so that it will not be carried over to the next lactation and to prevent new infections during the dry period [13]. According to the 2014 NAHMS of dairy herds study, 93% of cows in the U.S. received DCT. Among the operations that used DCT, more than half (58.1%) of them used cephapirin benzathine followed by ceftiofur 27.9%, and procaine penicillin G and dihydrostreptomycin sulfate combination (24.5%). A recent study also reported that beta-lactam antibiotics such as cephapirin, ceftiofur, and penicillin are the top three antibiotics used for DCT on U.S. farms [14].

Although total AMU in the U.S. cattle production, including dairy farming, is lower than that of other food animals such as pigs, most of the antibiotics used are important to treat infections in humans. Of all antibiotics classes approved for use in U.S. dairy cattle, at least eight are medically important (**Table 1**). These antibiotics used in both dairy and human medicine include aminoglycosides, cephalosporins, fluoroquinolones, lincosamides, macrolides, penicillins, sulfonamides, and tetracyclines [19]. These antibiotics are also used to treat other diseases of dairy cattle, such as respiratory and reproductive diseases and foot infections [7]. Some of these antibiotics are categorized by the World Health Organization (WHO) as critically important ones. Quinolones (enrofloxacin and danofloxacin) and extended-spectrum beta-lactams such as third-generation cephalosporins, which are heavily used in U.S. dairy farms for the treatment of mastitis, are considered as "highest priority critically important" classes of antibiotics [19]. The use of these antibiotics in dairy farms can exert selection pressure that may lead

Antimicrobial class	Antimicrobial agent	Indications	Importance for human medicine	References
Cephalosporins	Ceftiofur Cephalothin Cephapirin	DCT, BRD, mastitis, and metritis	Critically important	[8, 15–18]
Fluoroquinolones	Danofloxacin, Enrofloxacin	BRD	Critically important	[16, 17]
Aminoglycosides	Amikacin, Clindamycin Gentamicin, Apramycin Kanamycin, Neomycin	DCT, feet infections	Critically important	[16–18]
Penicillin	Amoxicillin/Clavulanic acid Ampicillin Penicillin Cloxacillin	DCT, mastitis, metritis, and other local infections	Important	[14, 16, 17]
Sulfonamide	Sulfamethoxazole, Sulfadimethoxine, Sulfisoxazole, Trimethoprim/ Sulfamethoxazole, Sulfamethoxazole/ Sulfisoxazole	Calf diarrhea	Highly important	[16–18]
Macrolides	Erythromycin, Tilmicosin Tulathromycin, Tylosin Tulathromycin and Gamithromycin, Tilmicosin,	BRD, foot rot, and metritis	Critically important	[16–18]
Amphenicols	Florfenicol	BRD	Highly important	[16–18]
Tetracyclines	Chlortetracycline Oxytetracycline Tetracyclines	BRD, metritis, bacterial scours, and eye infection	Highly important	[16–18]
Lincosamide	Pirlimycin, Lincomycin	DCT, Mastitis, BRD, and feet infections	Highly important	[16, 17]

BRD: Bovine respiratory disease; and DCT: Dry cow therapy.

Table 1.
Major antimicrobial classes used in the U.S. dairy cattle and their medical importance according to WHO classification.

to the emergence and spread of AMR pathogenic, opportunistic, and commensal bacteria from dairy farms to humans. Transmission may occur through direct contact between cattle and humans or indirectly through the food chain (milk and meat). The horizontal transfer of resistance genes may occur from bacteria of dairy cattle origin to human commensal or pathogenic bacteria in the gut [20]. Thus, the development of AMR that arises from the AMU in dairy farms could seriously impact the management of infectious diseases in the human population using antibiotics [21].

2. Antibiotics use in dairy farms and their implication to human health

There is considerable evidence that supports the view that the development of AMR in food animals such as dairy cattle is linked to the emergence of AMR bacteria that infected humans [22–24]. As one of the major consumers of antibiotics, dairy cattle production farms are likely to contribute to the rise of AMR bacteria in humans. Studies from outside of the U.S. [25–27] showed direct transmission of AMR from dairy cattle to humans through contact on farms or through indirect routes. The most common route of the spread of AMR bacteria and their resistome from dairy cattle farms to humans could be indirect through the food chain. In the U.S., the CC97 methicillin-resistant *S. aureus* (MRSA), the human pandemic clone, which claims the lives of thousands of people every year, was suggested to be originated from the dairy farm [28].

According to the U.S. centers for disease control and prevention (CDC), about 22% of infections (440,000 cases) caused by antibiotic-resistant pathogens in the U.S. are from a food of animal origin, such as milk [29, 30]. Most of these bacteria could be normal microflora that colonizes the gastrointestinal tract of the animal [24], but they could be pathogenic for humans or may also be commensal but may transfer resistance genes to other foodborne pathogens in the human gastrointestinal tract [23]. Additional routes of transmission of AMR bacteria and their resistome to humans is through contaminated dairy farm environments and other wastes entering the environment [31].

Multiple studies have linked the outbreak of foodborne AMR pathogens to animal and their products, including milk [25–27, 32]. Despite these reports, it should be noted that direct proof for AMR transmission through foods of animal origin or directly through contact is limited, especially from dairy cattle [33]. In the U.S., strong evidence for transmission of AMR isolates between dairy cattle and humans is not yet proven. Previous reviews that attempted to discern any linkage between AMU in dairy cows and AMR development in veterinary and human pathogens showed the absence of scientific proof to support this assumption [34]. However, there is ample evidence that the use of antibiotics in food-producing animals contributes to increased AMR [35]. Published literature showed that the risk of getting an infection from AMR zoonotic dairy pathogens seems less likely [36].

However, the absence of direct evidence of AMR bacteria or resistant determinant transmission does not mean there is no transmission between dairy cattle and humans. For instance, the current and future risk of acquiring AMR bacteria from milk is an important human health concern as the consumption of raw milk is increasing in some states in the U.S. [32]. Due to the presence of antibiotic-resistant foodborne or zoonotic bacteria in raw milk [29, 30], *an increasing trend in the consumption of raw* milk in the U.S. and other countries indicates public health risk [37]. Similarly, AMR bacteria present on meat from culled dairy cows should also be seen as an important human health risk since it can cause life-threatening infection if undercooked meat is consumed [34]. It is also unknown if pasteurization of milk or proper cooking of meat will prevent the AMR gene transfer especially in the gastrointestinal tract where horizontal gene transfer may occur.

2.1 Antimicrobial resistance in mastitis pathogens

Antibiotics are regularly used for the prevention and treatment of mastitis in dairy cows. Some review articles showed such uses had not been associated with a high risk of developing resistance in mastitis-causing pathogenic bacteria [7, 34]. The previous review on the impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens concluded that common

AMU in dairy farms did not lead to the widespread occurrence of resistance among mastitis pathogens against antibiotics frequently used in dairy production [34]. Nevertheless, there is no doubt that AMU in food-producing animals such as dairy cows contributes to the rise in AMR [7]. Recently Abdi et al. [38] reported a high prevalence (34.3%) of resistant *S. aureus* isolates from different dairy farms in Tennessee, U.S. suggesting a potential increasing trend of antimicrobial resistance in *S. aureus* isolates against some antibiotics.

Only a handful of studies investigated the impact of treatment of clinical or subclinical mastitis on AMR development. A controlled study by Levy et al. [39] measured AMR changes after antimicrobials were administered to a host; however, this study lacks mastitis treatment procedures [7]. However, some studies showed that AMU for mastitis treatment is linked to AMR development and changes in the diversity of mastitis pathogens [40, 41]. Pol and Ruegg [4] found a positive relationship between AMU such as pirlimycin, ampicillin, erythromycin, and tetracycline and increased resistance among gram-positive mastitis pathogens. Another U.S. study also reported a higher proportion of resistant mastitis pathogens recovered from conventional dairy farms than organic dairy farms [42], suggesting the effect of AMU.

3. Alternative approaches for the management of mastitis

There were no specific AMU data collected from U.S. dairy farms. Thus, it is not possible to know the doses of each antibiotic given to dairy cattle, the length of the treatment, and the diseases for which antibiotics were prescribed. However; there is no doubt that antibiotics have been administered for a considerable proportion of dairy cattle's lifetime in a farm, and dairy farm consumes a huge quantity of antibiotics, especially those of the medically important ones. The United States Food and Drug Administration (FDA) report showed more than 16,155 kg of medically important antimicrobials intended for IMM therapy were sold in 2019 [19].

The major concern is the use of critically important antibiotics for human medicine in dairy farms such as third-generation cephalosporins and fluoroquinolones. Both qualitative and quantitative studies that analyzed the risk of AMR in food animals such as dairy farms indicated that the continued use of these antimicrobials would increase the number and types of AMR bacteria and worsen the public health and animal health issues in the U.S. and beyond [43]. It is no longer deemed appropriate that antibiotics should be the only remedy to prevent disease, especially when other alternative disease control measures exist. Thus, it is important to look for potential alternative strategies that help to reduce AMU and prevent disease without heavily relying on antibiotics [7]. Some of the alternative approaches that can be explored to mitigate the rise of AMR bacteria include but are not limited to selective dry-cow therapy (SDCT) [44], good herd health management [45], vaccination [46], phage therapy [47], probiotics [48] antibacterial peptides [49], and nucleic acid-based antibacterial treatments such as CRSPR-Cas system [50].

3.1 Selective dry cow therapy (SDCT)

The number one reason for AMU in the U.S. dairy industry is to control mastitis. Studies showed that almost all U.S. dairy farms treat all cows in the farm (blanket dry cow therapy-(BDCT) with long-acting antibiotics at drying off to prevent mastitis during the dry period. The ideal dry period, the period between the end of the current lactation and the beginning of the next, for a profitable dairy producer is usually 60 days or 8 weeks [51]. A USDA survey of dairy farms reported that 85%

of conventional dairy farms used BDCT [15]. A study suggests that BDCT accounts for approximately one-third of the total AMU on conventional dairy farms in the U.S. [52].

Selective dry cow therapy (SDCT), unlike BDCT, uses a specific strategy to avoid treating every cow with antibiotics at dry off. In SDCT, only animals with IMI or high somatic cell count or cows with a health record showing a high probability of developing mastitis receive antibiotics. A teat sealant is applied to all cows at drying off. Using an internal teat sealant prevents entry of mastitis pathogens and decreases the prevalence of clinical mastitis, reducing the need for treatments for clinical cases [44]. To determine cows that require SDCT, bacterial culture, or somatic cell count (SCC) data of individual animals are required. A cow with a composite milk high SCC of ≥200,000 cells/mL of milk indicated the presence of subclinical mastitis and is eligible for IMM antibiotic infusion [52]. Studies [44, 53] showed that internal teat sealants, alone or when used with antibiotics can decrease the risk of acquiring new IMI after calving by as much as 25%. Internal teat sealants lowered the risk of IMI by 73% compared with cows that do not have teat sealants suggesting its potential use for managing mastitis [44].

3.2 Evidence-based treatment of mastitis

Before administering antibiotics, it is crucially important to isolate and identify mastitis-causing agents from infected udder quarters. Bacterial isolation and identification should be attempted at least in large dairy operations to make an evidence-based decision on whether to use antibiotics. Some investigations have confirmed that on-farm bacterial identification can decrease AMU by as much as 50% [40] since the use of antibiotics is not justified in some infections caused by gram-negative bacteria such as *E. coli* with high "spontaneous self-cure" [54, 55]. Another study also showed that the majority of (as high as 57%) milk samples collected from quarters of cows with negative culture results did not have bacterial DNA [56] suggesting that environmental factors such as trauma or viral infection may trigger an inflammatory response or infected animal was already fully recovered during sample collection. Failure to detect bacterial DNA could be due to bacteria elimination from the udder quarters by the host immunity [7]. In general, the possibility of a natural cure without the use of antibiotics against some bacterial pathogens is well documented in dairy cattle [57–61], and it is an important alternative to consider before deciding on antibiotic use.

3.3 Good dairy herd health management

Dairy herd health management is an essential component in the fight against AMR. The objectives of herd health management are to prevent and control mastitis and other diseases using appropriate hygienic and management practices [62]. AMU can be reduced by improving hygiene, frequent physical examination of animals, regular herd testing for common diseases, and quarantining all-new replacement animals before mixing with the herd [45]. In addition, dairy cattle should be managed to reduce stress and promote their welfare and immunity by providing suitable housing (good ventilation, appropriate humidity, low stocking densities, and good hygienic practices). Studies showed that hard flooring, poor bedding, and overcrowded conditions increase the chance of cows developing mastitis, lameness, and respiratory diseases [63, 64]. All efforts made to maximize herd health and welfare will enhance the host immune function and considerably reduce mastitis and other common dairy cattle diseases, reducing the need for antibiotics [65].

3.4 Vaccination

Vaccination against mastitis pathogens is recommended as one of the most important strategies to prevent new infections, which in turn reduce AMU in dairy farms [46]. Vaccination against mastitis-causing bacteria induces the cow's immune response that fights against subsequent infection and disease. Effective vaccine enhances adaptive humoral (antibody-mediated Th2 immunity) and cellular (cell-mediated- Th1 and Th17 immunity) immunity against mastitis pathogen that inhibits or restricts bacterial growth or kills bacteria upon its invasion of a mammary gland. The enhanced immunity cures the infection or reduces the number of invading bacteria, which reduces pathogen damage to milk-producing tissues and lessens the clinical severity of disease and production losses [66].

Vaccines can be classified into inactivated/killed, live/attenuated, chimeric live attenuated, subunit, and nucleic acid-based (DNA or mRNA) vaccines, each with advantages and disadvantages [66]. Live vaccines contain attenuated disease-causing agents capable of replicating within the host but do not cause disease because of attenuated pathogenicity. Modified live vaccines (MLV) are usually developed from the naturally occurring pathogen by (1) attenuation in cell culture, (2) use of variants from other species, and (3) development of temperature-sensitive mutants. Recombinant live attenuated vaccines include: (1) live attenuated vectored vaccines- pathogen's antigenic parts incorporated into a harmless carrier virus or bacteria, (2) chimeric live attenuated vaccines—genes from the target pathogen substituted for similar genes in a safe, but closely related organism, and (3) nucleic acid (DNA or mRNA) vaccines—a DNA vaccine is an immunogenic product encoding gene (DNA) cloned into a plasmid that can be injected into the host, where it will be transcribed and translated into an immunogenic product. The mRNA vaccine contains a messenger RNA (mRNA) molecule that encodes antigen that induces an immune response [67].

Inactivated/killed pathogen vaccines contain whole pathogens that have been inactivated with agents, such as phenol (bacteria) and formalin or beta-proprionolcatone (viruses). Inactivated/killed vaccines lack pathogenicity and can neither replicate nor spread between hosts and require multiple doses and regular boosters. The efficacy of inactivated/killed vaccines depends on the use of potent adjuvants. Bacterin is one of the killed/inactivated vaccines in which a suspension of killed whole bacterial cultures is used as a vaccine. Protein vaccines—include naturally produced proteins of pathogens and induce less injection site reactions than products containing the entire pathogen. Recombinant subunit vaccines—contain synthetically produced antigens that induce immunity to a specific pathogen. Adjuvants are one of the components of killed/inactivated vaccines that function to modulate and amplify the host immune response to the accompanying antigen and are critical to the success of inactivated vaccines.

Live-attenuated bacteria can multiply in the host, expressing a complete range of antigens [68]. However, the most important shortcomings of the live vaccine are their persistence in the animal body for an extended time, limited shelf life, potential for contamination, may cause abortion in pregnant animals, and safety concerns as the attenuated organism may revert to full virulence [69]. On the other hand, killed vaccines are safe, induce good colostral (lactogenic) immunity, have longer shelf lives but may interfere with passive immunity and are less immunogenic, and need adjuvants to enhance immune responses [70].

There is no effective vaccine against mastitis pathogens, and results of vaccine efficacy studies showed limited efficacy against mastitis-causing bacterial pathogens [66]. The most targeted udder pathogens for vaccine development include *S. aureus* [71–81], *Streptococcus uberis* [82, 83], *Streptococcus agalactiae* [66], and

E. coli [84–86]. Among mastitis pathogens, most vaccine trials were conducted against *S. aureus*, a major mastitis pathogen with a low cure rate by antibiotics, and remain undetected in the subclinical form in dairy cows [47, 87, 88]. Currently, there are two commercially available bacterin vaccines against *S. aureus* mastitis. These are lysigen® (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) in the United States and Startvac® (Hipra S.A, Girona, Spain) in Europe and some other countries. Several staphylococcal vaccine efficacy trials showed that vaccination with bacterin vaccines induced increased antibody titers associated with partial protection in the blood and milk in some studies [71, 74, 78, 81] or no protection at all in some other studies [72, 79, 80]. Neither of the two commercial vaccines against *Staphylococcus aureus* mastitis on the market, Lysigin®, and Startvac® [79] confers protection under field trials and controlled experimental studies [71–74]. Some studies reported that Lysigin® reduced somatic cell count (SCC), clinical mastitis, and chronic intramammary infection (IMI) [89–91], whereas other field-based studies concluded no such effect [72, 73, 75–77]. Similarly, some studies reported vaccination with Startvac®, reduced incidence, severity, and mastitis duration in vaccinated cows compared to non-vaccinated control cows [71, 74, 78]. Contrary to these observations, other studies failed to find an effect on improving udder health or showed no difference between vaccinated and non-vaccinated control cows [79, 80]. Overall, effective intramammary immune mechanisms against staphylococcal mastitis are still poorly understood.

Mastitis vaccine research has been conducted over the past several years, but to date, developing an effective vaccine has been a challenge due to the nature of the disease and the pathogens involved [92, 93]. For instance, an increased immune response may not always be beneficial in bovine mastitis unless increased immunity is followed by a decreased number of infecting pathogens, as the presence of a large number of bacteria in the presence of fighting immune cells is considered as an indication of mastitis which decreases milk quality [93]. Successful vaccination is challenging because the volume of milk present in the gland dilutes the number of immune effector cells available to fight off infection [92, 93]. In addition, fat and casein in the milk reduce the bactericidal abilities of the immune cells [93].

The development of an effective vaccine against mastitis pathogens is one of the sustainable alternatives to antibiotics. However, it may not be practically possible to develop an effective vaccine against all bacteria that cause mastitis [68]. Thus, combining effective vaccines with other infection control measures may considerably reduce the incidence of IMI and thereby reduce the need to use antibiotics [66].

3.5 Immunostimulants

Immunostimulants are compounds that activate any components of the host's innate immune system and help to enhance disease resistance. Immunostimulants directly stimulate innate immune responses by activating immune cells (phagocytes), complement system, and increased lysozyme activity [94, 95]. Currently, immunostimulants are increasingly used as an alternative to antibiotics [96]. Immunostimulants are broad ranges of substances including minerals (selenium and zinc); amino acids (leucine, arginine, and ubenimex); vitamins (A, E, C); plants and plant polysaccharides, bacterial components (β-glucan, peptidoglycan, lipopolysaccharide); hormones and hormone-like substances; nucleic acid preparations; chemical synthetics (imiquimod, cimetidine, levamisole, polyinosinic acid, pidotimod, and others); and biological cytokines (transfer factor, interferon, immune globulin, and interleukin) [68, 95, 97].

Bricknell and Dalmo [98] reported that the addition of immunostimulants in animal feed could enhance their innate defense and prevent infection during a period of high stress. Another group of researchers, Gertsch et al. [99], stated that applying plant-derived immunostimulants in animal feed boosts the immune system though they did not specify the mechanism. Similarly, Li et al. [100] administered polysaccharide chitosan to cattle and noted improved immune response and antioxidant activity. In 2010, Thacker [101] reported cytosine-phosphate-guanine (CpG), an oligo deoxynucleotides immune-stimulant, stimulating B-cell proliferation, cytokine production, and enhanced cytokines production and NK cell cytotoxic activity.

3.6 Cytokines

Cytokines are crucial for normal tissue functions, but their over- or under-expression is linked with pathological conditions [102]. They play a significant role in initiating, sustaining, and controlling the innate immune response and suggesting that they may have an excellent therapeutic effect for infectious disease treatment [103]. Toll-like receptors (TLRs), surface receptors that identify the structure of pathogens, also indirectly contribute to the secretion of cytokines by inducing a signaling cascade that leads to the secretion of cytokines controlling the adaptive immune response [104].

Cytokines, such as IL-6, TNF-α, and INF-γ, have also been proposed to treat bovine mastitis and endometritis. Hossain et al. [105] reported that cytokines alone or in combination with antibiotics significantly improve the rate of cure of bovine mastitis. Daley et al. [106] infused the mammary gland with recombinant bovine cytokines ((IL-1 and IL-2) and observed a rise in the proliferation of polymorphonuclear cells, with increased formation of oxygen radicals in the milk. The investigators also observed that the induced host natural defense system could prevent *S. aureus* infection in cattle. This suggests recombinant bovine cytokines are a promising candidate. Thus, further investigation is needed to identify the therapeutic potential of the cytokines for mastitis treatment and their possible use as an alternative to antibiotics.

3.7 Phage therapy

Phage therapy, which treats bacterial infections with bacteriophages, has been considered one strategy to manage mastitis [47]. Results of several studies showed that bacteriophages had antibacterial activity against a range of antibiotic-resistant bacteria with a considerable degree of specificity and potency [107]. Thus, the use of bacteriophages and their derivatives such as endolysins signifies a possible alternative for treating mastitis [108].

The bacteriophage works by inserting its genome into the bacterial cytoplasm, thereby the phage genome will incorporate itself into the host genome and reproduce along with the bacteria and produce endolysin, which break-down the bacterial cell wall and induce a cascade of bacterial lysis [108, 109]. Phages and endolysins are also known to destroy biofilms produced by major gram-positive and gram-negative mastitis pathogens, including *Staphylococcus* species*, E. coli, Klebsiella pneumonia,* and others [110].

Currently, interest in bacteriophages for the treatment of mastitis is rapidly growing [80]. Results from several *in vitro* experiments indicated that this method of treating mastitis is a viable option as phage therapy shows promising effectiveness against some mastitis pathogens, such as *S. aureus* [107, 108, 110–114]. However, a handful of clinical studies to evaluate the efficacy of bacteriophage for the treatment and prevention of mastitis showed limited efficacy of this approach, suggesting the

need for further study to improve its effectiveness [47, 112, 115]. Moreover, the practical use and broad application of phage therapy are limited by several factors. These include high specificity of phages, low effectiveness in eliminating the population of pathogenic bacteria, the need for a high dose of phage for effective therapy and its degradability in milk, and the emergence of phage resistance bacterial strains [108, 116]. Further clinical studies are needed to address these limitations and exploit the full potential of phage to prevent and treat mastitis.

3.8 Use of probiotics for the treatment of mastitis

The rise of AMR against antibiotics used in dairy farming demands the search for other alternative disease control measures. In this regard, probiotics have lately been considered a potential alternative for treating mastitis [49]. Probiotics are living microorganisms that give a health benefit to the recipient when given in sufficient amounts. This less precise definition includes several different well-identified microorganisms, safe for intended use, have proven health benefits when used in appropriate amounts and through the correct routes [117, 118].

Two mechanisms of action were suggested for mammary gland probiotics. The first mode of action is through the interactions between probiotics and the local microbiota (indirect mode) [48]. This model assumes that cows develop mastitis due to a lack of balance between the normal mammary gland microbiota and pathogenic bacteria causing mastitis. Therefore, modification of this imbalance with probiotics is suggested as an option to AMU [119]. The second proposed mode of action is a direct one, where probiotics interact directly with mastitis pathogen. Probiotic bacteria generate a range of antimicrobial substances such as short-chain fatty acids, lactic acid, nitric oxide, hydrogen peroxide, and bacteriocins, all of which may inhibit the growth and multiplication of mastitis-causing bacteria [120]. Rainard and Gilles [48] reviewed the use, mechanism of action, and *in vitro* and *in vivo* efficacy studies on probiotics used in mastitis treatment.

The selection and prophylactic or therapeutic use of mammary gland probiotic strains depend on the production of substances affecting the growth or survival of mastitis pathogens, the absence of known virulence factors, the absence of antibiotic resistance, and the ability to colonize mammary gland epithelium cells. The bacteria that meet these conditions are deemed promising for use as mammary probiotics [121]. Most studies investigated lactic acid bacteria as a potential probiotic for mastitis treatment and prevention. Few of these studies reported that probiotics are as effective as antibiotics for treating clinical mastitis [122]. In contrast, most other studies reported that the probiotics elicit a strong inflammatory response in the mammary gland or are neither effective nor safe [123, 124]. The current reports on the safety and efficacy of intramammary probiotics are generally conflicting, necessitating the need for further research to develop a conclusive recommendation on the use of probiotics for the management of mastitis.

3.9 Antimicrobial peptides

Antimicrobial peptides (AMPs), also known as cationic host defense peptides, are potent naturally occurring antibacterial agents with a broad spectrum of activities against both gram-negative and gram-positive bacteria. AMPs are found in all forms of life, from prokaryotes to eukaryotic cells. In contrast to most conventional antibiotics, AMPs often work in direct and indirect ways. They may directly kill the bacteria by disrupting cell membranes, thereby creating trans-membrane channels. They indirectly may also enhance host immunity as immunomodulators so that the host can clear the pathogen [49].

In vertebrates AMPs promote natural immunity and are a component of the first line of defense against pathogenic microorganisms. The crucial role of AMPs as innate immune modulators was shown in an experimental study in which the cnlp gene (encoding CRAMP) knockout mutant mice, a gene coding mouse analog of human LL-37 (encoded by camp) antimicrobial peptide, were very susceptible to infection [125]. In prokaryotes such as bacteria, the production and release of AMPs give a competitive advantage in a given environment by AMPs-mediated killing of other bacteria [126].

The mode of action of AMPs is recently reviewed [127] and seems different and related to the target bacterial pathogen. The positively charged AMPs interact with the negatively charged membranes of bacteria (lipopolysaccharides in gram-negative bacteria) and teichoic acids (in gram-positive bacteria). This strong electrostatic interaction between opposing charges (between AMPs and bacterial surface membranes) is the basis of the specificity of the action of AMPs on bacteria over other higher organisms. The "amphipathic" characteristics of AMPs help them to bind and penetrate the bacterial inner membrane causing leakage of bacterial cell contents and leading to cell death [128].

Currently, AMPs are considered as one of the promising classes of therapeutic agents as an alternative to conventional antibiotics. Several AMPs have been used as therapeutic agents for intravenous administration and topical application in human medicine owing to their short half-lives [129]. A recent study investigating the efficacy of specific AMPs against the AMR *S. aureus* in the mammary epithelial cells reported a very promising result. The study examined the intracellular activities of H_2 in the bovine mammary epithelial and mouse mammary glands infected with methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *S. aureus.* Results showed a 99% intracellular inhibition rate of the resistant *S. aureus* strains after treatment with the AMPs. The study finally concluded that H_2, the AMPs used in the study, "can be used as a safe and effective candidate for treating *S. aureus*-induced mastitis" [130]. This is an indication that AMPs-based treatment approaches may be used as one of the tools that may help in the fight against AMR pathogens. However, more studies are needed to generate information on the development of resistance to AMPs, challenges to their widespread use in dairy cattle.

3.10 Use of CRISPR-Cas system

The CRISPR-Cas system is a bacterial immune system that gives resistance to foreign genetic elements such as those that exist within plasmids and bacteriophages and provides a form of adaptive immunity [131]. In recent years, the use of the CRISPR-Cas system to treat AMR bacteria has received a considerable level of interest as the approach that can readily kill AMR bacteria in the same way as an antibiotic-sensitive bacterium [132]. Additionally, this system can be designed specifically so that it can only target pathogenic bacteria without disturbing commensal bacteria in the microbiota [50]. This bacterial immune system is commonly used for "genome editing" as it can selectively eliminate virulence and antimicrobial resistance genes from bacterial populations. The system uses small RNAs (sRNA) to detect and destroy specific sequences of DNA, including phages, transposons, and plasmids [133].

Nucleic acid-based antibacterial treatments can be used to control infections caused by resistant bacteria [134], including mastitis-causing pathogens. However, although *in vitro* studies on some resistant pathogens showed successful and promising results, *in vivo* study to treat mastitis pathogen has not yet been carried out [135]. Besides, despite its current potential, the sustainable application of CRISPR-Cas technology is complex. It needs an efficient delivery vector, developing an appropriate wide host

range vector, and using a multiplex method that includes CRISPR-Cas targeting different sequences to reduce the occurrence of resistance possibilities [136].

4. Conclusion

Mastitis is the most prevalent and economically important disease of dairy cattle responsible for the largest antibiotics used in the dairy industry. Most dairy farms in the United States use similar antibiotics used to treat various diseases in humans. Several studies have linked AMR to antibiotic use. Thus, the use of these classes of antibiotics in dairy cattle may speed up the development of AMR, which can also affect the successful treatment of infection in humans. Every effort must be made to avoid unnecessary use or reduce the use of antibiotics to prevent mastitis. Dairy farmers need to be educated on the importance of improving herd and udder health so that the incidence of clinical and subclinical mastitis will decrease, reducing the need to use antibiotics. The use of vaccines, probiotics, antimicrobial peptides, phage therapy, and CRISPR-Cas system are among the promising alternative options for mastitis management. To maintain dairy cattle health and productivity and preserve the effectiveness of antibiotics, these alternative approaches to antibiotic use must be thoroughly investigated and implemented for sustainable management of mastitis. *In vitro* studies showed promising results on the potential use of these approaches, but further *in vivo* studies are needed to make specific recommendations on their use. Research should focus on identifying good alternatives to antibiotics with important characteristics including but not limited to effectiveness against the target pathogens, safety toward the host, ease of elimination from the body, less harmful to normal flora, degradability in the environment, and cost. Thus, it is strongly recommended that researchers and funding organizations invest their resources and focus their effort on developing innovative and sustainable control tools that are easily adoptable by producers such as effective vaccines, probiotics, and others coupled with good herd health management practices.

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Edited by Oudessa Kerro Dego

Mastitis, an inflammation of the mammary glands, is the most costly disease in dairy farming, mainly caused by a broad range of bacteria categorized into contagious and environmental bacteria. This book is a concise summary of mastitis in dairy cattle, sheep, and goats, which mainly focuses on etiological agents, epidemiology, pathogenesis, clinical manifestation, pathological and histopathological changes, diagnosis, prevention, and control measures. This book serves as a textbook on mastitis in dairy cattle, sheep, and goats for dairy veterinarians, veterinary students, animal science students, dairy technicians, animal health professionals. Several researchers worldwide contributed to this book. This book contains the latest information on mastitis in dairy cattle, sheep, and goats and antimicrobial usage to prevent and control mastitis.

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