ABSTRACT

Title of Document: MILK AND BLOOD CONCENTRATIONS OF LIPOPOLYSACCHARIDE-BINDING PROTEIN IN COWS WITH NATURALLY-OCCURRING SUBCLINICAL AND CLINICAL MASTITIS

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The objective of this thesis was to evaluate the blood and milk concentrations of lipopolysaccharide-binding protein (LBP) from cows with naturally-occurring mastitis as biomarkers of this disease. Milk and blood samples were collected from 101 clinically healthy dairy cows and 17 dairy cows with clinical mastitis. The concentrations of LBP, haptoglobin and serum amyloid A (SAA) were determined in these samples by enzyme-linked immunosorbent assay. Both LBP and haptoglobin concentrations were higher in the milk and blood of quarters and cows with clinical mastitis respectively than in those that were healthy. Whereas haptoglobin concentrations differed between uninfected and subclinically-infected quarters, LBP concentrations only differed between them when milk somatic cell counts were low. Unlike haptoglobin and SAA, blood concentrations of LBP in cows with a subclinical intramammary infection were not significant from those of cows with all healthy quarters. Thus, haptoglobin may be a preferred biomarker of subclinical intramammary infection.

MILK AND BLOOD CONCENTRATIONS OF LIPOPOLYSACCHARIDE-BINDING PROTEIN IN COWS WITH NATURALLY-OCCURRING SUBCLINICAL AND CLINICAL MASTITIS

By

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Thesis submitted to the Faculty of the Graduate School of the

University of Maryland, College Park, in partial fulfillment

of the requirements for the degree of

Master of Science

2008

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Acknowledgements

The accomplishment of this experiment is a team work of all the members in the mastitis lab at USDA. I would like to thank all of them for their help in collecting samples and for making the lab a positive working environment. They are Dr. Douglas D. Bannerman, Dr. Max J. Paape, Elizabeth A. Cates, Jennifer Bilheimer, Manuela Rinaldi and Mary Bowman. I am grateful for being a member in such a great team.

I would also like to thank my advisors Dr. Brian J. Bequette and Dr. Douglas D. Bannerman for their extremely considerate guidance and enlightening training in every step towards this degree. The inspiration and encouragement they have provided over the years cannot be measured.

I also want to thank Dr. Xiaoping Zhu for his excellent teaching and active service on my committee.

To my family and friends, thanks for always being there and helping me in more ways than you probably know.

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Chapter 1: Literature Review

1.1 Introduction

Mastitis is one of the most costly and prevalent diseases affecting the dairy industry (Wells et al., 1998; Wellenberg et al., 2002). It is an inflammatory disease in the mammary gland, which is most commonly caused by bacterial infections. Depending on the degree of inflammation, intramammary infections can be classified as either subclinical or clinical type. Subclinical intramammary infections are more difficult to detect due to the absence of visible changes in the appearance of the milk or udder, while clinical infections are more readily detected by visual abnormalities in the milk and/or the presence of heat, pain and swelling in the infected gland (Bramley and Dodd, 1984; Sears and McCarthy, 2003a).

Clinical mastitis is accompanied by a large influx of blood leukocytes into the inflamed mammary gland, thereby, resulting in an increase in the milk somatic cell count (SCC) (Paape et al., 2003). Clinical mastitis is also commonly characterized by increased vascular permeability and breakdown of the blood-milk barrier, which leads to the dysregulated influx into milk of such blood components as albumin and cations (Guidry et al., 1983). Therefore, changes in milk SCC, bovine serum albumin (BSA), and electrical conductivity have all been used to assess the health status of the mammary gland. Relative to clinical mastitis, changes in these parameters during subclinical intramammary infection are often more subtle and their degrees of effectiveness at identifying these infections have been reported to vary (Verhoeff and Smith, 1981; Sheldrake et al., 1983b; Fernando et al., 1985; Mattila et al., 1986).

In response to infection or inflammation, a rapid and non-specific reaction known

as the acute phase response (APR) is generally elicited by the host (Suffredini et al., 1999). The APR is characterized by changes in concentrations of a large number of plasma proteins, termed acute phase proteins (APP's), which are produced predominantly by the liver (Pannen and Robotham, 1995). In cows with experimentally-induced and naturally-occurring mastitis, haptoglobin and serum amyloid A (SAA) concentrations have been shown to increase in both blood and milk, thus, serving as indicators of the inflammatory status of the udder (Eckersall et al., 2001; Ohtsuka et al., 2001; Pedersen et al., 2003). Correspondingly, both have been evaluated as biomarkers of intramammary infection and disease severity in cows with mastitis (Eckersall et al., 2001; Nielsen et al., 2004; Gronlund et al., 2005; Hiss et al., 2007).

Another APP that is upregulated during experimentally-induced mastitis in cows is lipopolysaccharide-binding protein (LBP). LBP is a 58 – 60 kDa protein that catalyzes the transfer of bacterial lipopolysaccharide, a highly pro-inflammatory component of the outer wall of Gram-negative bacteria, to CD14 (Tobias et al., 1999; Schumann and Latz, 2000). CD14, which exists as both a soluble and cell surface receptor, facilitates LPS presentation to Toll-like receptor-4. This, in turn, results in the activation of intracellular signaling pathways that promote the upregulation of pro-inflammatory cytokines and adhesion molecules, which are involved in the host innate immune response to invading pathogens. In addition to bacterial lipopolysaccharide, LBP has been reported to facilitate host recognition of, and activation by, cell wall products of Gram-positive bacteria (Fan et al., 1999; Schroder et al., 2003). Although detection of bacterial wall products is a key event in the activation of the innate immune response, excessive activation can lead to an overwhelming host response and the development of life-

threatening septic shock (Dinarello, 1997). Data from several studies suggest that LBP may also aid in the detoxification of bacterial wall products, thus, diminishing an excessive pro-inflammatory response that can be deleterious to the host (Wurfel et al., 1994; Wurfel and Wright, 1995; Vreugdenhil et al., 2003).

LBP concentrations increase in the blood and milk of cows following LPS challenge (Bannerman et al., 2003) and in response to experimentally-induced intramammary infections by such pathogens as *Escherichia coli*, *Klebsiella pneumoniae*, *Mycoplasma bovis*, *Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus*, and *Streptococcus uberis* (Bannerman et al., 2004a; Bannerman et al., 2004b; Bannerman et al., 2004c; Bannerman et al., 2005; Kauf et al., 2007). In these studies, LBP increases in milk temporally coincided with those in blood and occurred during a period of increased mammary vascular permeability. This finding suggests that the preponderance of LBP in milk during the APR to intramammary infection originates from leakage out of the blood vasculature. Interestingly, during experimentally-induced mastitis following intramammary infusion of *Escherichia coli*, *Mycoplasma bovis*, or *Pseudomonas aeruginosa*, LBP increases in blood have been shown to increase earlier, and remain elevated longer than SAA (Bannerman et al., 2005; Kauf et al., 2007; Bannerman et al., 2008), which indicates a good diagnostic value for LBP as a biomarker of underlying intramammary infection. To date, there have been no published studies investigating the use of LBP as a biomarker of naturally-acquired mastitis. Therefore, the objective of this thesis was to evaluate the blood and milk concentrations of LBP from cows with naturally-occurring subclinical intramammary infections and clinical mastitis, and to determine whether LBP concentrations could differentiate quarters or cows that

were subclinically infected from those that were healthy.

1.2 Review of Bovine Mastitis

1.2.1 Bovine Mastitis

Bovine mastitis remains as the disease causing the most economic losses in the dairy industry worldwide despite the extensive progress in optimizing control programs over the decades. Economic losses were estimated to be approximately $185 per cow per year in the U.S.A and about £175 in the U.K. These losses included reduced production, increased replacement costs, discarded milk, drug costs, veterinary fees, and labor costs (National Mastitis Council, 1996; Kossaibati, 2000). Due to the general agreement among consumers and processors that dairy products should be wholesome, nutritious, safe and produced from healthy cows, bovine mastitis has also become an issue of food safety and animal welfare (Klei et al., 1998; Wells et al., 1998; Boor, 2001; Oliver et al., 2005).

Bovine mastitis is an inflammation of the mammary gland that often results from an intramammary infection (IMI) by a microorganism (Bramley and Dodd, 1984; Bradley, 2002). More than 100 different microorganisms including bacteria, mycoplasma, yeasts and algae can cause mastitis, although the majority of cases are caused by bacteria (Watts, 1988).

1.2.2 Mastitis and Pathogens

Mastitis has been traditionally classified as contagious and environmental mastitis based on the primary reservoir of causative agent within cows that leads to subsequent infection (Blowey and Edmondson, 1995). Contagious mastitis is defined as IMI transmitted from cow to cow or quarter to quarter (Fox and Gay, 1993). Under this definition, *Streptococcus agalactiae*, *Corynebacterium bovis*, *Mycoplasma sp*. and *Staphylococcus aureus* are considered as contagious mastitis pathogens. The primary

reservoir for these pathogens is the infected udder, and infections are spread among cows or between quarters during the milking process by contaminated milking units (Bramley and Dodd, 1984). Whereas *Streptococcus agalatiae* responds well to the regular antibiotics therapy, no effective antibiotics are available for *Staphylococcus aureus* (Nickerson and Owens, 1993; Keefe, 1997; Barkema et al., 2006). However, the total eradication of *Strep. agalactiae* from dairy herds and a markedly reduced incidence of *Staph. aureus* have become true in some well managed dairy farms after meticulously following effective control programs directed at preventing new infections including post-milking disinfection of teat ends, strict milking time hygiene, dry cow therapy, and culling (Dodd, 1983; Eberhart, 1986; Fox and Gay, 1993; Keefe, 1997; Sears and McCarthy, 2003b). *Mycoplasma* species are contagious mastitis pathogens whose prevalence have been underestimated due to their long incubation periods before the onset of clinical signs, lack of clinical signs and long persistence after clinical signs (Jasper, 1981; Pfutzner and Sachse, 1996; Gonzalez and Wilson, 2003; Kauf et al., 2007). Among different *Mycoplasma* species that infect cattle, *Mycoplasma bovis* is the most common and might be the most pathogenic cause of mastitis (Pfutzner and Sachse, 1996; Nicholas and Ayling, 2003). For diagnosis of this disease, special bacteriological culture procedures are required and it takes 7-10 days of incubation to confirm a negative sample (National Mastitis Council, 1999). *Corynebacterium bovis* is a minor pathogen (Harmon and Langlois, 1986). Moderate inflammation and slight increase in SCC are associated with most infections by *C. bovis* (Ngatia et al., 1991; Djabri et al., 2002).

Environmental mastitis are caused by pathogens which can be found in virtually any surface area of the cow or her surrounding (Collier et al., 1982; Dodd, 1983; Smith,

1983; Hogan and Larry Smith, 2003). For this reason, the chance of environmental mastitis pathogens transferring among cows or quarters is minimal compared with the constant environmental exposure. Environmental mastitis pathogens consist of Gram-negative bacteria, including *Escherichia*, *Klebsiella*, *Enterobacter, Serratia* and *Pseudomonas* and some Gram positive bacteria including *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Enterococci*. Mastitis caused by *Escherichia*, *Klebsiella*, *Enterobacter* are also referred to as *coliform* mastitis and they are mainly responsible for the majority of peracute cases of clinical mastitis in a herd (Hogan and Larry Smith, 2003). The complete elimination of environmental mastitis is never a realistic goal. However, it is possible to reduce them to an economically acceptable incidence by implementing comprehensive control methods aimed at both decreasing exposure of teat ends and increasing the host resistance to environmental pathogens, which can include improvements in housing, the use of inorganic bedding, effective vaccines, adequate supplementation with vitamins and trace minerals and pre-dipping in some herds (Bushnell, 1984; Smith et al., 1985; Burvenich et al., 2003; Hillerton and Berry, 2003).

Although the above classification of pathogens reflects their different epidemiology in dairy herds and the possible effective control programs in control of them, mastitis should be treated as an evolving disease (Bradley, 2002). For example, *Streptococcus uberis*, once considered to be an environmental pathogen, has been found to behave as contagious pathogens such as *Staphylococcus. aureus* and can spread between cows by surviving in mammary epithelial cells (Baseggio et al., 1997; Phuektes et al., 2001; Almeida and Oliver, 2006; Tamilselvam et al., 2006). Similarly, *Escherichia coli*, traditionally associated with a transient infection, can persist in the mammary glands

(Bramley and Dodd, 1984; Dogan et al., 2006).

Historically, coagulase-negative species of *staphylococci* (CNS) were referred to as skin flora opportunists and minor pathogens of the mammary gland (Smith and Hogan, 1995). CNS include a variety of species other than *Staphylococcus aureus*. The predominant species isolated in most herds are *Staphylococcus chromogenes*, *Staphylococcus simulans and Staphylococcus. epidermidis, Staphylococcus hyicus, Staphylococcus hominis,* and *Staphylococcus xylosus* may also be found (Timms and Schultz, 1987). CNS were thought to cause only a mild inflammation of the mammary gland with modest increases in somatic cell count (SCC), and were infrequently associated with clinical mastitis (Jarp, 1991; Smith and Hogan, 1995; Taponen et al., 2006). In some countries, however, CNS have become the predominant pathogen in subclinical mastitis (Pitkala et al., 2004; Tenhagen et al., 2006). The CNS are also the primary cause of intramammary infection in heifers at calving (De Vliegher et al., 2003; Nagahata et al., 2006). Whether CNS contribute significantly to the somatic cell counts in milk is controversial, it might be species specific (Chaffer et al., 1999; De Vliegher et al., 2003). With a tendency of decreasing the upper legal limit for SCC, the elevation in SCC caused by the CNS has become an important issue (Rainard et al., 1990).

1.2.3 Clinical Mastitis and Subclinical Mastitis

Clinical mastitis is characterized by clinically visible signs. Typical examples of these signs include flakes, clots or other gross alterations in the appearance of the milk, swelling and discoloration of the udder, increased temperature or pain of the quarter. In general, the degree of swelling, severity of pain and the abnormal appearance of the milk from the infected quarters are correlating with the severity of infection and can be used as

a reference for the course of treatment. Obviously, no special methods are needed to detect clinical mastitis.

In contrast, subclinical mastitis often develops without any clinically visible signs. When quarter SCC is equal or above 200,000 cells/ml and bacteria are isolated in the absence of clinical signs, then the quarter is diagnosed as subclinical mastitis. Correspondingly, subclinical mastitis may remain undetected in routine veterinary inspection without monitoring milk SCC and performing bacterial examinations in labs.

1.3 Diagnostics of Subclinical Mastitis

1.3.1Bacterial Examination

Milk samples obtained aseptically from healthy mammary glands should not contain any microorganisms. Isolation of the same organism from duplicate milk samples collected consecutively represents a positive diagnosis of infection (National mastitis council, 2004). However, bacteriological cultures can be negative when the concentration of udder pathogens in milk is below the detection limitation either because bacteria can be readily cleared off from the mammary gland (eg. *E.coli*) or they can invade epithelial cells (eg. *S.aureus*) while they are undetectable in milk secretions (Erskine et al., 1991; Keefe, 1997; Kerro Dego et al., 2002). Also, the number of bacteria being shed at a given time point by infected animals is not stable, such as the cyclic shedding by *S.aureus*, multiple samples at different time points might be necessary under this condition. On the other hand, false positive results from contamination may occur considering that many common mastitis pathogens can be present everywhere in the environment. In addition, not all pathogens can be cultured on regular media and thereby general results from routine cultural procedures can only serve as preliminary evidence

for diagnostic purposes.

1.3.2 Milk Somatic Cell Count

Milk somatic cell counts refers to all cell types found in normal milk, which include lympocytes, macrophages and neutrophils from the blood and shedded epithelial cells from the lining of the gland. Macrophages are positioned in normal milk for immune recognition of invading pathogens and represent 66-88% of all cells in milk from non-infected quarters (Lee et al., 1980; Burvenich et al., 1994; Kelly et al., 2000). The proportion of neutrophils in normal milk is small (0-11%) but quickly become the predominant cell type (90%) in milk from infected quarters (Kehrli and Shuster, 1994; Paape et al., 2003).

Milk somatic cell count (SCC) has been widely accepted and used to indicate the health status of bovine mammary glands, the quality of milk for human consumption and the hygiene conditions of dairy farms in most developed countries and in some developing countries (Ruegg and Tabone, 2000; Schukken et al., 2003; Heeschen, 2005). Many studies have shown that inflammation is the predominant factor affecting the concentrations of somatic cell counts in milk (Dohoo and Meek, 1982; Schepers et al., 1997). Statistically, milk SCC over 300,000 cells/ml is a good indicator of abnormal milk and an infected or inflamed quarter. For this reason, herd bulk tank SCC (BTSCC) is monitored monthly to predict the udder health status in a herd in practice (Kehrli and Shuster, 1994; Jayarao and Wolfgang, 2003; Lee et al., 2003).

However, quarter milk SCC can be below 200,000 cells/ml for mastitis caused by some minor pathogens (Harmon and Langlois, 1986; De Vliegher et al., 2003). Clearly, the nature of the pathogen has a significant effect on the dynamics of elevating SCC. A

variety of other factors including heat stress, stage of lactation, lactation number, season and even diurnal variation can also contribute to the variations of SCC in normal milk to some extent (Paape et al., 1973; Sheldrake et al., 1983a; Harmon, 1994; Paape et al., 2003). In addition, SCC can remain elevated for days or weeks after the resolution of an inflammation or the clearance of bacteria from the mammary gland (Schultz, 1977; Olsson et al., 1986; Emanuelson et al., 1987; Emanuelson et al., 1988; Pyorala, 1988).

Somatic cell count can be determined accurately by automatic cell counters or estimated by performing a cow-side California Mastitis Test (CMT), or by measuring the adenosine triphosphate (ATP) (Olsson et al., 1986; Emanuelson et al., 1987; Emanuelson et al., 1988) or N-acetyl-ß-D-glucosaminidase (Kitchen et al., 1978) activity in milk. The CMT is based on the amount of cellular nuclear protein present in the milk sample. Because neutrophils are the predominant cell type present in milk, the CMT score correlates with the SCC level in the gland and is a widely used cow-side test for evaluation of SCC.

1.3.3 Electrical Conductivity

Electrical conductivity testing is based on the fact that mastitic milk has a higher electrical conductivity than normal milk due to the increase in sodium and chloride ions following the breakdown of the blood-milk barrier (Norberg et al., 2004). This method has promise of being incorporated into the on-line automatic milk system to detect the real-time quality of milk. However, this analysis is less efficient in detecting subclinical mastitis compared with clinical mastitis (Nielen et al., 1995).

1.4 Acute Phase Response and Acute Phase Proteins

The acute phase response (APR) is a systemic reaction of the host occurring within

the first 24-48 h in response to local or systemic disturbances caused by infection, tissue injury, trauma or immunological disorders, which enables the host to rapidly generate a nonspecific response to various stimuli before the adaptive immune response slowly develops (Heinrich et al., 1990; Koj, 1996; Gabay and Kushner, 1999; Gruys et al., 2005). One hallmark of the APR is that liver hepatocytes can adjust their synthesis of plasma proteins, known as acute phase proteins, in response to induced inflammatory cytokines including interleukin (IL)-1, IL-6 and TNFα (Heinrich et al., 1990; Fantuzzi and Dinarello, 1996; Moshage, 1997; Gruys et al., 2005).

The circulating concentrations of some APPs change substantially in inflammation, displaying a relation with the severity of infection or the extent of tissue damage, making them good markers of inflammation and infection in both medical and veterinary clinical pathology (Eckersall and Conner, 1988; Pyorala, 1988; Murata et al., 2004; Ceron et al., 2005; Gruys et al., 2006). Haptoglobin is more sensitive to inflammation than hematological screening in studies with dogs and sheep respectively (Solter et al., 1991; Skinner and Roberts, 1994). Serum amyloid A (SAA) and haptoglobin (Hap) have been shown to be good indicators to discriminate between acute and chronic inflammatory conditions in cattle (Horadagoda et al., 1999). Because there are species differences in the behavior of some APP (Gruys and Snel, 1994), the following section will only focus on those diagnostically useful APP in cattle (Petersen et al., 2004).

1.4.1 Serum Amyloid A

SAA is an apolipoprotein associated with high-density lipoprotein and is believed to modulate reverse cholesterol transport from dying cells at inflamed sites, thereby facilitating the uptake of free cholesterol by the hepatocytes (Coetzee et al., 1986;

Shephard et al., 1987; Liang and Sipe, 1995; Hayat and Raynes, 1997). SAA is also a potent chemoattractant in recruiting T lymphocytes, neutrophils and monocytes into inflammatory sites (Badolato et al., 1994; Xu et al., 1995). SAA at clinically relevant concentrations is known to induce IL-8 expression and release from neutrophis and monocytes, which results in a typical uncontrolled inflammatory reaction usually associated with the development of degenerative diseases (Ribeiro et al., 2003). In addition, SAA can function as an immune opsonin for Gram-negative bacteria, enhance phagocytic activity against bacteria by neutrophils, promote neutrophil survival by suppressing the apoptosis and assisting in the detoxification of endotoxin (Xu et al., 1995; Badolato et al., 2000; Shah et al., 2006; El Kebir et al., 2007).

Several isoforms of SAA have been reported with SAA-1 and SAA-2 produced in liver and SAA-3 in extra-hepatic tissues including macrophages, endothelial cells, intestinal epithelium and bovine mammary epithelial cells (DiBartola and Benson, 1989; Steel and Whitehead, 1994; McDonald et al., 2001; Vreugdenhil et al., 2003).

In cattle, the potential for using increased blood concentration of SAA in the assessment of herd health has been widely studied in the context of viral diseases, bacteria infection and complex stress such as weaning, mixing and transportation. In general, an increase in SAA blood concentration was observed although the magnitude and duration of elevated concentrations varies (Heegaard et al., 2000; Ganheim et al., 2003; Petersen et al., 2004; Lomborg et al., 2008).

1.4.2 Lipopolysaccharide Binding Protein

Lipopolysaccharide (LPS) is the main cell component of Gram-negative bacteria with no enzymatic or intrinsic activity. The “toxicity” of LPS is conferred entirely by the

injurious ways that the host senses the presence of LPS (Munford, 2005). Diverse as the effects of LPS, the recognition of LPS by the host uses the same core pathway which involves LBP-mediated sequential interaction with CD14, MD-2 and Toll-like-receptor 4(TLR4) followed by activation of the inflammatory host response (Wright et al., 1990; Shimazu et al., 1999; Visintin et al., 2003).

As the first biologically relevant receptor of LPS, LBP can catalyze the transfer of LPS to CD14 (Tobias et al., 1995), either membrane-bound (Wright et al., 1990) or soluble (Pugin et al., 1993), thus leading to activation of a wide variety of cell types including mCD14 –positive myeloid cells and mCD14-negative cells such as endothelial and epithelial cells. Whereas LBP knockout mice are less susceptible to LPS, LBP deficient mice are more likely to succumb to infections by whole live bacteria including *Salmonella* and *Klebsiella pneumoniae* characterized by a delayed PMN influx and uncontrolled bacteria growth (Gallay et al., 1994; Jack et al., 1997; Le Roy et al., 2001; Fierer et al., 2002).

In addition to this beneficial pro-inflammatory role, LBP can exert anti-inflammation effects by not yet understood mechanisms, depending on LBP concentration and the cellular environment. Administration of a high dose of recombinant LBP intraperitoneally protects mice from *Escherichia coli* peritonitis and from intraperitoneal LPS injection, indicating that high concentrations of LBP in extravascular fluids may help control responses to LPS (Lamping et al., 1998). Three inhibitory mechanisms have been proposed to explain this finding. First, LBP can redirect LPS away from monocytes and macrophages by transferring it to plasma lipoproteis (Wurfel et al., 1994; Vesy et al., 2000; Kitchens et al., 2003). Secondly, higher concentrations of

LBP can promote the removal of the mCD14-bound LPS and interfere with the subsequent interaction with MD-2 and TLR4 (Thompson et al., 2003). The third inhibitory mechanism involves the formation of larger extracelluar LBS-LBP complexes that have a reduced ability to stimulate cells (Gegner et al., 1995; Gioannini et al., 2003; Hamann et al., 2005).

Besides hepatocytes, lungs and intestinal epithelial cells represent minor additional sources of LBP (Su et al., 1994; Dentener et al., 2000; Vreugdenhil et al., 2003). Local production of LBP is also closely associated with a protective role through enhancing the inflammatory response to low doses of LPS while inhibiting the inflammatory response to high doses of LPS (Fierer et al., 2002; Yang et al., 2002; Knapp et al., 2006).

Finally, LBP may participate in the recognition of Gram-positive bacterium. Whereas LBP has been shown to play an indispensable role in the recognition of such cell wall components of Gram-positive bacterium such as peptidoglycan (PGN) or lipoteichoic acid (LTA) in vitro (Dziarski et al., 1998; Fan et al., 1999; Morath et al., 2001; Schroder et al., 2003), evidence for such a role in infections caused by Gram-positive bacteria in vivo is still conflicting (Dziarski et al., 1998; Fan et al., 1999; Morath et al., 2001; Schroder et al., 2003; Vreugdenhil et al., 2003; Weber et al., 2003; Branger et al., 2004; Brass et al., 2004; Knapp et al., 2008).

Bovine LBP is a 58 kDa glycoprotein and functions similar to human LBP to present LPS to host cells with comparable results (Tobias et al., 1986; Horadagoda et al., 1995; Tobias et al., 1995). Also as in humans, LBP participates in acute phase responses in cattle and has been proven to be a diagnostic marker in cattle infection (Schroedl et al., 2001; Bannerman et al., 2003; Nikunen et al., 2007).

1.4.3 Haptoglobin

Haptoglobin (Hap) is a plasma protein with an ability to bind free hemoglobin released from erythrocytes and thereby prevents free Hb in the circulation from passing through the glomerular filter and resulting in renal damage during intravascular hemolysis. During the process of binding hemoglobin, Hap also sequesters the iron within hemoglobin, making it unavailable to bacteria and thus is considered as a bacteriostatic agent and an APP (Barclay, 1985; Langlois and Delanghe, 1996). The Haptoglobin-hemoglobin complex is later cleared in the liver by the reticuloendothelial system through CD163 receptor-mediated endocytosis (Kristiansen et al., 2001; Graversen et al., 2002).

In addition to its classical role of binding hemoglobin, Hap has a broad range of anti-inflammatory activities. Hap antagonizes endotoxin effects by suppressing monocyte production of TNFα (Arredouani et al., 2005). It may also play a role in modulating immune cell response by binding receptors such as the β_2-integrin CD11b/CD18 on monocytes, granulocytes, natural killer cells, CD22 lectin on B cells and CD163 scavenger receptor on macrophages (El Ghmati et al., 1996; Wassell, 2000). Although no Hap receptor has been described for T cells, studies performed in vivo and in tissue culture indicated purified Hap had a moderate inhibitory effect on T cells (Arredouani et al., 2001; Arredouani et al., 2003; Arredouani et al., 2005). Results from Hap knockout mice show that Hap expression per se contributes to the normal development and differentiation of the immune system while inflammation-induced Hp fine-tunes an optimal immune response (Huntoon et al., 2008).

In cattle, increased Hap concentrations in blood are observed under a variety of

experimental infections with *P.multocida ,P.haemolytica*, bovine respiratory syncytial virus or natural infections with foot-and-mouth disease virus and clinical respiratory tract disease(Hofner et al., 1994; Wittum et al., 1996; Katoh and Nakagawa, 1999; Heegaard et al., 2000; Dowling et al., 2002). The concentration of Hap is reported to be correlated with clinical signs in cattle inoculated with bBovine herps virus 1 and *P. haemolytica* separately (Godson et al., 1996). Therefore, its role as a marker of various inflammatory diseases has long been recognized (Alsemgeest et al., 1995; Petersen et al., 2004).

1.5 Acute Phase Proteins in the Diagnosis of Mastitis

As in other inflammatory diseases, APP's are also induced in bovine mastitis. Hepatocyte derived APP's can also be found in milk from inflamed quarters as a result of breakdown of the milk-blood barrier. Recent research also shows that Hap and SAA can be induced in bovine mammary glands in response to IMI (Eckersall et al., 2001; Larson et al., 2005; Eckersall et al., 2006; Weber et al., 2006; Thielen et al., 2007).

Concentrations of SAA and Hap have been shown to increase in both blood and milk of cows with clinical and subclinical mastitis, either experimentally induced (Horadagoda et al., 1999; Gronlund et al., 2003; Hiss et al., 2004; Eckersall et al., 2006) or naturally occurring (Eckersall et al., 2001; Nielsen et al., 2004; Gronlund et al., 2005; Eckersall et al., 2006; O'Mahony et al., 2006; Akerstedt et al., 2007). However, there is no agreement regarding the relative diagnostic value of Hap and SAA as biomarkers for the identification of infection status of the mammary gland and the severity of inflammation.

LBP is another important APP in the bovine (Khemlani et al., 1994; Horadagoda et al., 1995) and its concentration displays a very early increase and a temporal change

coincident with that of the increases in milk SCC in experimentally induced clinical mastitis with LPS and several species of bacteria including *S.aureus*, *E.coli*, *S. marcescens* and *S. uberis* (Bannerman et al., 2003; Bannerman et al., 2004a; Bannerman et al., 2004b; Bannerman et al., 2004c; Bannerman et al., 2005).However, whether this correlation found in experimentally induced clinical mastitis also exists in cows with naturally occurring clinical mastitis and subclinical mastitis needs verification.

Chapter 2: Materials and Methods

2.1 Animals

Blood and milk samples were collected from lactating Holstein cows in the USDA-ARS Beltsville Area dairy herd. The use and care of all animals in this study was approved by the Beltsville Area Animal Care and Use Committee. Two experiments were conducted that involved sample collection from cows in the herd. In the first experiment, blood and milk samples were collected from 101 clinically healthy lactating cows. In the second experiment, all cows in the herd were monitored over a four month period for visible signs of clinical mastitis, including abnormal milk secretions and/or quarters that were red, swollen, or hard. During this period, a total of 17 cows were diagnosed with clinical mastitis and sampled.

2.2 Sample Collection

Milk samples were aseptically collected by spraying each teat with an iodine-based disinfectant, forestripping, wiping off the disinfectant with a paper towel, and scrubbing each teat with sterilized gauze pads saturated with 70% ethanol. Following cleaning and disinfection of the teats, milk samples were collected into sterile tubes. Blood samples were drawn from the coccygeal vein of each animal using a 20 gauge Vacutainer® needle and collected into glass tubes containing K_2 EDTA (Becton-Dickinson Corp., Franklin, Lakes, NJ).

2.3 Whey and Plasma Preparation

For the preparation of whey, milk samples were centrifuged at 44,000 × *g* at 4 °C for 30 min and the fat layer was removed with a spatula. The skimmed milk was centrifuged again for 30 min as above, and the translucent supernatant collected,

aliquotted, and stored at -70 °C. For the preparation of plasma, blood samples were centrifuged at 1,500 × *g* for 15 min, and the clear supernatant collected, aliquotted, and stored at -70 °C.

2.4 Detection of Milk SCC and Bacterial Examination

For the quantification of somatic cells, milk samples were heated to 60 °C and subsequently maintained at 40 °C until the cells were counted on an automated milk somatic cell counter (Bentley Instruments, Inc., Chaska, MN). For bacteriological analysis of milk samples collected during both experiments, all quarters were sampled twice over two consecutive days. Twenty and 100 µL of each sample were plated on blood and MacConkey agar plates (Becton-Dickinson Corp.), respectively. The plates were incubated for 24 h at 37 ºC and visually examined. If bacterial colonies were not evident, plates were incubated for an additional 24 h at 37 ºC and re-examined. Bacterial colonies were identified using standard microbiological procedures, including Gram staining and catalase and coagulase testing, in accordance with previously published guidelines (NationalMastitisCouncil, 1999). When inconsistent bacteriological results were obtained from two samples obtained from a given quarter, a third sample was collected and bacteriological analysis performed.

2.5 Enzyme-linked Immunosorbent Assays for BSA, LBP, SAA and Hap

BSA concentrations were assayed using a commercially available ELISA (Bethyl Laboratories, Inc., Montgomery, TX). The assay was performed as previously described (Bannerman et al., 2003) with the exception that wells were coated with 10 µg/ml of sheep anti-bovine BSA antibodies for 1 h at room temperature instead of overnight at 4 °C. Whey samples were diluted between 1:2,500 and 1:60,000 so that they were within

the linear range of the assay. The limit of detection of this assay was 0.98 µg/ml. The inter-assay and intra-assay coefficient of variance (CV) for BSA analysis was <5% and <10%. LBP concentrations were determined with a commercial ELISA kit (Cell Sciences, Inc., Canton, MA) as previously described (Bannerman et al., 2003). Whey samples were diluted between 1:10 and 1:9,000 and plasma samples diluted between 1:500 and 1:4,500 so that they were within the linear range of the assay. The limit of detection of this assay for whey and plasma samples was 0.008 µg/ml and 0.4 µg/ml respectively. The inter-assay and intra-assay CV for LBP analysis was <13% and <9% (Suojala et al., 2008).

Hap concentrations were determined with a commercial ELISA kit (Alpco Diagnostics, Salem, NH) according to the manufacturer's instructions. Whey samples were diluted between 1:10 and 1:500 and plasma samples diluted between 1:100 and 1:10,000 so that they were within the linear range of the assay. Plates were analyzed at a wavelength of 450 nm and a correction wavelength of 565 nm using a microplate reader (Bio-Tec Instruments, Inc., Winooski, VT). The limit of detection of this assay for whey and plasma was 0.02 µg/ml and 0.2 µg/ml respectively. The concentrations of Hap in the samples were calculated by extrapolation from a standard curve of known amounts of bovine Hap. The inter-assay and intra-assay CV for Hap analysis were both <10%.

SAA concentrations in plasma and whey were determined with two commercial ELISA kits respectively (Milk Amyloid A Assay and Serum Amyloid A Assay, Tridelta Development Ltd, Wicklow, Ireland). Whey samples were diluted between 1:10 and 1:500 and plasma samples diluted between 1:50 and 1:4,000 so that they were within the linear range of the assay. The limit of detection of the assay for whey and plasma samples was 0.047 µg/ml and 0.23 µg/ml respectively. The inter-assay and intra-assay CV for

milk Amyloid A analysis were <15% and 10% while those for SAA were <12% and 9% respectively.

2.6 Statistical Methods

A generalized linear model with lognormal distribution and identity link was fit to each dependent variable using SAS® Proc GLIMMIX (SAS Institute, 2006). For the analysis of the effects and interaction of bacteriological status (*i.e.*, non-infected or infected) and SCC (*i.e.,* ≤250,000 or >250,000 cells/ml) on a given dependent variable in samples derived from healthy and subclinically infected cows, a two-way ANOVA model was specified. For the analysis of the effects of the type of infection (*i.e.*, clinically infected, subclinically infected, or non-infected) on a given dependent variable, a one-way ANOVA model was specified. Significant differences, α=0.05, among treatment means were identified using the Extended Shaffer-Royen (ESR) multiple comparisons method (Westfall and Tobias, 2007) by specifying ADJUST=SIMULATE and STEPDOWN options in the LSMEANS statement (SAS Institute, 2006). A *P*-value of <0.05 was considered significant.

Chapter 3: Results

3.1 Milk Samples from Clinically Healthy Cows

3.1.1 Bacteriological Results and SCC

In order to evaluate the milk concentrations of APP in subclinically infected mammary glands, milk samples were collected from 393 quarters of 101 clinically healthy cows. Of the samples plated, 55 were positive for bacterial growth, and the quarters from which these samples were derived were classified as subclinically infected. Diagnostic microbiological testing identified coagulase-negative staphylococci (CNS) as the most prevalent pathogen among the subclinically infected quarters (Table 1). The second most prevalent pathogen isolated was *Staphylococcus aureus*. Together, these two pathogens accounted for 84% of the subclinical intramammary infections. Other pathogens isolated included *Streptococci* spp., Gram-negative bacilli, *Bacillus cereus*, *Corynebacterium bovis*, and yeast.

SCC was detected in milk from 316 (93%) non-infected quarters and all infected quarters and its concentrations ranged from 0 to 3361×10^3 cells/ml in non-infected quarters and from 2 to 13020×10^3 cells/ml (Table 2). The milk SCC of the 55 subclinically infected quarters ($851 \pm 319 \times 10^3$ cells/ml) were approximately 9-fold higher ($P < 0.0001$) than those of the non-infected quarters ($86 \pm 13 \times 10^3$ cells/ml) (Fig. 1A). For subsequent analysis of the effect of SCC on milk APP concentrations, samples were also analyzed on the basis of defined grouping by low (≤250,000 cells/ml) or high (>250,000 cells/ml) SCC regardless of infection status (Fig. 1B). Correspondingly, there was a significant difference in SCC between the two groups ($P < 0.0001$). Samples were also analyzed on the basis of both infection status and SCC grouping (Fig. 1C). Of the 347 quarters with

milk SCC $\leq$250,000 cells/ml, 36 (~10%) were positive for bacterial growth. The SCC of these quarters (94 ± 11 × 10^3 cells/ml) differed ($P < 0.0001$) from those of non-infected quarters (40 ± 3 × 10^3 cells/ml) in the low SCC group. Of the 46 quarters with milk SCC >250,000 cells/ml, 19 (~41%) were positive for bacterial growth. The SCC of these quarters (2,284 ± 841× 10^3 cells/ml) approached, but did not reach, a level that was significantly different ($P = 0.0865$) from those of non-infected quarters (616 ± 114 × 10^3 cells/ml) in the high SCC group.

Table 1. Bacteriological analysis of milk samples from cows that were either clinically healthy and had at least one subclinically infected quarter or had at least one quarter diagnosed with clinical mastitis.

	Clinically Healthy Cows	Clinical Mastitis Cows	
Bacteria[a]	Subclinically Infected[b]	Subclinically Infected[c]	Clinical Mastitis[d]
CNS	38[e]	11	3
S. aureus	8	3	2
Streptococci	3	1	1
Gram-negative	2	2	2
B. cereus	2	1	1
C. bovis	1	2	1
Yeast	1	1	1
No growth	NA	NA	7
Total	55	21	18

[a] CNS: coagulase-negative *Staphylococci*; *S. aureus*: *Staphylococcus aureus*; *B. cereus*: *Bacillus cereus*; *C. bovis*: *Corynebacterium bovis*; NA: not applicable

[b] Bacteriological results of milk samples obtained from subclinically infected quarters of clinically healthy cows.

[c] Bacteriological results of milk samples obtained from subclinically infected quarters of cows with one or more quarters diagnosed with clinical mastitis.

[d] Bacteriological results of milk samples obtained from quarters diagnosed with clinical mastitis.

[e] Data are reported as the number of quarters infected.

Figure 1

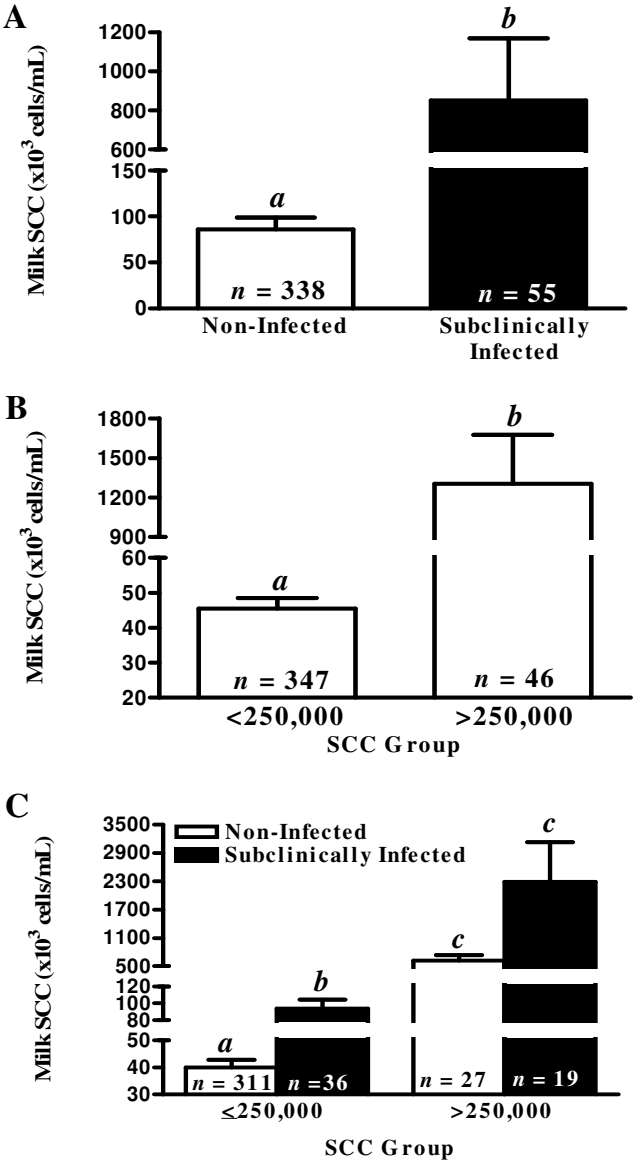


Fig. 1. Somatic cell counts (SCC) of milk samples obtained from clinically healthy cows. Milk samples were collected from 393 quarters of 101 clinically healthy cows and analyzed for SCC. Data are presented on the basis of infection status (non-infected versus subclinically infected) (A), SCC grouping (≤250,000 versus >250,000 cells/ml) (B), or both (C), and reported as mean (± S.E.) counts in thousands per milliliter. Different alphabets denote statistically significant ($P < 0.05$) differences between groups.

3.1.2 BSA Concentrations

As an indicator of increased vascular permeability and breakdown of the milk-blood barrier, milk BSA concentrations were analyzed as a marker of local inflammation in milk samples collected from the healthy and subclinically infected quarters of clinically healthy cows. BSA concentrations were above the limit of detection in all milk samples with concentrations ranging from 16.17 to 3258.65 μg/ml for non-infected quarters and from 12.32 to 944.66 μg/ml for subclinically infected quarters (Table 2). On the basis of infection status alone, there were no significant differences in the BSA concentrations of milk samples obtained from subclinically infected versus non-infected quarters (Fig. 2A). On the basis of SCC grouping alone, milk BSA concentrations were higher ($P = 0.0265$) in milk samples with SCC >250,000 cells/ml (388 ± 37 μg/ml) than in those with SCC ≤250,000 cells/ml (300 ± 14 μg/ml) (Fig. 2B). Analysis of samples on the basis of both SCC grouping and infection status identified comparable milk BSA concentrations among samples from subclinically infected and non-infected quarters in the low SCC group, as well as between those from subclinically infected and non-infected quarters in the high SCC group (Fig. 2C).

Table 2. Range of concentrations of somatic cell counts (SCC), bovine serum albumin(BSA), LPS binding protein(LBP), haptoglobin(Hap) and amyloid A(AA) in milk samples from clinically healthy cows.

	Non-Infected Quarters[b]		Subclinically Infected Quarters[c]	
	Samples detectable[a]	Range	Samples detectable	Range
SCC(10^3 cells/ml)	316/338	0-3361	55/55	2-13020
BSA(μg/ml)	338/338	16.17-3258.65	55/55	12.32-944.66
LBP(μg/ml)	337/338	<0.008-38.94	55/55	0.32-29.65
Hap(μg/ml)	278/338	<0.02-47.09	52/55	<0.02-85.85
AA(μg/ml)	15/338	<0.047-2.39	6/55	<0.047-1.08

[a]Number of milk samples with a concentration greater than the limit of detection of the assay used/ total number of milk samples.

[b] Data for milk samples from non-infected quarters of clinically healthy cows.

[c] Data for milk samples from subclinically-infected quarters of clinically healthy cows.

Figure 2

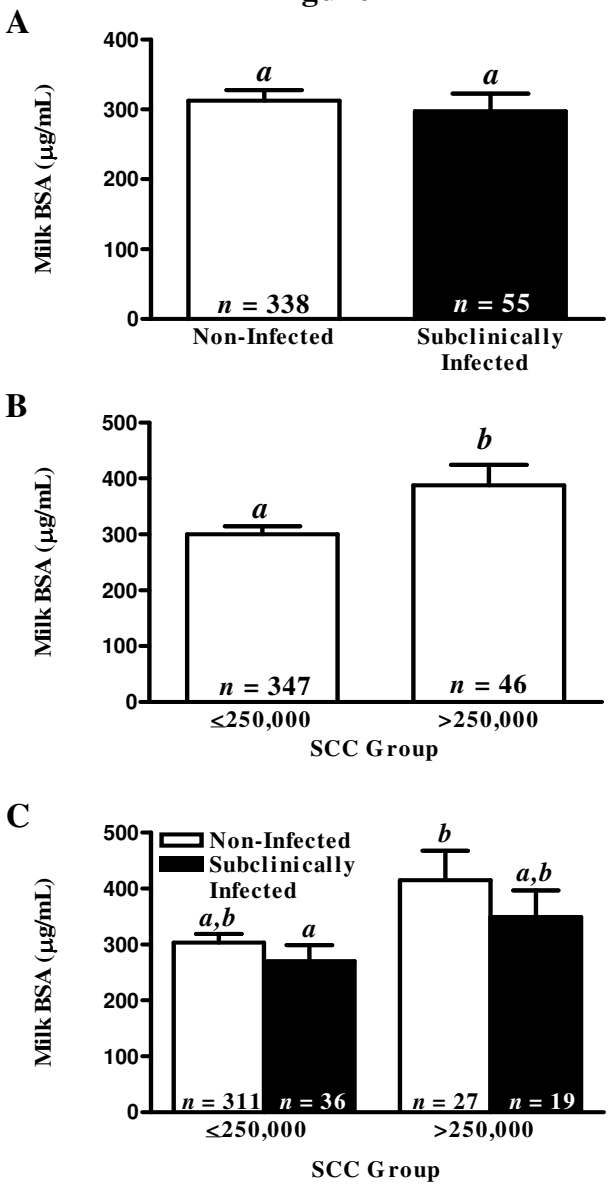


Fig. 2. Bovine serum albumin (BSA) concentrations in milk samples obtained from clinically healthy cows. The BSA concentrations of milk samples collected from 393 quarters of 101 clinically healthy cows were determined by ELISA. Data are presented on the basis of infection status (non-infected versus subclinically infected) (A), somatic cell count (SCC) grouping (≤250,000 versus >250,000 cells/ml) (B), or both (C), and reported as mean (± S.E.) milk BSA concentrations in micrograms per milliliter. Different alphabets denote statistically significant ($P < 0.05$) differences between groups.

3.1.3 LBP Concentrations

The concentrations of LBP were determined by ELISA in milk samples collected from the healthy and subclinically infected quarters of clinically healthy cows. LBP was detected in milk from 337 (99%) non-infected quarters and all infected quarters and their concentrations ranged from below 0.008 to 38.94 μg/ml for non-infected quarters and from 0.32 to 29.65 μg/ml for subclinically infected quarters (Table 2). On the basis of infection status alone, milk LBP concentrations were comparable between non-infected and subclinically infected quarters (Fig. 3A). On the basis of SCC grouping irrespective of infection status, milk LBP concentrations were higher (P = 0.0032) in milk samples with SCC >250,000 cells/ml (12.78 ± 1.39 μg/ml) than in those with SCC ≤250,000 cells/ml (6.20 ± 0.33 μg/ml) (Fig. 3B). Analysis of samples on the basis of both SCC grouping and infection status revealed a significant difference (P = 0.0357) in milk LBP concentrations between non-infected and subclinically infected quarters where SCC was ≤250,000 cells/ml (Fig. 3C). In samples with high SCC (>250,000 cells/ml), there were no significant differences (P = 0.1699) in milk LBP concentrations between non-infected and subclinically infected quarters.

Figure 3

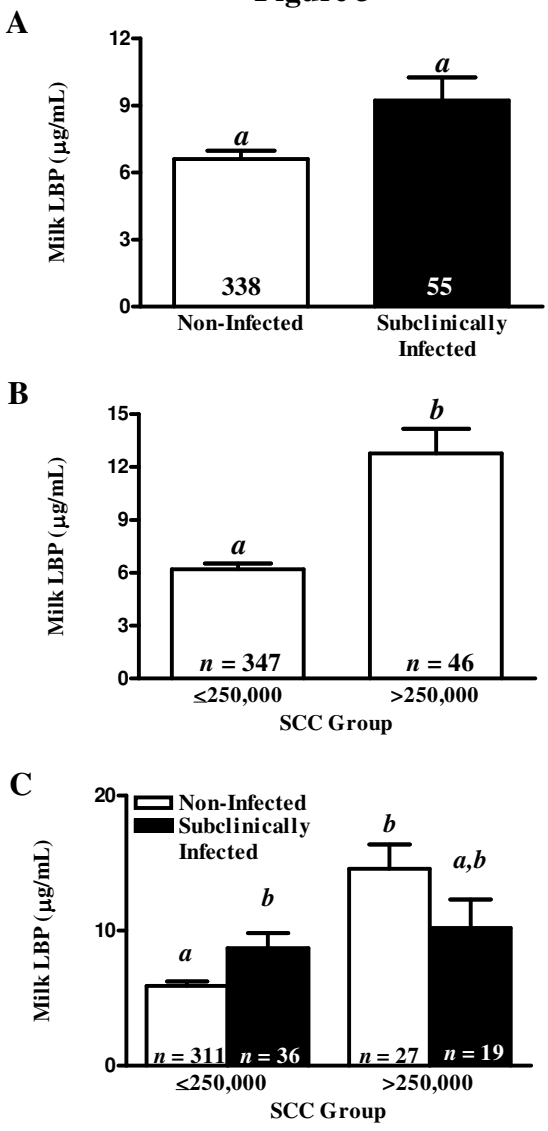


Fig. 3. Lipopolysaccharide-binding protein (LBP) concentrations in milk samples obtained from clinically healthy cows. The LBP concentrations of milk samples collected from 393 quarters of 101 clinically healthy cows were determined by ELISA. Data are presented on the basis of infection status (non-infected versus subclinically infected) (A), somatic cell count (SCC) grouping ($\leq$250,000 versus >250,000 cells/ml) (B), or both (C), and reported as mean (± S.E.) milk LBP concentrations in micrograms per milliliter. Different alphabets denote statistically significant ($P < 0.05$) differences between groups.

3.1.4 Milk haptoglobin concentrations

The concentrations of Hap were determined by ELISA in milk samples collected from the healthy and subclinically infected quarters of clinically healthy cows. Hap was detected in milk from 278 (82%) non-infected and 52 (94%) subclinically-infected quarters. Their concentrations ranged from below 0.008 μg/ml to 47.09 μg/ml for non-infected quarters and from below 0.008 μg/ml to 85.85 μg/ml for subclinically infected quarters (Table 2). On the basis of infection status alone, milk Hap concentrations were higher ($P = 0.0013$) in those quarters that were subclinically infected (4.12 $\pm$ 1.65 μg/ml) than in those that were uninfected (0.82 ± 0.21 μg/ml) (Fig. 4A). On the basis of SCC grouping irrespective of infection status, milk Hap concentrations were higher ($P < 0.0001$) in milk samples with SCC >250,000 cells/ml (7.18 ± 2.10 μg/ml) than in those with SCC $\leq$250,000 cells/ml (0.50 ± 0.15 μg/ml) (Fig. 4B). Analysis of samples on the basis of both SCC grouping and infection status revealed a significant difference ($P = 0.0001$) in milk Hap concentrations between non-infected and subclinically infected quarters where SCC was $\leq$250,000 cells/ml (Fig. 4C). In samples with high SCC (>250,000 cells/ml), there were no significant differences ($P = 0.1927$) in milk Hap concentrations between non-infected and subclinically infected quarters.

Figure 4

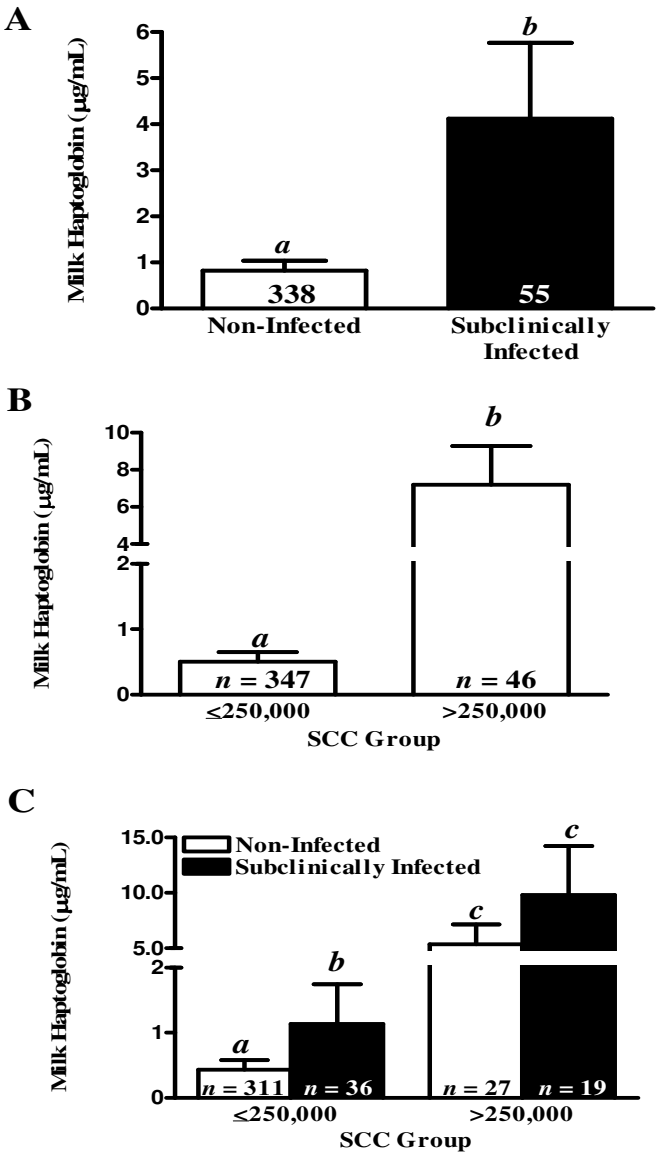


Fig. 4. Haptoglobin concentrations in milk samples obtained from clinically healthy cows. The haptoglobin concentrations of milk samples collected from 393 quarters of 101 clinically healthy cows were determined by ELISA. Data are presented on the basis of infection status (non-infected versus subclinically infected) (A), somatic cell count (SCC) grouping (≤250,000 versus >250,000 cells/ml) (B), or both (C), and reported as mean (± S.E.) milk haptoglobin concentrations in micrograms per milliliter. Different alphabets denote statistically significant ($P < 0.05$) differences between groups.

3.1.5. Milk Amyloid A Concentrations

The concentrations of milk Amyloid A were determined by ELISA in milk samples collected from the healthy and subclinically infected quarters of clinically healthy cows. Milk Amyloid A was detected from 15 (4.4%) non-infected and 6 (10.9%) subclinically infected quarters and their concentrations ranged from below 0.047 to 2.39 µg/ml for non-infected quarters and from below 0.047 to 1.08 µg/ml for subclinically infected quarters (Table 2). On the basis of infection status alone, there were no significant differences (P =0.8561) in the SAA concentrations of milk samples obtained from subclinically infected versus non-infected quarters (Fig. 5A). On the basis of SCC grouping alone, milk SAA concentrations were higher (P = 0.0094) in milk samples with SCC >250,000 cells/ml (0.12 ± 0.057 µg/ml) than in those with SCC ≤250,000 cells/ml (0.0085 ± 0.0027µg/ml) (Fig. 5B). Analysis of samples on the basis of both SCC grouping and infection status identified comparable milk SAA concentrations among samples from subclinically infected and non-infected quarters in the low SCC group, as well as between those from subclinically infected and non-infected quarters in the high SCC group (Fig. 5C).

Figure 5

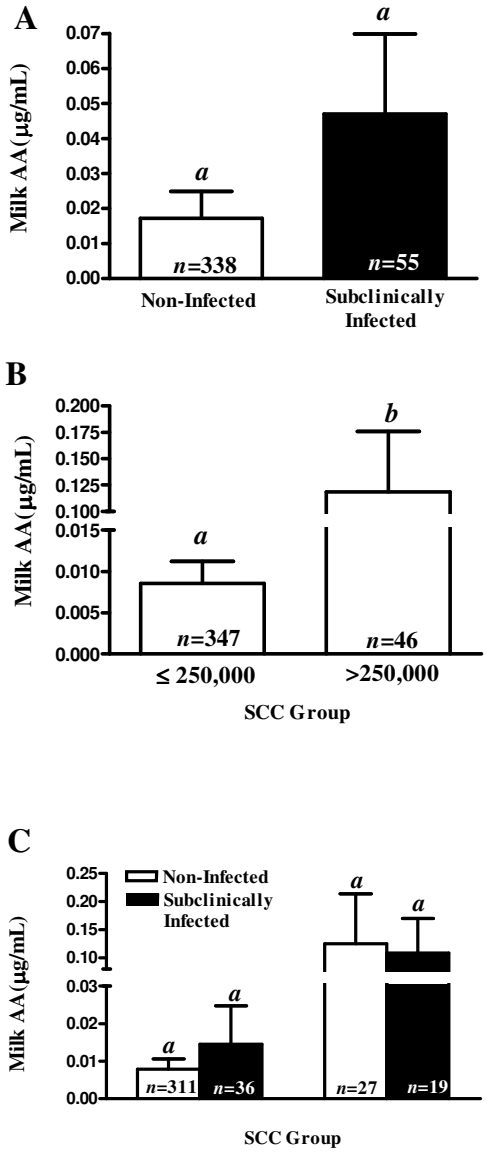


Fig. 5. Amyloid A concentrations in milk samples obtained from clinically healthy cows. The Amyloid A concentrations of milk samples collected from 393 quarters of 101 clinically healthy cows were determined by ELISA. Data are presented on the basis of infection status (non-infected versus subclinically infected) (A), somatic cell count (SCC) grouping ($\leq$250,000 versus >250,000 cells/ml) (B), or both (C), and reported as mean (±S.E.) milk Amyloid A concentrations in micrograms per milliliter. Different alphabets denote statistically significant ($P < 0.05$) differences between groups.

3.2 Milk Samples from Cows with Clinical Mastitis

3.2.1 Bacteriological Results, SCC, and BSA Concentrations

In order to evaluate the milk concentrations of APP in mammary glands showing clinical signs of disease, milk samples were collected from 17 cows with naturally-occurring clinical mastitis. Samples were obtained at the first sign of clinical mastitis during the morning or evening milking and collected from all quarters. With the exception of one cow that had clinical symptoms in two quarters, all other cows sampled had only one quarter with clinical mastitis. Of the milk samples isolated from the remaining quarters showing no clinical signs of disease, 21 were positive for bacterial growth and the corresponding quarters classified as subclinically infected. Among the quarters of these cows that showed either clinical mastitis or were subclinically infected, CNS, *S. aureus*, and Gram-negative bacilli were the most prevalent bacteria recovered (Table 1). Twenty-two quarters were free of infection and had milk SCC $\leq$250,000 cells/ml. These quarters were classified as healthy based on the absence of infection and inflammation.

Among the cows with clinical mastitis, the milk SCC was detected in all milk samples and its concentration ranged from 8×10^3 to 179×10^3 cells/ml, 1.5×10^3 to 2525×10^3 cells/ml and 26×10^3 to 157750×10^3 cells/ml for healthy quarters, subclinically infected quarters and clinically infected quarters, respectively (Table 3). The milk SCC of the quarters with clinical signs ($18{,}553 \pm 9{,}596 \times 10^3$ cells/ml) were approximately 42-fold higher ($P = 0.0002$) than those of subclinically infected quarters ($434 \pm 175 \times 10^3$ cells/ml) (Fig. 6A). The SCC of the healthy quarters of these cows ($44 \pm 10 \times 10^3$ cells/ml) were lower than those of the quarters showing clinical signs ($P <$

0.0001) or that were subclinically infected (P = 0.0189). BSA was detected in all milk samples (Table 2) and its concentrations ranged from 36.67 to 3905.6 µg/ml, 100.20 to 710.97 µg/ml and 125.85 to 18508 µg/ml for healthy quarters, subclinically infected quarters and clinically infected quarters respectively (Table 3). The milk BSA of these cows were higher in the quarters with clinical mastitis (2,143 ± 1,054 µg/ml) than in those quarters that had a subclinical infection (329 ± 37 µg/ml; P = 0.0204) or were healthy (374 ± 170 µg/ml; P = 0.0021) (Fig. 6B). There was no difference (P = 0.2371) between the milk BSA concentrations of the non-infected and subclinically infected quarters of these cows.

Table 3. Range of concentrations of somatic cell counts (SCC), bovine serum albumin (BSA), LPS binding protein (LBP), haptoglobin (Hap) and amyloid A (AA) in milk samples from cows with clinical mastitis.

	Non-Infected SCC<250,000[b]		Subclinically Infected Quarters[c]		Clinically Infected Quarters[d]	
	Samples detectable[a]	Range	Samples detectable	Range	Samples detectable	Range
SCC(10^3 cells/ml)	22/22	8-179	21/21	1.5-2525	18/18	26-157750
BSA(μg/ml)	22/22	36.7-3905.6	21/21	100.2 -710.9	18/18	125.9-18508
LBP(μg/ml)	22/22	0.39-42.3	21/21	1.06-41.73	17/18	<0.4-47.8
Hap(μg/ml)	22/22	0.026-48.8	20/21	<0.02-61.17	18/18	1.77-841.1
AA(μg/ml)	7/22	<0.047-6.9	15/21	<0.047-7.71	13/18	<0.047-129.4

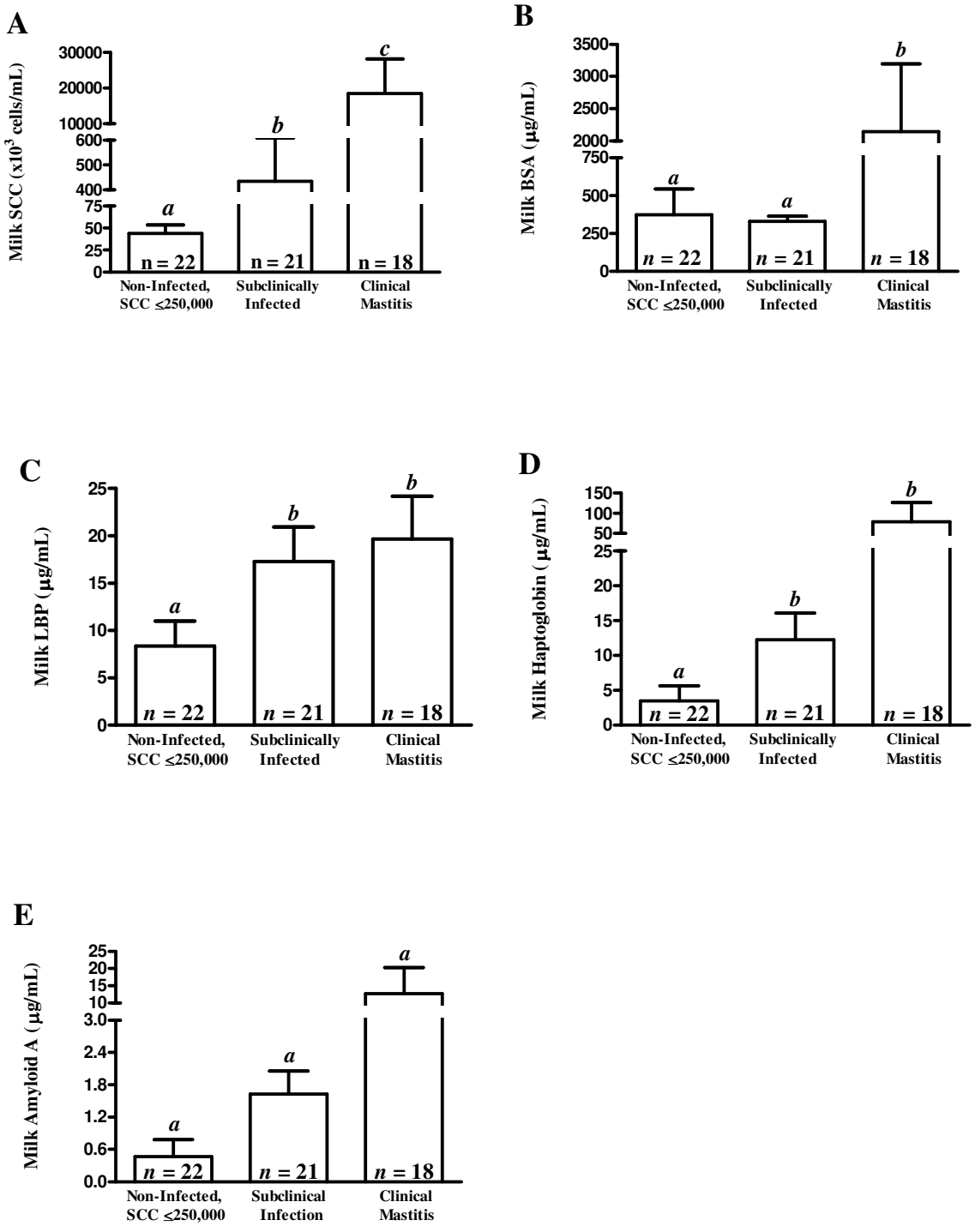
[a] Number of milk samples with a concentration greater than the lower detection limit of the assay used/ total number of milk samples.

[b] Data for milk samples from quarters that were free of infection and with SCC below 250,000 cells/ml of cows with clinical mastitis.

[c] Data for milk samples from subclinically infected quarters of cows with clinical mastitis.

[d] Data for milk samples from quarters with clinical signs of cows with clinical mastitis.

Figure 6


A
Milk SCC (x10^3 cells/mL)
30000
20000
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600
500
400
75
50
25
0
a
b
c
n = 22
n = 21
n = 18
Non-Infected, SCC ≤250,000
Subclinically Infected
Clinical Mastitis
B
Milk BSA (µg/mL)
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3000
2500
2000
750
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n = 22
n = 21
n = 18
Non-Infected, SCC ≤250,000
Subclinically Infected
Clinical Mastitis
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Milk LBP (µg/mL)
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n = 22
n = 21
n = 18
Non-Infected, SCC ≤250,000
Subclinically Infected
Clinical Mastitis
D
Milk Haptoglobin (µg/mL)
150
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n = 22
n = 21
n = 18
Non-Infected, SCC ≤250,000
Subclinically Infected
Clinical Mastitis
E
Milk Amyloid A (µg/mL)
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2.4
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1.2
0.6
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n = 22
n = 21
n = 18
Non-Infected, SCC ≤250,000
Subclinical Infection
Clinical Mastitis

Fig. 6. Measurement of indices of inflammation in milk samples obtained from cows with clinical mastitis. Milk samples were collected from all quarters of 17 cows diagnosed with clinical mastitis in one or more quarters. Milk samples from the quarters were divided into three groups on the basis of: (1) absence of bacterial growth after plating, somatic cell counts (SCC) $\leq$250,000 cells/ml, and absence of any clinical signs of disease in the quarter from which the sample was collected; (2) presence of bacterial growth and absence of any clinical signs of disease in the quarter from which the sample was collected (subclinically infected); and (3) presence of clinical signs of disease in the quarter from which the sample was collected (clinical mastitis). The milk SCC of the samples were determined and reported as mean (± S.E.) counts in thousands per ml (A). The bovine serum albumin (BSA) (B), lipopolysaccharide-binding protein (LBP) (C), haptoglobin (D) and amyloid A (E) concentrations in the samples were determined by ELISA and reported as mean (±S.E.) concentrations in micrograms per milliliter. Different alphabets denote statistically significant ($P < 0.05$) differences between groups.

3.2.2 LBP, Haptoglobin and Amyloid A Concentrations

In cows with clinical mastitis, LBP was detected in all milk samples except one from clinically infected quarter and its concentrations ranged from 0.39 to 42.30 µg/ml, 1.06 to 41.73 µg/ml and <0.4 to 47.88 µg/ml for healthy quarters, subclinically infected quarters and clinically infected quarters respectively (Table 3). Those quarters that showed clinical signs of disease (19.64 ± 4.50 µg/ml) or were subclinically infected (17.29 ± 3.64 µg/ml) had higher (P = 0.0464 and 0.0489, respectively) milk concentrations of LBP than corresponding healthy quarters (8.37 ± 2.61 µg/ml) (Fig. 6C). There was no difference (P = 0.5979), however, in the milk LBP concentrations of the quarters of these cows that showed clinical signs versus those quarters that were subclinically infected. Haptoglobin was detected in all milk samples except one from subclinically infected quarter and its concentration ranged from 0.026 to 48.83 µg/ml, <0.02 to 61.17 µg/ml and 1.77 to 841.01 µg/ml for healthy quarters, subclinically infected quarters and clinically infected quarters respectively (Table 3). Similar to LBP, milk haptoglobin concentrations in quarters with clinical signs (78.72 ± 47.28 µg/ml) or subclinical infections (12.27 ± 3.80 µg/ml) were higher (P < 0.0001 and P = 0.0003, respectively) than those in the healthy quarters (3.45 ± 2.20 µg/ml) of the same cows (Fig. 6 D). The haptoglobin concentrations of milk samples obtained from the quarters of these cows that were subclinically infected versus those that demonstrated clinical signs differed by a level that approached (P = 0.0666) statistical significance. Milk amyloid A was detected in 7 (32%) healthy quarters, 15 (71.42%) subclinically infected quarters and 13(72.22%) infected quarters and its concentration ranged from <0.047 to 6.92 µg/ml, <0.047 to 7.71 µg/ml and <0.047 to 129.42 µg/ml for healthy quarters, subclinically

infected quarters and clinically infected quarters respectively (Table 3).However, there was no difference in the milk SAA concentrations between quarters that showed clinical signs and those subclinically infected ($P = 0.0626$) or healthy quarters($P = 0.1229$) as well as between subclinically infected quarters and healthy quarters($P = 0.2822$)(Fig. 6E).

3.3 Blood Samples from Clinically Healthy Cows and Cows with Clinical Mastitis

In order to evaluate the effect of naturally-occurring clinical mastitis on APP blood concentrations, blood samples were obtained from the 17 cows diagnosed with clinical mastitis. For comparison, blood samples obtained from the 101 clinically healthy cows were segregated into two groups, those from cows with all 4 quarters that were free of infection and had milk SCC $\leq$250,000 cells/ml ($n = 47$) and those from cows with at least one quarter that was subclinically infected ($n = 39$).

LBP was detected in all blood samples and its concentration ranged from 0.16 to 101.33 µg/ml, 1.79 to 180.48 µg/ml and 11.72 to 259.59 µg/ml for cows with all healthy quarters, subclinical intramammary infections, or clinical mastitis respectively(Table 4). Blood concentrations of LBP were higher in cows with clinical mastitis (113.12 ± 17.48 µg/ml) than in those with subclinical intramammary infections (35.10 ± 6.31 µg/ml; $P < 0.0001$) or all healthy quarters (23.16 ± 3.19 µg/ml; $P < 0.0001$) (Fig. 7A). There was no difference ($P = 0.1390$) between the blood LBP concentrations of non-infected cows and those with subclinical intramammary infections. Hap was detected in blood from 33(70%) cows with all healthy quarters, 29(74%) cows with subclinically infected quarters and all 17 cows with clinical mastitis and its concentration ranged from <0.2 to 28.66 µg/ml, <0.2 to 422.97 µg/ml and 1.18 to 4843.52 µg/ml for these three groups

correspondingly(Table 4). Similar to LBP, blood haptoglobin concentrations were significantly higher in cows with clinical mastitis than in cows with all healthy quarters or at least one quarter with a subclinical infection (Fig. 7B). In contrast to LBP, the concentrations of haptoglobin were higher (P = 0.0499) in the blood of cows with a subclinical intramammary infection versus those with all healthy quarters. The blood concentrations of haptoglobin in cows with all healthy quarters, subclinical intramammary infections, or clinical mastitis, were 1.45 ± 0.7 µg/ml, 23.31 ± 13.16 µg/ml, and 1,732.61 ± 314.27 µg/ml, respectively.

In contrast to its relatively low detection rate in milk samples, SAA was detected in all blood samples and its concentration ranged from 0.81 to 154.23 µg/ml, 1.57 to 469.32 µg/ml and 20.0 to 323.61 µg/ml for cows with all healthy quarters, subclinical intramammary infections, or clinical mastitis respectively. (Table 4). Similar to haptoglobin and LBP, blood SAA concentrations were higher(163.15 ± 25.47 µg/ml) in cows with clinical mastitis than in cows with all healthy quarters(31.95 ± 4.71 µg/ml; P <0.0001) or at least one quarter with a subclinical infection(85.19 ± 18.40 µg/ml; P = 0.0012) (Fig. 7 C). Similar to haptoglobin and in contrast to LBP, the concentrations of SAA were higher (P = 0.0086) in the blood of cows with a subclinical intramammary infection versus those with all healthy quarters.

Table 4. Range of concentrations of LPS binding protein (LBP), haptoglobin (Hap) and serum amyloid A (SAA) in plasma samples from clinically healthy cows and cows with clinical mastitis.

	Non-Infected, SCC<250,000[b]		Subclinically Infected [c]		clinically Infected [d]	
	Samples detectable [a]	Range	Samples detectable	Range	Samples detectable	Range
LBP(μg/ml)	47/47	0.16-101.33	39/39	1.79-180.48	17/17	11.72-259.59
Hap(μg/ml)	33/47	<0.2-28.66	29/39	<0.2-422.97	17/17	1.18-4843.52
SAA(μg/ml)	47/47	0.81-154.23	39/39	1.57-469.32	17/17	20.0-323.61

[a] Number of milk samples with a concentration greater than the lower detection limit of the assay used/ total number of milk samples.

[b] Data for blood samples from cows with all quarters that were free of infection and had SCC below 250,000 cells/ml.

[c] Data for blood samples from cows with one or more quarters that were subclinically infected.

[d] Data for blood samples from cows with one or more quarters with clinical mastitis.

Figure 7

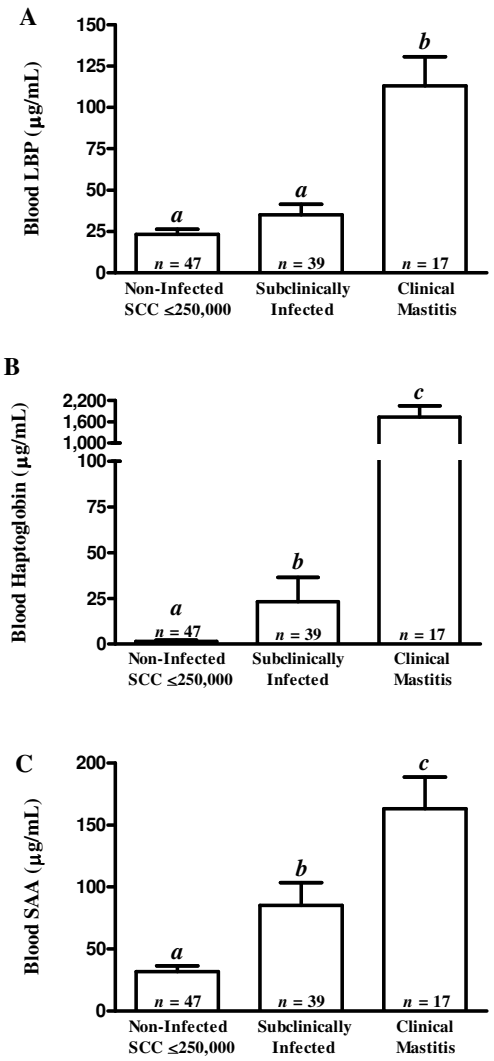

Fig. 7. Measurement of acute phase protein concentrations in blood samples obtained from cows with subclinical intramammary infections or clinical mastitis. Blood samples were obtained from: (1) clinically healthy cows that were free of infection and had milk somatic cell counts (SCC) ≤250,000 cells/ml in all quarters; (2) clinically healthy cows with at least one quarter that was subclinically infected; and (3) cows that had at least one quarter diagnosed with clinical mastitis. The lipopolysaccharide-binding protein (LBP) (A), haptoglobin (B) and serum amyloid A(SAA) (C) plasma concentrations were determined by ELISA and reported as mean (± S.E.) concentrations in micrograms per milliliter. Different alphabets denote statistically significant ($P < 0.05$) differences between groups.

Chapter 4: Discussion

The current study investigated the effects of naturally-occurring subclinical intramammary infection and clinical mastitis on the milk and blood concentrations of APP's in dairy cows. In various surveys, CNS, *S. aureus*, and *Streptococcus* spp. have been identified as the most frequent bacterial isolates from herd milk samples (Wilson et al., 1997; Makovec and Ruegg, 2003). In milk samples obtained specifically from clinical cases of mastitis, CNS, Gram-negative bacilli, *S. aureus*, and *Streptococcus* spp. have been reported in four surveys to be among the most prevalent bacterial isolates, although the absolute rank order of prevalence differs among the surveys (Erskine et al., 1988; Barkema et al., 1998; Sargeant et al., 1998; Bradley et al., 2007). In the current study, the frequency distribution of the pathogens identified in the milk samples from subclinically infected cows and those with clinical mastitis are consistent with these previously published surveys (Table 1). Further, the predominance of the absence of bacterial growth in the plated milk samples from quarters with clinical mastitis is consistent with a previous report (Bradley et al., 2007). Thus, the distribution of bacterial pathogens isolated from infected quarters in this study was reflective of that observed in other herds.

Factors such as cyclical bacterial shedding (Sears et al., 1990), milk bacterial concentrations below the level of detection (*i.e.*, <50 CFU/ml when plating 20 μL of sample), or infection by fastidious pathogens not readily cultured by traditional methods (*e.g.*, *Mycoplasma bovis*), can result in failure to detect an intramammary infection. Elevated SCC can serve as an indicator of intramammary infection, and the infection status of the gland is one of the most important factors affecting milk SCC (Dohoo and

Meek, 1982; Reneau, 1986). Consistent with this notion, the milk SCC of subclinically infected quarters sampled in this study were higher than those of non-infected quarters (Fig. 1A). Several other studies have suggested that a SCC threshold value of 200,000 to 300,000 cells/ml has diagnostic value for distinguishing between uninfected and infected quarters, and consequently, non-inflamed versus inflamed quarters (Dohoo and Meek, 1982; Reneau, 1986; Schepers et al., 1997; Schukken et al., 2003). In the current study, APP concentrations were analyzed in samples from clinically healthy cows on the basis of bacterial growth in plated milk samples, as well as on the basis of an SCC threshold of 250,000 cells/ml, the latter of which was chosen based on the findings of the previously mentioned studies. In cows with clinical mastitis, the APP concentrations in samples from quarters showing clinical signs were compared to those in samples from quarters of the same cows that either had a subclinical infection *(i.e.,* positive for bacterial growth on plated milk samples) or had SCC $\leq$250,000 cell/ml and no bacterial growth. This experimental design allowed for the comparison of APP concentrations from quarters showing clinical signs or that were subclinically infected to APP concentrations in healthy quarters as defined by the combination of both low SCC and absence of bacterial growth.

Associations between milk BSA concentrations and infection status of the mammary gland have also been previously investigated, and the findings of these studies have cast doubt on the utility of BSA as a single diagnostic marker of intramammary infection (Verhoeff and Smith, 1981; Mattila et al., 1986; Huszenicza et al., 1997). The findings in the current study that milk BSA concentrations are equivalent in non-infected and subclinically infected quarters (Fig. 2A) and higher in quarters with clinical mastitis

(Fig. 5B), are consistent with previous reports (Verhoeff and Smith, 1981; Huszenicza et al., 1997; Bannerman et al., 2003; Bannerman et al., 2008). Further, the milk BSA concentrations reported here are comparable with those in the aforementioned studies. The finding that milk BSA concentrations analyzed on the basis of SCC alone were higher in samples with SCC >250,000 cells/ml compared to those with SCC less than this amount (Fig. 2B) is consistent with the previous reports establishing a positive correlation between milk BSA and SCC (Kitchen et al., 1980; Poutrel et al., 1983).

Several reports have suggested that APP's, such as haptoglobin and SAA, may have diagnostic value in identifying cows with subclinical or clinical intramammary infections (Eckersall et al., 2001; Nielsen et al., 2004; Gronlund et al., 2005; Hiss et al., 2007). Comparison of the temporal onset and duration of increased concentrations of LBP to other APP's has been reported in blood, but not milk. Relative to other APP's, increases in blood LBP concentrations have been shown to increase earlier, and remain elevated for longer, in response to experimental intramammary infection (Bannerman et al., 2005; Kauf et al., 2007; Bannerman et al., 2008). The findings from these studies suggest that LBP could be a more sensitive marker of underlying intramammary infection because elevated concentrations would be more likely to be detected due to its prolonged elevation. Because clinical signs of mastitis are often readily detected by visual observation of the gland and/or milk secretions, the greatest utility for LBP and other APP's would be as diagnostic markers for subclinical intramammary infection. Results from the current study demonstrate that relative to cows with all healthy quarters, blood LBP concentrations are elevated in cows with clinical mastitis but not in those with subclinical infections (Fig. 6A). In clinically healthy cows with low SCC, milk LBP

concentrations were higher in subclinically infected quarters than in non-infected quarters (Fig. 3C). However, when SCC was not taken into consideration, milk LBP concentrations did not differ between subclinically infected and uninfected quarters in clinically healthy cows (Fig. 3A). Interestingly, LBP concentrations were higher than those of healthy quarters in the subclinically infected quarters of cows with clinical mastitis (Fig. 5C). This may be due, in part, to the increased availability of LBP that can diffuse into these quarters as a result of higher circulating blood concentrations of LBP in cows with clinical mastitis (Fig. 6A).

The current study is the first to report on the milk and blood concentrations of LBP in cows with subclinical intramammary infections and cows with naturally-occurring clinical mastitis. The milk (~7 μg/ml) and blood (~23 μg/ml) concentrations of LBP identified in the current study in healthy quarters and cows, respectively, are comparable to those that have been previously reported (Bannerman et al., 2003; Bannerman et al., 2004b; Kauf et al., 2007). Further, in cows with naturally-occurring clinical mastitis, the milk (~20 μg /ml) and blood (~113 μg /ml) LBP concentrations detected in this study are within the range of those reported during the course of experimentally-induced clinical mastitis (Bannerman et al., 2003; Bannerman et al., 2004b; Bannerman et al., 2004c; Bannerman et al., 2005).

For comparison with LBP, the current study also investigated the influence of subclinical intramammary infection and clinical mastitis on milk and blood concentrations of haptoglobin and SAA, two APPs which have been widely studied as a diagnostic marker of udder health (Salonen et al., 1996; Hirvonen et al., 1999; Eckersall et al., 2001; Gronlund et al., 2003; Nielsen et al., 2004; Gronlund et al., 2005; Eckersall

et al., 2006; Akerstedt et al., 2007; Hiss et al., 2007). Consistent with these studies, milk haptoglobin concentrations were increased in quarters that were subclinically infected or showed clinical signs (Fig. 4A and 5D). Similar to LBP, milk haptoglobin concentrations differed between non-infected and infected quarters with low SCC (Fig. 4C). In contrast to LBP, milk haptoglobin concentrations in clinical quarters approached a level that statistically differed ($P = 0.0666$) from those in subclinically infected quarters (Fig. 5D). The range of haptoglobin concentrations in milk has been shown to vary greatly across studies. Previously reported mean or median milk haptoglobin concentrations of 3.8 µg /ml (Gronlund et al., 2005), 4.4 µg /ml (Hiss et al., 2007), 7.8 µg /ml (Akerstedt et al., 2007), and 9.7 µg /ml (Gronlund et al., 2003) in subclinically infected quarters are comparable with the 4.1 and 7.2 µg /ml concentrations detected in this study in quarters that were subclinically infected (Fig. 4A) or had SCC >250,000 cells/ml (Fig. 4B), respectively. Similarly, in quarters with clinical mastitis, the mean milk haptoglobin concentration of 79 µg /ml detected in this study is within the range of mean values (40 – 110 µg /ml) reported by others (Eckersall et al., 2001; Gronlund et al., 2003; Nielsen et al., 2004).

In all but one previous studies (Jacobsen et al., 2005), whole milk was used to detect the concentration of amyloid A in secretions from mammary glands, based on the assumption that the main part of Amyloid A, an apolipoprotein, will be more likely to be associated with the milk fat (Malle et al., 1993; Akerstedt et al., 2007). However, we found similar concentrations of amyloid A in whole milk and whey from same udder quarters in a preliminary study. In support of our observations, Jacobsen found comparable concentrations of amyloid A in different milk fractions including whole milk,

skimmed milk and whey (personal communication). Thus, we used whey instead of whole milk for the detection of SAA as well as Haptoglobin, LBP and BSA in this study. Another difference from all previous studies was that a specific kit dedicated for detection of amyloid A in milk was used in our study whereas previous studies used the same kit for detection of amyloid A in both blood and milk(Gronlund et al., 2003; Pedersen et al., 2003; Nielsen et al., 2004; Akerstedt et al., 2007). However, comparable concentrations of milk amyloid A were obtained in a preliminary test with both kits since detection antibody from the kit can cross react with different isoforms of amyloid A in blood and milk. Therefore, the relatively low concentrations of milk amyloid A in subclinically infected quarters from clinically healthy cows in our study are more likely due to factors other than the aforementioned differences.

In clinically healthy cows, the detection rate of amyloid A in milk samples from non-infected quarters 4.4%(15/338) was similar to 2.5% (1/24) for healthy quarters in a previous study(Gronlund et al., 2003). The range of amyloid A concentrations in milk samples from non-infected quarters (0.23–2.39 ug/ml) is also comparable with the range (0.2–0.54 µg/ml) reported by Eckersall (2001) for healthy quarters. However, the detection rate (5.4% versus 49% or 54%) and concentration range of amyloid A (0.23–1.08 µg/ml versus 0.9–151 µg/ml) in milk samples from subclinically-infected quarters were much lower than previous findings about clinically healthy quarters or naturally subclinically infected quarters (Gronlund et al., 2005; Akerstedt et al., 2007). These discrepancies can be partly explained by the different pathogen profile isolated from milk samples from subclinically infected quarters in the previous study, in which *S.aureus* accounted for 46% (13/28), *Streptococcus dysgalactiae* 36% (10/28),

Streptococcus uberis 14% (4/28), CNS 14% (4/28) (Gronlund et al., 2005). The specific profile of APPs induced by each pathogen is somehow mediated by the repertoire of pro-inflammatory cytokines it can induce in each infection (Baumann et al., 1989; Horadagoda et al., 1994; Alsemgeest et al., 1996), which seems to be a pathogen-specific case demonstrated by increasing data from experimentally induced IMI (Riollet et al., 2000; Bannerman et al., 2004a; Bannerman et al., 2004b; Bannerman et al., 2005).

In clinical mastitis cows, the range of amyloid A concentrations (0.25–129.42 µg/ml) in quarters with clinical mastitis were consistent with the range (0.2–95 µg/ml) reported by Eckersall (2001) for quarters with naturally occurring mastitis. The mean (12.73 µg/ml) is also close to 40.2 µg/ml reported by Nielsen (2004) for quarters with clinical mastitis considering the remarkable variations in Amyloid A concentrations across studies(Eckersall et al., 2001; Nielsen et al., 2004).

In contrast to LBP, blood concentrations of haptoglobin were higher in cows with a subclinical infection than in those with all healthy quarters (Fig. 6). Similar to LBP, blood concentrations of haptoglobin were higher in cows with clinical mastitis than in cows with either a subclinical intramammary infection or all healthy quarters. In cows with clinical mastitis, the mean haptoglobin blood concentration of 1,733 µg /ml detected in this study is within the range of 740–1,820 µg /ml reported in previous studies of clinical mastitis (Salonen et al., 1996; Hirvonen et al., 1999; Eckersall et al., 2001; Gronlund et al., 2003; Nielsen et al., 2004).

The mean of SAA concentrations (163.14 µg/ml) in blood samples from cows with clinical mastitis, is between two median values(13.8 µg/ml & 752 µg/ml) in previous studies of cows with naturally clinical mastitis (Eckersall et al., 2001; Nielsen et al.,

2004). Similarly, the mean of SAA concentration (31.9µg/ml) in blood samples from cows with all healthy quarters is between two median values (5.1 µg/ml & 75 µg/ml) reported in the above mentioned studies.

The sharp contrasts between milk and blood samples from clinically healthy cows in terms of the detection rates and concentrations of amyloid A indicated that milk amyloid A and blood amyloid A might be regulated both locally and systematically using different mechanism, as suggested by other studies (Eckersall et al., 2001; McDonald et al., 2001; Pedersen et al., 2003; Lehtolainen et al., 2004; Jacobsen et al., 2005; O'Mahony et al., 2006; Suojala et al., 2008). In support of our finding, substantial numbers of quarters had undetectable levels of MAA in the milk though high level of amyloid A were detected in blood in a previous study (O'Mahony et al., 2006). It was possible that conditions such as stress and /or extramammary inflammation existed in our study which induced a production of acute phase protein in plasma in the absence of intrammary infection (Nielsen et al., 2004; Lomborg et al., 2008).

During clinical mastitis, increased hepatic synthesis of APP's and inflammation-induced vascular permeability are most likely to be the predominant events contributing to increased milk concentrations of LBP and haptoglobin (Riollet et al., 2000; Bannerman et al., 2003; Bannerman et al., 2004c). However, mammary gland derived origins for these proteins cannot be excluded. Epithelial cells of the respiratory and intestinal tracts have been demonstrated to produce LBP in response to pro-inflammatory cytokines that are upregulated during mastitis (Vreugdenhil et al., 1999; Dentener et al., 2000; Bannerman et al., 2004c). Thus, one cannot rule out the possibility that the mammary epithelium is equally capable of serving as a local source of LBP. It is known that bovine

mammary epithelial cells can synthesize haptoglobin (Thielen et al., 2007). Moreover, haptoglobin is synthesized and stored in the specific granules of neutrophils, and released upon their activations (Theilgaard-Monch et al., 2006). Because milk concentrations of neutrophils can approach 5 ×107 cells/ml during mastitis (Bannerman et al., 2004c), one cannot exclude these cells as contributing sources of haptoglobin within the inflamed gland.

To our knowledge, this is the first study to evaluate milk and blood concentrations of LBP in cows with naturally-occurring mastitis. Specifically, this study demonstrated that: (1) LBP concentrations are increased in the milk and blood of quarters and cows, respectively, with clinical mastitis; (2) in clinically healthy cows with quarters with low SCC, milk concentrations of LBP are increased in quarters with subclinical infections; and (3) in clinically healthy cows, milk LBP concentrations are higher in quarters with SCC >250,000 cells/ml than in those with lower SCC. In contrast to the APP haptoglobin, blood concentrations of LBP in cows with a subclinical intramammary infection could not be differentiated from those of cows with all healthy quarters. Thus, although previous studies have demonstrated a more rapid and prolonged induction of LBP in response to experimental intramammary infection than other APP's, data from this study suggest that blood haptoglobin concentrations may serve as a better diagnostic biomarker of naturally-occurring subclinical intramammary infection.

Implications

Increased blood concentrations of APP is of marginal value in defining a cow with naturally occurring mastitis compared with experimentally induced IMI since various extra-mammary gland inflammation can also cause an increase in the blood APP concentrations (Saini and Webert, 1991; Nielsen et al.,2004). In contrast, increased amyloid A and haptoglobin concentrations are observed earlier in milk than in blood and their increase in milk would be a more specific indicator of IMI (Hiss et al., 2004; Jacobsen et al., 2005; Suojala et al., 2008). Furthermore, it is a less invasive procedure to collect milk samples compared with blood samples. In addition, hepatocyte-derived APP and non-hepatocyte derived APP have been suggested to have different immunological roles in mice against lung inflammation (Knapp et al., 2006). To this end, additional studies are needed to discriminate APPs of different origins and compare their relative diagnostic values for the identification of IMI.

On the other hand, some APPs have similar functions in immune response. Similar to LBP, SAA can bind to LPS and haptoglobin can participate in the detoxification of LPS (Schroedl et al., 2001; Arredouani et al., 2005). So the concept of APP index which takes in account into several APP's simultaneously might be an alternative to individual APP as an indicator of IMI. Further, LBP of different concentrations can exert opposing effects with low concentration of LBP related to a pro-inflammatory role and high concentration to an anti-inflammatory role. SAA can work as an opsonin for Gram-negative at normal concentration (Shah et al., 2006). Therefore, a thorough understanding of the immunological roles of APPs under various concentrations would allow us to draw more relevant conclusions concerning the incidence of mastitis from increased

concentrations of APPs in milk and blood.

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