

ABSTRACT

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CATANIONIC SURFACTANT VESICLE
VACCINE FOR BOVINE MASTITIS.

Prarthana Vasudevan, Master of Science, 2014.

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17-20% of the world's cows have had bovine mastitis at some point in their lives. Mastitis is the inflammation of mammary glands caused by infection, such as by the bacterium *Escherichia coli*. The focus of this thesis research is the immunopotency of a novel catanionic surfactant vesicle vaccine for *E. coli* mastitis that could theoretically resemble an 'artificial pathogen.' To this end, serum studies analyzing antibody titers and immunogenic profiles were conducted. These studies demonstrate that there was no significant increase in total *E. coli* specific-IgG in vaccinated cows post-vaccination and that there may be variation in immunogenic profiles post-vaccination.

IMMUNOPOTENCY OF A NOVEL CATIONIC SURFACTANT VESICLE
VACCINE FOR BOVINE MASTITIS.

By

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List of Abbreviations

APC: antigen presenting cell

BM: bovine mastitis

CDC: U.S. Centers for Disease Control and Prevention

CIM: cytoplasmic/inner membrane protein fraction

CNS: Coagulase-negative staphylococci

CO₂: carbon dioxide

CTAT: cetyltrimethylammonium tosylate

DAEC: diffusely adherent *E. coli*

E. coli: *Escherichia coli*

EAEC: enteroaggregative *E. coli*

EDTA: ethylenediaminetetraacetic acid

EHEC: enterohaemorrhagic *E. coli*

EIEC: enteroinvasive *E. coli*

ELISA: Enzyme-linked immunosorbent assay

EPEC: enteropathogenic *E. coli*

ETEC: enterotoxigenic *E. coli*

FAO: Food and Agricultural Office of the United Nations

FDA: Food and Drug Administration

GI: gastrointestinal

H₂O: water

hr: hour

Ig: immunoglobulin

IgG: immunoglobulin G

IL-1: interleukin 1

IL-6: interleukin 6

IL-8: interleukin 8

kD: kiloDalton

Kdo: 3-deoxy-D-manno-octulosonic acid

KM: Kaleidoscope Marker

LB: Luria Broth

LBP: lipopolysaccharide binding protein

LPS: lipopolysaccharide

MAC: membrane attack complex

Mb: megabase

mCD14R: membrane-bound Cluster of Differentiation 14 receptor

mg: milligram

ml: milliliter

MPEC: mammary pathogenic *E. coli*

NASDA: National Association of State Development Agencies

nm: nanometer

O.D.: optical density

OM: outer membrane protein fraction

PO: post-vaccination

PR: pre-vaccination

PVDF: polyvinylidene fluoride

rpm: rotations per minute

RT: room temperature

SCC: somatic cell count

SDBS: sodium dodecylbenzenesulfonate

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate- polyacrylamide gel electrophoresis

sec: second

spp.: species

TLR-4: Toll-like Receptor 4

TMB: 3, 3', 5, 5'-tetramethylbenzidine

TNF- α : Tumor Necrosis Factor α

TP: total protein fraction

UD: undiluted

USDA: United States Department of Agriculture

μ g: microgram

μ l: microliter

Chapter 1: Introduction

Bovine Milk

Dairy Industry in the Global and Domestic Markets

The dairy industry is a major player in global and domestic markets. In 2008, a study conducted by the Food and Agriculture Organization of the United Nations (FAO) analyzing global milk production in 2007 determined that India, the United States, China, Pakistan, and Brazil were the top 5 milk-producing countries that year.¹ This calculation took cow, buffalo, goat, sheep, and camel milk into consideration, but also specified that milk derived from cattle represents 83% of the world's milk.¹ The same study concluded that between 2002 and 2007 world milk production increased 13% to 697 million tons.¹

As of 2014, milk production in the United States is projected to reach 93.6 million tons.² Currently, the United States ranks as the third-highest dairy exporter in the world, following New Zealand and the European Union.² In fact, the United States Department of Agriculture (USDA) states that for fiscal year 2013 the US dairy industry reported exports worth a recording-breaking \$6.1 billion.³ Domestically, the US dairy industry is responsible for \$140 billion in economic output, \$29 billion in household earnings, and more than 900,000 jobs.⁴ Clearly, the US dairy industry has economic significance internationally and domestically.

Bovine Milk

As defined by FAO, milk is "the normal mammary secretion of milking animals without either addition to it or extraction from it, intended for consumption as liquid milk or for further processing."⁵ Liquid milk ranks as one of the most identifiable products of the dairy industry alongside powdered milk, cream, cheese, and butter.² Last year, the US dairy industry produced 201,218 million pounds of liquid milk (USDA- National Agricultural Statistics Service).^{6, 7}

Milk composition and regulations

Liquid milk comprises of approximately 88% water, 3-4% fat, 3.5% protein, and 5% lactose.⁸ Cows' milk contains over 400 different fatty acids, which derive from feed and microbial activity in the cow's rumen.⁹ Fatty acids components include conjugated linoleic acid and vaccenic acid, which contains trace elements of natural *trans*-fatty acids that are unique to ruminants.¹⁰ The major proteins found in cows' milk are casein and whey. Casein (α_1 - α_2 , β -casein, and κ -casein) constitute 78% of total milk protein.¹⁰ Whey constitutes the other 17% of milk protein and includes β -lactoglobulin, α lactalbumin, serum albumin, immunoglobulins (Ig), glycomacropeptides, lactoferrin, insulin-like growth factor (IGF), and the lactoperoxidase system.¹⁰ The major carbohydrate component of milk is lactose, a disaccharide made of glucose and galactose.¹⁰ Given its physiochemical characteristics, milk is a popular source of nutrition for cows and humans alike.

Strict federal regulations and safety procedures are enforced by agencies like the USDA, the US Food and Drug Administration (FDA), and the National Association

of State Departments of Agriculture (NASDA) to ensure safe milk for human consumption. Consumable milk must contain no debris, off-flavors, abnormal coloring or odors, must be low in bacterial counts, contain little to no chemicals like antibiotics or detergents, and must retain normal acidity and composition.⁸ As milk is a natural growth medium for microbes, spoilage by organisms in milk and transmission of food-borne pathogens¹¹ are safety concerns that are largely addressed by pasteurization. Pioneered by the microbiologist Louis Pasteur, pasteurization remains the gold-standard in maintaining milk safety in the US and abroad.

Milk can never be completely sterile once it leaves the udders.¹² Pasteurization is a process that decontaminates but does not completely sterilize milk, as it reduces the number of viable pathogens in milk but does not necessarily kill them all.¹² This process reduces the number of milk-borne microbes, yeasts, and molds and extends shelf life.⁸ Pasteurization techniques include holding vat pasteurization, flash pasteurization, and ultra-high temperature pasteurization.¹² In the US, each state has the authority to regulate pasteurization¹² though there are overarching federal agencies like the FDA, USDA, and the US Centers for Disease Control and Prevention (CDC) that also monitor milk safety. According to the 2011 revision of the FDA's Pasteurized Milk Ordinance, it is imperative that milk does "not contain levels of deleterious substances, harmful pathogenic organisms, or other toxic substances which are secreted in the milk at any level, which may be deleterious to human health."¹³ Thus, milk's safety is inextricably linked to milk's pasteurization.

Pathogenic Contamination

Pasteurization helps reduce milk-borne pathogens. These pathogens include human-derived *Streptococcus pyogenes* and *Salmonella typhi* and bovine-derived *Brucella abortus*, *Mycobacterium bovis*, *Listeria monocytogenes*, *Salmonella* species (spp.), *Campylobacter* spp., and *Escherichia coli* (*E. coli*) O157:H7.^{12, 14} When produced and stored in healthy udders, milk is sterile.^{12, 15} however, upon exiting the udders, milk becomes contaminated by commensal or pathogenic microbes of the udder or teat canal, by fecal contamination of the udders, by contamination of milking equipment, or by commensal or pathogenic microbes belonging to human milk-handlers.^{12, 15, 16} Of specific relevance to this study is the contamination of milk caused by infections of the udders.¹⁶

Bovine Mastitis

Mastitis is the inflammation of the mammary glands caused by infection.¹⁷ Of the estimated 250 million dairy cows in the world¹⁸, 17-20% have had mastitis at some point in their lives.¹⁹ In 2007, the disease ranked first among health problems found in cows (**Figure 1**).²⁰ Over 135 pathogenic species can cause bovine mastitis (BM), including bacteria and fungi.²¹ BM can be classified as subclinical or clinical and contagious or environmental. BM causes changes in composition and physiochemical characteristics¹⁹ that render mastitic milk unsuitable for human consumption, and as such is a costly disease that has relevance to public health and safety.

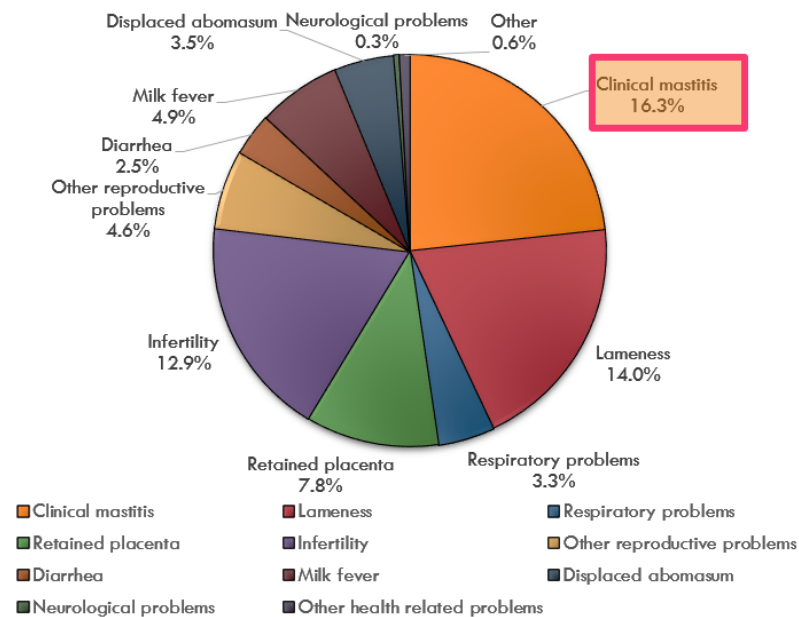


Figure 1. Percent of cows by health problems. Data was collected related to prevalence of cow health problems in the year 2007 published in a National Animal Health Monitoring System study in 2010. This figure was constructed based on statistics provided in that document.²⁰

Etiological Agents

Mastitis can be caused by bacterial pathogens. These bacterial pathogens can be contagious or environmental (**Table 1**).²² Contagious pathogens are those that live on mammary gland epithelia and teat lesions as well as on milking machines, milkers' hands, fomites, and sponges.²¹ These species include *S. aureus*, *S. agalactiae*, mycoplasmas and *M. bovis*.^{21,22} Contagious pathogens can be transmitted between cows or even between quarters during milk.^{22, 23} Environmental pathogens derive from the cows' environment and enter the mammary gland by contaminating the teat.²² These species include *E. coli*, *S. dysgalactiae*, *S. uberis*, *K. pneumonia*, and *Bacillus* spp.²² Reservoirs for environmental pathogens include feces, soil, bedding, and manure.²¹ *Corynebacterium* species and coagulase-negative staphylococcus (CNS) are emerging pathogens.²¹ The most common BM-associated pathogens are *S.*

aureus, CNS, and *E. coli*.²³ Contagious and environmental pathogens can cause different signs and symptoms in infected cows. BM can also be described, based on signs and symptoms, as either subclinical or clinical (**Table 1**). Clinical signs of BM will be discussed in the next subsection.

Bovine Mastitis	Classified by transmission	Environmental pathogens	Environmental <i>Strep.</i> (not <i>agalactiae</i>) Coliforms • <i>Klebsiella pneumoniae</i> • <i>Klebsiella oxytoca</i> • <i>Enterobacter aerogenes</i> • <i>Escherichia coli</i>
		Contagious pathogens	<i>Streptococcus agalactiae</i> <i>Staphylococcus aureus</i> <i>Mycoplasmas</i>
	Classified by signs and symptoms	Subclinical Mastitis	Asymptomatic
		Clinical Mastitis	Symptomatic Inflammation

Table 1. The Two Classifications of Bovine Mastitis. BM can be identified as environmental/ contagious or subclinical /clinical.^{24,25}

Signs and symptoms

The severity of a BM case relates to the physical signs and symptoms exhibited by the infected cow. BM can be classified as subclinical (asymptomatic) or clinical (symptomatic). In both cases, cows with BM present elevated somatic cell counts (SCC). Somatic cells are white blood cells (macrophages, lymphocytes, polymorphonuclear cells) and mammary epithelial cells that shed from the infected mammary gland into milk upon milking.²⁶ Elevated SCC can alter the appearance and composition of milk as well as encourage milk spoilage. Healthy milk contains

somatic cells but at a very low concentration, typically a SCC of <100,000 cells/ml of milk.²⁶ In contrast, mastitic milk typically contains >200,000 cells/ml of milk to an upper limit of millions of cells.^{26, 27} A SCC of >200,000 cells/ml clearly indicates mammary gland inflammation and is synonymous with the diagnosis that the animal producing that milk suffers from subclinical or clinical mastitis. Cows that suffer from subclinical mastitis present elevated SCC in milk but no other symptoms. Cows that suffer from acute mastitis present elevated SCC in milk and observable symptoms.

Observable symptoms can be assessed by considering the state of the cows' udders, the physical appearance of milk collected from cows', and the cows' overall health. In cases of clinical mastitis, udders frequently appear swollen and hard.^{28, 29, 30} Mastitic milk can appear clotted, thick, serous to yellow colored, flakey, and purulent as well as contain blood.^{28, 29, 30} Cows with clinical mastitis exhibit high fevers, decreased rumen contractions, appetite loss, decreased milk yield, elevated rectal temperatures, dullness, diarrhea, depression, and weakness.^{28, 29, 30} The observable symptoms of clinical mastitis render this form of the disease more easily identifiable.

The consequences clinical mastitis can be severe. Clinical mastitis oftentimes becomes systemic, which leads to death. Cows afflicted with clinical mastitis die from their condition naturally or are forcibly killed (culled) to prevent further spread of the disease.^{17, 19, 21, 22, 23, 28, 30, 31} Pregnant cows (periparturient) are especially at risk for clinical mastitis.³¹ Clinical mastitis is an expensive problem that challenges

the dairy industry: economic losses include reduced milk production losses, cost of discarded milk, increased cull rates, costs associated with pharmaceutical treatment, and increased labor.³²

Management, treatment, and control

Bovine mastitis is an economically expensive health challenge for the dairy industry. Managing, treating, and controlling this disease is a top priority for dairy farmers and scientists. Practices include “establishing good management [of cows] on the farm, treating infected animals, and slaughtering chronic cases.”¹⁹ Poor udder hygiene, dirty udders, and teats close to the ground are all potential risk factors for mastitis.³¹ In terms of management practices, disinfecting teats with germicidal dipping, disinfecting milking equipment before milking, and reducing cow densities per unit area all help reduce risk.^{31,34} Additionally, mastitic cows can be quarantined. Cows with chronic mastitis that are especially contagious can be forcibly killed or culled. Treatments include novel bacteriophage methods and antibiotics.²¹ Control methods include administering killed whole-cell vaccines to cows during their dry periods.²¹ Current commercial vaccines for *E. coli*-associated bovine mastitis will be discussed later in this chapter.

Significance

Bovine mastitis is a disease of public health and economic significance due to its impact on human consumption of milk. Milk is a nutritious source of calcium, magnesium, selenium, riboflavin, and vitamin B12.¹ However, milk contaminated with mastitis-causing bacteria can cause fever, nausea, vomiting, diarrhea, and

abdominal pain in humans.¹⁵ Veterinary drugs and antibiotic residues used to treat mastitis can be secreted into milk and can influence milk processing, inhibit acid production in starting cultures for cheese, and contribute to poor aging of further processed milk products like cheese and butter.¹¹ The presence of antibiotic residues in milk decreases milk quality as well as raises debate regarding excessive antibiotic use and the emergence of antibiotic-resistant bacterial species within bovine and human populations.^{11, 35} Additionally, the presence of somatic cells in milk secretions decreases milk and cheese yields as well as influences milk's nutritional qualities.^{30, 35} Specifically, proteolytic and lipolytic enzymes promote enzymatic breakdown of milk protein and fat.³⁵ Overall, mastitis is estimated to cost \$200 per cow affected per year, including costs for cow feed, management, treatment, antibiotics, labor, and milk.³⁰ This figure is projected to be a \$2 billion loss per year for the dairy industry.³⁰ Therefore, controlling the spread of bovine mastitis is in the interest of public health and economics.

E. coli Mastitis

E. coli is the most commonly isolated pathogen in cases of environmental (coliform) mastitis and frequently causes acute clinical mastitis.^{23,30}

***E. coli* and *E. coli* strain P4**

E. coli belongs to the *Enterobacteriaceae* family.³⁰ It is a Gram-negative, rod shaped, lactose fermenting, facultative anaerobe that is commonly found in the gastrointestinal (GI) tracts of animals and humans. Common pathogenic strains include enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC),

enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli*, (EIEC) and diffusely adherent *E. coli* (DAEC), which cause GI and urinary tract diseases in humans.³⁶ *E. coli* is classified into serotypes based on shared O (lipopolysaccharide) and H (flagellar) antigens.^{30,36} General virulence factors include lipopolysaccharide (O antigen), fimbriae, capsules (K antigen), and flagella (H antigen).³⁷

P4 is a mammary pathogenic *E. coli strain* (MPEC) responsible for inducing mastitis in bovines.^{38,28} P4 was first isolated by Andrew John Bramley in 1976 from a clinical case.³⁹ P4's natural reservoirs include the bovine GI tract, soil surroundings in the dairy barn, and bedding.³³ P4 utilizes lactose found in milk as a carbon source to grow in mammary secretions.³³ Today, P4 is considered a model mastitis-producing strain and is used to induce experimental mastitis.

Virulence factors

P4 was first isolated in 1976, but it was not fully characterized bioinformatically until 2012 by Blum *et al.* The Blum group determined that P4 belonged to serotype O32:H37.⁴⁰ In addition to lipopolysaccharide (LPS), within the 5.2 megabases (Mb) genome the following virulence factors were identified: aerobactin siderophores, enterobactin siderophores, protein secretion system type II, yidE mediator (for hyperadherence), type I fimbriae, type I pili, and curli pili.⁴⁰ LPS, flagella, and fimbriae are depicted in **Figure 2**.

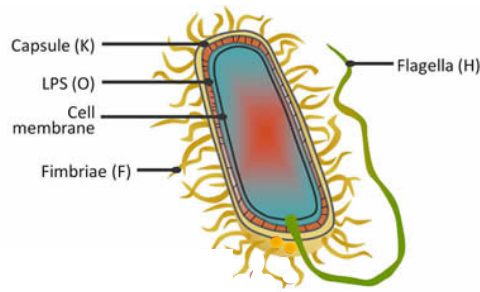


Figure 2. Selected *E. coli* virulence factors. Adapted from source 81.

Lipopolysaccharide (LPS) endotoxin plays a pivotal role in the structure and function of Gram-negative outer membranes.⁴¹ The molecule consists of 3 regions: the lipid A region, the core polysaccharide region, and the O-polysaccharide region as illustrated in **Figure 3**.⁴¹ The general architecture of a core polysaccharide, made of inner and outer cores, and a lipid A portion is highly conserved among Gram-negative species.⁴² More than 180 O-antigen serotypes have been identified and these can be divided into the R1, R2, R3, R4, and K-12 groups.⁴² The R1 group is commonly found in *E. coli* that colonize humans and bovines.

LPS can be divided into three regions: the O-polysaccharide region, the core polysaccharide region, the 3-deoxy-D-manno-octulosonic acid (Kdo) residues, and the lipid A region.⁴² First, the O-polysaccharide region is made of repeating units of 1 to 8 glycosyl residues.⁴² This region is highly variable due to structural diversity in sugars, sequence, chemical linkage, substitution, and ring formations.⁴² The O-polysaccharide is the most exterior facing portion of the LPS molecule and as such is highly antigenic.^{41, 42} The O-polysaccharide also assists in conferring serum resistance as it prevents the membrane attack complex (MAC) of the complement pathway from penetrating the outer membrane.⁴² Next, the core polysaccharide is

made of an outer and inner core. The outer core is made of hexose sugars like glucose, galactose, N-acetyl galactosamine, and N-acetylglucosamine.⁴² The inner core is made of a highly conserved sequence consisting of heptose and Kdo.^{41,42} *E. coli* LPS's lipid A region contains acyl chains of C12-C14 length and seems to be optimally structured to bind and activate Toll-like Receptor 4 (TLR-4).⁴² The lipid A moiety is the part of the LPS molecule responsible for its endotoxicity.^{41, 42} The LPS molecule is amphiphilic, meaning it has a hydrophilic and hydrophobic domain. The lipid A region of the molecule is embedded in the outer membrane while the O-polysaccharide faces the extracellular milieu.

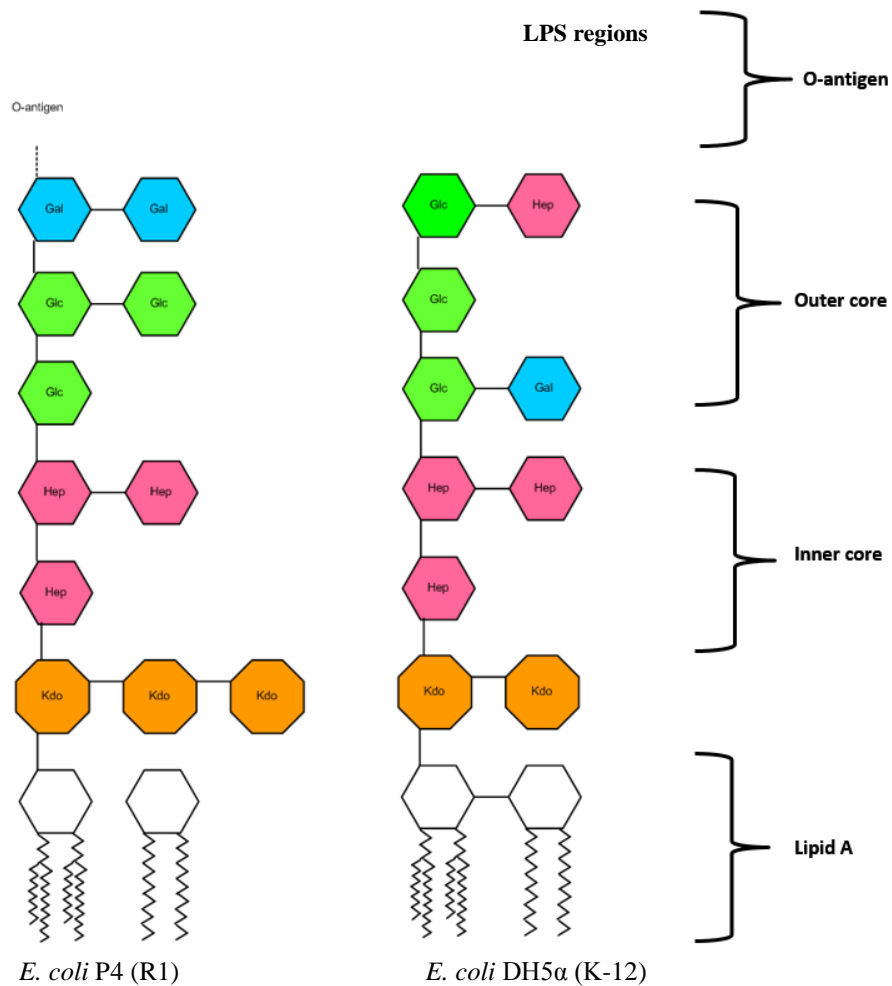


Figure 3. *E. coli*'s LPS. These figures were adapted from sources 41 and 76.

LPS is an important factor in determining the severity of *E. coli* mastitis.⁴³ The molecule is found on Gram-negative bacteria and is released upon bacterial lysis.⁴³ Once released, LPS enters the teat and udder cistern, where it induces the expression of pro-inflammatory cytokines, including Interleukin 6 (IL-6), Interleukin 1 (IL-1), Interleukin 8 (IL-8), and Tumor Necrosis Factor (TNF- α).⁴⁴ This results in leukopenia, leukocytosis, complement activation, macrophage activation, and increased vessel permeability, and inflammation.⁴³ Injecting *E. coli* LPS alone into healthy bovine mammary glands is enough to induce mastitis.^{38,44} LPS is recognized by TLR-4 as well as by membrane-bound CD14 receptors (mCD14R) found on macrophages.⁴⁴ LPS can directly bind mCD14Rs and the binding is facilitated by LPS binding protein (LBP), which transfers lipids, promotes LPS disassociation into monomers, and catalytically transfers those monomers to mCD14R.⁴⁴ LBP expression is upregulated by IL-1 and IL-6.⁴⁴ In an experiment by Yunhe *et al* comparing innate responses elicited by *E. coli* and *S. aureus*, it was determined that *E. coli* LPS activated the NF κ B pathway in mammary epithelial cells.⁴⁵

While P4 *E. coli* is highly virulent in cows, it causes mild or latent mastitis in mice that is self-curing.³⁸ These results suggest that P4 is highly host specific. P4's niches in the bovine mammary gland include stratified epithelial lining, luminal walls, teat canal, teat cistern, and the specialized aveoli.³⁸

The Mammary Gland and *E. coli* Pathogenesis

Anatomy of the Mammary Gland

The udders are the milk producing organs of bovines. Each udder is made of 4 separate mammary glands, each with their own teat end.⁴⁶ Mammary gland structure is depicted in **Figure 4**. Significant features include the teat end, teat cistern, gland cistern, alveoli, and ducts.

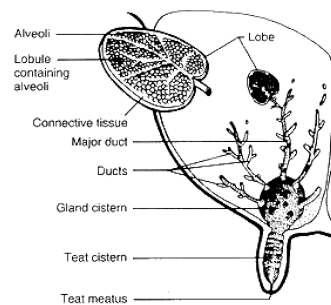


Figure 4. Bovine mammary gland. Adapted from source 82.

Teats, which allow milk to exit the mammary gland, are made of a teat muscle sphincter, a teat canal and a teat cistern.⁴⁶ The teat sphincter is the first anatomical barrier against mammary infection.²² The teat sphincter keeps the gland closed between milkings and therefore helps prevent bacterial invasion.⁴⁷ The teat canal connects the teat cistern to the teat end. The teat canal contains keratin, a protein formed by stratified squamous epithelium.⁴⁷ Keratin contains bacteriostatic fatty acids like myristic acid, palmitoleic acid, and linoleic acid, a defense against bacterial invasion.⁴⁷ The teat cistern is a cavity where milk collects before it is expelled through the teat end.⁴⁶

Milk is synthesized in alveoli by milk-secreting cells. An alveolus is a sac-like spherical structure of milk-secreting cells and many alveoli come together to form lobules.⁴⁸ The overall structure of a lobule resembles the structure of a lung.⁴⁸ Milk is continuously made and stored in alveoli as well as transported in milk ducts to the gland cistern and the teat cistern.⁴⁸ Alveoli respond to the hormone oxytocin, which induces milk to be squeezed out of alveoli into small tubes called ducts, which carry milk into the gland cistern.⁴⁸ The gland cistern is another collection area for milk, which connects directly to the teat cistern, where milk is stored prior to expulsion by milking the teat end.⁴⁶

***E. coli* Pathogenesis**

E. coli pathogenesis is a multi-step process, involving colonization, evasion of host defense, and host damage.³⁶ P4 breaches the anatomical defenses of the mammary gland and colonizes mammary epithelial cells. Due to the constant presence of lactose in milk, which is ubiquitous in the mammary gland, bacteria are able to grow rapidly, doubling every 20 minutes.^{37, 49} Bacteria colonize epithelial cells in the teat cistern and then migrate into the udder cistern.³⁵ As it grows, P4 releases LPS into the teat and udder cisterns. As the bacteria invade epithelial cells along milk-collecting ducts and cisterns, it kills milk-secreting cells, whose contents permeate blood vessels and attract leukocytes.³⁰ Blockage of the teat end ultimately ensues and any milk trapped inside the gland converts milk-secreting cells into resting cells, resulting in shrinkage of alveolis.³⁰ Eventually, *E. coli* destroy milk-secreting tissues, increasing somatic cell counts in milk and decreasing milk yield.³⁰

Neutrophils are the first-responders to *E. coli* infection. In fact, robust neutrophil response is the hallmark of *E. coli* mastitis cases.³⁰ Neutrophils account for 90% of all mammary gland leukocytes.⁴⁷ Neutrophils migrate from the blood to the site of infection, where they are involved in bactericidal killing by phagocytosis and respiratory bursts via release of superoxide ions, hypochlorites, and hydrogen peroxide.^{30, 47} Neutrophil recruitment usually occurs 16 hr post-infection and precedes increased TNF- α , IL-1 β , and C5a production.²² In healthy lactating mammary glands, neutrophils are found in a concentration of $<10^5$ cells/ml but in mastitic glands neutrophil concentrations rise to $>10^6$ cells/ml.³⁴ Complement killing is not a very effective response to *E. coli* infection as many strains, like P4, are inherently complement-resistant. Serum and milk complement components like C3b and C3bi are too low in concentration to contribute significantly to phagocytosis by neutrophils.⁴⁹

After neutrophils, macrophages dominate the immune response.⁴³ Macrophages make up the majority of cells in milk and tissue of healthy, lactating mammary glands.⁴⁷ However, macrophages are much less in concentration than neutrophils and have fewer Fc receptors than neutrophils.³⁴ Macrophages are involved in bactericidal killing by phagocytosis and in antigen processing and presentation by way of their MHC class II molecules.⁴⁷ Macrophages express TLR-4, LBP and mCD14 receptors.^{34, 43} TLR-4 recognizes the lipid A portion of LPS.³³ mCD14 receptors also bind LPS and initiate TNF- α expression.⁵⁰ Activated macrophages recruit other macrophages and neutrophils.³⁸ Activated macrophages also release prostaglandins,

leukotrienes, and cytokines like IL-1, TNF- α , and IL-8, which all enhance the local inflammatory response.^{33, 34} Lymphocytes, cytokines, and chemokines all contribute to the local inflammatory response, which is characterized by heat, redness, pain, and swelling of the mammary gland.³⁵ The fat and casein found in milk dilute recruited immune factors such as neutrophils and macrophages. Neutrophils and macrophages can experience 'phagocytic overload,' which means instead of ingesting bacterial pathogen, they ingest casein micelles and fat globules in milk. This means the population of neutrophils and macrophages that would otherwise recognize and phagocytize bacteria is reduced.⁴⁹ Therefore greater amounts of these factors must be continually recruited to infection sites.⁴⁹

Following macrophage recruitment, adaptive immunity is initiated. Healthy glands are mostly populated by CD8+T cells, whereas mastitic glands are mostly populated by CD4+ T cells.²² In the mastitic response, CD4+ T cells are activated by antigen-MHC II complexes on antigen presenting cells (APCs).⁴⁷ B cells secrete antibodies and irreversible class-switching occurs from IgM to IgG₁ and IgG₂ populations.³³ IgM, IgG₁, and IgG₂ populations all play a role in opsonization.^{33,34} IgM could act as an opsonin for to recruit milk neutrophils to clear bacterial cells, however to do so IgM must first fix complement and the concentration of complement in milk is very low.⁴⁹ IgG is the predominant phenotype in milk.⁵¹ IgG₁ is the major isotype in healthy milk whereas IgG₂ is the major isotype in mastitic milk.^{47,52} IgG₁ is the major isotype in healthy milk because in healthy milk, massive neutrophil response is not required.⁴⁹ Bovine neutrophils usually do not express high affinity F_c receptors for

IgG₁.⁴⁹ Bovine neutrophils do express high affinity F_c receptors for IgG₂, which is why IgG₂ is the major isotype in mastitic milk.⁴⁹ Post infection, the blood-milk barrier becomes altered.⁵¹ Sloughed epithelial, P4 bacteria, and blood components (immunoglobulins, neutrophils, macrophages, and lymphocytes) cross into milk, rendering that milk mastitic.^{47,51}

Despite a robust bovine immune response, P4 bacteria have developed ways to evade host defenses. Firstly, P4 is inherently complement-resistant.⁵² Therefore, it can evade bactericidal killing by host classical and adaptive complement pathways. Additionally, P4 can form biofilms and intracellular microcolonies that adhere to mammary alveolar epithelial cells, which may have implications the mediation of inflammatory responses as well as the process of microbial invasions.³⁸ P4 is also resistant to high concentrations of nitric oxide.³⁸ In these ways, P4 can evade host defenses and establish niches in the mammary glands.

Vaccines for *E. coli* mastitis

Four vaccines are currently available for coliform mastitis: the J5 Bacterin vaccine, the MastiguardTM vaccine, the JVAC[®] vaccine, and the STARTVAC vaccine. These vaccines are administered in the non-lactating stages but their effects tend to wane over time.²¹ They are based on antigens of *E. coli* LPS.²¹ Research into the J5 Bacterin, long considered the gold-standard in the field of coliform mastitis control, and STARTVAC vaccines will be discussed as there are not many published studies specifically on the MastiguardTM and JVAC[®] preparations.

The J5 Bacterin vaccine is derived from LPS mutant *E. coli* strain O111:B4.⁵³ The J5 strain is a rough LPS mutant, meaning its LPS does not have an O-side chain but does have an intact core.⁵⁴ J5 has been extensively studied since 1989. In a 1989 study González *et al* showed that in a sample population of 218 vaccinated and unvaccinated cows, only 35 (6 vaccinated, 25 unvaccinated) developed clinical mastitis.⁵⁵ *E. coli* was successfully isolated from 2.5% of the vaccinated and 12.77% of the unvaccinated cows.⁵⁵ In this study, the J5 vaccine was prepared by heat-killing a 24 hr bacterial culture, resuspending the culture in 0.9% sodium chloride solution, and emulsifying the antigen by sonication.⁵⁵ 5 ml of emulsified J5 antigen was mixed with 1 ml of Freund's incomplete adjuvant.⁵⁵ This emulsion was sonicated once more time prior to injection.⁵⁵

In a 2007 study by Wilson *et al* no difference was observed in antibody concentrations between vaccinated and unvaccinated cows.⁵⁶ Wilson *et al* conducted a 2009 study of J5- Bacterin, hypothesizing that vaccination would increase J5-specific antibodies in sera.⁵⁷ The authors collected sera samples for J5-specific ELISA assays.⁵⁷ They observed that prior to vaccination, there was no difference in titer for serum-specific IgM and IgG₂ between vaccinates and control cows.⁵⁷ After vaccination and calving, the authors observed an increase in J5-specific IgG₁ and IgG₂ in vaccinate cow sera compared to control sera.⁵⁷ A 2014 study by Tomita *et al* concluded that J5 immunization can induce cross-reactive antibodies against other coliforms.⁵³

STARTVAC, manufactured by HIPRA, contains inactivated *E. coli* J5 and inactivated *S. aureus* SP 140.⁵⁸ SP 140 is a *S. aureus* strain that expresses the slime and biofilm forming phenotype.⁵⁸ STARTVAC is given in 3 doses of 2 ml: the first dose is 45 days before parturition, the second dose 35 days after the first dose, and the third dose 62 days after the second dose.⁵⁸ In a study examining 386 cows (198 vaccinated and 188 control cows) 7 vaccinated cows and 31 control cows developed coliform mastitis.⁵⁸ 11.42% of vaccinated cows produced abnormal milk.⁵⁸ March *et al* concluded the 2 ml dosage and immunization schedule to be effective.⁵⁸ The STARTVAC vaccine is marketed to protect against *E. coli*, *S. aureus*, and CNS.⁵⁹

Although there are already 4 commercial vaccines for *E. coli* mastitis, the market could benefit from the addition of a fifth vaccine that is based on cationic surfactant vesicles. All 4 current vaccines must be refrigerated. A cationic surfactant vesicle does not need to be refrigerated. A cationic surfactant vesicle vaccine is thermodynamically stable at RT for years. A cationic surfactant vesicle vaccine can be autoclaved or pasteurized and so, a single aliquot can be used for many years. Also, a cationic surfactant vesicle vaccine is prepared with relatively inexpensive reagents. Finally, the cationic surfactant vesicle allows for the incorporation of LPS without the risk of molecule's toxicity harming or killing hosts. Thus, there could be demand for a cationic surfactant vesicle vaccine due to its stability, its potential for sterilization, its relatively inexpensive preparation, and its ability to present LPS in a form with decreased toxicity.

Eighty percent of all coliform mastitis cases are caused by *E. coli*.⁶⁰ However, mastitis-causing *E. coli* has become resistant to many favored antibiotics like ampicillin, streptomycin, tetracycline, and sulfonamides.⁶⁰ Therefore, immunization against *E. coli* is an attractive endeavor as the goal of a bovine mastitis vaccine is to increase sera levels of IgM and IgG₂.⁵⁶ Thus, developing new vaccines that can confer long-term protective immunity is highly anticipated by dairy farmers and scientists. The purpose of this thesis is to investigate one such new vaccine: the catanionic surfactant vesicle vaccine.

Catanionic surfactant vesicles

Catanionic surfactant vesicles are derived from colloidal systems.⁶¹ Coined in 1861 by Thomas Graham, colloids are substances of 1 μm to 1 nm size.⁶¹ Colloids were characterized by lack of sedimentation under the influence of gravity and low diffusion rates.⁶¹ Today, colloids are defined as systems in which one substance finely disperses into another.⁶¹

There are 3 major classes of colloidal systems: lyophobic, lyophilic, and association colloids.⁶¹ Lyophobic colloids are made of immiscible components.⁶¹ An example of a commonly encountered lyophobic colloid is milk, wherein liquid fat droplets are dispersed in an aqueous phase.⁶¹ Lyophilic colloids are spontaneous, thermodynamically stable solutions of solute molecules.⁶¹ A major branch of lyophilic colloid research is polymer research.⁶¹ Association colloids, which are also

lyophilic, are aggregates of amphiphilic molecules.⁶¹ Association colloids are commonly referred to as surface-active agents or surfactants.⁶¹

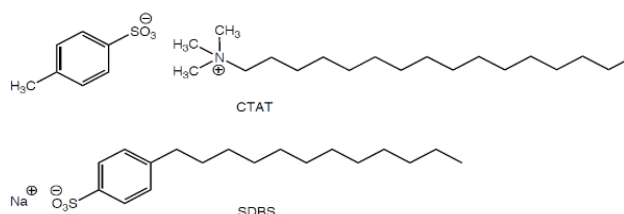
Surfactants are organic molecules that can move towards surface interfaces when dissolved in solvent at low concentrations.⁶¹ Such interfaces include liquid/liquid, solid/liquid, and gas/liquid.⁶¹ In the case of surfactants, their amphiphilic nature allows them to orient at interfaces in such a way that their lyophobic moiety is sequestered from strong solvent interactions while their lyophilic moiety remains in solution.⁶¹ As water is the most common solvent, surfactants' lyophobic and lyophilic moieties are referred as hydrophilic heads and hydrophobic tails.⁶¹

Surfactants can be classified by their head groups into the following classes: cationics/anionics, non-ionics, and zwitterionics.⁶¹ A catanionic surfactant is a mixture of cationic and anionic surfactants.⁶¹ These oppositely charged surfactants spontaneously aggregate, forming thermodynamically-stable vesicles in aqueous mixtures.⁶² Relevant to this study is the use of the cationic surfactant cetyltrimethylammonium tosylate (CTAT) and the anionic surfactant sodium dodecylbenzylsulfonate (SDBS) in preparing catanionic surfactant vesicles. These molecules are shown in **Figure 5A**.

Surfactants are known for moving towards surface interfaces and for self-assembling in bulk phase.^{61, 62} They can be emulsified or even made into detergents.⁶¹ Surfactants can adsorb and aggregate because of the hydrophobic effect: water-water

intermolecular interactions are much stronger than water-tail interactions.⁶¹ Surfactants can aggregate as micelles, liquid crystals, bilayers, microemulsions, liposomes, and vesicles.⁶² Liposomes, known as lipid vesicles, are made of phospholipid molecules and are formed by the supramolecular interactions of 2 hydrophobic tails and 1 hydrophilic head group.⁶² Advances in colloid and liposome research have paved the way for recent studies evaluating the use of surfactant vesicles as a drug delivery platform.

A. Structure of CTAT and SDBS.



B. Formation of catanionic surfactant vesicles.

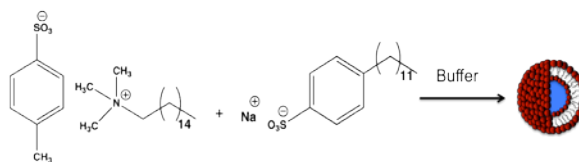


Figure 5. General structure of the catanionic surfactant vesicle vaccine.

Figures were adapted from sources 72 and 83.

In the early 1960s, A.D. Bangham's work related to liposomes advanced the field of colloid science. Based on his work, liposomes became accepted as a model for biological membranes.⁶² In the 1970s, research was conducted into encapsulating liposomes, allowing these structures to be applied toward drug delivery and

pharmaceutical endeavors.⁶² Then, in the 1980s, research into catanionic surfactants came to the forefront. The term ‘catanionic’ was first used by Jokela *et al*⁶³. Kaler *et al* noted the spontaneous formation of catanionic vesicles using three different catanionic systems, one of them being the CTAT/SDBS system.^{64, 65} Hargreaves and Dreamer were the first to characterize catanionic vesicles’ ability to carry molecules.⁶² Since then, many other studies have been undertaken to investigate catanionic vesicles’ unique properties, especially investigating their applications as drug delivery systems and vaccines (Bramer *et al* 2006; Bramer *et al*, 2007; Boudier *et al*, 2011; Gosh *et al* 2013; Stein *et al*, 2013; Richard *et al* 2014; Richard *et al*, 2014)^{66, 65, 67,68,69,70,71} The DeShong group at the University of Maryland, College Park has shown that a surfactant vesicle vaccine prepared from a mixture of CTAT/SDBS (see **Figure 5B**) can elicit antibodies against the LPS molecule of *Francisella tularensis*, a Gram-negative coccobacillus and the causative agent of tularemia.⁷⁰

Catanionic vesicles have many advantages over liposomes as a vaccine platform. The main advantage is that catanionic vesicles form spontaneously from inexpensive reagents and are thermodynamically stable.^{71, 72, 73} For example, liposomes must be made from phospholipids like egg yolk phosphatidylcholine (\$177/X) whereas catanionic vesicles can be made from less expensive ionic surfactants like SDBS (\$1.10/X).⁷² Another advantage of vesicles is that they can be sterilized by pasteurization at 65°C or by autoclaving.⁷² With liposomes, proteins can become denatured when incorporated into membranes.⁷² Catanionic vesicles vaccines can be prepared in such a way as to extract immunogenic bacterial membrane components

into the membranes of the vesicles, creating an ‘artificial pathogen’ whereby proteins may be presented on membrane surfaces in their natural conformations.⁷² In Gram-negative infections, such as those caused by *F. tularensis* and *E. coli*, LPS is a potent and critical immunostimulatory molecule. The surfactant vesicle vaccine platform allows incorporation of LPS without the toxicity associated with the molecule.

Maintaining the conformation of immunogenic surface proteins as well as decreasing the endotoxicity of LPS may help elicit long-term protective immunity.

Thesis focus

The focus of this thesis research is the immunopotency of a novel cationic surfactant vesicle vaccine for *E. coli* (P4) bovine mastitis. In 2000, a liposomal complete core LPS vaccine intended for humans was developed from a LPS cocktail of 4 Gram-negative bacteria (*E. coli* K-12, *E. coli* R1, *Pseudomonas aeruginosa* PAC 608, and *B. fragilis*).⁷⁴ However, to date an *E. coli* mastitis vaccine that incorporates the entire LPS molecule as well as other potential immunogenic surface components has not yet been developed. The cationic surfactant vesicle is one such potential vaccine platform.

As depicted in **Figure 6**, the outer surface of this vaccine could resemble the outer membrane surface of a Gram-negative bacterium with LPS, phospholipids, porins, and outer membrane proteins in the outer membrane of the vesicle. The lipid A moiety of LPS could incorporate into the inner leaflet of the vesicle’s outer membrane. The oligosaccharide and O-antigen moieties could be in the outer leaflet of the vesicle’s outer membrane. In this way, the highly immunogenic LPS molecule,

which does not readily functionalize in vaccine preparations, can be incorporated.⁷⁵

The cationic surfactant platform provides the potential for immunizing cows against whole-cell *E. coli* P4, thereby potentially providing a means to prevent rampant cases of *E. coli* mastitis.

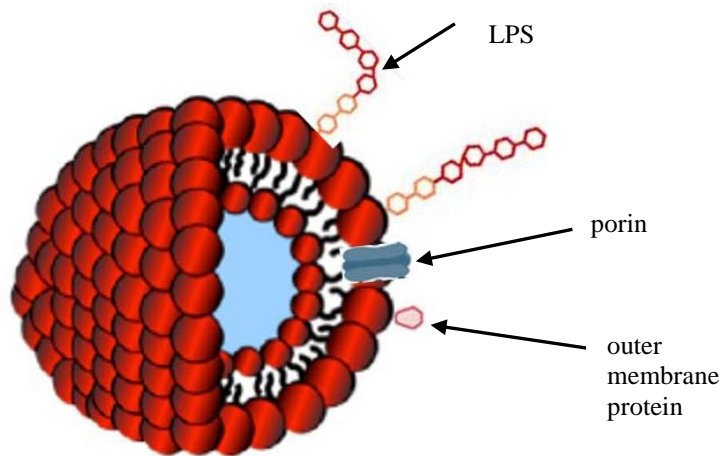


Figure 6. Putative outer membrane surface of the cationic surfactant vesicle vaccine.

Adapted from source 72 and 84.

Hypothesis

The bovine mastitis vaccine induces a strong antibody response to antigens found on the outer membrane surface of *E. coli* P4.

Specific Aim 1

Generate an antibody titer comparing pre- and post- vaccination sera.

Specific Aim 2

Generate an immunogenic profile comparing pre- and post-vaccination sera.

Chapter 2: Materials and Methods

Preparation of catanionic surfactant vesicle vaccine

The bovine mastitis vaccine was developed based on technology described in U.S. Patent 20110165067 (Carbohydrate Functionalized Catanionic Surfactant Vesicles for Drug Delivery).⁷⁵ *E. coli* strain P4 was grown and collected by centrifugation. Centrifugation allowed outer membrane proteins to disassociate from the outer membrane but kept the peptidoglycan layer intact. *E. coli* was then mixed with surfactant reagents. Within 12 hr, vesicles formed spontaneously from mixing the anionic surfactant SDBS and the cationic surfactant CTAT in a 7: 3 ratio.⁷⁵ The 180 nm vesicles were purified on sepharose columns, quantified, and normalized to contain 20 mg of LPS.

Preliminary *in vivo* vaccine study

Preliminary *in vivo* studies were conducted in partnership with the USDA. Three pregnant heifers were selected. Heifers are cows that are giving birth (calving) for the first time. Heifers were preferred over cows because these animals had never suckled calves before. Suckling can cause tissue damage to the udders, thereby creating opportunities for microorganisms to invade and infect the mammary gland, inducing mastitis. Therefore, unlike cows' udders, heifers' udders would have minimal exposure to environmental pathogens. Additionally, heifers were selected because cows are at a significantly higher risk of developing mastitis during parturition.³⁴ Using heifers allowed researchers to ascertain whether this vaccine had any toxic

effects on gestation and the delivery of calves. All 3 heifers in this study delivered healthy calves, suggesting this vaccine is safe for pregnant cows.

Heifers were vaccinated with 50 µl of the whole-cell cationic surfactant vesicle vaccine, 45 days prior to their expected calving date (“d 0”). Each dose of vaccine was normalized to contain 20 mg of LPS. Heifers were then administered a second booster dose approximately 21 days into the study (“d 21”). During this time, blood and milk samples were collected from the animals at regular intervals, following the schedule outlined in **Table 2**. Animals were monitored until 14 days after calving.

Three heifers (3635, 3629, 3643) were vaccinated with the P4 vaccine. Blood samples were collected at regular intervals. Pre-vaccination blood was collected on d0. Post-vaccination blood was collected the final day of the study (clv d 14). Serum was isolated from the blood samples by collaborators at the USDA.

Date	Cow 3635	Cow 3629	Cow 3643
03-08-2013	d 0		
03-15-2013	d 7		
03-22-2013	d 14	d 0	
03-29-2013	d 21	d 7	
04-05-2013	d 28	d 14	d 0
04-10-2013	clv d 0		
04-11-2013	clv d 1		
04-12-2013	clv d 2	d 21	d 7
04-13-2013	clv d 3		
04-17-2013	clv d 7		
04-18-2013		clv d 0	
04-19-2013		clv d 1	d 14
04-20-2013		clv d 2	
04-21-2013		clv d 3	
04-23-2013			clv d 0
04-24-2013	clv d 14		clv d 1
04-25-2013		clv d 7	clv d 2
04-26-2013			clv d 3
04-30-2013			clv d 7
05-02-2013		clv d 14	
05-03-2013			clv d 10
05-07-2013			clv d 14

	Vaccination
	Booster
	Calving

Table 2. Vaccination schedule.

Growth and maintenance of bacterial strains

Bacterial strains used in this study are shown in **Table 3**. Media employed are shown **Table 4**. *E. coli* strains P4 and DH5 α were grown from glycerol stock on Luria Broth (LB) agar plates at 37°C for 16-24 hr. Strains were confirmed to be P4 and DH5 α by streaking them onto MacConkey's agar plates, incubating overnight at 37°C and observing the lactose utilization patterns. P4 ferments lactose and DH5 α does not. Bacterial strains were maintained by subculturing onto fresh LB agar plates daily and used for experimentation within 18-24 hr. *N. gonorrhoeae* strain MS11 was grown from glycerol stock on GCK agar plates at 37°C with 5% CO₂ for 16-24 hr. Strains were streaked onto fresh GCK agar plates as needed and used for experimentation within 18-24 hr.

Bacterial Species	Strain	Description	Phenotype/ Genotype	Use in this study
<i>Escherichia coli</i>	P4 serotype 108	Pathogenic mastitis strain isolated by Bramley <i>et al</i> ⁷⁸ and used to induce experimental mastitis	O32: H37 ⁴⁰	Experimental coliform strain
<i>Escherichia coli</i>	DH5 α - mcr	Laboratory strain	fhuA2 lac(del) U169 phoA glnV44 Φ 80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 ⁷⁹	Control coliform strain
<i>Neisseria gonorrhoeae</i>	MS11	Pathogenic strain used in laboratory studies		Control gonococcal strain

Table 3. Bacterial strains used in this study. *E. coli* P4 was used as the experimental coliform strain, *E. coli* DH5 α as the control coliform strain, and *N. gonorrhoeae* as the control gonococcal strain.

Media	Composition
GCK agar	36g Difco TM GC Medium Base (Becton, Dickson, and Co.) 5g bacteriological agar (U.S. Biological Co.) 1L Elix water 10ml 100X Kellogg's supplement (5g glutamine, 5g ferric nitrate anhydrous, 0.02g thiamine pyrophosphate, 1L Elix water, 400g glucose)
LB agar	40g LB agar base (U.S. Biological Co.) 1L Elix water
LB agar + Ampicillin	40g LB agar base (U.S. Biological Co.) 1L Elix water 30µg/L ampicillin
LB broth	40g LB Broth Base (U.S. Biological Co.) 1L Elix water
MacConkey's agar	17g peptone 3g proteose peptone 10g lactose 5g NaCl 1.0 mg crystal violet 3.0mg neutral red 1.5g bile salt 13.5g agar 1L Elix water pH 7.1

Table 4. Media used in this study.

Reagent/Buffer	Composition
<u>Preparation of Cytoplasmic/Inner Membrane/Outer Membrane/ Total Proteins</u>	
Buffer 1	0.2M Tris-HCl, pH 8.0; 1 M sucrose, 1 mM EDTA
Buffer 2	50 mM Tris-HCl, pH 8.0; 2% (w/v) Triton X-100; 10 mM MgCl ₂
1X Lysing Buffer	10% glycerol; 1M Tris-HCl, pH 6.8; 2% SDS; 4% β-mercaptoethanol
<u>SDS-PAGE</u>	
10X Tris-Glycine+ SDS Running Buffer	Dissolve in 500ml Elix water: 15.15g Tris; 72g glycine; 5g SDS. pH 8.3
1X Running Buffer	Dilute 10X Running Buffer 1:10
1X Lysing Buffer	10% glycerol; 1M Tris-HCl, pH 6.8; 2% SDS; 4% β-mercaptoethanol
<u>Silver Staining</u>	
Fixing Solution	200ml 95% ethanol; 300ml Elix water; 25ml glacial acetic acid
Periodic Acid Solution	0.83g periodic acid in 100ml Elix water
Silver Nitrate Stain	Solution 1: 1.25ml NH ₄ OH + 1 NaOH pellet + 25ml Elix water Solution 2: 0.8g silver nitrate/ 4ml Elix water Add Solution 2 dropwise to Solution 1 using a stir bar. Balance pH by adding NH ₄ OH dropwise. Bring final volume to 100ml using Elix water.
Developing Solution	1ml citric acid (25mg/ml); 95μl formaldehyde; 500ml Elix water
<u>Western/Immunoblotting</u>	
10X Tris-Glycine Buffer	15.15g Tris; 5g SDS; 72g glycine; 500ml Elix water. pH 8.3
Transfer Buffer	100ml 10X Tris-Glycine Buffer; 200ml 100% methanol; 700ml Elix water
10X PBS	To 800ml Elix water: 80g NaCl; 2g KCl; 7.62g Na ₂ HPO ₄ ; 0.77g KH ₂ PO ₄ . pH to 7.37 Fill 1L.
1X PBS	Dilute 10X PBS
PBST	1L 1X PBS; 0.05% Tween-20™
Blocking Buffer	5g powdered milk dissolved in 100ml PBST
<u>ELISA</u>	
Blocking Buffer	5g BSA in 100ml 1X PBS
ELISA Wash Buffer	1L 1X PBS; 0.05% Tween-20™

Table 5. Buffers and reagents used in this study.

Preparation of Outer Membrane, Inner Membrane, and Cytoplasmic Fractions

Cytoplasmic/inner/outer membrane protein fractions were obtained using lysozyme/EDTA lysis and selective detergent isolation following Method 1 developed by Thein *et al.*⁸⁰ The protocol can be divided into three parts: collection of bacterial cells after growth; induction of spheroblast formation; and isolation of cytoplasmic/inner membrane and outer membrane proteins.

Collection of bacterial cells after growth

P4 and DH5 α were grown from glycerol stocks in LB broth for 16-24 hr at 37°C in 15 ml centrifuge tube (VWR International). Bacterial cultures were grown overnight in a rolling drum at 37°C. The next day, 1 ml overnight culture was added to 24 ml of LB broth, and the cultures incubated at 37°C, 220 rotations per minute (rpm), until O.D₆₀₀~0.9. Bacterial cells were collected by centrifugation in a Sorvall RC-53 Refrigerated Super-speed centrifuge for 10 min at 12,000rpm, 4°C. The supernatant was discarded and the pellet, containing whole cell bacteria, was kept for resuspension.

Induction of spheroblast formation

Pellets were resuspended in 500 μ l of Buffer 1 (0.2M Tris-HCl, pH 8.0; 1 M sucrose; 1 mM EDTA). The resuspension was transferred to a Beckman Ultra-clear centrifuge tube. Lysozyme (100 μ l of a 5mg/ml stock solution dissolved in Elix H₂O) was added to each centrifuge tube, which was flicked gently. The resuspension was incubated at room temperature (RT) for 5 min, and 1.76 ml of Elix H₂O added. This resuspension was incubated at RT for 20 min until spheroblasts were observed.

Following spheroblast formation, 3 ml of Buffer 2 (50 mM Tris-HCl, pH 8.0; 2% (w/v) Triton X-100; 10 mM MgCl₂) and 30 µl of TURBOTM DNase (Ambion®) were added. The suspension was incubated at RT until it cleared.

Isolation of cytoplasmic/inner membrane and outer membrane proteins

The suspension was clarified by ultracentrifugation at 26,500 rpm for 30 min, 4°C in a SW 55 Ti rotor (Beckman Coulter OptimaTM L-90 K Ultracentrifuge). The supernatant, containing cytoplasmic and inner membrane proteins, was collected and stored at -20°C. The pellet, which contained the outer membrane, was re-suspended in 750 µl of Buffer 2 and repelleted by ultracentrifugation at 27,000 rpm for 20 min at 4°C. The pellet containing the outer membrane fraction was washed with 500 µl of Elix H₂O and collected by ultracentrifugation at 27,000rpm for 20 minutes at 4°C. The membrane was washed 3 times and the final wash was stored at -20°C.

Preparation of whole-cell protein fractions

Whole-cell protein lysates were prepared for each *E. coli* strain from culture grown for 16-24 hr in LB broth. The remaining 1ml of overnight culture was concentrated using a tabletop centrifuge (eppendorf Centrifuge 5417C) at 10,000rpm for 10 min at RT. Pellets were resuspended in 50 µl of Lysing Buffer and stored at -20°C. To all protein fractions, protein inhibitors were added.

SDS-PAGE/western blot procedures

Aliquots of membrane fractions were analyzed by gel electrophoresis through a 10-20% SDS-PAGE gel. Half of the gel was silver stained and the other half was immunoblotted using Pre-Vaccination and Post-Vaccination sera as primary antibodies.

Preparation of protein fractions for SDS-PAGE

The amount of protein contained in each fraction was determined using a Nanodrop (ND-100 Spectrophotometer) by loading 2 μ l of protein fraction and measuring the absorbance at A_{280} . A concentration was given in mg/ml and each fraction was then normalized to 10 μ g of protein: 10 μ l samples of each fraction were prepared for SDS-PAGE analysis in 1X Lysing Buffer.

Separation of protein fractions by SDS-PAGE

Prior to loading, samples were boiled at 100°C for 10 min and vortexed for 30 sec. Samples were loaded on a 10-20% Criterion Tris-HCl gel (Bio-Rad) in the pattern shown in **Figure 7**. Experimental samples (10 μ l) were loaded alongside 1 μ l of Precision Plus Protein TM Kaleidoscope Standard (Bio-Rad). The gel was placed in a Criterion cell, immersed in 1X Running Buffer, and run at constant current (100 mA) on ice for 2-4 hr. After electrophoresis, the gel was cut and used immediately for immunoblotting and silver staining.

Silver stain protocol

The gel was fixed in Fixing Solution (500 ml) overnight at RT. The next day, the gel was oxidized with Periodic Acid (100 ml) for 5 min and then washed in Elix H₂O for

2 hr. During the wash period, water was changed every 30 min. Silver Stain was applied for 15 min at RT. The gel was washed 3 times, 30 min each, with 250 ml of Elix. Staining was detected by allowing the gel to soak in developing Solution until bands became visible. The gel was given a final water rinse and then imaged using the Bio-Rad ChemiDoc™ XRS Gel Documentation system and Bio-Rad Quantity One ® software.

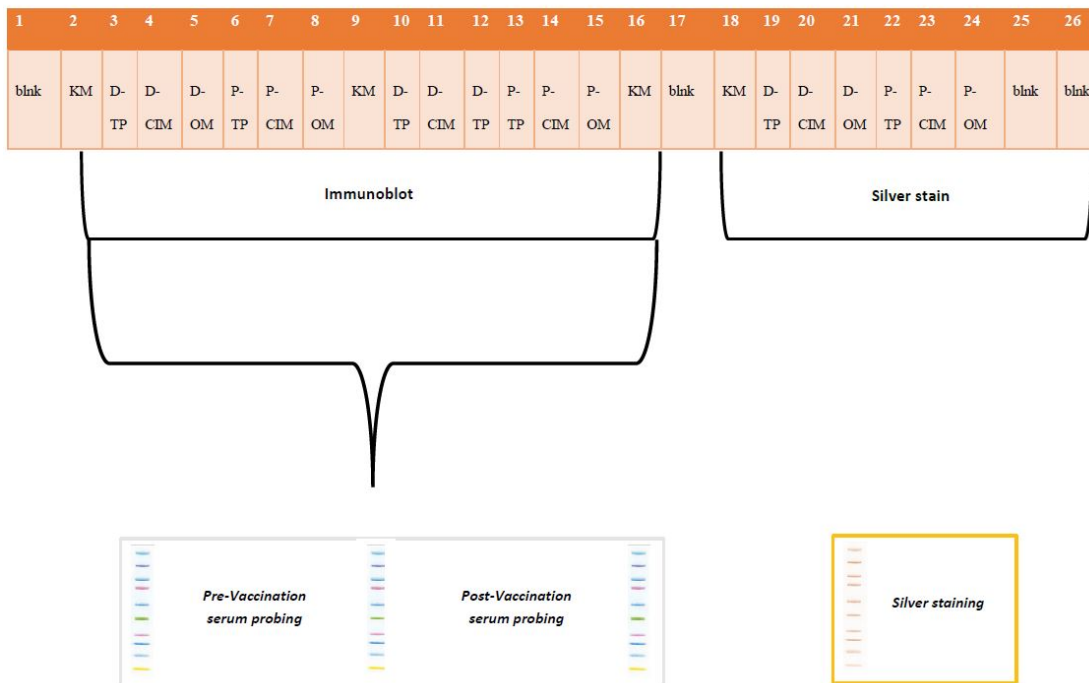


Figure 7. Gel loading pattern and silver stain/immunoblotting workup. 10 µl of each sample was run in the following pattern: Kalediscope Marker (KM), DH5α Total Protein (D-TP), DH5α Cytoplasmic/Inner Membrane (D-CIM), DH5α Outer Membrane (D-OM), P4 TP (P-TP), P4 CIM (P-CIM), P4 OM (P-OM), KM. Empty lanes (blnk) contained 10 µl of 1X Lysing Buffer. The gel was run at constant 100 mA and following electrophoresis, was cut along lane 16. The first gel was used for immunoblotting and the second gel was used for silver staining.

Immunoblotting protocol

Immediately following electrophoresis, the gel was transferred in Transfer Buffer to a 0.45 µm pore PVDF membrane (Immobilon-P membrane) at 100 V on ice for 1 hr.

The membrane was blocked overnight at 4°C in Blocking Buffer. The next day, the membrane was cut along lane 9 (Kaleidoscope Marker). The left half was probed with pre-vaccination (PR) cow serum and the other half with post-vaccination (PO) cow serum. The membranes were incubated in 10 ml of their respective primary antibodies (see **Table 7**) overnight at 4°C. The next day, the membrane was washed 3 times in PRST for 15 min and rinsed in Elix H₂O 3 times. The membrane was blocked again for 1 hr at RT, probed with 50 ml of secondary antibody at RT for 1 hr, washed in PRST 6 times, and rinsed in Elix H₂O 3 times. The blot was developed using 2 ml of the Western Blotting Luminol Reagent system (Santa Cruz Biotechnology) at RT for 5 min. Finally, the blot was imaged using audiography or the LAS-3000 Imaging System (FUJIFILM) and quantified using ImageJ software (National Institutes of Health).

ELISA procedures

Enzyme-linked immunosorbent assays (ELISAs) were performed on pre- and post-vaccination sera.

ELISA plate setup

Control gonorrheal strain MS11 wild-type and experimental coliform strain P4 were grown overnight. Bacteria were swabbed from the plate using a Dacron swab and resuspended in 4 ml of 1X PRS to a turbidity of 100, as measured with a Klett Summerson Photoelectric Colorimeter. Bacteria (100 µl) was added to 96 well microtiter plates (nuncTM Maxisorp flat bottom plate) following the design outlined in

Table 6. Plates were incubated at 37°C for 3 days to allow bacteria to adsorb. The experimental controls were a blank control (wells A1-3, E1-3) and two sample controls (wells A5-7 and A9-12). The controls were duplicated in Rows A and D. The experimental pre-vaccination samples were triplicated in Rows B, E, and G. The experimental post-vaccination samples were triplicated in Rows C, F, and H.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank TMB control	Blank TMB control	Blank TMB control	empty	<i>E. coli</i> P4 N _{go} 1' N _{go} 2'	<i>E. coli</i> P4 N _{go} 1' N _{go} 2'	<i>E. coli</i> P4 N _{go} 1' N _{go} 2'	empty	N _{go} MS11 N _{go} 1' N _{go} 2'	N _{go} MS11 N _{go} 1' N _{go} 2'	N _{go} MS11 N _{go} 1' N _{go} 2'	empty
B	<i>E. coli</i> P4 PR 1:100 Cow 2'	<i>E. coli</i> P4 PR 1:200 Cow 2'	<i>E. coli</i> P4 PR 1:400 Cow 2'	<i>E. coli</i> P4 PR 1:800 Cow 2'	<i>E. coli</i> P4 PR 1:1600 Cow 2'	<i>E. coli</i> P4 PR 1:3200 Cow 2'	<i>E. coli</i> P4 PR 1:6400 Cow 2'	<i>E. coli</i> P4 PR 1:12800 Cow 2'	<i>E. coli</i> P4 PR 1:25600 Cow 2'	<i>E. coli</i> P4 PR 1: 51200 Cow 2'	<i>E. coli</i> P4 PR 1:102400 Cow 2'	<i>E. coli</i> P4 PR UD Cow 2'
C	<i>E. coli</i> P4 PO 1:100 Cow 2'	<i>E. coli</i> P4 PO 1:200 Cow 2'	<i>E. coli</i> P4 PO 1:400 Cow 2'	<i>E. coli</i> P4 PO 1:800 Cow 2'	<i>E. coli</i> P4 PO 1:1600 Cow 2'	<i>E. coli</i> P4 PO 1:3200 Cow 2'	<i>E. coli</i> P4 PO 1:6400 Cow 2'	<i>E. coli</i> P4 PO 1:12800 Cow 2'	<i>E. coli</i> P4 PO 1:25600 Cow 2'	<i>E. coli</i> P4 PO 1: 51200 Cow 2'	<i>E. coli</i> P4 PO 1:102400 Cow 2'	<i>E. coli</i> P4 PO UD Cow 2'
D	Blank TMB control	Blank TMB control	Blank TMB control	empty	<i>E. coli</i> P4 N _{go} 1' N _{go} 2'	<i>E. coli</i> P4 N _{go} 1' N _{go} 2'	<i>E. coli</i> P4 N _{go} 1' N _{go} 2'	empty	N _{go} MS11 N _{go} 1' N _{go} 2'	N _{go} MS11 N _{go} 1' N _{go} 2'	N _{go} MS11 N _{go} 1' N _{go} 2'	empty
E	<i>E. coli</i> P4 PR 1:100 Cow 2'	<i>E. coli</i> P4 PR 1:200 Cow 2'	<i>E. coli</i> P4 PR 1:400 Cow 2'	<i>E. coli</i> P4 PR 1:800 Cow 2'	<i>E. coli</i> P4 PR 1:1600 Cow 2'	<i>E. coli</i> P4 PR 1:3200 Cow 2'	<i>E. coli</i> P4 PR 1:6400 Cow 2'	<i>E. coli</i> P4 PR 1:12800 Cow 2'	<i>E. coli</i> P4 PR 1:25600 Cow 2'	<i>E. coli</i> P4 PR 1: 51200 Cow 2'	<i>E. coli</i> P4 PR 1:102400 Cow 2'	<i>E. coli</i> P4 PR UD Cow 2'
F	<i>E. coli</i> P4 PO 1:100 Cow 2'	<i>E. coli</i> P4 PO 1:200 Cow 2'	<i>E. coli</i> P4 PO 1:400 Cow 2'	<i>E. coli</i> P4 PO 1:800 Cow 2'	<i>E. coli</i> P4 PO 1:1600 Cow 2'	<i>E. coli</i> P4 PO 1:3200 Cow 2'	<i>E. coli</i> P4 PO 1:6400 Cow 2'	<i>E. coli</i> P4 PO 1:12800 Cow 2'	<i>E. coli</i> P4 PO 1:25600 Cow 2'	<i>E. coli</i> P4 PO 1: 51200 Cow 2'	<i>E. coli</i> P4 PO 1:102400 Cow 2'	<i>E. coli</i> P4 PO UD Cow 2'
G	<i>E. coli</i> P4 PR 1:100 Cow 2'	<i>E. coli</i> P4 PR 1:200 Cow 2'	<i>E. coli</i> P4 PR 1:400 Cow 2'	<i>E. coli</i> P4 PR 1:800 Cow 2'	<i>E. coli</i> P4 PR 1:1600 Cow 2'	<i>E. coli</i> P4 PR 1:3200 Cow 2'	<i>E. coli</i> P4 PR 1:6400 Cow 2'	<i>E. coli</i> P4 PR 1:12800 Cow 2'	<i>E. coli</i> P4 PR 1:25600 Cow 2'	<i>E. coli</i> P4 PR 1: 51200 Cow 2'	<i>E. coli</i> P4 PR 1:102400 Cow 2'	<i>E. coli</i> P4 PR UD Cow 2'
H	<i>E. coli</i> P4 PO 1:100 Cow 2'	<i>E. coli</i> P4 PO 1:200 Cow 2'	<i>E. coli</i> P4 PO 1:400 Cow 2'	<i>E. coli</i> P4 PO 1:800 Cow 2'	<i>E. coli</i> P4 PO 1:1600 Cow 2'	<i>E. coli</i> P4 PO 1:3200 Cow 2'	<i>E. coli</i> P4 PO 1:6400 Cow 2'	<i>E. coli</i> P4 PO 1:12800 Cow 2'	<i>E. coli</i> P4 PO 1:25600 Cow 2'	<i>E. coli</i> P4 PO 1: 51200 Cow 2'	<i>E. coli</i> P4 PO 1:102400 Cow 2'	<i>E. coli</i> P4 PO UD Cow 2'

Antibodies used in the ELISAs:
N. go 1' = goat α *Ngo* IgG bleed 3 antiserum (1:50)
N. go 2' = donkey α goat IgG-HRP (1:10,000)
 Cow 1' = PR or PO serum diluted as outlined
 Cow 2' = rabbit α bovine IgG- HRP (1:30,000)

Serum dilution abbreviations:
 PR= Pre-vaccination
 PO= Post-vaccination
 UD= Undiluted

Table 6. ELISA setup.

Antibodies

Cow primary antibody solutions were prepared in 2 ml quantities following a 12 two-fold serial dilution scheme. Dilutions were prepared from 1:100 to 1:102,400. Cow secondary antibody was prepared in a 45 ml quantity and diluted 1:30,000.

N. gonorrhoeae primary antibody (goat α *N. gonorrhoeae* IgG bleed 3 antiserum) was prepared in 1 ml quantities and diluted 1:50. *N. gonorrhoeae* secondary antibody (donkey α goat IgG-HRP) was prepared in 1 ml quantities and diluted 1:10,000. Antibodies were used at RT and stored at -4°C. The antibodies used in this thesis are summarized in **Table 7**.

Description	Designation	Stock Volume; Dilution in PRST
Heifers 3643, 3629, 3635 Pre- and Post-Vaccination sera	1' antibody	Immunoblot: 10ml; 1:500 ELISA: 2ml; 1:100 to 1:102,400
Rabbit α bovine IgG (whole molecule)-peroxidase	2' antibody	Immunoblot: 50ml; 1:50,000 ELISA: 45ml; 1:30,000
Goat α <i>N. gonorrhoeae</i> IgG bleed 3 antisera	1' antibody	Immunoblot: 1ml; 1:50
Donkey α goat IgG-HRP	2' antibody	Immunoblot: 1ml; 1:10,00

Table 7. Antibodies used in this study.

ELISA readings

Wells were blocked with 250 μ l Blocking Buffer for 1 hr at RT. Wells were rinsed with PRST 3 times. Wells were probed with primary antibody (50 μ l) for 1 hr at RT, rinsed with PRST 3 times, and dried by aspiration. Wells were probed with secondary antibody (70 μ l) for 1 hr at RT and rinsed with PRST 6 times. Finally, wells were developed with trimethylbenzidine (TMB) (Sigma) (100 μ l) for 5 min at RT. The KC4 Plate Reader program was employed to measure absorbance at 645 nm every min for 20 min. For analysis, readings were averaged across a 1 min time frame.

Chapter 3: Results

Verification of strain phenotypes

P4 and DH5 α *E. coli* were swabbed on MacConkey's agar and allowed to grow for 18 hr. MacConkey's agar contains lactose as its carbon source. As seen in **Figure 8**, only P4 is able to utilize and ferment lactose, generating an acidic by-product that renders the agar on which it grows pink. DH5 α is a lactose operon deletion mutant and as such cannot utilize the lactose on the MacConkey's plate as its carbon source. Instead, DH5 α uses the peptones in the agar, generating ammonia as a by-product, raising the pH of the agar and rendering colonies with a white appearance.

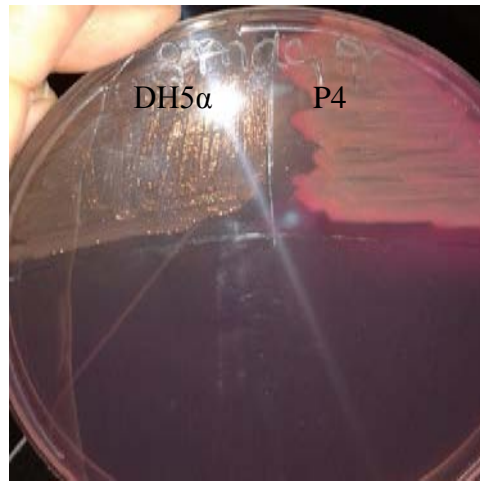


Figure 8. Verification of strain phenotypes.
P4 ferments lactose (pink colonies) and
DH5 α does not (white colonies).

Determination of pre--vaccination and post-vaccination antibody titers

The concentrations of IgG antibodies in sera pre- and post-vaccination were analyzed by performing direct ELISAs. Antigen (whole-cell P4) was adsorbed to wells of a microtiter plate. Antiserum (PR and PO sera) was diluted 2-fold from 1:100 to 1:100,240 and probed with a secondary horseradish peroxidase (HRP)-conjugated antibody that recognizes bovine IgG molecules. HRP cleavage of the substrate TMB forms a blue-colored product, the absorbance of which was measured at O.D. 645nm. An example of a representative ELISA plate is shown in **Figure 9**, which follows the same setup depicted in **Table 6**.

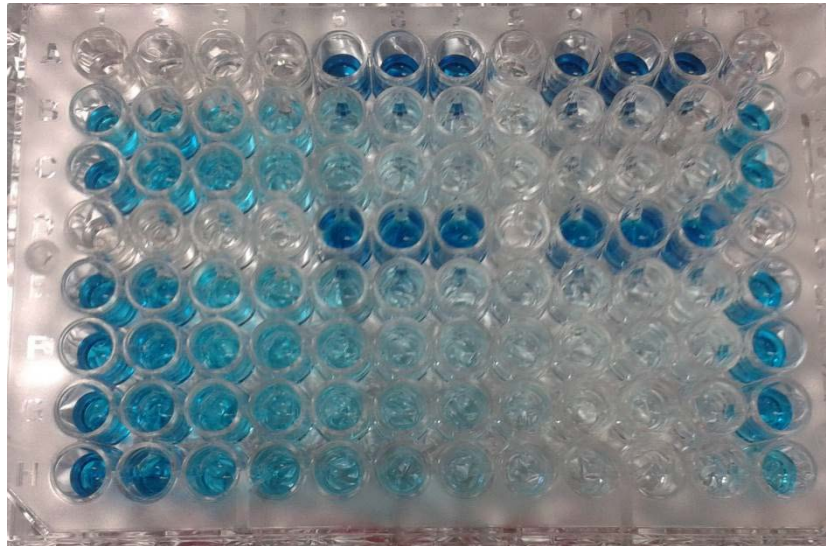


Figure 9. Representative ELISA plate.

The absorbance readings for PR and PO sera of cows 3643, 3629, and 3635 are given in **Table 8**. These data are corrected values after the values for the blanks were subtracted out. Absorbance measurements of PR and PO sera were plotted as \log_2

graphs to determine antibody titers. Graphs are shown as follows: Cow 3643 (**Figure 10**), 3629 (**Figure 11**), and 3635 (**Figure 12**).

	Absorbance measurements at O.D. ₆₄₅					
	Cow 3643		Cow 3629		Cow 3635	
Antisera Dilution Factor	PR	PO	PR	PO	PR	PO
1	0.555	0.451	0.399	0.322	0.558	0.567
100	0.516	0.567	0.371	0.416	0.490	0.491
200	0.443	0.507	0.270	0.290	0.415	0.460
400	0.319	0.323	0.210	0.211	0.285	0.302
800	0.198	0.181	0.136	0.153	0.176	0.203
1,600	0.124	0.102	0.088	0.102	0.106	0.103
3,200	0.063	0.057	0.061	0.065	0.067	0.075
6,400	0.044	0.034	0.044	0.051	0.042	0.054
12,800	0.026	0.025	0.036	0.038	0.033	0.039
25,600	0.022	0.018	0.035	0.031	0.023	0.032
51,200	0.021	0.023	0.026	0.030	0.027	0.030
102,400	0.020	0.037	0.027	0.029	0.034	0.037

Table 8. Absorbance measurements pre-vaccination and post-vaccination.

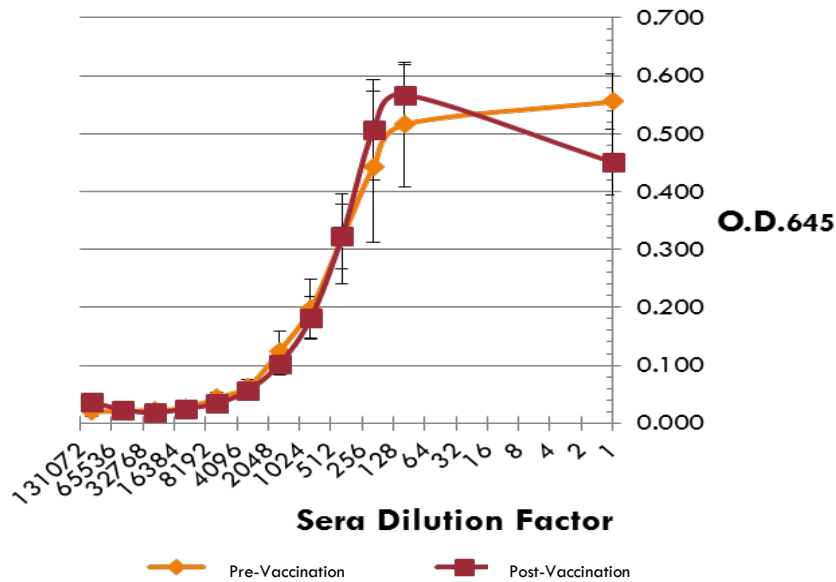


Figure 10. P4-specific IgG antibodies in Cow 3643 pre-and post-vaccination sera.

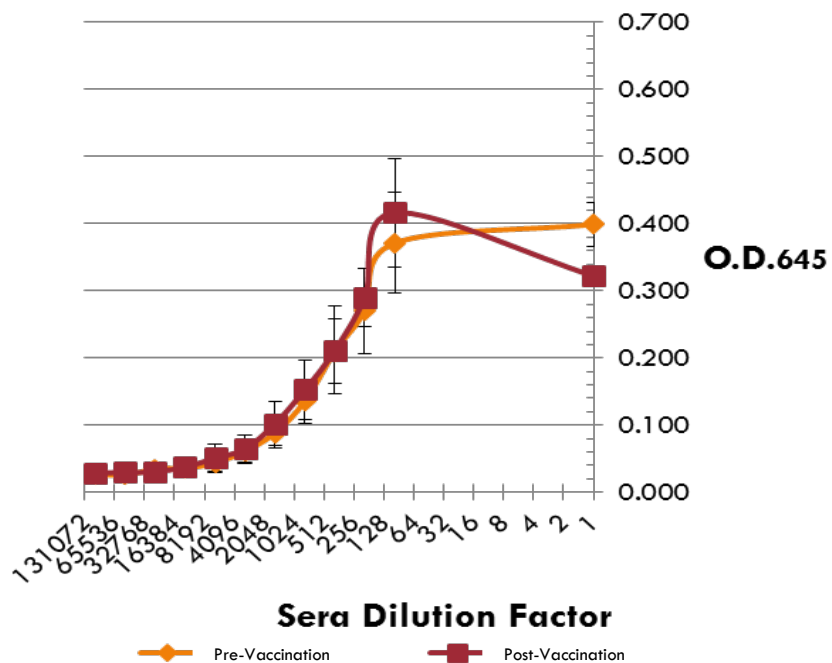


Figure 11. P4-specific IgG antibodies in Cow 3629 pre-and post-vaccination sera.

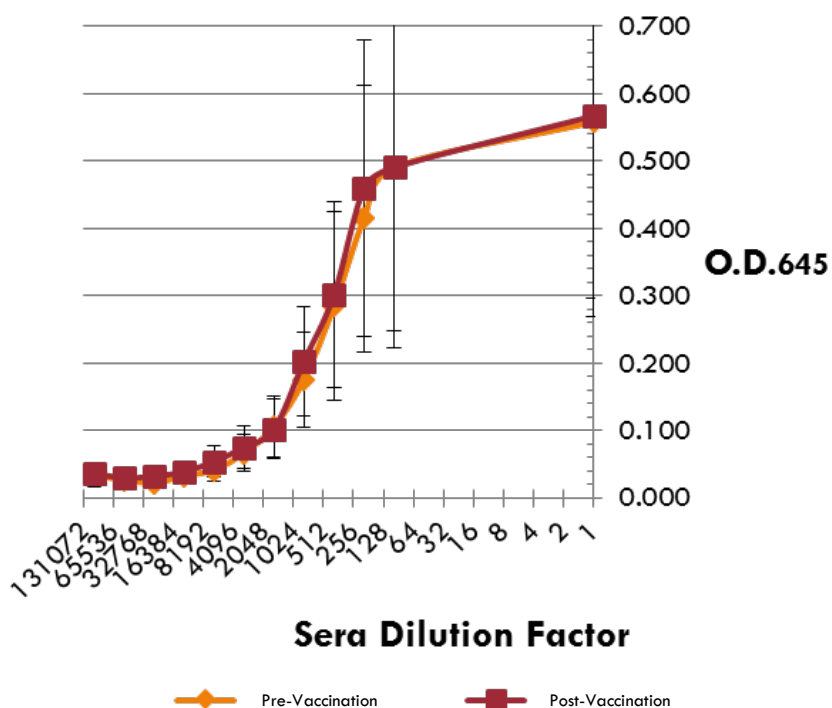


Figure 12. P4-specific IgG antibodies in Cow 3635 pre- and post-vaccination sera.

For all 3 cows, background existed in the ranges of dilution factors 12,800 to 102,240. Antibody titer is defined as the greatest dilution of antisera that still allows for detection. Titer for all 3 cows' pre-and post-vaccination sera was 6,400. For cow 3643, detection was observed between absorbance 0.340 to 0.325. For cow 3629, detection was observed between absorbance 0.044 to 0.300. Finally, for cow 3635 detection was observed between absorbance 0.042 to 0.302. Plateaus of saturation were observed for all cows from dilution factors 200 to undiluted. Based on titer, conclusions can be made about antibody concentration in sera pre-and post-vaccination. These conclusions will be discussed in the following chapter.

Matched two-tailed student t-tests were performed on all 3 sets of ELISA data. The P value for cow 3643 was 0.858. The P-value for cow 3629 was 0.728. Finally, the P value for cow 3635 was 0.012. The meaning of these P values will be discussed in the following chapter.

Identification of potential vaccine antigens

Immunoblot analyses were conducted to identify potential P4 antigens that had incorporated into cationic surfactant vesicles. Outer membrane proteins were thought to have been extracted from P4 *E. coli* into the outer membrane of the cationic surfactant vesicle vaccine. It was hypothesized that the vaccine could stimulate strong antibody production to P4 outer membrane antigens.

Thus, subfractionation of *E. coli* cells was conducted to assist in identifying which P4 antigens incorporated where in the cationic surfactant vesicle. The following fractions were prepared and analyzed: total proteins (TP), cytoplasmic/inner membrane proteins (CIM), and outer membrane proteins (OM). *E. coli* DH5 α protein fractions were used as controls. *E. coli* P4 protein fractions were used as experimental samples. To aid in identifying potential antigens in the vaccine, immunogenic profiles were generated. Each immunogenic profile consists of a silver stain, an immunoblot using pre-vaccination and post-vaccination sera as primary antibody, and histograms of the distribution by size of protein antigens in each fraction. Histograms, a densitometric analysis, relate detection to number of proteins. Profiles are shown for cows 3643 (**Figure 13**) and 3635 (**Figure 14**).

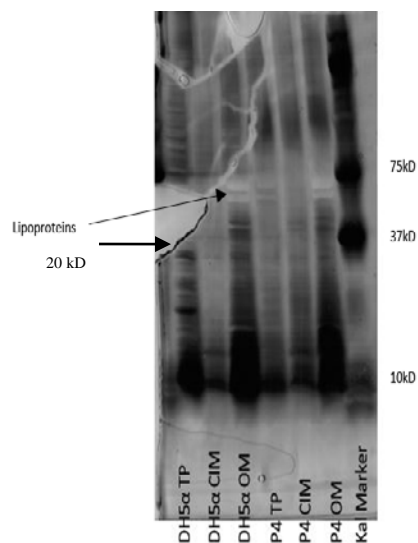
Cow 3643's silver stain revealed the presence of lipoproteins, the "blank bands" in the gel. The lipid portions of these proteins do not take up silver stain completely. A doublet of lipoproteins was observed in the 60 kiloDalton (kD) range in P4 and DH5 α OM fractions. CIM proteins were observed from 100 kD and below. CIM proteins were strongly seen in the 37 to 10 kD range. A 20 kD band was observed in all fractions: TP, CIM, and OM of P4 and DH5 α . The relative density of this band was calculated across the fractions with the DH5 α -TP band as the control. The data presented in **Table 9** was collected in reference to this 20 kD band.

Lane	Area	Percent	Relative Density
1	221340.9	18.245	1.00
2	156914	12.934	0.71
3	155296	12.801	0.70
4	155655.3	12.83	0.70
5	156293	12.883	0.71
6	156765	12.922	0.71

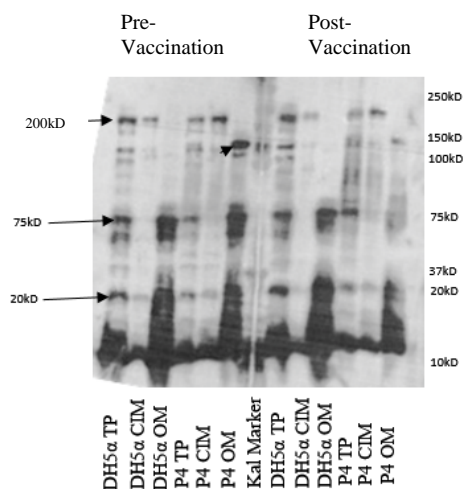
Table 9. Relative density of 20 kD band across all protein fractions (Silver Stain, Cow 3643).

Shown in **Figure 13**, an immunogenic profile for Cow 3643 was generated. The immunoblot showed a prominent band in the 75 kD range in all DH5 α and P4 fractions but the CIM fraction. A band was observed in the 200 kD range in all fractions except in the OM fractions. A band in the 150 kD range was strongly observed in the P4-OM fraction by pre-vaccination probing. This ~150kD band was not strongly observed in the P4-OM fraction by post-vaccination sera probing. Again, in the immunoblot a 20 kD band was observed in all fractions. Normalization of the 200 kD, 150 kD, and 75 kD bands relative to the 20 kD was not pursued due to the saturation already present in the immunoblot. Histogram analysis of the immunoblotted protein fractions showed protein detection across pre-and post-vaccination sera did not differ significantly, except for in the P4 OM fractions. The histograms reveal a slight shift in peak distribution towards higher molecular weight proteins in the post-vaccination serum probing of P4 OM fractions. Specifically, the mode for pre-vaccination serum detection was 52 and the mode for post-vaccination serum detection was 207.

A Silver Stain of Protein Fractions



B Immunoblot of Pre-and Post-Vaccination Sera (Cow 3643)



Histograms of Pre-and Post-Vaccination Sera Protein Distribution (Cow 3643)

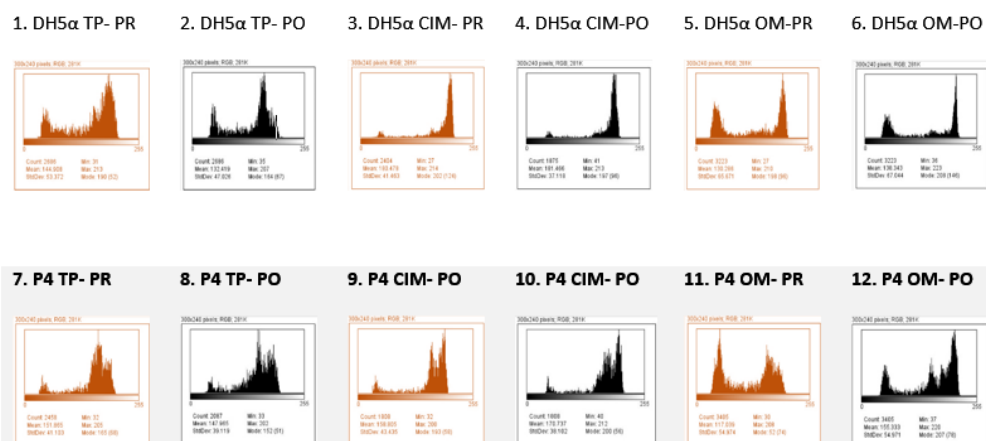
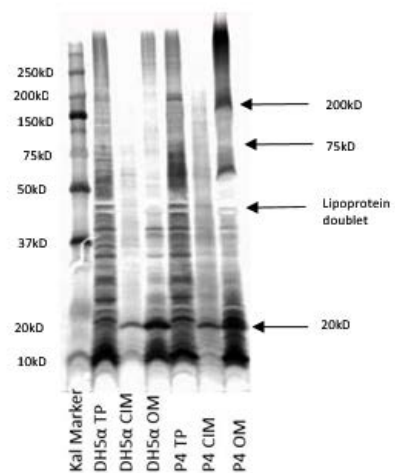


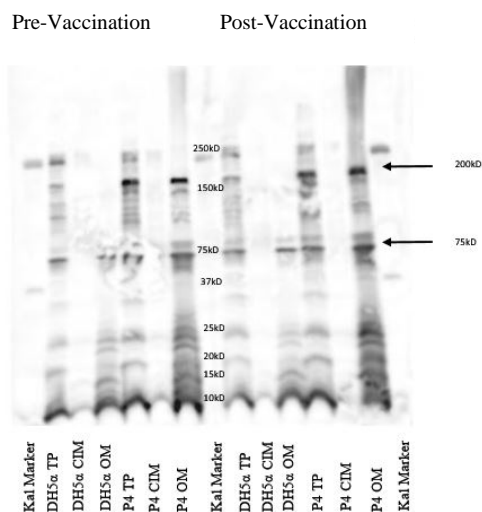
Figure 13. Immunogenic profile for Cow 3643.

Shown in **Figure 14**, an immunogenic profile for Cow 3635 was generated. A silver stain of cow 3635 protein fractions was performed. Again, a band around 75 kD was observed in all fractions. A strong doublet lipoprotein band was observed in the TP fractions in the 50 to 37 kD range. A prominent band was observed in all fractions at 20 kD as was seen in the previous silver stain. Bands in the 200 kD range were also observed in TP and OM fractions. Looking at the immunoblot, a prominent 200 kD band was observed in P4-TP and-OM fractions pre-and post-vaccination. A 75 kD band was observed in all fractions but CIM. CIM bands were observed only in the 25 to 10 kD range on the immunoblot. By histogram analysis, the only significant differences in detection were between detection of P4 OM fractions pre-and post-vaccination. Pre-vaccination serum detected a high number of protein counts in the 30-100 peak range. Post-vaccination serum detected a high number of protein counts in the 200-230 peak ranges. Specifically, the mode for pre-vaccination serum detection was 174 and the mode for post-vaccination serum detection was 226.

A Silver Stain of Protein Fractions



B Immunoblot of Pre-and Post-Vaccination Sera (Cow 3635)



C Histograms of Pre-and Post-Vaccination Sera Protein Distribution (Cow 3635)

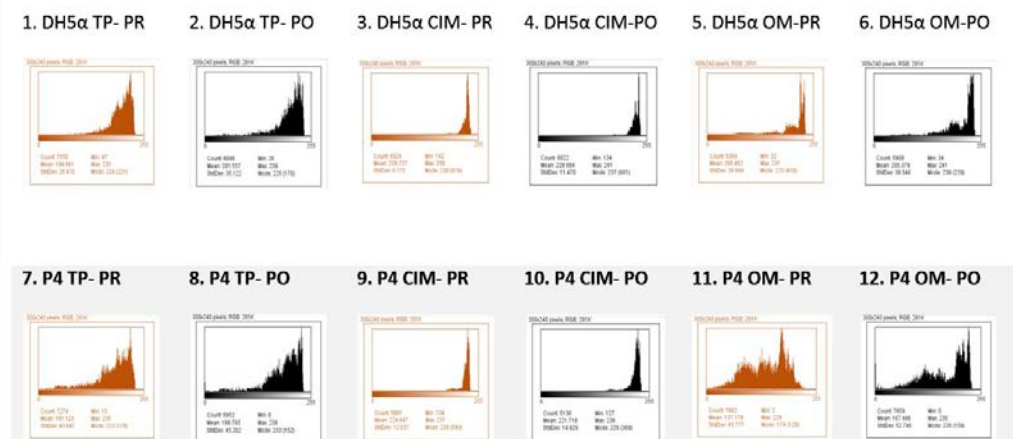


Figure 14. Immunogenic profile for Cow 3635.

Chapter 4: Discussion

The goal of this project was to investigate the immunopotency of a cationic surfactant vesicles as a potential vaccine platform. This project focused on investigating if a cationic surfactant vesicle vaccine could elicit antibody response against *E. coli*, a causative agent of bovine mastitis. Specifically, it was hypothesized that the vaccine could elicit strong antibody response against *E. coli* outer membrane antigens. It was predicted that data generated may offer insight into whether the vaccine could elicit protective immunity against *E. coli*. The goals of this particular vaccine were 1) determining if the vaccine formulation decreased the inherent toxicity of incorporating the lipid A portion of LPS and 2) determining what, if any, effect the vaccine formulation had on antibody production in cow serum.

Based on these goals, success could be framed in the context of survival of animals that received the vaccine. The 3 heifers that were vaccinated in the study all survived vaccination and were alive at the conclusion of day 14. Additionally, success could be framed in the context of the delivery of healthy offspring. All heifers in this study birthed healthy calves. By this perspective, the vaccine was a success.

Another context by which success could be defined is by the vaccine formulation's effects on bovine antibody production in serum. At the very least, success could be thought of as the vaccine's ability to generate an antibody response. To validate this perspective, serum studies were performed. These studies were ELISAs and immunoblots.

A vaccine was developed using *E. coli* as the bacterium causes 80% of all coliform mastitis cases.⁶⁰ Moreover, *E. coli* was chosen over other causative agents like *S. aureus* as *E. coli* is a Gram-negative bacterium. Previous published data from the DeShong group⁷⁰ and unpublished data from the Stein group⁶⁹ have demonstrated the suitability of a cationic surfactant vesicle platform in eliciting antibody against LPS/LOS. This thesis utilized DH5 α and P4 *E. coli* as the control and experimental coliform strains.

DH5 α was selected as the control coliform strain. DH5 α shares structural and antigenic characteristics with P4. However as a laboratory strain, DH5 α has been genetically modified and no longer expresses some characteristics that contribute towards virulence such as lactose utilization⁷⁹ and an O-antigen.⁴² This strain belongs to the K-12 O-antigen serotype 42, meaning it does not express O-antigen, rendering bacteria sterically susceptible to complement-mediated killing (serum sensitivity). Additionally, the lack of O-antigen renders bacteria less antigenic than R-1 strains like P4. DH5 α can be considered an avirulent *E. coli* strain whereas P4 is a highly virulent (pathogenic) *E. coli* strain. P4 was chosen for study as it is frequently isolated

in cases of clinical mastitis.^{39, 78} and is used to induce experimental mastitis. Responses to DH5 α were to be compared with responses to P4 in the hopes that surface antigens unique to P4 could be discerned. Overlap in responses could be attributed to shared structural features and the response to DH5 α could be ‘subtracted out,’ giving further insight into vaccine-induced P4-specific responses.

The ELISA experiments, under the conditions described in this thesis, suggest there was no significant difference in bovine IgG concentration after vaccination. For example, significant difference is defined as $P < 0.01$. P values for each cow’s ELISA readings are as follows: Cow 3643 ($P = 0.858$), Cow 3629 ($P = 0.728$), and Cow 3635 ($P = 0.012$). Based on the parameters of the experiment conducted, it could be concluded that 1) there was no observable difference in IgG concentrations after vaccination and 2) perhaps the vaccine formulation was unable to elicit an IgG response. These observations are in stark contrast to Wilson *et al*’s J5 Bacterin vaccine study, which showed increase in J5-specific IgG post-vaccination.⁵⁷

On the other hand, the argument can be made that differences in IgG concentration may be obscured by a high background of non-specific binding. This could be concluded because whole-cell P4 reacted strongly to *Neisserial* primary and secondary antibody, as observed qualitatively by comparing intensity of blue color development between P4 and MS11 control wells. Blue color was equally intense between P4 and MS11 wells. Ideally, P4 would have little to no specificity for *Neisserial* antibodies.

E. coli response to *Neisserial* antibodies may have been observed because of improper blocking of wells. Perhaps wells could have been blocked overnight at 4°C instead of for 1 hr at RT. Another explanation for non-specific binding could be the choice of 5% BSA solution as the blocking buffer. Cows naturally produce BSA in their sera. Blocking with BSA may not then have been the most choice, given that this protein is already highly prevalent in bovine sera and given that in these experiments bovine sera were used as primary antibodies. In these studies BSA, casein, or powdered milk should not be used as blocking reagents. These reagents could interact with antibodies present in bovine sera. An alternative experiment could be to repeat the ELISA procedures using a reagent like fish gelatin as a blocking buffer.

The use of additional positive and negative controls could help troubleshoot the ELISA studies. Since P4 belongs to *E. coli* serotype O32: H37, ⁴⁰ antibodies for O32 O-antigen or H37 flagellar antigen could be used as positive controls to probe specifically for P4. Theoretically, only wells with P4 should have TMB development. Since these antigens are highly specific for P4, wells with MS11 should have no TMB detection. One negative control could be probing bovine antisera with bovine secondary antibody. As these wells would contain no bacteria, theoretically antisera would not bind the well, preventing the binding of secondary antibody and the cleavage of TMB substrate. Little to no TMB should be detected in a well with only bovine antisera and bovine secondary antibody. Similarly, to assess non-specificity another negative control could be probing MS11 with bovine antisera and bovine secondary antibody. Theoretically, heifers would not have been exposed to *Neisseria*

at any point in their lives and therefore should not produce antibodies against this bacterial species. Ideally, little to no TMB should be detected in wells with *Neisseria*, bovine antisera, and bovine secondary antibody. In the future, ELISA studies could be repeated with these positive and negative controls. ELISAs could be optimized to reduce the high background of non-specific binding and perhaps in this new context, differences between IgG concentrations pre- and post-vaccination could be seen.

Based on the immunogenic profiles generated in this thesis, it could be concluded that post-vaccination, antibodies were produced to high molecular proteins found mostly in P4's outer membrane. For example, there was an antibody response to a high-molecular weight protein on P4's OM surface in the post-vaccination serum of one cow (Cow 3635). The presence of that 200 kD band may warrant further study. To better identify that protein, bands could be excised from the gel, analyzed, and structurally characterized by mass spectrophotometry. One interpretation of the data could be that vaccination may elicit antibodies to P4 OM antigens and that in this way, the vaccine may potentially hold promise as an alternative commercial therapeutic for decreasing symptoms of clinical mastitis and improving the quality of milk obtained from mastitic cows.

However, another interpretation of the immunogenic profiles generated in this thesis is that high background response to common DH5 α and P4 antigens obscures P4-specific antibody binding. This could be concluded from comparing histogram distributions of detection of DH5 α and P4 protein fractions. There was a high, non-

specific bovine serum antibody response to DH5 α proteins. Non-specific antibody response to DH5 α proteins could be minimized in future studies by conducting an antibody absorption experiment. Bovine antisera could be absorbed with DH5 α whole cell antigen to eliminate non-specific binding. Immunoblotting could be repeated using absorbed pre-and post-vaccination primary antibodies. Immunoblots probed with absorbed antisera should theoretically demonstrate P4-specific binding. Histogram analyses of these immunoblots should reveal little to no detection of protein bands in the DH5 α fractions and detection of some unique protein bands in the P4 fractions. These changes could be applied to future immunoblot studies and perhaps in these studies, detection of P4-specific antigens could be seen.

Data generated from this study can be compared to what is known about current vaccines for bovine mastitis. The well-studied vaccine available is J5 Bacterin. In previous studies related to J5 Bacterin, Wilson *et al* observed no differences in antibody concentrations prior to vaccination between vaccinated and unvaccinated cows.⁵⁶ Another study showed vaccination increased J5-specific IgG₂ antibodies.⁵⁷ In this thesis study, P4-specific IgG antibodies remained relatively unchanged. But perhaps, IgG₂ and IgG₁ populations differed pre-and post-vaccination. Knowing this, further ELISA studies could be done to determine whether vaccination promoted P4-specific IgG₂ production while reducing IgG₁ production. Secondary antibodies that distinguish between the two IgG isotypes could be used in these studies. Additionally, perhaps the vaccine was unable to elicit class-switching from IgM to IgG. If that is the case, perhaps post-vaccination bovine serum contains high levels of IgM. To

determine the validity of this hypothesis, ELISAs could be conducted specifically for bovine IgM instead of IgG.

A major limitation of this study could be the 3 heifers chosen for the preliminary trial. Under the parameters of this thesis, even if these animals had exposure to P4 *E. coli* prior to the study this may not have been observed with the high background of non-specific binding. Optimizing the ELISA and immunoblot experiments to minimize non-specific binding would help in determining what effect exposure to *E. coli* prior to vaccination has on the data. Theoretically, vaccines should elicit highly specific and robust responses despite prior exposure.

Overall, this study could benefit from further optimization to reduce the non-specific binding of bovine sera antibodies. At this time, given the parameters of this thesis, conclusive statements regarding the immunopotency of the cationic surfactant vesicle as a vaccine platform cannot be made. Nonetheless, cationic surfactant vesicles could hold potential as a vaccine platform as they are relatively inexpensive to prepare, can be stored long-term at room temperature, can be autoclaved and pasteurized, and allow the incorporation of LPS without inducing toxicity in hosts.

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