Chapter 3

Dairy *Staphylococcus aureus*: Epidemiology, Drug Susceptibilities, Drug Modulation, and Preventive Measures

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Additional information is available at the end of the chapter

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Abstract

Staphylococcus aureus is an emerging pathogen from dairy animals' mammary glands. Among various risk factors associated with this pathogen are unhygienic milking procedures, improper preventive techniques, and lack of germicidal teat dipping before and after milking. Methicillin-resistant *S. aureus*, coagulase positive *S. aureus*, vancomycin-resistant *S. aureus*, and biofilm-producing *S. aureus* are common strains of *S. aureus* being isolated from dairy milk these days. They have huge economic and public health concerns. Trials of antibiotic susceptibility proposed variable responses, while drug modulation and drug synergistic proved to be hope for its treatment. Some of the plant derivative, phages, and nanoparticles are non-antibiotic sources to treat *S. aureus*. Various attempts to treat *S. aureus* at the world level have been carried out but require more researches to be undertaken in order to prevent it. The chapter concludes that *S. aureus* from dairy needs equal attention as is given to *S. aureus* from the human origin, and researches are required to probe solutions.

Keywords: *Staphylococcus aureus*, prevalence, public health, antibiotic susceptibility, prevention strategies

1. Introduction

Staphylococcus aureus is a Gram-positive coccus, non-motile, non-spore-forming, catalase positive, coagulase positive, and facultative anaerobic bacteria that is responsible for all kinds of mastitis in dairy animals. The pathogen has developed the capability to resist action of most of the antibiotics used in disease management. The inflammation in mammary

glands of dairy animals is a worldwide issue, origin of which may be infectious or non-infectious. The latter is less frequent that, however, occurs due to physical insult to mammary glands during or after milking. The bacterial contaminants cover most of the part of the infectious causes of mastitis. The pathogenic pattern of *S. aureus* involves adherence to mammary epithelial cells and to the extracellular components. Subsequent to this comes the mammary epithelial invasion where they remain in membrane-bound vacuoles of the mammary gland's epithelial cells. The phagocytic activity of the phagosome is bypassed to induce apoptosis. The recurrent subclinical infections occur because of bacteria dwelling in epithelial cells in that they inflict injury there by the endocytic process. Not only are the economic and health challenges limited to bovine, but potential zoonosis exists due to *S. aureus*. A clonal complex 398 representative of livestock-associated methicillin-resistant Staphylococcus aureus (MRSA) has proven the ability of colonization and serious health consequences in humans who are in close contact with animals.

2. Prevalence of *Staphylococcus aureus* from dairy milk

2.1. Cattle and buffalo milk

Bovine mastitis has been reported with more than 140 bacterial species in addition to minor prevalence owned by fungi, algae, and virus where *S. aureus* stands in an average number as the first causative agent for this malady. *S. aureus* prevalence is variable, starting from less than 10% to as high as 65%. The staphylococcal isolates from the bovine subclinical mastitis have been tuned to 85% in Pakistan. Recent studies in Canada reported a 46% *S. aureus* prevalence at herd level. The pathogen is invariably present in both buffalo and cattle but some of the studies report higher prevalence in buffalo than cattle. Variation in prevalence of *Staphylococcus aureus* within and among different dairy species might be because of bacterial survival in keratin layer of mammary glands where various immune evasive techniques like biofilm production are the reasons for lower shedding of bacteria from the mammary gland's environment. Other factors include geographic area variation, breed, specie, and farm management. The prevalence of mastitis in buffalo is higher than that of cattle in various studies. The fact behind might be higher nutritive values of its milk that favor growth of bacteria. The longer teats with pendulous shape also support bacterial invasion which is comparatively higher than that of cattle [1]. Some of salient features for spread of this pathogen are regarded as Milker's hands, flies, and towels spread these pathogenic bacteria to clean udders during milking practices.

2.2. Camel milk

Studies about camel diseases reported lower prevalence of mastitis before the twentieth century. The reason for not prioritizing camel mastitis was that higher contents of lactoferrin are recognized as antibacterial. However, later studies identified various aspects of mastitis. The studies on the microbial involvement find *S. aureus* invariably present with various percentages. Its prevalence has been noted as lowest as 1.8% in Saudi Arabia and as high as 83% from Kenya. Pakistan has reported 74.04% of *S. aureus* prevalence in the camel community from the desert. The majority of studies reported a non-comparable higher prevalence of *S. aureus*.

However, some of the studies report it to be second major pathogen after *Streptococcus agalactiae,* thus, meaning that the prevalence of this pathogen was noted to be 20.35% at the world camel community so far. Variation in prevalence has been attributed to the irregular shedding pattern of this bacteria, different hygienic standards at farms, unhygienic milking process, and lower than required inoculum (0.1 mL) for streaking on growth media and biofilm production. Unhygienic conditions are dominant in the desert environment which results in heifer-harboring intra-mammary bacteria that upon giving birth keep shedding in milk. Use of devices to stop calf suckling, tick infestation, udder deformities inflicted by thorny bushes, and camel pox favors the spread of mastitis. All these factors are unleashing *S. aureus* incidences. Some diagnostic screening techniques have been attempted for early identification of this pathogen that otherwise requires biochemical protocols. Sensitivity of the California mastitis test is reported to be 68% in a study. In case of camel milk, the California mastitis test is difficult to perform in that large numbers of cellular fragments surrounded by plasma membrane having rough endoplasmic reticulum and mitochondria but lacking nucleus are found normally in milk. Presence of these cellular fragments creates false positive results that normally require lymphocyte, neutrophils, and macrophages that are markers of inflammation.

2.3. Goat milk and sheep milk

S. aureus prevalence in caprine milk has been tuned to 66% [2]. Raw milk cheese and unpasteurized milk is consumed on traditional grounds. Apart from quality and quantity of milk deterioration, bulk milk contamination with *S. aureus* reflects the severity of farm's subclinical and clinical mastitis. This could be a reason of high observation of *S. aureus* from bulk milk. Swiss dairy farms reported 30% of goat and sheep herds having been identified with *S. aureus*. The virulence of *S. aureus* observed was same both for caprine and for ovine in terms of *splE* and *sdrD*. Higher prevalence of *spLE* was observed in goat milk while *lukM* was observed in sheep milk. Genes that code superantigen-like proteins (*ssl*) were observed to be immunoevasive as they interfere the toll-like receptor system. The biofilm-forming gene (Q7A4X2) was observed in addition to *sdrD*, *splE*, and *lukM* that are mainly virulent factors of *S. aureus* isolated from small ruminants. Studies have reported that the *Staphaurex* latex agglutination test is a more effective diagnostic tool in case of caprine and ovine *S. aureus*. This test was reported to present 51% of results as false negative when used as a diagnostic test for bovine *S. aureus*. Information is limited on raw bulk milk contamination with *S. aureus*. There are limited studies reporting ewe's bulk milk tank contamination with *Staphylococcus aureus*. The heterogeneity in *S. aureus* reporting exists with peak percentage during the 2003 studies that reports 33.3% [3, 4]. However, meat of ewes is reported to have 20–94% of *S. aureus* incidences [5].

2.4. Risk factors

The animals in older age are more prone to mammary gland infection due to dilated teats, previous repeated exposure to infection, and lower immune response [6]. The animals in old age are at double the risk with mastitis than animals of younger age. On the other hand, some of the studies did not find age as a risk factor for mastitis. The unhygienic conditions at farms along with other risk factors may result in infection to animals irrespective of age. Lactating animals are more prone to Staphylococcal infection because at lactation state spread of contagious pathogen increases if hygienic measures are not adopted. The periparturition period

is most susceptible to disease because of lower immune response during this period. Some of the studies report higher prevalence of disease in late lactation with reasons of lower immune response [7]. Early lactation was also found susceptible in some of the studies with reasons of higher milk production which is positively correlated with spread of mastitis. Ticks work to spread the pathogen from one animal to other. They create a suitable environment to aid microbial pathogenesis. Most of the studies have reported higher prevalence of mastitis in cases where ticks were infecting.

The higher parity was found more susceptible to infection. This was justifiable with carryover of infection from one parity to the next. Some of the researchers did not find the correlation of mastitis with parity number [8]. *S. aureus* being contagious is positively correlated with an unhygienic milking system. Fore milking stripping is found with *S. aureus* that may spread to other animals if hygiene is not adopted [9]. While conducting studies on prevalence of *S. aureus*, it is advised to discard the first few strippings of milk. However, spread of environmental mastitogens is not linked with fore milking stripping. The farms where teat dipping before and after milking with chlorhexidine and iodine is being practiced are reported to have reduced chances of disease [10]. They are discovering alter resistance of antibiotics against foodborne bacteria [11].

2.5. Types of *S. aureus* strains isolated from dairy milk

Staphylococcus aureus comes from the family Staphylococcaceae and genus *Staphylococcus*. The *Staphylococcus* genera is reported to have 42 species that are further categorized based on coagulase production. There are some of species of this genus that are normal inhabitants of the skin and mucus membrane. The species other than *Staphylococcus aureus* that produce coagulase and are found in etiologies of mastitis include *Staphylococcus intermedius* and *Staphylococcus hyicus*. The production of coagulase may not be strictly adherent to these strains due to advent of genetic variation. In addition to this phenotypic identification, results' interpretations exist [12]. This invites nucleic acid target-based techniques for the sake of identification and classification. The virulent genes namely spa igG binding, icaA, icaD, agrI-agrIII, cap. fnbA, fnB, hla, hlb, clfA, nuc, and spa X-region are linked to bovine mastitis. Added to these are *mecA* gene, *blaZ* gene, vancomycin-resistant genes, and hyper-virulent genes that increase diagnostic labor [13]. Salient virulence factors that include hemolysin (alpha, beta, gamma, delta), heat-shock protein, enzymes (nuclease, lipases, protease, staphylokinase, esterase), capsular polysaccharides, slime, cell-adhered proteins (fibronectin-binding protein, elastin-binding protein, collagen binding protein, and protein A) have been frequently identified from dairy milk. They have direct effects on public health (14, 13). Methicillin-resistant *S. aureus* not only spreads to animals but also has been reported to develop outbreak in humans [14].

Biofilm-producing strains in subclinical and clinical mastitis are also one the rise. These are sessile microbial-derived community of cells that get attached to substrate or to the each other whereby they are embedded in self-produced extracellular polymeric substance of diverse constituents like DNA, protein, carbohydrate and so on [15]. Identification of these strains from *S. aureus* has been tuned to 61%, and this may increase in environment where suitable risk factors are observed. The intra-mammary infections settled for long periods call for adhesive colonies' aggregation that are surrounded by the self-created exopolysaccharide matrix, the biofilm. The biofilms evade phagocytosis because of higher size. The matrix of biofilm varies from specie to

specie, and also the environmental circumstances play a role in determining the complexity of the biofilm's matrix. Biofilms have proven resistance to ultraviolet light, antibacterial drugs, biocides, biodegradability, and amplified genomic diversity, diversified degradability, and higher production of secondary metabolites [16]. The resistance to antibiotics is attributed to the physical barrier (exopolysaccharide), limited growth of bacteria in biofilm, accumulation of antibiotic-degrading enzymes in the matrix, and transformation of protein in the cell wall of bacteria.

2.6. Public health concern

Staphylococcal food poisoning (SFP) cases have been reported by Centers for Disease Control (CDCs) in the USA to be as high as 240,000 [17], while Europe observed 386 outbreaks in 2014 (Anonymous, 2015). The outbreaks are characterized with diarrhea and violent vomiting soon after ingesting SFP food. Analysis realized the involvement of enterotoxins and super antigens; some of those were classical enterotoxins like SEA-SEE and others were newly identified [18]. The necessitation of identification of *S. aureus* from domestic animals is impartial because of their residency in animals that act as a reservoir for onward infection. The feature is in addition to their role in compromised livestock economy [19]. The spread to public health presented new strains entitled LA-MRSA (livestock-associated methicillin-resistant *Staphylococcus aureus*). The frequent isolation of LA-MRSA has been observed by farmers, veterinarians, and farm workers' family members [20]. *S. aureus* produces heat-resistant enterotoxins that are one of the leading food poisoning causes. They are actually of 26900–29600 Da, molecular weight moiety, that up till the moment is nearly 20 different kinds of isolated entitled as staphylococcal enterotoxins (SE) and staphylococcal enterotoxin-like proteins (SEI). The prevalence of enterotoxins is rising in various dairies. These enterotoxins may be effective in milk even when *S. aureus* is not viable [21]. In Turkey, 46.9% of SEs of one or more types were isolated from subclinical bovine mastitis [22]. The Samsun province of Turkey presented 75% enterotoxins from raw milk [23], while 68.4% of strains isolated from bovine raw and pasteurized milk were positive for SE genes. The toxic proteins of bacteria exploit host tissues to produce nutrients for their growth. Staphylococcal enterotoxins are hypothesized to induce emesis. They are associated with inflammatory mediators like prostaglandin E_2, 5-hydroxyeicosatetraenoic acid, and leukotriene B4. The observed areas of inflammation in gastrointestinal tract appear with upper part involving stomach and intestine. The observable pathogenesis includes exudate in duodenum.

Not only had the raw but processed milk also reflected 10.4% of *S. aureus* prevalence, the analysis isolated five virulent genes encoding Paton-Valentine leukocidin, staphylococcal enterotoxin, toxic-shock syndrome toxin-1, methicillin resistance, and exfoliative toxin. More than 60% of strains presented greater than one virulent factor. The strains show variable response to various classes of antibiotics and even to the members of each class. Cheese made of goat milk may have this pathogen as some of the studies have detected 9.5% of this pathogen's involvement that was characteristically enterotoxigenic, coagulase positive, and methicillin resistant. The studies found six new alleles (glpf-500, pta-440, aroe-552, aroe-553, yqil-482, and yqil-496) and five newer sequence types (STs) that is to say ST 3431, ST 3440, ST 3444, ST 3445, and ST 3461 in *S. aureus* from goat milk. Isolation of novel alleles in *Staph areus* from goat is thought normal than those of bovine and humans in that more focused studies are scarce in case of goats.

2.7. Economic damages

Economic damages that are outcomes of clinical and subclinical mastitis are entitled as reduced milk yield, spoiled milk, lower milk quality, unstable taste, reduced milk processing, lower shelf life, and decreased yield of milk products. The ancillary economic burden includes treatment costs, spread of disease, culling, veterinarian fee, and labor costs. For staphylococci, losses to dairy in the Dutch dairy system were noted to be €293 per cow clinical mastitis. Dairy cattle per cow clinical cases were anchored to estimated €277 for the first three month's post-calving and €168 onward to the end of lactation. In US dairy circumstances, the estimated economic damages in dollars are estimated to be $1.8 billion/9 million dairy cows on an annual basis, exclusive of antibiotic residual in human diet, costs used to control milk's nutritive quality, and degradation of milk.

2.8. Drug susceptibilities and drug modulation

2.8.1. Susceptibility

The susceptibility of *S. aureus* from bovine mastitis is variable in the increase or decrease in resistance against antibiotics. Somewhere, *S. aureus* is noted to be pan-susceptible to antibiotics in studies from goats, while pan-resistance from bovine milk is also on record. The report of a retrospective study concludes two times the reduction of *S. aureus* resistance against penicillin while six times resistance against erythromycin over a period of 6 years [24]. This was not true in reports encompassing results of studies conducted in other geographical locations where resistance to the antibacterial drug increased to double of what was reported 12 years ago [1, 25]. The studies later to 2001, however, mention increase in general resistance of *S. aureus* strains against antibiotics. The difference in trends is attributed to evolution of resistance against local microflora being under therapy selection, traditions of farmers, drug regulation of country, local antibiotic therapy protocols, and number of processed samples in the study. Bacteria use horizontal gene transfer from resistant to sensitive strains [26]. The prevalent resistance genes noted in *S. aureus* encode for oxacillin (mecA), erythromycin (ermA, ermB, erm C), gentamicin (aac-6/aph-2), and tetracycline (tetK and tetM), penicillin (blaZ), and vancomycin [27].

Penicillin and cephalosporin group of antibiotics are found to be generally resistant against *Staphylococcus aureus* from bovine and camel milk. However, susceptibility varies from species to specie, region to region, strains of *Staphylococcus aureus*, and frequent exposure to antibiotics. Cefoxitin- and vancomycin-resistant strains are emerging. Linezolid is however effective in current dates against *Staphylococcus aureus* strains of bovine milk. The antibiotic trials have presented ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, chloramphenicol and tetracycline effective against *Staphylococcus aureus* that originates from various dairy animals. Higher susceptibility of *S. aureus* could be because of infrequent use of antibiotics in that area. Pan-susceptibility is noted higher than all dairy animals in that the drugs that usually face resistance by *S. aureus* of other dairy animals are quite effective in case of ovine *S. aureus*. Penicillin resistance is extensively noted while limited resistance was found when tested against *S. aureus* of ovine milk. The current status of ovine-based *S. aureus* was 100% susceptible at Greece farms, which thus reflects an absence of methicillin-resistant strains. The feature is attributed to very low pressure of antibiotic use at sheep farms in Greece. Traditional

farming is mostly on organic farming so they are safe from MRSA infection that in turn draws attention toward ovine milk as safe food.

2.8.2. Drug combinations

The increased resistance has been noted against all kinds of antimicrobials and no introduction of any new drugs has invited the use of newer drug combinations. Some of the drugs from the aminoglycoside group are although effective but reportedly linked to ototoxic and nephrotoxic effects due to their continued use. The drug combination requires antibiotics to target at different sites. Penicillin group in conjunction with aminoglycoside was reported as potent, effective, and safe. Combination of cephalosporin (cefaroxil) and penicillin (amoxicillin) showed synergistic effects against 80% of resistant isolates. Within the drug class, for example, β-lactam with ß-lactam combinations, efficient results were presented as well [28], and in vivo trials have also proved their effectiveness. Aminoglycosides are potent drugs that create fissures in the outer portion of bacterial cell wall by binding with 30S ribosomal subunit, thus misreading mRNA. Penicillin in combination with chloramphenicol has been reported synergistic in some of the studies while antagonistic results have also been reported. Antagonism reported in some studies claim penicillin to activate while chloramphenicol to deactivate murein hydrolase that in its function is responsible for lysis of bacteria. The general concept describes bactericidal and bacteriostatic to be antagonistic which is now true in other studies [29]. This trend might be because of diversification of genetic variation in modern pathogens.

2.8.3. Plant derivative effects/drug modulation

Plants have various antimicrobial peptides like c-thionin and thionin Thi 2.1 tested against intracellular *S. aureus* of bovine mastitis. These peptides in addition to their antibacterial activity work as immune modulators. The extracts from other plants like ethanolic extract of propolis (EEP), a resinous mixture obtained by honeybees from plants, are reported to be highly biologically active against *S. aureus* mastitis. There is limitation attached with this in terms of lower minimum inhibitory concentration (MIC) when tested in milk environment. However, authors have suggested its in vivo activity against mastitis. Monolaurin, a coconut oil derivative made of glycerol monoester of lauric acid, has also presented antibacterial activity against *S. aureus*. Extracts of *Tabernaemontana divaricata* (L.) have shown significant efficacy against a group of microorganisms of bovine mastitis origin which demand further research to be undertaken [30]. The bovine clinical mastitis-based *S. aureus* showed sensitivity against crude extracts of *Combertum molle* and *Commicarpus pedenculosus* medicinal plants [7].

The development of resistance demands some alternative ways to combat *S. aureus*. The bacterial resistance takes place due to impairment in binding as a consequence of genetic mutations, enzyme production, for example, hydrolyzing that impaired amide bond, and efflux extrusion which is responsible for reduction in drug concentration inside the cell [31, 32]. The constituents of plant extracts modulate resistant mechanism techniques of bacteria to the extent where they become sensitive. Various in vitro trials have been reported with promising results against multidrug-resistant *S. aureus*. Some of the plants naturally growing in animal-rearing areas are featured with antimicrobial characteristics. A few among 500 plant species are explored in documentation with proven antibacterial effects. There is wider scope yet to

be explored as an alternative source of bactericidal. *Calotropis procera* and *Eucalyptus globolus* have proven activity against *S. aureus*. These plants are salt and drought resistant growing in wider quantities in the surroundings of animal-rearing far areas. Plant extracts in synergy with antibacterial drugs target various sites of *S. aureus,* thus modifying phenotypic resistance to sensitivity [33]. The antibacterial activity is attributed to flavonoids, alkaloids, saponins, glycosides, phenols, and tannins. The active ingredient gives rise to the porous cell wall, thus releasing contents from cytoplasm, electron-transport chain inhibition, and interference with sphingolipid inhibition [34]. The activity may vary depending on the variation in solvents for extraction, the stage of plant's cultivation, geographical area, method of extraction, and specific mode of action [32].

2.8.4. Nanoparticles

The recent few years have presented nanoparticles (NPs) which have emerged as a cost-effective potential antibacterial against various pathogens. Nanoparticles (NPs) are small particles of 1–100 mm size and work by disruption of cell membrane, simultaneous activation of multiple mechanisms, and action as antibiotic carriers. They break physical barriers made of biofilms to reach bacterial cells embedded inside whereby antibiotics cannot reach alone. Oxidative stress, non-oxidative stress, and metal-ion release mechanisms are used by Ag, Mg, NO, ZnO, CuO, Cu_2O, Fe_2O_3, FeO, and many others to kill bacteria. Multiple drug-resistant *S. aureus* showed a 177 mm zone of inhibition at 80 μL of silver nanoparticles. Nitric oxide nanoparticles are not only effective against *S. aureus* but also play a role in prevention of mastitis in dairy animals. They alone and in combination with antibiotic preparation are evaluated in vitro targeting *S. aureus* and also the wound healing. Nanoparticles that work as drug delivery include liposomal NPs, inorganic NPs, polymer-based NPs, terpenoid-based NPs, and polymer micelle NPs. These nanoparticles coat antibiotics and effectively reach to the site where the drug mechanism does not work. The encapsulation of antibiotics with nanoparticles makes drugs express their potential that in alone are unable to impart their effect. Tilmicosin-solid lipid and amoxicillin are sometimes unable to deliver their effects alone but encapsulation with nanoparticles complements their activity at full bloom. Hydrogel-coated nanoparticles, for example, sliver hydrogel coated, proved to be superior in antibacterial activity, viscosity, and drug release. Several studies have proven their efficacy in terms of wound healing, normal skin appearance, and hair growth. These particles help make production of hydrogen per oxide and reactive oxygen species at wound site that help cure infection/mastitis. The small-size particles confer cell death and reduction of bacterial resistance.

2.8.5. Other alternatives

Phages are alternative sources where no other therapeutic action against pathogens is workable. The staphylococcal species may effectively be lysed with phage K. Moreover, phage K can be used prophylactically against intra-mammary infections endorsed by *S. aureus*. Phage K is reported as a pocket rocket against mastitis by some researchers. On the other hands, phages are vulnerable to mammary glands' immune system and whey protein of milk that render phages ineffective [35]. Studies are needed to rule out pharmacokinetics and pharmacodynamics in addition to the challenges of their administration into tissues. Another polyvalent

virulent phage, MSA6, is isolated from cow mastitis that is being used as a potential universal anti-staphylococcal agent [36]. This particular phage is applicable against a wider host range, superior lytic action, and importantly are thermo stable. The peptidase derived from the bacteriophage, $CHAP_K$, of cow mastitis is effective both at prophylactic and at therapeutic ends. Biofilm-producing strains of *S. aureus* may be effectively prevented from biofilm production and disruption of already established biofilms. Stress can affect bacteriophage activity. Some bacteriophages including Sabp-P1, Sabp-P2, and Sabp-P3 are resistant to environmental stress [13]. Apart from limitations, phages resistant to stress can be best applicable for futuristic staphylococcal mastitis treatment.

Cytokines are proteins with a definitive role in cell signaling. Some of the recombinant cytokines of bovine origin like IL-2, IFn-c, and TNF-alpha stimulate both kinds of immunity (innate and acquired) in mammary glands. However, their effect in combination with antibiotic therapy is additive against mastitis [37]. Beta-lactoglobulin protein is normally present in mammal's whey while lactoferrin is present in milk, bronchial mucus, saliva, and tears. Both molecules have proven activity against *S. aureus*-based mastitis. These proteins complement a higher spectrum of antimicrobial activity either applied alone, in combination with each other, or in combination with antibiotics. There are other animal-derived sources like marine sponges that exhibit antibacterial activity against a wider range of Staphylococcal species when used in extracts. These sponges include species from Cinachyrella, Haliclona, and Petromica that were effective antimicrobial agents against 61% of tested microorganisms [38].

2.8.6. Bacteria with probiotics

Mechanisms of persistence of *S. aureus* in intra-mammary environments still need to be explored but evasion of host immune system and adherence to epithelial cells of mammary glands are some of the known in this regard [39]. Some bacteria like *Weisella confuse* and *Lactobacillus casei* are reported to produce certain compounds that are active against internalized persistence of *S. aureus*. Lactic acid bacteria have the ability of adherence to epithelial cells, thus resisting *S. aureus* pathogenicity by its competitive adhesion ability, production of H_2O_2, competition in nutrition utilization, and host immune modulation [40]. Continuous use of Weissella strains and their metabolites are reported to be effective alternatives of antibiotics in control and prevention of mastitis [41].

2.9. Prevention strategies against dairy *S. aureus*

Controlling *S. aureus* in dairy products is needful for commercial and profitable small-scale cow farming for improving milk quality to consumers as well as dairy industries. Although a significant progress has been done in over the last 30 years, *S. aureus* seems to be still severe in dairy animals around the world. The lack of effectiveness of the current strategies (principally based on antiseptic teat dipping after milking and antibiotic therapy during the dry period) to suppress *S. aureus* has promoted in the sense of vaccine preparation against *S. aureus* which is a reasonable/alternative approach for the control of these microorganisms associated with mastitis. Studies have reported higher prevalence coupled with increased resistance to antibiotics in *S. aureus* isolates of camel mastitis [42, 43]. The emergence of discrepancies in resistance

identification has also added to increased resistance in terms of unjustified use of antibiotics to combat *S. aureus* [44, 45]. Resistance to antibiotics and the phagocytosis phenomenon leads to treatment failure against *S. aureus,* so the vaccine development against mastitis is an exigent to prevent new infections by *S. aureus* for commercial dairy farms. Anti-*Staphylococcus aureus* vaccines give different results, depending on the type of vaccine, the adjuvant used, and some other factors involved.

2.9.1. Vaccinal targets in S. aureus

Several studies have shown that different soluble and cytotoxic factors are involved which increase the *S. aureus* pathogenicity by using different pathogenic factors, for example, pseudocapsules, toxins, clumping factors, protein A, and fibronectin-binding protein. It has been suggested that these pathogenic factors should be considered for preparing mastitis vaccine to be used in field conditions. Furthermore, it has recently been suggested that the *S. aureus* vaccine may be much effective if it is multicomponent integrated with surface proteins, toxins, and surface polysaccharides. Recently, it has been proposed that more than 99% of the world's bacteria exist as biofilm producers. Experts at disease control and prevention centers, the USA, estimate that 65% of human bacterial infections are involved in biofilm production [46]. The term "biofilm" for bacteria refers to a structured population of bacterial cells enclosed in a self-produced polymeric matrix and attached to an inert or living surface that forms a protected growth pattern that allows surviving in harsh environments. Biofilm-forming microorganisms produce a particular mechanism to attach the surface to form a microbial community, producing a three-dimensional structure of mature biofilms [47]. Their growth rate, composition, and resistance to anti-biocides, antibiotics, and antibodies are all different because they up-regulate and/or down-regulate about 40% of the genes. This makes it difficult to eliminate the infections due to such microorganisms with therapeutic doses of antimicrobials. A better understanding of the mechanisms by which they can evolve and survive in sessile environments can help in designing control strategies against *S. aureus* [48].

2.9.2. Vaccines in action

There is growing evidence that *S. aureus* can form biofilms in the udder of dairy cows affected by mastitis. Biofilms not only affect the host's immune system but also prevent the action of antibacterial drugs, leading to persistent infection. *S. aureus* causes chronic infections, resulting in significant financial losses in most of the cases [49]. Biofilm is an important factor in the virulence of *S. aureus* [50]. It has been demonstrated that the active immunization of exopolysaccharides extracted with strongly adherent *S. aureus* isolates provokes the defensive immunity against mastitis [51]. The use of antibiotics to treat and prevent *Staphylococcus aureus* mastitis has driven mastitis researchers in preventing udder infections through vaccine due to high costs, low cure rates, high antibiotic resistance, and consumer concerns about antibiotic residues in milk and meat [52]. Various mastitis vaccines have been studied including inactivated whole cell, live vaccines, cell wall components, bacterin toxoid, and antigen extracts with or without adjuvants. Findings of some researchers are summarized later. Israeli workers [53] supervised a large number of field trials with commercially available vaccine (MSTIVAC

I; Patent No. PTC/IL 98/00627) for *S. aureus* mastitis. The authors observed a 42–54% reduction in first and second lactation in SCCs and 0.5 Kg/day/animal increase in milk production as compared to unvaccinated (control) cows. In the vaccinated group, only 3 out of 228 animals (1.3%) while in the control group 6 out of 224 (2.7%) was detected. No statistical analysis was conducted as these numbers were low for statistical analysis between vaccinated animals and non-vaccinated (control) animals. Later on, findings of Athar [54] at the Department of Clinical Medicine and Surgery of the University of Agricultural Faisalabad (UAF), Pakistan, confirmed that the locally developed polyvalent vaccine for mastitis (incorporated with killed *S. aureus*, *S. agalactiae* and various *E. coli*) provided protection for new infections as well as eliminated existing infections in dairy buffaloes. Similarly, other authors have also observed such findings with locally prepared *S. aureus* vaccines (plain bacterin, oil-adjuvant bacterin, live attenuated vaccine and dextran sulfate-adjuvant bacterin) [47]. Brouillette et al. [55] conducted a DNA immunization study against the *Staphylococcus aureus* aggregation factor A (CIF-A). It has been found that preincubation of *S. aureus* with serum obtained from vaccinated mice reduces the ability of pathogens to bind up to 92% of fibrinogen. These preincubated bacteria were phagocytosed by elevated macrophages in vitro, whereas, in in vivo trials, these were less toxic when evaluated experimentally in a mouse-mastitis model. However, DNA-immunized mice could not resist the challenges caused by the intraperitoneal route. The results showed that DNA immunization can be used as a new method to prevent *S. aureus* infection.

2.9.3. Current scenario of vaccines

In this new era, mastitis has been one of the imperative diseases in dairy cows, despite tremendous advances in improving overall udder health. Epidemiological studies have showed a lot of variations in biological cure rates (from 0 to 80%) following antibiotic treatment, but these do not show the significant loss of antibiotic activity of the major classes. Repeated infections often lead to the formation of biofilms in bacteria. In the case of microorganisms, biofilm formation is caused by subsequent physiological and significant genetic changes resulting in loss of sensitivity to antibiotics, thus leading to development of resistance to antibiotics of different classes. Ahmad and Muhammad [56] conducted a study on the preparation and evaluation of *S. aureus* and *S. agalactiae* aluminum hydroxide adjuvant mastitis vaccine in rabbits. Biocharacterization of both bacteria was done from 95 milk samples collected aseptically from mastitic buffalos. Immunogenicity, pathogenicity and susceptibility testing of antibiotics was performed. Bivalent aluminum hydroxide adjuvant vaccine was developed in the Mastitis Research Laboratory at Clinical Medicine and Surgery Department, University of Agriculture Faisalabad-Pakistan. The vaccine was proved stable, sterile, and safe to use. Rabbits were used to evaluate the quality of the vaccine and the antibody response. For this purpose, rabbits were divided into two (GA and GB) groups, having 10 rabbits in each. Rabbits in the GA group were injected with *S. aureus* and *S. agalactiae* aluminum hydroxide-adjuvant mastitis vaccine, while the rabbits in second group (GB) remained non-vaccinated. To check the antibody titers in rabbits of group GA, indirect hemagglutination inhibition assay (IHA) was performed. GA rabbits had the highest anti-*S. aureus* serum antibody titer (GMT) which was 78.8 at the 45th day, dropping slightly to 73.3 on day 60 post-vaccination. IHA titer gradually increased for *S. agalactiae* at days 45 and 60 after the inoculation of vaccine. The cumulative mean antibody

titer (CMT) for the vaccinal *S. aureus* was 44.94 and CMT for the vaccinal *S. agalactiae* was 46.56 as compared to the control group. The CMT was significantly higher in vaccinated group at days 45 and 60 after the vaccination than the control group. The study showed that the bivalent aluminum hydroxide-adjuvant vaccine was immunogenic in rabbits. To evaluate the *S. aureus* bacterin, Middleton [57] used a lactating cow model to study the ability of this vaccine to prevent intra-mammary infections (IMI) of staphylococcal (*S. aureus* and coagulase-negative staphylococci (CNS)). Assessment parameters were the vaccination effects on somatic cell count (SCC) and the effects of vaccine on the antibody isotype of milk. For this purpose, 90 lactating cows of Holstein-Friesian were selected and divided into two groups. One group (n-44) served as vaccinated group and the second group (n-46) was the control group. First group received 5 mL of bacterin vaccine, 2 shots, 14 days apart. Milk samples were collected from individual quarters for bacterial culture before each shot and then collected monthly for 6 months. For determining IgG1, IgG2, IgM, IgA, and SCC, composite samples of milk were collected on days 0, 14, 28, 49, and 70. The authors did not observe any new IMI in any group and this was not different significantly between the groups ($p > 0.05$) of mammary quarter infection. The vaccine in herds having been reported with coagulase-negative staphylococcal prevalence (30%) and *S. aureus* prevalence (3%) in intra-mammary infection did not respond well to newer *Staphylococcal* infections. Another study has evaluated a multicomponent vaccine to eradicate staphylococcal biofilm infections [58]. Selected antigens including glucosaminidase (hypothetical conserved protein), an ABC transporter lipoprotein, and conserved lipoproteins have been found in previous studies to sustain and up-regulate expression in biofilms both in vitro and in vivo. For these antigens, the antibody was first used in a microscopic study to determine its expression in an in vitro biofilm. In biofilms, each of the four antigens exhibits heterologous production at different locations within a complex biofilm community. The four antigens were delivered simultaneously as a quadrivalent vaccine. As vaccine antigens were specific for biofilms, antibiotic treatments were also used to remove residual and non-adhered planktonic cells. The results showed that the clinical and radiographic symptoms were reduced to 67 and 82%, respectively, when the vaccine was given with vancomycin treated in biofilm rabbit models with chronic osteomyelitis. It was compared with animals infected or not treated with vancomycin. In contrast, only vaccination resulted in a modest and insignificant reduction.

Recently, Raza [47] evaluated the role of a bacterin toxoid prepared from a strong biofilm-producing *S. aureus* in effective immunization of rabbits. The strong biofilm-producing *S. aureus* selected from 64 isolates of staphylococci was used to prepare bacterin toxoid, and aluminum hydroxide gel was added as an adjuvant. The vaccine was evaluated in rabbits by challenge protection assay and humoral immune response. The mortality rates in control and vaccinated groups were 80% and 10% at day 7 post-challenge and 100 and 20% at day 15 post-challenge, respectively. Serum antibody titer (GMT) was significantly higher (294.0) in vaccinated group as compared to the control group rabbits (2.63) at day 45. The results showed an increased antibody production in the vaccinated group that was capable of preventing establishment of new *S. aureus* infection in rabbits as compared to the control group. Based on the results of the present study, a short-term clinical trial was conducted in dairy cows and buffaloes which also showed effectiveness of vaccine as indicated by a significant difference in the prevalence and incidence of mastitis, high level of variation in the microbiological examination of milk, reduced

intra-mammary infections, and somatic cell counts between vaccinated and control groups of dairy cows and buffaloes.

3. Conclusions

Staphylococcus aureus from dairy animal origin has obtained more serious attention than that of human origin in terms of pathogenicity, strain variability, response to antibiotics, public health concern, and economic losses to the dairy industry. Apart from bovines, camel and caprine are noted with surged prevalence since last few years. Being contagious in nature, *S. aureus* has been found to be emerging due to the increase in the span of risk factors. As there is an increase in antibiotic resistance against *S. aureus*, the hope in the form of non-antibiotics like nanoparticles, plant derivatives, bacterial, and phage based-remedies exists. Vaccines, as a preventive strategy, have been implemented at local and commercial levels. The research is required for a comprehensive approach both at preventive and at therapeutic levels.

Conflict of interest

Authors declare no conflict of interest

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