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Coliform mastitis

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Abstract – Gram-negative bacteria that commonly cause bovine mastitis are classified as environmental pathogens. The point sources of coliform bacteria that cause infections include bedding materials, soil, manure and other organic matter in the environment of cows. Rates of coliform mastitis increase during climatic periods that maximize populations in the environment. The portal of entry into the mammary gland for Gram-negative bacteria is the teat canal. Once in the gland, bacteria must utilize available substrates in the mammary secretion to replicate and evade host defenses. Rates of coliform mastitis are greater during the transitional phases of the non-lactating period than during lactation. The ability to infect the non-lactating gland is directly related to the ability of bacteria to acquire iron from the mammary secretion. The primary host defense against coliform mastitis during lactation is the elimination of bacteria by neutrophils migrating into the gland in response to inflammation. Damage to the host is mediated by the release of endotoxin. The severity and duration of clinical signs associated with coliform mastitis are reduced by the use of core-antigen bacterins.

coliform mastitis / virulence factor / risk factor / core antigen vaccine

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1. INTRODUCTION

Gram-negative bacteria are the etiological agents most often isolated from acute clinical cases of mastitis. The term *coliform mastitis* frequently is used incorrectly to identify mammary disease caused by all Gram-negative bacteria. Genera classified as coliforms are *Escherichia*, *Klebsiella*, and *Enterobacter* [37]. Other Gram-negative bacteria frequently isolated from intramammary infections include species of *Serratia*, *Pseudomonas*, and *Proteus*.

Gram-negative bacteria are considered environmental mastitis pathogens [34]. Transfer of Gram-negative bacteria from the mammary glands of infected cows to uninfected cows appears minimal compared with the constant environmental exposure. Coliform bacteria occupy many habitats in the cow's environment. *Escherichia coli* are normal inhabitants of the gastrointestinal tract of warm blooded animals. Both *Klebsiella* spp. and *Enterobacter* spp. populate soils, grains, water, and intestinal tracts of animals. *Serratia marcescens* share many environmental sources with *Klebsiella* spp. and *Enterobacter* spp. *Pseudomonas* spp. and *Proteus* spp. commonly contaminate drop hoses used to wash udders before milking. Gram-negative bacteria may be isolated from virtually any surface area of the cow or her surrounding and cause a host of diseases other than mastitis. Coliform bacteria are among the aetiological agents commonly responsible for infectious respiratory and

urogenital diseases in dairy cows. However, the spread of Gram-negative bacteria from other regions of the body to the mammary gland via the vascular or lymphatic systems appears minimal. Intramammary infections caused by Gram-negative bacteria typically result from the bacteria traversing the teat canal and multiplying in the gland. Although the mammary gland is not considered a natural habitat for coliform bacteria, many strains are capable of surviving and multiplying in the mammary gland.

2. ETIOLOGY

2.1. Diagnoses of infections

Diagnoses of intramammary infections caused by Gram-negative bacteria offers a number of unique challenges compared with other mastitis pathogens [34]. Colony-forming units in milk often are less than 100 cfu/mL for Gram-negative bacteria isolated in the later phase of clinical disease or from subclinical glands. Therefore, volumes of milk larger than 0.01 mL, traditionally plated on primary isolation media, are needed for isolating Gram-negative bacteria. Confounding this potential problem of low shedding rates is the fact that Gram-negative bacteria are common contaminants in milk samples taken for bacteriological examination. The use of enrichment procedures and pre-incubation of milk samples is discouraged as any contaminating

Gram-negative bacteria will proliferate and reduce the specificity of accurately diagnosing intramammary infections. Extreme care must be exercised to assure aseptic techniques are used during sample collection to avoid contamination and allow for accurate diagnoses of infections.

2.2. Primary isolation

Coliforms are heterotrophs capable of oxidizing organic compounds as a source of energy and grow readily on simple nutrient media. Blood agar is recommended for primary isolation of coliforms from milk of infected mammary quarters [34]. Coliform bacteria appear on blood-agar as grey to brown colonies ranging in size from 3 to 5 mm in diameter. A fecal odor is characteristic of colonies produced by these species. Less than 15% of *E. coli* are hemolytic and both *Klebsiella* spp. and *Enterobacter* spp. are non-hemolytic.

Colonies of *Serratia*, *Pseudomonas*, and *Proteus* on blood-agar appear quite distinct from coliform species. Following incubation at 37 °C, *Serratia marcescens* colonies on blood agar are 2 to 3 mm in diameter, grey to yellow, and resemble staphylococci. *Pseudomonas* spp. produce white to grey colonies with irregular edges. *Pseudomonas* spp. are usually hemolytic and produce a distinctive grape-like odor. *Proteus* spp. produce grey swarming colonies that can emit a putrid odor.

Selective and differential media, such as McConkey agar, can be used for isolation of coliforms and presumptive identification of genera [17]. McConkey agar is selective for Gram-negative bacteria and coliform bacteria produce pink to red colonies resulting from the utilization of lactose. *Escherichia coli* appear as pink to red, flat colonies surrounded by a pink zone of precipitated bile salts. *Enterobacter* spp. growth on McConkey agar results in pink, dry colonies, but lack a zone of precipitated bile salts as produced by *E. coli*. *Klebsiella* spp. produce large pink-yellow mucoid colonies on McConkey agar. Other Gram-

negative bacteria produce translucent colonies on McConkey agar. *Serratia marcescens* often produce red-pigmented colonies when incubated at 25 °C.

2.3. Biochemical identification

The use of triple-sugar-iron (TSI) test reaction, citrate utilization, and motility is a simple biochemical scheme for presumptive identification of Gram-negative bacteria commonly isolated from bovine mastitis [17]. Coliform bacteria are differentiated from other Gram-negative bacilli by the ability to ferment lactose within 18 h at 37 °C with the production of acid and gas. The TSI reaction of coliforms is acid slant (aerobic utilization of lactose), acid butt (anaerobic fermentation of lactose), and the production of gas. The TSI reactions of *Serratia* spp. are alkaline slant, acid butt, and no gas production. *Proteus* spp. produce a TSI reaction of an alkaline slant and acid butt with black precipitate resulting from hydrogen sulfide production. *Pseudomonas* spp. produce an alkaline slant, alkaline butt, and no gas as a TSI reaction.

Genera of coliform bacteria can be characterized by mobility and utilization of citrate [34]. *Klebsiella* are non-motile and can utilize citrate as the sole carbon source in a medium. *Enterobacter* are motile and also utilize citrate. *Escherichia* can not utilize citrate as a carbon source and greater than 90% of strains are motile. Biochemical testing schemes more elaborate than that described above are necessary to biotype strains for epidemiological surveys and research. In general, commercially produced miniaturized biochemical tests developed for identification of isolates from human clinical isolates offer an array of tests and have been successful for delineating Gram-negative bacterial strains isolated from bovine mammary glands.

2.4. Serotyping and colicin sensitivity

Serotyping and testing for colicin production of Gram-negative bacteria from

bovine intramammary infections have aided little in epidemiological studies to determine association of phenotype and pathogenicity [1, 6, 52, 63]. The wide distribution of O and H sero-antigens among coliforms isolated within a herd appears to reflect the same distribution of antigens among isolates collected from the herd's environment. Serotyping and colicin production do offer an identification pattern that may be useful as a means to substantiate the serial isolation of a strain from a mammary quarter.

2.5. Fingerprinting

The use of genetic fingerprinting procedures has had limited success in identifying virulence factors or establishing epidemiological patterns within herds [38]. Similar to phenotypic typing schemes, fingerprinting has the greatest value in delineating strains [4].

3. VIRULENCE FACTORS

Gram-negative bacteria isolated from bovine intramammary infections possess a myriad of virulence factors. Gram-negative bacteria isolated from bovine intramammary infections are opportunistic pathogens that reflect the population inhabiting the animals' environment. The only apparent prerequisite for a strain to cause mastitis is the ability to grow and multiply in mammary secretions. *Escherichia coli* and *Klebsiella pneumoniae* are the species for which virulence factors have been most completely characterized.

3.1. Traversing the teat canal

The portal of entry for Gram-negative bacteria into the mammary gland is the teat canal. The manner that coliform bacteria traverse the teat canal is unknown, but probably involves an opportunistic entry into the gland whereby at least a portion of the canal is bypassed. The bovine teat

canal is not susceptible to colonization by coliform bacteria [35]. The teat canal appears to provide a physically restrictive area for high concentrations of antibacterial systems in milk. Virulence factors that allow for growth and multiplication in the teat canal may be related to those responsible for multiplication and evading host defenses in the mammary gland [32].

3.2. Multiplication in the mammary gland

Adherence of *E. coli* and *K. pneumoniae* to epithelial tissue does not play a major role in the pathogenesis of bovine mastitis [15, 47]. Coliform bacteria do not appear to colonize inside the mammary gland, but multiply in the secretion without attachment to epithelial surfaces. The more rapidly that coliforms can adjust metabolically to mammary secretion, the more rapidly bacterial numbers increase and disease can occur. Therefore, the severity of clinical disease and peak coliform counts in mammary secretions are positively correlated. Two important virulence factors for coliforms are the ability to utilize lactose as an energy source and the ability to survive at near anaerobic conditions. Lactose is the principal carbohydrate in milk and the oxygen tension in the gland is very low. Coliforms that can metabolize the constituents of milk in the micro-environment of the gland can reach populations exceeding 10^8 colony-forming units per milliliter of milk [31]. In contrast, Gram-negative bacteria not capable of fermenting lactose, such as *Serratia* spp. and *Pseudomonas* spp., seldom exceed 10^4 colony-forming units per milliliter of milk.

Secretions from fully involuted mammary glands do not readily support growth and multiplication of coliform bacteria [46]. The limiting nutritional factor for many coliform bacteria in the dry mammary gland is iron [54]. Lactoferrin is an iron binding protein that increases in mammary secretion during involution and remains elevated until colostrogenesis.

Klebsiella pneumoniae are more capable than most strains of *E. coli* to overcome the inhibitory effects of lactoferrin and infect involuted mammary glands [58]. Coliform bacteria that can multiply in the secretion of involuted glands probably overcome the effects of lactoferrin by utilization of a high affinity iron acquisition systems. The enterobactin iron acquisition systems is commonly expressed by Gram-negative bacteria isolated from involuted mammary glands. The enterobactin system plays a vital role in pathogenesis during the dry period. Growth of *E. coli* can be inhibited in secretion from involuted glands by blocking iron uptake with antibody specific for the enterobactin receptor [41].

3.3. Evading cellular defenses

The primary cellular defense of the bovine mammary gland against coliform mastitis is the phagocytosis and killing of bacteria by neutrophils [18, 60]. The peak bacterial numbers in the gland and clinical severity of disease are often dependent on the speed and efficiency of the neutrophil response. The ability of a strain to evade neutrophils is a key virulence factor for coliform bacteria. Differences in susceptibility to phagocytosis among coliform strains is related to variability of surface exposed antigens. Capsules produced by *K. pneumoniae* isolated from bovine intramammary infections block deposition of complement and camouflage against antibody-mediated opsonization [62]. Capsule producing strains of *E. coli* are more likely to create intramammary infections of longer duration than are non-encapsulated strains of *E. coli* [19]. *Pseudomonas* spp. and *Proteus* spp. produce capsular material associated with reduced phagocytosis and chronicity of disease.

The expression of cell surface components other than capsule can affect susceptibility to phagocytosis. *Escherichia coli* strains within O serotype groups O8 and O9 possess antiphagocytic factors that are not related to capsule [19]. Expression of

outer membrane proteins induced by specific substrates and other environmental factors may alter the susceptibility of isolates to phagocytosis. As the composition of the mammary secretions changes with the functional status of the gland, the distribution of *E. coli* able to evade phagocytosis and establish disease also changes. *Escherichia coli* isolated from intramammary infections originating during the periparturient period were more resistant to phagocytosis than isolates from infections originating in the early dry period or during lactation [29]. These differences are not related to O serotype or capsule. Many coliform bacteria isolated from the bovine mammary gland are capable of expressing cytotoxins and hemolysins, but the production of exotoxins does not appear to be critical for evading host defenses [42].

3.4. Serum susceptibility

Resistance to the bactericidal activity of serum is a virulence factor common to many coliform bacteria causing clinical mastitis, but is not a prerequisite for pathogenicity [14, 26, 36, 52]. The relationship between in vitro resistance to serum and virulence in vivo appears spurious. Bactericidal activity of serum is due to complement. Complement activity is greater in secretions from involuted mammary glands than in milk collected during lactation [51]. In addition, milk diminishes the bactericidal activity of complement. The differences in complement activity among secretions collected from glands in differing physiological stages suggest serum resistance is a phenotypic trait offering a selective advantage to isolates infecting involuted mammary glands. However, serum resistance does not differ between coliforms isolated from intramammary infections originating during the dry period and strains creating infections that originate during lactation [26]. The percentage of isolates from intramammary infections susceptible to the bactericidal and bacteriostatic activities of bovine serum is similar

to the percentage of serum susceptible coliform isolates existing in the environment of the cow [5]. Therefore, serum resistant coliforms apparently have no selected advantage over serum susceptible coliforms for creating naturally occurring intramammary infections.

3.5. Endotoxin

Endotoxin is the primary virulence factor of Gram-negative bacteria responsible for damage to the cow. Endotoxin refers to the lipopolysaccharide portion of the Gram-negative bacterial wall. Endotoxin is released from the bacteria at the time of cell death initiating an inflammatory response. Locally, endotoxin does not directly effect secretory cell but disrupts the blood flow [53]. Systemic signs of clinical mastitis include anorexia, fever, dehydration, and diarrhea. Decreased milk production during clinical coliform mastitis results both directly and indirectly from the local and systemic effects of endotoxin [3, 21, 22]. Coliform mastitis can result in bacteremia and septicemia as the blood-milk barrier is destroyed [61]. Septicemia resulting from coliform mastitis is rare, but is often fatal when it occurs.

4. EPIDEMIOLOGY

Gram-negative bacteria are frequently the leading cause of clinical mastitis in well-managed dairies with low bulk tank milk somatic cell counts. Coliform pathogens generally account for the majority of peracute cases of clinical mastitis in a herd. Specifically, *E. coli* and *K. pneumoniae* are the coliform species most commonly isolated from intramammary infections and clinical mastitis.

4.1. Dry period

Rates of new intramammary infections caused by coliforms are greater during

the dry period than during lactation [12]. During the dry period, susceptibility to intramammary infections is greatest the two weeks after drying off and the two weeks prior to calving [56]. Many infections acquired during the dry period persist to lactation and become clinical cases. Research has shown that 65% of coliform clinical cases that occur in the first two months of lactation are intramammary infections that originated during the dry period [55]. Coliforms are adept at infecting the mammary gland during the transitional phase from lactating to fully involuted mammary gland. However, *K. pneumoniae* are more capable than *E. coli* at surviving in the mammary gland from the onset of involution until calving. Distribution of infections reveals that the greatest proportion of *K. pneumoniae* infections present at calving originated in the first half of the dry period. *Escherichia coli* infections present at calving and early lactation most often originate during the last two weeks of the dry period [59].

4.2. Lactation

Rate of coliform intramammary infections during lactation is highest at calving and decreases as days in milk advances. The prevalence of coliform mastitis in a herd seldom exceeds five percent of quarters because coliform infections tend to be of short duration during lactation. The average duration of *E. coli* intramammary infections during lactation is less than ten days [59]. Duration of intramammary infections caused by *K. pneumoniae* average about 21 days [55]. Chronic infections of greater than 90 days caused by *E. coli* or *K. pneumoniae* are relatively rare. A major difference between intramammary infections caused by coliform bacteria and those caused by other Gram-negative bacteria is the duration that bacteria persist in the mammary gland. Intramammary infections caused by *Serratia* spp. and *Pseudomonas*

spp. often are chronic infections that may persist multiple lactations [25].

The high frequency of clinical cases and relatively short duration of Gram-negative bacterial intramammary infections render the use of individual cow SCC and bulk tank SCC as poor indicators of the prevalence of disease caused by these bacteria [13, 20, 25, 57]. Prevalence of intramammary infections caused by Gram-negative bacteria seldom exceeds 5% of quarters in a herd, however greater than 25% of cows in well-managed herds are annually diagnosed with clinical mastitis caused by coliforms. The prevalence of intramammary infections caused by these bacteria is seldom great enough to cause bulk tank somatic cell counts (SCC) greater than 400 000/mL, but approximately 85% of coliform infections will cause clinical mastitis. Therefore, even low SCC herds can still have mastitis problems and these problems generally involve clinical cases of mastitis.

Recording the number of clinical cases and documenting the seasons and stage of lactation when they occur will aid in determining when cows are at greatest risk to clinical coliform mastitis. Gram-negative bacteria were the bacterial group most commonly isolated from clinical cases of mastitis in many surveys. The percentage distribution of Gram-negative bacteria causing clinical mastitis is herd dependant, but studies in the United States and Europe consistently report that appropriately 40% of clinical cases are the result of Gram-negative bacteria [25, 27, 55, 57]. Rate of clinical cases caused by Gram-negative bacteria average approximately 20 cases per 100 cows per year in these studies. The severity of clinical cases caused by coliform bacteria ranges from mild local signs to severe systemic involvement. The vast majority of clinical coliform cases are characterized by abnormal milk and a swollen gland. Only about 10% of clinical coliform cases result in systemic signs including fever, anorexia, and altered respiration [25,

55]. Despite the relatively low percentage of clinical coliform cases yielding systemic signs, coliform bacteria have an exaggerated reputation for causing peracute mastitis. The basis for this distinction originates from the point that the coliforms are the most common cause of systemic illness resulting from mastitis. Survey averages suggest that coliform bacteria are the culprits of 60 to 70% of peracute clinical cases [25, 55]. Therefore, the general conclusions concerning severity of clinical coliform cases are that few coliform intramammary infections cause systemic clinical signs, but the majority of clinical cases resulting in systemic signs are caused by coliform bacteria.

Although clinical mastitis caused by species of *Serratia*, *Pseudomonas*, and *Proteus* tend to occur much less frequently than clinical coliform mastitis, sporadic herd outbreaks involving Gram-negative bacteria other than the coliforms have been reported [23]. Intramammary infections caused by these bacteria develop into clinical disease less often and clinical cases tend to be less severe than coliform clinical cases.

4.3. Seasonal effects

Season of the year influences rates of new coliform infection during the dry period and lactation. Rates of new infection and clinical mastitis are highest in summer months for confinement-housed cows [59]. A shift toward increased rates of clinical mastitis coincides with an increase in Gram-negative bacterial counts in bedding during warm weather months. When cows are housed in dry lots or pastured, rates of clinical mastitis are generally elevated during periods of rainy, wet weather. Increases in clinical coliform mastitis caused by heat stress directly affecting susceptibility of the mammary host defenses to Gram-negative bacteria is conceivable, but control data is lacking to demonstrate a clear effect.

4.4. Parity

In general, older cows have a higher rate of clinical mastitis caused by Gram-negative bacteria compared with primiparous cows [55]. An interaction between age, season of the year, and lactation status effects susceptibility to clinical mastitis caused by Gram-negative bacteria in total confinement herds. Older cows calving during the summer months are commonly the population of animals at greatest risk to coliform clinical mastitis [55].

5. MANAGEMENT PROCEDURES

Mastitis management practices intended to control mastitis caused by Gram-negative bacteria often were formulated with the assumption that these bacteria comprised a homogenous group with shared phenotypic characteristics and point sources of contamination. More recent findings have revealed that Gram-negative bacteria are a more heterogenous set of pathogens than previously recognized and the variability among strains may help explain why management practice intended to control the disease have been minimally successful.

5.1. Bedding

Gram-negative bacteria are inept at surviving and multiplying on teat skin. Therefore the number of Gram-negative bacteria on teat skin is a reflection of the cow's recent exposure to the contaminating environment. Common sources of exposure include feedstuffs, manure, water, and soil. A primary source of bacterial contamination is bedding. Populations of Gram-negative bacteria in bedding are related to the number of Gram-negative bacteria on teat ends and rates of clinical mastitis. Reducing the number of bacteria in bedding generally results in a decrease in clinical mastitis caused by Gram-negative bacteria. Use of the inorganic bedding materials sand and

crushed limestone exposes cows to fewer Gram-negative bacteria than the use of organic materials such as wood products and straw [24]. Wood shavings, straw, chopped newspaper, recycled manure, and corn fodder are commonly used bedding materials that often sustain coliform populations greater than 10^6 colony forming units/gram of bedding [24, 27]. Total coliform counts do not differ greatly among organic bedding materials, however counts of specific pathogens do vary among organic bedding materials. For example, outbreaks of clinical mastitis caused by *Klebsiella pneumoniae* are common in herds using finely chipped sawdust [45].

5.2. Vaccination: core antigen bacterins

The use of Gram-negative core antigen vaccines effectively reduces the incidence and severity of clinical mastitis caused by Gram-negative bacteria. Most of these vaccines use either *Escherichia coli* J5 or *Salmonella typhimurium* Re17 [43] as the antigens. These rough mutants lack the O-polysaccharide chains of lipopolysaccharides, thereby exposing the core antigens of lipopolysaccharides. Protection provided by the vaccines is thought to be afforded by immunoglobulins specific for the core portions of lipopolysaccharides which are structurally and antigenically conserved among Gram-negative bacteria. Most commercially available Gram-negative core antigen vaccines specify efficacy against only *Escherichia coli*. Data from field trials suggest that these vaccines also reduce clinical cases of mastitis caused by species in the genera *Klebsiella*, *Pseudomonas*, *Serratia*, and *Proteus* [16, 28]. Cost-benefit modeling indicates vaccination is an economically sound strategy on well-managed dairies with clinical coliform mastitis problems.

Rate of clinical mastitis caused by Gram-negative bacteria was 4 to 5-fold lower in vaccinated cows compared with controls in controlled field trials [16, 28].

A consistent result was that the use of these bacterins did not prevent intramammary infections. For example, immunization did not reduce prevalence of Gram-negative bacterial intramammary infections at calving, but did reduce incidence of clinical mastitis. Therefore, these data imply that the core antigen vaccines do not prevent the occurrence of mastitis, but do reduce the severity of the disease. The most striking difference between vaccinated and non-vaccinated cows was that 66.7% of coliform intramammary infections in unvaccinated cows became clinical during early lactation compared to 20% in vaccinated cows [28].

The mechanisms by which immunization reduces the incidence of clinical mastitis is not known. Protective mechanisms suggested include: (1) core LPS specific antibodies neutralize the toxic effects of LPS; (2) increased complement-mediated bacteriolysis; (3) antibodies promote clearance of bacteria through opsonization and enhanced phagocytosis; and (4) enhancing neutrophil diapedesis [11]. Evidence is limited to support that immunization either results in increased neutralization of free LPS in the mammary gland or that vaccination accentuates complement-mediated bacteriolysis. Enhanced opsonization of wild strains of coliform bacteria by serum and whey of vaccinated cows was correlated with elevated IgM titers [30]. A working hypothesis is that improved opsonization leads to reduced bacterial numbers in infected glands, less severe clinical signs, and subsequently a reduction in clinical cases of mastitis. Experimental challenge trials have supported this thesis. Bacterial counts in milk following intramammary challenge were reduced by vaccination. Duration and severity of clinical signs were positively correlated with bacterial counts in milk following intramammary challenge with either virulent or avirulent strains. Vaccination reduced the peak bacterial counts in infected quarters, duration of intramammary infections by 25%, and

duration of clinical signs of mastitis by 50% compared with unvaccinated cows [33].

5.3. Antibiotic therapy

Currently available antibiotics have minimal effect on shortening the duration of intramammary infections caused by coliform bacteria. The use of antibiotics administered by intramammary or systemic routes for treating *E. coli* clinical cases is virtually useless because of the short duration of infections and high spontaneous cure rate [55]. Treatment of peracute clinical coliform mastitis often involves supportive therapy including oral or intravenous fluids and anti-inflammatory agents. The use of antibiotics for treatment of mammary glands at the end of lactation has no effect on the prevalence of coliform infections at calving [2].

5.4. Sanitizers

The use of germicidal sanitizers on teats immediately prior to milking (predipping) can reduce the incidence of new coliform infections during lactation [48]. However, the use of post-milking teat antiseptics as a means to control coliform mastitis is unsuccessful [49]. Germicidal teat antiseptics often kill a large percentage of coliform bacteria on teat skin at the end of milking, however the antibacterial properties of the germicides diminish rapidly after contact with teat skin and milk. Contamination of teats by coliforms continues between milkings after the germicide is ineffective. Products designed to form a physical barrier between the teat and the environment between milkings have been minimally successful in persistence and they lack efficacy in reducing new intramammary infections [44].

Coliform bacteria have not been reported to have acquired tolerance or resistance to disinfectants and sanitizers commonly used in milking hygiene procedures. However, *Serratia* spp. and *Pseudomonas* spp. often

are resistant to the bactericidal activity of chlorhexidine gluconate [8, 39].

6. FUTURE RESEARCH AREAS

6.1. Immunization schemes

Immunization schedules for Gram-negative core antigen vaccines have involved a primary systemic immunization at the end of lactation with systemic boosters during the dry period and at calving. The aim of this schedule was to maximize protection during the periparturient period when the rates of new coliform intramammary infections and clinical mastitis are highest. Active immunization of cows with Gram-negative core antigens increased titers specific to conserved antigens. Titers responsive to immunization were negatively correlated with severity of clinical signs of mastitis following intramammary challenge [33]. However, alternative immunization schemes to optimize host defenses against coliform mastitis have been proposed [7, 50]. Future trials to optimize doses, adjuvants, and immunization schedules are needed to maximize the profitability of using core antigen vaccines.

6.2. Fe regulated outer membrane protein vaccines

Controlling coliform mastitis during the dry period may be accomplished by preventing the uptake of essential nutrients by bacteria once they penetrate into the gland. A limiting nutritional factor for many coliform bacteria in secretion from involuted mammary glands is iron. Iron is essential for most coliform bacteria to fulfill normal metabolic processes. The protein lactoferrin binds iron and makes the element unavailable to bacteria [54]. The ability of *E. coli* and *K. pneumoniae* to cause mastitis is related to the ability of these isolates to overcome the inhibitory properties of lactoferrin [58]. Coliforms may overcome the inhibitory effects of lactoferrin with

one of several iron acquisition systems, including enterochelin, aerobactin, citrate, and ferrichrome systems. Coliform isolates that infect involuted mammary glands probably do so as a result of one or more of these systems. Coliforms isolated from intramammary infections shared a specific enterochelin-iron retrieval system that included a protein on the cellular surface named FepA. FepA was determined to be an excellent protein from which to formulate vaccines because FepA was expressed on all clinical isolates tested [40]. A FepA vaccine caused an immune response in cows and the antibody blocked growth of *E. coli* in synthetic medium and dry cow secretion. While vaccination with FepA was effective against *E. coli*, the immune response did not affect growth of *Klebsiella* in secretion from involuted glands [41]. Subunit vaccines to promote production of specific antibodies to block nutrient uptake offer either an alternative or augmenting approach to core antigen bacterins for enhancing resistance to coliform mastitis.

6.3. Chronic *E. coli* infections

Recurrent or chronic *E. coli* infections have been reported as accounting a relatively small percentage of intramammary infections in survey herds [25, 55]. Recent reports suggested an increase in the frequency of chronic *E. coli* infections in selected herds [4, 9]. Additional trials are needed to confirm this proposed epidemiological shift and to substantiate in vitro trials [10] suggesting novel virulence factors allowing intracellular survival of strains from chronic infections.

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