5-2004

Identification and Association of Bovine CXCR2 Single Nucleotide Polymorphisms with Clinical and Subclinical Mastitis in Holstein and Jersey Cattle

Sara Marie Youngerman
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Sara Marie Youngerman entitled "Identification and Association of Bovine CXCR2 Single Nucleotide Polymorphisms with Clinical and Subclinical Mastitis in Holstein and Jersey Cattle." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Gina M. Pighetti, Major Professor

We have read this thesis and recommend its acceptance:

Arnold M. Saxton, Stephen P. Oliver

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Sara Marie Youngerman entitled "Identification and Association of Bovine CXCR2 Single Nucleotide Polymorphisms with Clinical and Subclinical Mastitis in Holstein and Jersey Cattle." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Gina M. Pighetti
Major Professor

We have read this thesis
and recommend its acceptance:

Arnold M. Saxton

Stephen P. Oliver

Accepted for the Council:

Anne Mayhew
Vice Provost and Dean of Graduate Studies

(Original signatures are on file with official student records.)

IDENTIFICATION AND ASSOCIATION OF BOVINE CXCR2 SINGLE NUCLEOTIDE POLYMORPHISMS WITH CLINICAL AND SUBCLINICAL MASTITIS IN HOLSTEIN AND JERSEY CATTLE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Sara Marie Youngerman

May 2004

DEDICATION

This thesis is dedicated to my husband Casey Youngerman, who is my soul mate. Thank you for your love, support, encouragement, and especially for reminding me to look at the big picture and not sweat the small stuff. I love you with all my heart.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my major professor, Dr. Gina Pighetti, for giving me the opportunity to continue my graduate education at The University of Tennessee. I wish to thank her for all of her guidance, enthusiasm, patience and encouragement throughout the completion of my education, and also for being a great person and friend. I would also like to thank Dr. Stephen Oliver and Dr. Arnold Saxton for critically reviewing this thesis and for all of their invaluable knowledge and suggestions.

I wish to send a gigantic thank you to Eileen Gallion, who was a great friend and invaluable help during my first semesters as a graduate student. A huge thank you also goes out to Jamie Gilreath, Rose Clift, Barbara Gillespie and Susan Headrick for all of their help during the completion of this project. I would also like to thank Doug Luther, Joan Cooper and Mary Roberts for all of their time, helpfulness and friendship.

Finally, I would like to thank all of the graduate students, especially Magdalena Rambeaud, Fernando Scenna, Rebecca Adcock and S. Kristin Asbury for all of their love, humor, advice and caring.

ABSTRACT

Mastitis is the most economically devastating disease affecting the dairy industry. A genetic marker associated with inflammatory responses during mastitis could aid in selection for mastitis resistant dairy cattle. Objectives of this experiment were to identify single nucleotide polymorphisms (SNPs) and resulting haplotypes in the bovine CXCR2 gene for two breeds of dairy cattle, determine SNP and haplotype frequencies, and identify genotype associations with subclinical and clinical mastitis. Genomic DNA was isolated from whole blood collected from Jersey and Holstein cows. A 311 bp segment located within the coding region of exon 3 of the CXCR2 gene was amplified and sequenced. Five SNPs were expressed in both breeds of cattle. Strong linkage disequilibrium was exhibited for both breeds among all five SNPs ($P<0.001$). Four SNPs resulted in synonymous nucleotide changes, while one nonsynonymous nucleotide substitution (CXCR2+777 G→C) resulted in a Gln→His substitution at amino acid residue 245. Breed was shown to have a significant effect on frequencies for four of the five SNPs ($P<0.001$). Ten haplotypes were observed from the five polymorphisms. Six haplotypes were common between the two breeds, while Holsteins and Jerseys each uniquely expressed two haplotypes.

We then examined the association of CXCR2 SNPs and haplotype genotypes with subclinical and clinical mastitis. Subclinical mastitis was defined

as the presence of the same pathogen in at least two out of three consecutive samples obtained during lactation. Clinical mastitis was defined as the presence of abnormal milk, and/or an abnormal udder, and/or systemic signs of intramammary infection that warranted treatment with intramammary therapies and/or use of anti-inflammatory agents. A significant difference was detected between CXCR2 SNP +777 genotypes and percentages of subclinical mastitis cases in Holstein cows. Holsteins with CXCR2 SNP +777 genotype GG had decreased percentages of subclinical mastitis (~22%), while genotype CC cows had increased percentages of subclinical mastitis (~37%). In summary, results from these experiments demonstrated that the CXCR2 gene is highly polymorphic in Jersey and Holstein cattle. CXCR2 SNP +777 genotypes were associated with subclinical mastitis in Holstein cows, indicating that the CXCR2 amino acid 245 may be involved in cellular signaling and function during mastitis. However, with observed levels of linkage disequilibrium, other genes near the CXCR2 locus may also be involved with cellular functions during mastitis. This research is promising for dairy producers as this approach may represent an effective means of marker assisted selection for mastitis resistance and other inflammatory diseases involving neutrophils.

TABLE OF CONTENTS

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

LIST OF ABBREVIATIONS

BoLA	bovine leukocyte antigen
BTA	*Bos taurus* autosome
BTSCC	bulk tank somatic cell count
CNS	coagulase-negative Staphylococci
DES	dairy experiment station
DHIA	Diary Herd Improvement Association
gDNA	genomic DNA
HAS	*Homo sapien* autosome
IL	interleukin
IMI	intramammary infection
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LSM	least squares means
LTB	leukotriene B
MHC	major histocompatability complex
MTES	Middle Tennessee Experiment Station
NRAMP	natural resistance associated macrophage protein
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyte
QTL	quantitative trait loci
RFLP	restriction fragment length polymorphism
SCC	somatic cell count
SCS	somatic cell score
SNP(s)	single nucleotide polymorphism(s)
TNF	tumor necrosis factor

Part I

INTRODUCTION

Mastitis, an inflammation of the mammary gland caused predominantly by infiltration of the teat by bacteria, continues to be the most economically devastating disease affecting the dairy industry. Although control measures such as maintaining a sanitary environment and use of pre- and post-milking teat disinfectants and dry-cow therapies help control mastitis, mastitis continues to be a formidable problem. Furthermore, sire and dam selection for decreased mastitis has not been successful due to low heritability and lack of relevant phenotypic information. Thus, the ability to identify and cull susceptible animals would be of great benefit, as producers could eliminate costly treatment regimens as well as provide a healthier and safer milk supply for consumers.

The ability to select disease resistant cattle based on genetic information has been much discussed in recent years. Genes associated with neutrophil function are potential genetic markers for mastitis as neutrophil migration from blood to the site of infection is essential for resolution of mastitis (Kehrli, 2001; Paape, 2000). The bovine CXCR2 gene is a potential candidate gene for mastitis resistance as it encodes the CXCR2 chemokine receptor, which is involved in neutrophil activation and chemotaxis during infection. The bovine CXCR2 gene lies in close proximity on BTA 2 to the natural resistance associated macrophage protein (NRAMP)-1, which has been associated in mice with susceptibility to infections by intracellular pathogens (Blackwell, 2000). Several single nucleotide polymorphisms (SNPs) have been identified within both the human and bovine CXCR2 gene. Human CXCR2 SNPs have been associated with susceptibility to inflammatory diseases (Kato, 2000; Renzoni,

2000). To date, there have been no studies examining the association of bovine SNPs with disease incidence.

Therefore, the bovine CXCR2 gene is an excellent candidate genetic marker for mastitis resistance and/or susceptibility in dairy cattle. Due to the close location of interleukin-8 receptor gene loci to NRAMP-1, the importance of CXCR2 in immune function, and the polymorphic nature of the bovine CXCR2 gene, we hypothesize that interleukin-8 receptor haplotypes formed by SNPs, exist in various frequencies in Jersey and Holstein cattle, and these haplotypes are associated with mastitis resistance or susceptibility. **To test this hypothesis, we had three specific objectives: 1) to identify SNPs and resulting haplotypes within a segment of bovine CXCR2; 2) determine SNP and haplotype CXCR2 frequencies; and 3) identify relationships of these SNPs/haplotypes with subclinical and clinical mastitis incidence.** Identification of a genetic marker associated with mastitis susceptibility or resistance would allow producers to decrease costs associated with mastitis by improving herd health through sire selection. Furthermore, definitive separation of mastitis resistant or susceptible cattle populations would promote further research aimed at better understanding of immune mechanisms involved in mastitis and would allow development of more effective therapies and preventive treatments for mastitis.

REFERENCES

Blackwell, J.M., Searle, S., Goswami, R., Miller, E.N. 2000. Understanding the multiple functions of NRAMP1. Microbes and Infection 2, 317-321.

Kato, H., Tsuchiya, N., Tokunaga, K. 2000. Single nucleotide polymorphisms in the coding region of human CXC-chemokine receptors CXCR1, CXCR2 and CXCR3. Genes and Immunity.

Kehrli, M.E., Jr., Harp, J.A. 2001. Immunity in the mammary gland. Veterinary Clinics of North America: Food Animal Practice 17, 495-516.

Paape, M.J., Shafer-Weaver, K., Capuco, A.V., van Oostveldt, K., Burvenich, C. 2000. Immune survalence of mammary tissue by phagocytic cells. Advances in Experimental Medicine and Biology 480, 259-277.

Renzoni, E., Lympany, P., Sestini, P., Pantelidis, P., Wells. A., Black, C., Welsh, K., Bunn, C., Knight, C., Foley, P., du Bois, R. 2000. Distribution of novel polymorphisms of the interleukin-8 and CXC receptor 1 and 2 genes in systemic sclerosis and cryptogenic fibrosing alveolitis. Arthritis and Rheumatism 43, 1633-1640.

Part II

LITERATURE REVIEW

I. MASTITIS AND THE MAMMARY GLAND

A. Mastitis-Causing Pathogens

Bovine mastitis is a dynamic disease and continues to be the most economically devastating disease affecting the dairy industry each year. Economic losses related to mastitis result from discarded milk, decreased milk yield, treatment costs, culling or death of affected cows (Fetrow, 2000; Gilmore, 1977; Hansen, 1979; Jones, 1994; Shanks, 1977; Short, 1990). Mastitis has been estimated to affect all herds in the United States, regardless of location, climate, or breed of cow. In 1995, producers reported 26.7% of culled cows and 16.3% of adult-cow deaths were due to mastitis or mastitis related problems (AIPL, 2003).

Several factors influence cow susceptibility to infection, including type of pathogen, climate, season, parity, stage of lactation and individual cow genetics (Oliver, 1983; Ruegg, 2003; Smith, 1985). Due to these many variables, the incidence of mastitis varies among herds as well as between different breeds of dairy cattle. Older cows have been shown to have increased somatic cell scores, which are associated with increased mastitis (Eberhart, 1972; Natzke, 1972). Stage of lactation and season of sampling have also been shown to influence mastitis incidence. Studies have shown that cows have increased somatic cell counts (SCC) during the first two weeks of lactation, as well as during the summer and autumn months (Blackburn, 1969; Miller, 1976; Simensen, 1976). Genetics also play a large role in mastitis prevalence, as cows selected for

increased milk yield, such as cows within the Holstein breed, have been associated with increased mastitis (AIPL, 2003). Good management practices are critical for mastitis prevention and control (Gill, 1990; Jayarao, 2003; Ruegg, 2003). Cleanliness of freestall areas, sanitation during udder preparation and milking procedures, as well as types of bedding all have been shown to affect mastitis prevalence (Bushnell, 1984; Hogan, 1989; Pankey, 1993). While sanitary practices reduce mastitis, complete elimination of infections is not a realistic goal due to the non-sterile dairy environment and competitive nature of intramammary pathogens (Erksine, 1989; Oliver, 1984).

The diversity of mastitis-causing bacteria contributes to difficulties surrounding the control of mastitis. Mastitis pathogens are categorized as contagious or environmental pathogens based on their modes of transmission (Bramley, 1990; Smith, 1983). Contagious pathogens are adapted to survive and multiply within the mammary gland. Transmission occurs from infected cow to uninfected cow primarily during milking and has been linked to unsanitized milking equipment and poor hygienic practices (Fox, 1993; Neave, 1969). In contrast, environmental pathogens are found everywhere in the dairy environment and teat infection can occur at any time (Smith, 1993). As contagious pathogens are eliminated from a herd, environmental pathogens are more apt to cause infection and are the most prevalent causes of intramammary infections (IMI) in well-managed dairy herds (Eberhart, 1977; Erksine, 1989; Oliver, 1984; White, 2001; Wilson, 1997). Better understanding of specific

pathogen and host interactions during IMI will provide greater insight to developing improved management and preventative strategies.

1. Contagious pathogens

The primary reservoir of contagious intramammary bacteria is the mammary gland and resulting infections are generally subclinical in nature. Contagious pathogens include *Streptococcus agalactiae, Staphylococcus aureus*, and *Mycoplasma* spp., and *Corynebacterium bovis* (Bramley, 1984; Crist, 1997; Fox, 1993). Bulk tank somatic cell counts (BTSCC) >300,000 cells/ml, frequent recurrent clinical mastitis, and >15% of cows in a herd with a Dairy Herd Improvement Association (DHIA) somatic cell score (SCS) ≥ 5 indicates the presence of contagious organisms within the herd (Bramley, 1990; Crist, 1997; Erksine, 1989; Hogan, 1988).

Contagious infections usually occur during milking due to poor hygienic practices when handling the udder and milking equipment (Ruegg, 2003; Sischo, 1993; Wilson, 1997). Use of pre- and/or post-milking teat disinfectants and antibiotic dry cow therapy helps control mastitis due to contagious pathogens. Factors contributing to increased mastitis include injury to the teat, diets deficient in selenium and vitamins A and E, in addition to poor maintenance of milking equipment (Bramley, 1984; National Mastitis Council, 2001). Infected cows, newly introduced into the herd, are also sources of contagious pathogens (Fox, 1993). Thus, proper udder preparation, testing new herd-mates, and balanced diets are extremely important in the control of contagious mastitis (Fox, 1993).

Several mechanisms enable contagious bacteria to evade mammary gland immune defenses and establish infection. Once exposed to the teat, bacteria colonize the teat end and streak canal before migrating and attaching to epithelial cells within the teat and mammary gland (Calvinho, 1998; Dodd, 1975; Filipssen, 1990; Harmon, 1986; Notermans, 1979; Razin, 1992; Sutra, 1994). Contagious pathogens may then be internalized by epithelial cells, which may help protect the organism from phagocytic cells such as macrophages and neutrophils (Calvinho, 1998; Fernard, 1982; Almeida; 1996; Isberg, 1991). Several virulence factors also enable contagious bacteria to bind to epithelial cells and escape phagocytosis. Capsules, protein A, and other toxins allow *Staph. aureus* to evade neutrophil phagocytosis, while *Mycoplasma* spp. produce cell surface proteins that decrease activation of mammary humoral and cellular immune responses (Bennett, 1978; Haraldsson, 1984).

Timely and effective treatment of contagious mastitis is necessary as prolonged infections can cause permanent destruction mammary tissue (National Mastitis Council, 2001). Severe *Strep. agalactiae* infections can scar milk producing tissues through destruction of mammary cisterns and ductal tissues during the inflammatory process (National Mastitis Council, 2001; Keefe, 1997). *Staphylococcus aureus* infections produce deep abscesses in the ductal tissue, contributing to permanent tissue damage and decreased antibiotic effectiveness (Gudding, 1984). *Mycoplasma bovis* infections are difficult to treat and damage secretory tissues by causing fibrosis and abscess formation within the udder (National Mastitis Council, 2001; Gonzalez, 1992; Pfutzner, 1996). Thus,

producers incur mastitis-related costs from culling chronically infected cows, increased antibiotic treatment and decreased milk production from damaged mammary tissues. The ability to select cattle that exhibit natural resistance to contagious pathogens will decrease economic losses and improve herd health.

2. Environmental pathogens

Unlike contagious bacteria, environmental pathogens are present everywhere in the dairy cow's surroundings. Infection can occur at any time in the cow's life. Increased rates of clinical mastitis, especially during hot and wet periods of weather, indicate the presence of environmental bacteria within the herd (Crist, 1997; Hogan, 1988; Smith, 1985). Environmental pathogens include streptococci, coagulase-negative staphylococci (CNS), coliforms, and gram-negative bacteria (Hogan, 1999; Jayarao, 1999; Jayarao, 2003). The most commonly isolated environmental bacteria include *Streptococcus uberis*, *Streptococcus agalactiae* subsp. *dysgalactiae*, *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., and CNS (Crist, 1997; Smith, 1993).

Cows express increased susceptibility to environmental mastitis during the first three weeks of the dry period and a few weeks before to a few weeks after calving due to physical and hormonal changes associated with involution and parturition (Crist, 1997; Oliver, 1989; Smith, 1985; Todhunter, 1995). Following the last milking of lactation, milk accumulates in the udder, increasing mammary pressure and aiding in the induction of mammary involution. Increased mammary pressure and leakage due to milk accumulation, as well as length of

involution may increase the mammary gland's susceptibility to mammary infections (Hurley, 1987; Oliver, 1989). After approximately three weeks, mammary involution is complete and the risk of mastitis decreases. However, the mammary gland's susceptibility to mastitis increases again 2-3 weeks prior to parturition (Eberhart, 1986). The mammary gland prepares for the onset of lactation by increasing mammary tissue growth and secretions. These changes increase mammary pressure and leakage, thereby compromising the protective barrier of the closed teat end and increasing susceptibility to new infections (Eberhart, 1986).

Management practices that reduce teat exposure to bacteria help minimize infections (Smith, 1983). The same hygienic management practices associated with decreasing contagious infections apply for controlling environmental mastitis pathogens. Extra efforts to maintain a clean and dry environment, such as removing manure and providing fresh bedding, also contribute to controlling infections. Pre- and post-milking teat disinfectants are extremely important in minimizing infection (Crist, 1997; Smith, 1985; Todhunter, 1995). If teat ends are not properly sanitized before milking, bacteria can enter the teat through the streak canal.

Once inside the teat, pathogens bind to epithelial cells and begin gland colonization (Lammers, 2001). Several studies have identified numerous bacterial surface proteins that aid in the binding and internalization of bacteria to epithelial cells, colonization of the mammary gland, and avoidance of the immune system for *Strep. agalactiae* subsp. *dysgalactiae*, *Strep. uberis* and *E. coli*

(Calvinho, 1998; Oliver, 1998; Kaipainen, 2002; Riberio, 2002). *Streptococcus* spp. and CNS attach to epithelial cells within the teat cistern and can lead to infections in the ductal and alveolar tissues (Calvinho, 1998; Wattiaux, 1999). Coliform bacteria differ from other environmental pathogens as some strains attach to epithelial tissues, while others do not bind to target cells. Rather, they multiply rapidly in milk and secrete toxins that are absorbed in the blood stream (Lammers, 2001; Wattiaux, 1999).

If environmental mastitis infections are left untreated, permanent damage can occur to milk-producing tissues. Difficulties surround therapeutic efforts against environmental pathogen infections, as dry-cow antibiotic treatments are less effective against environmental streptococci when compared with cure rates for contagious pathogens. Furthermore, both antibiotic treatments and dry-cow therapies are ineffective against coliform infections (Eberhart, 1986; Hogan, 1987; Smith, 1985). Guterbock et al. (1993) determined that bacterial cure rates for clinical non-coliform mastitis cases ranged only from 27.3 - 61.1% within three commercial herds treated with amoxicillin or cephapirin. Cows treated with oxytocin exhibited similar cure rates within the same experiment. However, antibiotic treatments may lead to variable withdrawl periods for residues in milk and meat, as well as contribute to bacterial antibiotic resistance (Erksine, 1993; Wilson, 1995; Wilson, 1996). Other types of preventative treatments, such as teat disinfectants, have been shown to reduce environmental streptococci and CNS infections at calving (Eberhart, 1983; Hogan, 1987; Smith, 1985). While sanitary management practices, dry-cow therapies and antibiotics may help

control environmental mastitis infections, the ability to select cows that exhibit natural resistance to mastitis pathogens would minimize health care costs and decreased production due to discarded milk during antibiotic withdrawl periods and permanently scarred mammary tissues.

B. Neutrophil Function

Polymorphonuclear leukocytes (PMN), or neutrophils, are a component of innate immunity. Neutrophils migrate from blood to mammary tissue during lactation, providing an immediate defense to bacterial infection (Paape, 1977). As milk is produced, neutrophils are flushed into the alveolar lumen and milk. However, once in milk, neutrophils have a short life-span (1-2 days) before undergoing apoptosis (Carlson, 1973; Paape, 1979). Apoptosis is necessary to help eliminate less-active cells as the ingestion of fat and casein in milk decreases neutrophil phagocytic abilities. However, neutrophils also exhibit decreased viability during early stages of lactation, which may be associated with a decreased ability to maintain neutral cytosolic pH (Jankowski, 2002; Mayer, 1989). The milking process thus provides a continuous immune defense through the constant attraction of healthy neutrophils from blood to mammary tissue and removal of dead neutrophils through milking (Paape, 2002; Paape, 1977).

Although neutrophils are present in mammary tissue and milk before infection, their numbers are relatively low. Studies have shown that neutrophils make up only 3-23% of cells found in milk from healthy mammary glands (Lee, 1980; Miller, 1993; Ostensson, 1988). However, once infection occurs,

macrophages ingest bacteria and release inflammatory mediators. This release initiates the further release of inflammatory mediators and soluble factors by epithelial cells and lymphocytes, as well as neutrophil diapedesis and chemotaxis from the circulating blood to the site of infection (Craven, 1983; Paape, 1979; Riollet, 2000). Neutrophils make up approximately 90-95% of somatic cells collected from milk within an infected quarter (Lee, 1980; Paape, 1981). Neutrophils then phagocytose and kill intramammary bacteria through reactive oxygen species generation and degranulation (Parham, 2000). Once an infection is cleared, neutrophils undergo apoptosis and are phagocytosed by macrophages (Savill, 1989). This rapid clearance is important as neutrophil lysosomal enzymes and respiratory burst products can damage mammary gland tissue (Capuco, 1986; Harmon, 1982; Paape, 2000). However, without quick and efficient neutrophil migration to the mammary gland, infections can persist and lead to severe clinical mastitis, severe tissue damage and potentially death of the cow.

1. Neutrophil chemotaxis

Neutrophils develop and mature in bone marrow before their release into blood (Bainton, 1992). Neutrophil migration during the early stages of infection is critical for the resolution of most IMI (Kehrli, 2001). The direction of neutrophil diapedesis is controlled by adhesion molecules, as well as release of chemoattractants from invading pathogens, injured cells within infected tissues, and phagocytic cells at the site of infection (Ben-Baruch, 1995; Zhelev, 2002).

The binding of adhesion molecules allows neutrophils to migrate towards the site of infection. Inactivated neutrophils constantly search for inflammatory mediators on vascular endothelium surfaces by rolling along the surface. Rolling slows when L-selectin (CD62L) receptors on neutrophil surfaces bind to target molecules on endothelial cells and allows neutrophils to detect the presence of E- and P-selectin on endothelial cell surfaces (Burton, 2003; Diez-Fraile, 2002a; Kansas, 1996). Upon infection, endothelial cells upregulate E- and P-selectins, CD62E and CD62P, which bind to neutrophils rolling along blood vessels and concentrating them to sites of inflammation (Burton, 2003). Proinflammatory mediators, such as interleukin (IL) -1α, β, -6, -8, tumor necrosis factor (TNF) -α, complement factor 5a, platelet activating factor and leukotriene B_4 (LTB_4), migrate to the surface of endothelial cells and interact with neutrophils, causing down regulation of CD62L and upregulation of β_2-integrins, and intercellular and vascular cell adhesion molecules (Diez-Fraile, 2002b; Shuster, 1993). β_2-integrins such as CD11a, b and CD18, as well as intercellular and vascular adhesion molecules, are involved in adherence of neutrophils to endothelial surfaces and aid in neutrophil diapedesis and chemotaxis (Diez-Fraile, 2002b; Ley, 2002).

Once through the cardiovascular endothelium, activated neutrophils follow chemoattractant gradients to the site of infection (Huges, 1998; Mackay, 1993; Zhelev, 2002). Neutrophil activation is caused by cell signaling pathways that lead to coupling and uncoupling of adhesion molecules, F-actin polymerization, which is required for neutrophil motility, proteolytic enzyme release and

superoxide anion production (Ali, 1999; Arai, 1996). However, as of yet, activation of signal transduction pathways that result in neutrophil chemotaxis is not completely understood (Zhelev, 2002).

2. Neutrophil-interleukin-8 interaction

The interaction between neutrophils and interleukin-8 is critical for neutrophil migration and chemotaxis (Frendeus, 2000). Interleukin-8 is a chemokine released by monocytes, macrophages, neutrophils, T lymphocytes, epithelial cells, endothelial cells, fibroblasts, keratinocytes, synovial cells, chondrocytes, and mitogen-stimulated T cells (Gregory, 1988; Schroeder, 1988). These cells release IL-8 after stimulation by IL-1α, β, and TNF-α from monocytes and macrophages following bacterial phagocytosis (Lindley, 1998). Interleukin-8 forms a concentration gradient within the extracellular matrix from the infection site to the vascular endothelium. Neutrophils recognize IL-8 and become activated, causing changes in neutrophil shape, regulation of surface adhesion molecule expression, adherence to cytokine-activated endothelial tissues and chemotaxis (Feniger-Barish, 2003; Lindley, 1998; Peveri, 1988; Thelen, 1988). Neutrophils then phagocytose foreign pathogens, generate reactive oxygen species and release other inflammatory mediators to elicit and maintain inflammation (Riollet, 2000).

Neutrophils recognize IL-8 through two surface receptors, CXCR1 and CXCR2 (Lee, 1992; Skelton, 1999). These two surface receptors belong to the seven transmembrane protein family, characterized by three intracellular and

three extracellular loops (Murphy, 1994). The amino terminus is located on the extracellular region of the neutrophil membrane, while the carboxyl terminus is located intracellularly in the cytoplasm. Structurally similar, the IL-8 receptor amino acid sequences express 77% homology, but exhibit different binding characteristics. CXCR1 binds only IL-8 with high affinity. CXCR2 also binds IL-8 with high affinity, as well as other structurally and functionally related peptides such as monocyte-granulocyte stimulating antigen, growth related oncogene-α and neutrophil activating peptide-2 (LaRosa, 1992; Lee, 1992). The amino terminus determines receptor-binding specificity. Peptides that interrupt the amino terminus inhibit both CXCR1 and CXCR2 recognition and binding of IL-8 (LaRosa, 1992; Suzuki, 1994). In contrast, the ability of the receptor ligand complex to internalize and initiate signal transduction has been related to the cytoplasmic carboxyl terminus of IL-8 receptors. Signal transduction activates coupling of G-proteins attached to the third internal loop of the CXCR2 receptor (Ben-Baruch, 1995; Richardson, 2003). Activation of the signal pathway results in phagocytosis of the foreign pathogen and respiratory burst (Baggiolini, 1992).

3. Neutrophil phagocytosis

Upregulation of antigen-recognition receptors is required for neutrophil phagocytosis of pathogens in the mammary gland. During chemotaxis, neutrophil receptors that recognize both non-opsonized fragments of bacterial cell walls and opsonized bacterial particles are upregulated due to continuous exposure to proinflammatory cytokines such as LTB_4, IL-8, granulocyte-

monocyte-colony simulating factor, TNF-α and interferon-γ (Mitchell, 2003; Wright, 1992). Several different receptors responsible for recognizing foreign antigens and bacteria are required, as mastitis-causing pathogens do not elicit the same intramammary response. Neutrophil complement receptors, CR3 and CR1, bind to bacterial particles coated with C3b or C3bi complement fragments, while neutrophil-Fc receptors bind to IgG_2 opsonized bacteria (DiCarlo, 1996; Worku, 1994). Lipopolysaccharide-binding protein (LBP) opsonizes lipopolysaccharide (LPS), which is present on gram-negative bacterial cell surfaces. Neutrophil CD14 surface receptors bind to the LBP-LPS complex which signals the presence of gram-negative pathogens, initiates upregulation of C3R and CD18 surface molecules, increasing activation of neutrophils (Paape, 1996; Wright, 1992).

Binding of opsonized microbes and particles to neutrophil receptors initiates phagocytosis through a series of complex signal transduction pathways (Dewitt, 2002; Garcia-Garcia, 2002; Zhong, 2003). Once bacteria and particles are engulfed within a phagosome, neutrophils undergo a large respiratory burst, intaking large amounts of oxygen to produce toxic oxygen radicals including superoxide anions, hydrogen peroxides, and hydroxyl radicals (Babior, 1973; Klebanoff, 1992). Following phagocytosis and the oxidative burst, lysosomes within the cytoplasm bind to phagosomes and undergo degranulation, releasing antimicrobial proteins and enzymes into the phagosome to further neutralize intramammary pathogens (Burton, 2003; Chen, 2003).

Mature neutrophils have a relatively short lifespan and live in tissues only 1-2 days (Lee, 1993; Wang, 2003; Whyte, 1993). Although inflammatory mediators such as IL-8 and GRO-α have been shown to delay the onset of programmed cell death, neutrophils in tissues eventually exhibit decreased migration, phagocytosis, degranulation and respiration abilities, which have been linked to neutrophil apoptosis (Glynn, 2002; Kettritz, 1998; Whyte, 1993; Yagi, 2002). During apoptosis, cell and nucleus structures change until the cell breaks apart into membrane bound vesicles called apoptotic bodies (Paape, 2000). Macrophages and other phagocytes recognize neutrophil apoptotic bodies and ingest them through phagocytosis. Apoptotic bodies are then neutralized and degraded, preventing harmful and toxic molecules and substances from neutrophil lysosomes and granules being released and damaging surrounding mammary tissue (Adams, 1992; Paape, 2002; Paape, 2000).

Neutrophils are a critical component of the innate immune system and their presence within the mammary gland is crucial for the resolution of infection. While the milking process creates a continuous presence of neutrophils, clearance of infection requires a large influx of neutrophils from blood to the mammary gland. Neutrophil identification of intramammary pathogens, migration to sites of infection, bacterial phagocytosis, and apoptosis are important factors in clearance of mastitis pathogens. Several pro-inflammatory mediators and chemoattractants are released from phagocytic and other cells that activate neutrophils and initiate chemotaxis. The interaction of neutrophils with the chemoattractant IL-8 regulates many of these steps. The ability to select cows

exhibiting more efficient neutrophil responses due to IL-8 interactions may contribute to more efficient immune responses to IMI, and thus, increased mastitis resistance.

II. GENETIC IMPROVEMENT OF MASTITIS

A. Current Selection Methods in Dairy Production

Genetic improvement is viable when an easily identifiable and heritable trait is associated with a desirable and measurable phenotype (Shook, 1993). Selection to improve herd performance within the dairy industry has historically focused on production and conformational traits such as milk yield, SCS, udder type and body size (Hansen, 2000; Nash, 2002). Improvement for these traits also occurs within a relatively short time as heritabilities for these traits range from 0.15 to 0.40 (Dickinson, 1985). With the advent of artificial insemination in the dairy industry, improvement of selected traits has occurred rapidly (Hansen, 2000; Shook, 1993).

Selection for performance traits has both directly and indirectly lead to increased milk yield across all dairy breeds (AIPL, 2003). Unfortunately this also has increased mastitis incidence as milk yield and mastitis are correlated (0.20-0.30) (Hansen, 1979; Rogers, 1998; Shook, 1993; Shook, 2001; Strandberg, 1989). In efforts to reduce mastitis incidence, measurable traits associated with mastitis, including severity and incidence of clinical mastitis, SCS and udder type characteristics, were examined (Detilleux, 1995; Heringstad, 2000; Rogers,

1991). Although clinical mastitis is a direct measure of mastitis susceptibly, it exhibits low heritabilities and is difficult to measure accurately. Clinical mastitis data also represent only one aspect of mastitis, as the majority of infections are subclinical. This method of selection is further limited because very little herd data exist for clinical or subclinical mastitis incidence within the United States (Ruegg, 2003; Heringstad, 2000; Nash, 2002; Odegard, 2003). Because of these limitations, SCS and udder type traits are often used in combination as the primary selection criterion for mastitis resistance as they are more heritable and correlate with clinical mastitis occurrence (Lund, 1994; Nash, 2002; Rogers, 1991; Shook, 1994). However, as both SCS and udder type traits are indirect measures of mastitis, a more accurate predictor of mastitis resistance is still needed.

1. Selection utilizing mastitis records

Accuracy of most available clinical mastitis records is problematic as there are no standardized descriptions of symptoms, duration, detection of clinical signs, or treatment methods (Detilleux, 2002; Roberson, 2003; Ruegg, 2003; Shook, 1993). Thus, due to lack of mastitis histories within the United States and variabilities associated with available records, selection based on mastitis records does not appear to be a viable option.

2. Selection utilizing somatic cell counts and scores

Estimates of subclinical mastitis can be derived from BTSCC or herd SCC from DHIA surveys. However, these estimates of subclinical mastitis may not be entirely reliable as BTSCC and DHIA SCC estimates may vary from actual herd SCC due to calculation methods, sampling errors (Jayarao, 2003; Ruegg, 2003). Furthermore, subclinical mastitis prevalence may be difficult for producers to recognize as BTSCC may rise and fall slowly and at irregular intervals (Ruegg, 2003).

Somatic cell scores are the logarithmic transformation of somatic cell counts. SCS are more reliable measures of mastitis than raw SCC, as SCS exhibit increased heritabilities compared to SCC (Shook, 1993). Somatic cell scores are widely available and easily obtainable due to the participation of the majority of dairy herds within the United States (AIPL, 2003; Shook, 1993). Several studies have determined a strong genetic correlation between clinical mastitis and SCS, ranging from 0.60 to 0.80 (Emmanuelson, 1988; Rogers, 1993; Rogers, 1995; Shook, 1993). In 1994, the United States Department of Agriculture Animal Improvement Programs Laboratory began publishing genetic evaluations for SCS, in efforts to aid in the selection process against mastitis (Schutz, 1995; Shook, 1994).

Although selecting for SCS has been effective in reducing the ever-increasing incidence of mastitis, it still has considerable limitations. First, SCS exhibit a heritability of approximately 0.10 (Banos, 1990; Boettcher, 1992; Heringstad, 2000). As such, improvements due to selection of low SCS have

occurred slowly over several generations. Low heritability is not surprising as SCS most likely respond to a variety of stimuli, including management practices, age, stage of lactation, and parity (Castillo-Juarez, 2000; Castillo-Juarez, 2002; Heringstad, 2000; Shook, 1993). Somatic cell scores also are affected by the specific pathogen causing mastitis as mammary gland response varies to different pathogens (Heringstad, 2000; Schukken, 1999; Ward, 1972). Furthermore, mastitis incidence based on SCS may be underestimated because the short duration of increased SCC characteristic of clinical mastitis were not evident in monthly sampling. Shook and Schutz (1994) estimates that only 10-20% of clinical mastitis cases are detected by monthly sampling for SCC. Overall, SCS are highly associated with clinical mastitis, widely available and easily obtainable. However, this indirect method of selection against mastitis is limited due to low heritabilities and dependency upon several factors.

3. Selection utilizing conformation and udder type

Conformational traits are utilized in dairy selection because they are highly heritable (0.10 - 0.41) and allow for genetic improvement to occur relatively quickly (Gengler, 1998). Boettcher et al. (1993) determined that cows selected for high milk yield were taller, stronger and deeper, had more dairy appearance and had improved rear udders and rear clefts. Studies also have shown large frame, high-production cows incur more costs due to digestive disorders, increased feed and labor, as well as mastitis (Hansen, 1979; Mahoney, 1986).

However, increased body size has also been associated with decreased herd life (Cruickshank, 2002).

With respect to mastitis and injury, udder morphology is important. Udder composite scores, which are comprised of udder depth, cleft, height, teat spacing and teat diameters, were moderately correlated with clinical mastitis (Rogers, 1998). Low-hanging udders, wide-set teats, and large, flat teat ends have been associated with increased mastitis incidence due to their physiologic predisposition for exposure to mastitis pathogens (Monardes, 1990; Rogers, 1991; Seykora, 1985; Seykora, 1986). Large teat diameters and longer teat ends also have been associated with liner slips during milking, which may contribute to mastitis (Rogers, 1991). As such, selection for higher placed udders and closely spaced teats has been correlated with reduced clinical mastitis (Rogers, 1993).

Nash et al. (2002) determined daughters of sires that transmitted strongly attached fore udders were associated with more severe *Streptococcus* spp. infections during the first lactation. Daughters of sires that transmitted shallow udders were associated with increased severity of streptococcal infections as well as longer clinical CNS infections during the first lactation. However, daughters with shallow udders also were associated with less severe clinical infections for all tested pathogens during the second lactation. Closely spaced teats were associated with more severe streptococcal infections and longer duration of clinical mastitis for several pathogens during the first lactation. Longer teats were associated with a decrease in severity of clinical mastitis caused by coliforms, while daughters of sires that transmitted shorter teats were

associated with more severe streptococcal infections. Thus, Nash and colleagues concluded that while selection for improved udder traits may minimize mastitis and associated costs, many of these traits are variable and other influences affecting mastitis severity and duration exist.

B. Marker-Based Selection Methods

Breeding selectively for resistant phenotypes is necessary due to the huge economic impact of mastitis on the dairy industry. Identification of genetic loci linked to mastitis would allow producers to select herd replacements carrying desirable molecular markers such as quantitative trait loci (QTL) and single gene markers. Several QTL have been associated with health and production traits in dairy cattle (Ashwell, 1998; Ashwell, 1997; Heyen, 1999; Kalm, 1998; Klungland, 2001). However, associating a single QTL with disease and immunity is difficult as several QTL on different chromosomes have been associated with the same trait (Heyen, 1999; Kalm, 1998). Single gene markers associated with immune function in dairy cattle include the NRAMP1 and bovine leukocyte antigen (BoLA) (Ables, 2002; Grosse, 1999; Heaton, 2001; Horin, 1999; Ostergard, 1989). However, studies examining these marker loci have had contradicting results regarding their impact on immunity and disease resistance. Identification of loci associated with efficient immune responses to mastitis would lead to a better understanding of genetic mechanisms underlying immune function.

1. Quantitative trait loci

Quantitative trait loci are regions on chromosomes associated with measurable characteristics such as production and health traits (Crawford, 2000). Genetic markers used in the identification of QTL primarily include microsatellites and restriction fragment length polymorphisms, as they are the most abundant markers available.

Many QTL have been identified for dairy cattle traits such as milk yield, protein percentages, and conformation traits (Ashwell, 1998; Ashwell, 1999; Kuhn, 2003; Schrooten, 2000; Van Tassell, 2000). Quantitative trait loci were first associated with SCC and were later expanded to identify other economic traits including milk production, calving ease, and conformational traits (Da, 1994). Studies have identified many QTL associated with several of these economic traits, including markers on chromosomes 3, 4, 6, 7, 9, 10, 12, 14 and 27 (Ashwell, 1998; Klungland, 2001; Van Tassell, 2000). In particular, QTL on chromosomes 10 and 12 were associated with increased SCC. However, fewer loci have been associated with clinical mastitis and other disease traits due to lack of health records (Ashwell, 1998; Ashwell, 1999; Ashwell, 2000; Heyen, 1999; Kalm, 1998; Klungland, 2001; Van Tassell, 2000). Klungland et al. (2001) identified several QTL on chromosomes 3, 4, 6, 14 and 27 associated with clinical mastitis. However, SCC were not affected by these same loci, indicating that loci associated with SCC may be useful indicators of immune function but not necessarily informative markers for mastitis. This may be related to the large chromosome region encompassed by QTL, creating a great deal of variability.

This is supported by several studies that have shown that QTL for specific traits such as clinical mastitis and milk yields may vary between different families within the same cattle breed (Ashwell, 1998; Klungland, 2001). Thus, the need to identify smaller, more localized markers or genes directly related to specific function exists. This may be possible through specific gene markers.

2. Bovine leukocyte antigen

The bovine major histocompatability complex (MHC), also known as the bovine leukocyte antigen, encodes highly polymorphic cell-surface glycoproteins (Amorena, 1978; Spooner, 1978; Van Eijk, 1992). The MHC genes are divided into three classes. Class I and II genes encode proteins responsible for antigen presentation to T cells, while class III encodes several complement components. All nucleated cells express class I peptides, which present antigenic proteins to cytotoxic T cells. Class II proteins present antigenic peptides to helper T cells and are expressed primarily on immune cells such as macrophages, B cells and some T cells.

Numerous alleles within the BoLA complex, especially for MHC class I and II, have been characterized in different breeds of dairy cattle (Aarestrup, 1995; Dietz, 1997; Gillespie, 1999; Kelm, 1997; Sharif, 1998; Siite, 2002; Spooner, 1978; Vage, 1992; Van Eijk, 1992; Weigel, 1990). Several studies have related BoLA alleles with SCC and incidence of clinical mastitis (Table 1). However, BoLA alleles expressed across different breeds of cattle showed contradicting associations with mastitis incidence and SCC. While both Dietz et al. (1997) and

Table 1. BoLA haplotypes associated with mastitis parameters.

Breed	BoLA Haplotype	Phenotype	Reference
Canadian Holstein	DRB3.2*16	Decreased SCC	Sharif et al., 1998
	DRB3.2*23	Increased Coliform mastitis incidence	Sharif et al., 1998
U.S. Holstein	DRB3.2*16	Increased mastitis incidence	Dietz et al., 1997
	DRB3.2*16	Increased SCC	Kelm et al., 1997
	DRB3.2*11 *23 *3	Decreased clinical mastitis incidence	Kelm et al., 1997
	BoLA Class I CA42	Increased risk of *S. aureus* mastitis	Mallard et al., 1995
	BoLA Class I W11	Decreased clinical mastitis incidence	Weigel et al., 1990
Swedish Red And White	BoLA Class I DQ1A	Increased clinical mastitis susceptibiliy	Lunden et al., 1990

Kelm et al. (1997) associated allele DBR3.2*16 in U.S. Holsteins with increased mastitis incidence and increased SCC, respectively, Sharif et al., (1998) associated the same allele with decreased SCC in Canadian Holsteins. Furthermore, Kelm et al. (1997) identified allele DRB3.2*23 with decreased clinical mastitis incidence in U.S. Holsteins, but was associated with increased coliform mastitis in Canadian Holsteins. This variability may indicate that other breed-specific genes may be more influential on mastitis incidence than the BoLA loci.

Mastitis is a complex trait governed by genes responsible for several immune mechanisms. The large number of alleles and variety of immune functions associated with BoLA alleles indicates other genetic loci may strongly affect immune function. Furthermore, the MHC-antigen presenting mechanism may not be the most reliable and efficient mechanism on which to base mastitis selection. Other cellular functions, such as macrophage or neutrophil activities during mastitis, may be more strongly related to the immune response. These may provide genetic markers more strongly related to the immune response during mastitis.

3. Natural resistance associated macrophage protein (NRAMP) 1

NRAMP1 is a potential candidate gene that may be associated with immune response to mastitis infection. NRAMP1 is a cation transporter and aids in maintaining an acidic environment in macrophage lysosomes (Flemming, 1998; Gruenheid, 1999; Kuhn, 1999). This mechanism is important in

macrophage activation during the early stages of infection as it helps regulate expression and release of IL-1β, inducible nitric oxide synthase, nitric oxide, TNF-α, and MHC II molecules (Blackwell, 1989; Blackwell, 1996; Blackwell, 1994; Blackwell, 1991). The NRAMP1 gene is highly involved in macrophage activation and functional responses of cattle to *Salmonella dublin, Mycobacterium bovis,* and *Brucella abortus* infection (Bellamy, 1999). Microsatellite and nucleotide polymorphisms have been detected within different regions of the NRAMP1 gene in cattle (Bellamy, 1999; Ables, 2002; Horin, 1999). Various alleles of NRAMP1 have been associated with resistance or susceptibility to intracellular pathogens including *M. bovis*, and *Leishmania donovani* in mice (Blackwell, 2000; Vidal, 1993). While the NRAMP1 gene has the potential to be a genetic marker for immune function and mastitis resistance, more research is necessary to understand the functional effects of these polymorphisms and how they directly affect bovine immunity.

4. Interleukin-8 receptor (CXCR1/2)

The IL-8 receptor is a critical component in the activation of neutrophils during IMI. Thus, mutations within the IL-8 receptor genes may affect neutrophil function during mastitis. Interleukin-8 receptor genes may be potential genetic markers for mastitis resistance as the gene complex is located near the NRAMP-2 gene, which has been associated with disease susceptibility.

Human IL-8 receptor genes (CXCR1 and CXCR2) are both clustered on chromosome 2q35, and have been mapped approximately 130 kb telomeric to

the NRAMP1 gene (Kato, 2000; White, 1994). Furthermore, evidence of the potential of the CXCR2 gene to serve as a marker for mastitis is the association of a CXCR2 RFLP with rheumatoid arthritis (White, 1994). Bovine CXCR2 genes also are located close to NRAMP1 on bovine chromosome 2 (Grosse, 1999)

Single nucleotide polymorphisms (SNPs) have been detected for both human IL-8 receptors (Kato, 2000). However, a separate study indicated that these particular SNPs were not associated with resistance or susceptibility to rheumatoid arthritis, systemic lupus erythematosus, or scleroderma, Behcet disease, or Sjogren's syndrome (Kato, 2000). However, these SNPs may be associated with other inflammatory diseases.

Grosse et al. (1999) determined sixteen SNPs exist for a cluster of bovine IL-8 receptor loci. Four SNPs conformed to Mendelian inheritance patterns between parents and offspring, and produced five haplotypes. These four polymorphisms occurred within the third exon of CXCR2. Three single nucleotide polymorphisms resulted in silent mutations; however, the fourth polymorphism replaced glutamine with histidine at amino acid 245. This amino acid substitution occurs within the third intracellular loop of the receptor and may affect the ability of neutrophils to internalize the receptor-ligand complex, as well as neutrophil phagocytic ability (Damaj, 1996). However, no studies have analyzed neutrophil immune function with these polymorphisms. Furthermore, genetic analysis of the bovine IL-8 receptor has only occurred in commercial beef cattle and not within dairy breeds. Thus, different polymorphisms associated with neutrophil function may exist for dairy breeds. Further study of the IL-8

receptor's potential as a genetic marker is warranted due to the IL-8 receptor's polymorphic nature, its importance in neutrophil function, and its close relationship to other immune related genes.

REFERENCES

Aarestrup, F.M., Jensen, N.E., Ostergard, H. 1995. Analysis of associations between major histocompatability complex (BoLA) class I haplotypes and subclinical mastitis of dairy cows. Journal of Dairy Science 78, 1684-1692.

Ables, G.P., Nishibori, M., Kanemaki, M., Watanabe, T. 2002. Sequence analysis of the NRAMP1 gene from different bovine and buffalo breeds. Journal of Veterinary Medical Science 64, 1081-1083.

Adams, D.O., Hamilton, T.A. 1992. Macrophages as destructive cells in host defense. In: Gallin, J.I., Goldstein, I.M., Snyderman, R. (ed.), Inflammation: Basic Principles and Clinical Correlates. Raven Press, Ltd., New York, NY, pp. 637-662.

Animal Improvement Programs Laboratory, 2003 AIPL USDA Dairy Information. USDA Animal Improvement Programs Laboratory. <http://www.aipl.arsusda.gov>. access date: 8-01-03.

Ali, H., Richardson, R.M., Haribabu, B., Snyderman, R. 1999. Chemoattractant receptor cross desensitization. Journal of Biological Chemistry 274, 6027-6030.

Amorena, B., Stone, W.H. 1978. Serologically defined (SD) locus in cattle. Science 201, 159-160.

Arai, H., Charo, I.F. 1996. Differential regulation of G-protein-mediated signalling by chemokine receptors. Journal of Biological Chemistry 271, 21814-21819.

Ashwell, M.S., Da, Y., Van Tassell, C.P., Vanraden, P.M., Miller, R.H., Rexroad, C.E.J. 1998. Detection of putative loci affecting milk production and composition, health, and type traits in a United States Holstein population. Journal of Dairy Science 81, 3309-3314.

Ashwell, M.S., Rexroad, C.E., Milller, R.H., VanRaden, P.M., Da, Y. 1997. Detection of loci affecting milk production and health traits in an elite U.S. Holstein population using microsatellite markers. Animal Genetics 28, 216-222.

Ashwell, M.S., Van Tassell, C.P. 1999. Detection of putative loci affecting milk, health, and type traits in a U.S. Holstein using population using 70 microsatellite markers using a genome scan. Journal of Dairy Science 82, 2497-2502.

Ashwell, M.S., Van Tassell, C.P., Sonstegard, T.S. 2000. A genome scan to identify quantitative trait loci affecting economically important traits in a U.S. Holstein population. Journal of Dairy Science 84, 2535-2542.

Babior, B.M., Kipnes, R.S., Curnutte, J.T. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. Journal of Clinical Investigation 52, 741-744.

Baggiolini, M., Dewald, B., Walz, A. 1992. Interleukin-8 and related chemotactic cytokines. In: Gallin, J.I., Goldstein, I.M., Snyderman, R. (ed.), Inflammation: Basic Principles and Clinical Correlates. Raven Press, Ltd., New York, NY, pp. 247-263.

Bainton, D.F. 1992. Developmental biology of neutrophils and eosinophils. In: Gallin, J.I., Goldstein, I.M., Snyderman, R. (ed.), Inflammation: Basic Principles and Clinical Correlates. Raven Press, Ltd., New York, NY, pp. 303-324.

Banos, G., Shook, G.E. 1990. Genotype by environment interaction and genetic correlations among parities for somatic cell count and milk yield. Journal of Dairy Science 73, 2563-2573.

Bellamy, R. 1999. The natural resistance-associated macrophage protein and susceptibility to intracelluar pathogens. Microbes and Infection 1, 23-27.

Ben-Baruch, A., Bengali, K.M., Biragyn, A., Johnson, J.J., Wang, J., Kim, J., Chuntharapai, A., Michiel, D.F., Oppenheim, J.J., Kelvin, D.J. 1995. Interleukin-8 receptor beta: the role of the carboxyl terminus in signal transduction. Journal of Biological Chemistry 270, 9121-9128.

Bennett, R.H., Jasper, D.E. 1978. Immunologic and pathologic responses of cows to naturally occurring *Mycoplasma bovis* mastitis. Veterinary Microbiology 2, 325-340.

Blackburn, P.S. 1969. The incidence of infection of the udder in a herd of cows over a period of years. British Veterinary Journal 125, 504-510.

Blackwell, J.M. 1989. The macrophage resistance gene Lsh/Ity/Bcg. Research in Immunology 140, 767-828.

Blackwell, J.M. 1996. Structure and function of the natural-resistance-associated macrophage protein (Nramp1), a candidate protein for infectious and autoimmune disease susceptibility. Molecular Medicine Today 5, 205-211.

Blackwell, J.M., Barton, C.H., White, J.K., Roach, T.I.A., Shaw, M.A., Whitehead, S.H., Mock, B.A., Searle, S., Williams, H., Baker, A.-M. 1994. Genetic regulation of leishmanial and mycobacterial infections: The Lsh/Ity/Bcg gene story continues. Immunology Letters 43, 99-107.

Blackwell, J.M., Roach, T.I.A., Atkinson, S.E., Ajioka, J.W., Barton, C.H., Shaw, M.A. 1991. Genetic regulation of macrophage priming/activation: the Lsh gene story. Immunology Letters 30, 241-248.

Blackwell, J.M., Searle, S., Goswami, R., Miller, E.N. 2000. Understanding the multiple functions of NRAMP1. Microbes and Infection 2, 317-321.

Boettcher, P.J., Hansen, L.B., VanRaden, P.M., Ernst, C.A. 1992. Genetic evaluation of Holstein bulls for somatic cells in milk of daughters. Journal of Dairy Science 75, 1127-1137.

Boettcher, P. J., Hansen, L.B., Chester-Jones, H., Young, C.W. 1993. Responses of yield and conformation to selection for milk in a designed experiment with a control population. Journal of Dairy Science 76, 267-273.

Bramley, A. 1990. Environmental mastitis. In: AABP/NMC International Symposium on Bovine Mastitis, Indianapolis, IN, pp. 158-170.

Bramley, A., Dodd, F. 1984. Reviews of the process of the progress of dairy science: Mastitis control-progress and prospects. Journal of Dairy Research 51, 481-512.

Burton, J.L., Erksine, R.J. 2003. Immunity and mastitis: some new ideas for an old disease. Veterinary Clinics of North America: Food Animal Practice 19, 1-45.

Bushnell, R.B. 1984. Symposium. Bovine mastitis: The importance of hygienic procedures in controlling mastitis. Veterinary Clinics of North America: Food Animal Practice 6, 361-370.

Calvinho, L.F., Almeida, R.A., Oliver, S.P. 1998. Potential virulence factors of *Streptococcus dysgalactiae* associated with bovine mastitis. Veterinary Microbiology 61, 93-110.

Capuco, A.V., Akers, R.M. 1986. In vitro study of polymorphonuclear leukocyte damage to mammary tissues of lactating cows. American Journal of Veterinary Research 47, 663-668.

Cardon, L.R., Palmer, L.J. 2003. Population stratification and spurious allele association. The Lancet 361, 598-604.

Carlson, G.P., Kaneko, J.J. 1973. Isolation of leukocytes from bovine peripheral blood. Proceedings of the Society for Experimental Biology and Medicine 142, 853-853.

Castillo-Juarez, H., Oltenacu, P.A., Blake, R.W., Mcculloch, C.E., Cienfuegos-Rivas, E.G. 2000. Effect of herd environment on the genetic and phenotypic relationships among milk yield, conception rate, and somatic cell score in Holstein cattle. Journal of Dairy Science 83, 807-814.

Castillo-Juarez, H., Oltenacu, P.A., Cienfuegos-Rivas, E.G. 2002. Genetic and phenotypic relationships among milk production and composition traits in primiparous Holstein cows in two different herd environments. Livestock Production Science 78, 223-231.

Chen, Q., Powell, D.W., Rane, M.J., Singh, S., Butt, W., Klein, J.B., McLeish, K.R. 2003. Akt phosphorylates p47phox and mediates respiratory burst activity in human neutrophils. Journal of Immunology, 5302-5308.

Craven, N. 1983. Generation of neutrophil chemoattractants by phagocytosing bovine mammary macrophages. Research in Veterinary Science 3, 310-317.

Crawford, A.M., Dodds, K.G., McEwan, J.C. 2000. DNA markers, genetic maps and the identification of QTL: general principles. In: Axford, R.F.E., Bishop, S.C., Nicholas, F.W., Owen, J.B. (ed.), Breeding for Disease Resistance in Farm Animals. CABI Publishing, New York, NY, pp. 3-26.

Crist, W.L., Harmon, R.J., O'Leary, J., McAllister, A.J. 1997 Mastitis and it's control. University of Kentucky, College of Agriculture, Lexington, KY, pp. 1-14.

Cruickshank, J., Weigel, K.A., Dentine, M.R., Kirkpatrick, B.W. 2002. Indirect prediction of herd life in Guernsey dairy cattle. Journal of Dairy Science 85, 1307-1313.

Da, Y., Ron, M., Yanai, A., Band, M., Everts, R.E., Heyen, D.W., Weller, J.I., Wiggans, G.R., Lewis, H.A. 1994. The dairy bull DNA repository: A resource for mapping quantitative trait loci. In: 5th World Congress on Genetics and Applied Livestock Production, Guelph, ON, Canada, pp. 229-232.

Damaini, G., Florio, S., Budelli, E., Polla, P., Caroli, A. 2000. Single nucleotide polymorphisms (SNPs) within Bov-A2 SINE in the second intron of bovine and buffalo k-casein (CSN3) gene. Animal Genetics 31, 277-279.

Damaj, B.B., McColl, S.R., Neote, K., Na, S.Q., Ogborn, K.T., Hebert, C.A., Naccache, P.A. 1996. Identification of G-protein binding sites of the human interleukin-8 receptors by functional mapping of the intracellular loops. FASEB Journal 10, 1426-1434.

Detilleux, J.C. 2002. Genetic factors affecting susceptibility of dairy cows to udder pathogens. Veterinary Immunology and Immunopathology 88, 103-110.

Detilleux, J.C., Kehrli, M.E.J., Freeman, A.E., Fox, L.K., Kelley, D. 1995. Mastitis of periparturient Holstein cattle: a phenotypic and genetic study. Journal of Dairy Science 78, 2285-2293.

Dewitt, S., Hallett, M.B. 2002. Cytosolic free Ca^{2+} changes and calpain activation are required for beta integrin-accelerated phagocytosis by human neutrophils. Journal of Cell Biology 159, 181-189.

DiCarlo, A.L., Paape, M.J. 1996. Comparison of C3b binding to bovine peripheral blood and mammary gland neutrophils (PMN). American Journal of Veterinary Research 57, 151-156.

Dickinson, F.N. 1985. Genetic Improvement of Dairy Cattle. NCDHIP Handbook.

Dietz, A.B., Cohen, N.D., Timms, L., Kehrli, M.E.J. 1997. Bovine lymphocyte antigen class II alleles as risk factors for somatic cell counts in milk of lactating dairy cows. Journal of Dairy Science 80, 406-412.

Diez-Fraile, A., Meyer, E., Duchateau, L., Burvenich, C. 2002a. L-selectin and beta2-integrin expression on circulating bovine polymorphonuclear leukocytes during endotoxin mastitis. Journal of Dairy Science 86, 2334-2342.

Diez-Fraile, A., Meyer, E., Duchateau, L., Burvenich, C. 2002b. Regulation of adhesion molecules on circulating neutrophils during coliform mastitis and their possible immunomodulation with drugs. Veterinary Immunology and Immunopathology 86, 1-10.

Dodd, F.H., Griffin, T.K. 1975. The role of antibiotic treatment at drying off in the control of mastitis. Bull Int Dairy Fed 85, 232-302.

Eberhart, R.J. 1986. Management of dry cows to reduce mastitis. Journal of Dairy Science 69, 1721-1732.

Eberhart, R.J., Buckalew, J.M. 1972. Evaluation of a hygiene and dry period therapy program for mastitis control. Journal of Dairy Science 55, 1683-1691.

Eberhart, R.J., Buckalew, J.M. 1977. Intramammary infections in a dairy herd with a low incidence of *Streptococcus agalactiae* and *Staphylococcus aureus* infections. Journal of American Veterinary Medicine Association 171, 630-634.

Eberhart, R.J., LeVan, P.L., Griel, L.C., Kesler, E.M. 1983. Germicidal teat dip in a herd with low prevalence of *Streptococcus agalactiae* and *Staphylococcus aureus* mastitis. Journal of Dairy Science 66, 1523-1526.

Emanuelson, U., Danell, B., Philipsson, J. 1988. Genetic parameters for clinical mastitis, somatic cell counts, and milk production estimated by multiple-trait restricted maximum likelihood. Journal of Dairy Science 71, 467-476.

Erksine, R.J., Eberhart, R., Grasso, J., Scholz, R.W. 1989. Induction of Escherichia coli mastitis in cows fed selenium-deficient or selenium-supplemented diets. American Journal of Veterinary Research 50, 2093-2100.

Erksine, R.J., Kirk, J.H., Tyler, J.W., DeGraves, F.J. 1993. Advances in therapy of mastitis. Veterinary Clinics of North America: Food Animal Practice 9, 499-562.

Feniger-Barish, R., Yron, I., Meshel, T., Matityahu, E., Ben-Baruch, A. 2003. IL-8 induced migratory responses through CXCR1 and CXCR2: association with phosphorylation and cellular redistribution of focal adhesion kinase. Biochemistry 42, 2874-2886.

Fernard, G.W. 1982. Immunologic interactions between host cells and mycoplasmas: an introduction. Review of Infectious Diseases 4, S201-S204.

Fetrow, J. 2000. Mastitis: An economic consideration. In: Council, N.M. (ed.), 39th Annual Meeting of the National Mastitis Council, Madison,WI, pp. 3-47.

Filippsen, L.F., Valentin-Weigand, P., Blobel, H., Preissner, K.T., Chhatwal, G.S. 1990. Role of complement S protein (vitronectin) in adherence of *Streptococcus dysgalactiae* to bovine epithelial cells. American Journal of Veterinary Research 51, 861-865.

Flemming, M.D., Romano, M.A., Su, M.A., Garrick, L.M., Garrick, M.D., Andrews, N.C. 1998. Nramp2 is mutated in the anemic Belgrade (b) rat: Evidence of a role for Nramp2 in endosomal ion transport. Proceedings of the National Academy of Sciences 95, 1148-1153.

Fox, L.K., Gay, J.M. 1993. Contagious mastitis. Veterinary Clinics of North America: Food Animal Practice 9, 475-487.

Frendeus, B., Godaly, G., Hang, L., Karpman, D., Lundstedt, A.C., Svanborg, C. 2000. Interleukin-8 receptor deficiency confers susceptibility to acute experimental pyelonephritis and may have a human counterpart. Journal of Experimental Medicine 6, 881-890.

Garcia-Garcia, E., Rosales, C. 2002. Signal transduction during Fc receptor-mediated phagocytosis. Journal of Leukocyte Biology 72, 1092-1108.

Gengler, N., Wiggans, G.R., Wolfe, C.W., Wright, J.R. 1998. Genetic evaluations for breeds other than Holstein. USDA Animal Improvement Programs Laboratory. <http://www.aipl.arsudsa.gov>

Gill, R., Howard, W.H., Leslie, K.E., Lissemore, K. 1990. Economics of mastitis control. Journal of Dairy Science 73, 3340-3348.

Gillespie, B.E., Jayarao, B.M., Dowlen, H.H., Oliver, S.P. 1999. Analysis and frequency of bovine lymphocyte antigen DRB3.2 alleles in jersey cows. Journal of Dairy Science 82, 2049-2053.

Gilmore, J.A., McDaniel, B.T. 1977. Health costs of dairy cattle, its variation, and its associations with milk yield and type scores. Journal of Dairy Science 60, 76-80.

Glynn, P.C., Henney, E., Hall, I.P. 2002. The selective CXCR2 antagonist SB272844 blocks interleukin-8 and growth-related oncogene-a mediated inhibition of spontaneous neutrophil apoptosis. Pulmonary Pharmacology and Therapeutics 15, 103-110.

Gonzalez, R.N., Merrill, R.A., Sears, P.M. 1992. Shedding of *Mycoplasma bovis* from the udder of naturally infected cows and its importance for the diagnosis of bovine intramammary infections. Journal of Dairy Science 75, 259-265.

Gregory, H., Young, J., Schroeder, J.M., Mrowietz, U., Christophers, E. 1988. Structure determination of a human lymphocyte derived neutrophil activating peptide (LYNAP). Biophysical and Biochemical Research Communication 151, 883-890.

Grosse, W.M., Kappes, S.W., Laegreid, W.W., Keele, J.W., Chitko-McKown, C.G., Heaton, M.P. 1999. Single nucleotide polymorphism (SNP) discovery and linkage mapping of bovine cytokine genes. Mammalian Genome 10, 1062-1069.

Gruenheid, S., Canonne-Hergaux, F., Gauthier, S., Hackam, D.J., Grinstein, S., Gros, P. 1999. The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes eith transferrin in recycling endosomes. Journal of Experimental Medicine 189, 831-841.

Gudding, R., McDonald, J.S., Cheville, N.F. 1984. Pathogenesis of *Staphylococcus aureus* mastitis: Bacteriologic, histologic, and ultrastructural pathologic findings. American Journal of Veterinary Research 45, 2525-2531.

Guterbock, W.M., Van Eenennaam, A.L., Anderson, R.J., Gardner, I.A., Cullor, J.S., Holmberg, C.A. 1993. Efficacy of intramammary antibiotic therapy for treatment of clinical mastitis caused by environmental pathogens. Journal of Dairy Science 76, 3437-3444.

Hannson, B., Westerberg, L. 2002. On the correlation between heterozygosity and fitness in natural populations. Molecular Ecology 11, 2467-2474.

Hansen, L.B. 2000. Consequences of selection for milk yield from a geneticist's viewpoint. Journal of Dairy Science 83, 1145-1150.

Hansen, L.B., Young, C.W., Miller, K.P., Touchberry, R.W. 1979. Health care requirements of dairy cattle. I. Response to milk yield selection. Journal of Dairy Science 62, 1922-1931.

Haraldsson, I., Jonsson, P. 1984. Histopathology and pathogenesis of mouse mastitis induced with *Staphylococcus aureus* mutants. Journal of Comparative Pathology 94, 183-196.

Harmon, R.J., Christ, W.L., Hemkin, R.W. 1986. Prevalence of minor udder pathogens after intramammary dry treatment. Journal of Dairy Science 69, 843-849.

Harmon, R.J., Heald, C.W. 1982. Migration of polymorphonuclear leukocytes into the bovine mammary gland during experimentally induced *Staphylococcus aureus* mastitis. American Journal of Veterinary Research 43, 992-998.

Heaton, M. P., Chitko-McKown, C.G., Grosse, W.M., Keele, J.W., Keen, J.E., Laegreid, W.W. 2001. Interleukin-8 haplotype structure from nucleotide sequence variation in commerical populations of U.S. beef cattle. Mammalian Genome 12, 219-226.

Heaton, M.P., Grosse, W.M., Kappes, S.M., Keele, J.W., Chitko-McKown, C.G., Cundiff, L.V., Braun, A., Little, D.P., Laegreid, W.W. 2001. Estimation of DNA sequence diversity in bovine cytokine genes. Mammalian Genome 12, 32-37.

Heringstad, B., Klemetsdal, G., Ruane, J. 2000. Selection for mastitis resistance in dairy cattle: a review with focus on the situation in the Nordic countries. Livestock Production Science 64, 95-106.

Heyen, D.W., Weller, J.I., Ron, M., Band, M., Beever, J.E., Feldmesser, E., Da, Y., Wiggans, G.R., Van Raden, P.M., Lewin, H.A. 1999. A genome scan for QTL influencing milk production and health traits in dairy cattle. Phsyical Genomics 3, 165-176.

Hogan, J., Hoblet, K., Smith, K., Todhunter, D.A., Schoenberger, P.S., Hueston, W.D., Pritcherard, D.E., Bowman, G.L., Heider, L.E., Brockett, B.L. 1988. Bacterial and somatic cell counts in bulk tank milk from nine well managed herds. Journal of Food Protection 51, 930-934.

Hogan, J., Hoblet, K., Smith, K., Todhunter, D.A., Schoenberger, P.S., Hueston, W.D., Pritcherard, D.E., Bowman, G.L., Heider, L.E., Brockett, B.L. 1989. Bacterial counts in bedding materials used on nine commercial dairies. Journal of Dairy Science 72, 250-258.

Hogan, J.S., Gonzalez, R.N., Harmon, R.J., Nickerson, S.C., Oliver, S.P., Pankey, J.W., Smith, K.L. 1999 Laboratory handbook on bovine mastitis. 1st ed. National Mastitis Council, Inc., Madison, WI.

Hogan, J.S., Pankey, J.W., Smith, K.L. 1987. Staphylococcus species other than *Staphylococcus aureus*. In: Council, N.M. (ed.), 26th Annual Meeting of the National Mastitis Council, Orlando, FL, p. 21.

Horin, P., Rychlik, I., Templeton, J.W., Adams, L.G. 1999. A complex pattern of microsatellite polymorphism within the bovine NRAMP1 gene. European Journal of Immunogenetics 26, 311-313.

Huges, P.E., Pfaff, M. 1998. Integrin affinity modulation. TRENDS in Cell Biology 8, 359-364.

Hurley, W.L. 1987. Mammary function during the nonlactating period: enzyme, lactose, protein concentrations, and pH of mammary secretions. Journal of Dairy Science 70, 20-28.

Isberg, R.R. 1991. Discrimination between intracellular uptake and surface adhesion of bacterial pathogens. Science 252, 934-938.

Jankowski, A., Scott, C.C. Grinstein, S. 2002. Determinants of the phagosomal pH in neutrophils. Journal of Biological Chemistry 277, 6059-6066.

Jayarao, B.M., Wang, L. 1999. A study on the prevalence of coliforms and non-coliforms in bulk tank milk. Journal of Dairy Science 82, 2620-2624.

Jayarao, B.M., Wolfgang, D.R. 2003. Bulk-tank milk analysis. A useful tool for improving milk quality and udder health. Veterinary Clinics of North America: Food Animal Practice 19, 75-92.

Jones, W.P., Hansen, L.B., Chester-Jones, H. 1994. Response of health care to selection for milk yield of dairy cattle. Journal of Dairy Science 77, 3137-3152.

Kaipainen, T., Pohjanvirta, T., Shpigel, N.Y., Shwimmer,A., Pyorala, S., Pelkonen, S. 2002. Virulence factors of *Escherichia coli* isolated from bovine clinical mastitis. Veterinary Microbiology 85, 37-46.

Kalm, E., Reinsch, N., Xu, H. 1998. Mapping QTL on cattle chromosome 2, 5, 10, 16, 18 and 23. In: Proceedings of the XXVIth International Conference on Animal Genetics, E009, Auckland, New Zealand.

Kansas, G.S. 1996. Selectins and their ligands: current concepts and controversies. Blood 88, 3259-3287.

Kato, H., Tsuchiya, N., Tokunaga, K. 2000. Single nucleotide polymorphisms in the coding region of human CXC-chemokine receptors CXCR1, CXCR2 and CXCR3. Genes and Immunity.

Keefe, G.P. 1997. *Streptococcus agalactiae* mastitis: a review. Canadian Veterinary Journal 38, 429-437.

Kehrli, M.E., Jr., Harp, J.A. 2001. Immunity in the mammary gland. Veterinary Clinics of North America: Food Animal Practice 17, 495-516.

Kelm, S.C., Detilleux, J.C., Freeman, A.E., Kehrli, M.E. Jr., Dietz, A.B., Fox, L.K., Butler, J.E., Kasckovics, I., Kelley, D.H. 1997. Genetic association between parameters of innate immunity and measures of mastitis in periparturient Holstein cattle. Journal of Dairy Science 80, 1767-1775.

Kettritz, R., Gaido, M.L., Haller, H., Luft, F.C., Jenette, C.J., Falk, R.J. 1998. Interleukin-8 delays spontaneous and tumour necrosis factor-a-mediated apoptosis of human neutrophils. Kidney International 53, 84-91.

Klebanoff, S.J. 1992. Oxygen metabolites from phagocytes. In: Gallin, J.I., Goldstein, I.M., Snyderman, R. (ed.), Inflammation: Basic Principles and Clinical Correlates. Raven Press, Ltd., New York, NY, pp. 541-588.

Klungland, H., Sabry, A., Heringstad, B., Olsen, H.G., Gomez-Raya, L., Vage, D.I., Olsaker, I., Odegard, J., Klemetsdal, G., Schulman, N., Vilkki, J., Ruane, J., Aasland, M., Ronningen, K., Lien, S. 2001. Quantitative trait loci affecting clinical mastitis and somatic cell count in dairy cattle. Mammalian Genome 12, 837-842.

Konfortov, B.A., Licence, V.E., Miller, J.R. 1999. Re-sequencing of DNA from a diverse panel of cattle reveals a high level of polymorphism in both intron and exon. Mammalian Genome 10, 1142-1145.

Koss, K., Fanning, G.C., Welsh, K.I., Jewell, D.P. 2001. Interleukin-10 gene promoter polymorphism in English and Polish healthy controls. Polymerase chain reaction haplotyping using 3' mismatches in forward and reverse primers. Genes and Immunity 1, 321-324.

Kuhn, C., Bennewitz, J., Reinsch, N., Xu, N., Thomsen, H., Looft, C., Brockmann, G.A., Schwerin, M., Weimann, C., Hiendleder, S., Erhardt, G., Medjugorac, I., Forster, M., Brenig, B., Reinhardt, F., Reents, R., Russ, I., Averdunk, G., Blumel, J., Kalm, E. 2003. Quantitative trait loci mapping of functional traits in the German Holstein cattle population. Journal of Dairy Science 86, 360-368.

Kuhn, D.E., Baker, B.D., Lafuse, W.P., Zwilling, B.S. 1999. Differential iron transport into phagosomes isolated from the RAW264.7 macrophage cell lines transfected with Nramp1Gly169 or Nramp1Asp169. Journal of Leukocyte Biology 66, 113-119.

Lammers, A., van Vorstenbosch, C.J., Erkens, J.H.F., Smith, H.E. 2001. The major bovine mastitis pathogens have different cell tropisms in cultures of bovine mammary gland cells. Veterinary Microbiology 80, 255-265.

LaRosa, G.J., Thomas, K.M., Kaufman, M.E., Mark, R., White, M., Taylor, L. 1992. Amino terminus of of the interleukin-8 receptor is a major determinant of receptor subtype specificity. Journal of Biological Chemistry 267.

Lazarus, R., Klimecki, W.T., Raby, B.A., Vercelli, D., Palmer, L.J., Kwiatkowski, D.J., Silverman, E.K., Martinez, F., Weiss, S.T. 2003. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics 81, 85-91.

Lee, A., Whyte, M.K.B., Haslett, M.B. 1993. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. Journal of Leukocyte Biology 54, 283-288.

Lee, C.S., Wooding, F.B., Kemp, P. 1980. Identification, properties, and differential counts of cell populations using electron microscopy of dry cows secretions, colostrum and milk from normal cows. Journal of Dairy Research 1, 39-50.

Lee, J., Horuk, R., Rice, G.C., Bennett, G.L., Camerato, T., Wood, W.I. 1992. Characterization of two high affinity human interleukin-8 receptors. Journal of Biological Chemistry 267, 16283-16287.

Ley, K. 2002. Integration of inflammatory signals by rolling neutrophils. Immunological Reviews 186, 8-18.

Lindley, I.J.D. 1998. Interleukin-8. In: Cytokines. Academic Press, pp. 125-140.

Lund, M.S., Miglior, F., Dekkers, J.C.M., Burnside, E.B. 1994. Genetic relationships between clinical mastitis, somatic cell count, and udder conformation in Danish Holsteins. Livestock Production Science 39, 243-255.

Lunden, A., Sigurdardottir, S., Edfors-Lilja, I., Danell, B., Rendel, J., Andersson, L. 1990. The relationship between bovine major histocompatability complex class II polymorphism and disease studied by use of bull breeding values. Animal Genetics 21, 221-232.

Mackay, C.R., Imhof, B.A. 1993. Cell adhesion in the immune system. Immunology Today 14, 99-103.

Mahoney, C.B., Hansen, L.B., Young, C.W., Marx, G.D., Reneau, J.K. 1986. Health care of Holsteins selected for large or small body size. Journal of Dairy Science 69, 3131-3139.

Mallard, B. A., Leslie, K.E., Dekkers, J.C.M., Hedge, R., Bauman, M., Stear, M.J. 1995. Differences in bovine lymphocyte antigen associations between immune responsiveness and risk of disease following intramammary infection with Staphylococcus aureus. Journal of Dairy Science 78, 1937-1944.

Mayer, S.J., Keen, P.M., Craven, N., Bourne, F.J. 1989. Regulation of phagolysome pH in bovine and human neutrophils: the role of NADPH oxidase activity and an Na^{2+}/H^{+} antiporter. Journal of Leukocyte Biology 45, 239-248.

Meenagh, A., Williams, F., Rodd, O.A., Patterson, C., Gorodezky, C., Hammond, M., Leheny, W.A., Middelton, D. 2002. Frequency of cytokine polymorphisms in populations from Western Europe, Africa, Asia, the Middle East and South America. Human Immunology 63, 1055-1061.

Miller, R.H., Owen, J.R., Moore, E.D. 1976. Incidence of clinical mastitis in a herd of Jersey cattle. Journal of Dairy Science 59, 113-119.

Miller, R.H., Paape, M.J., Fulton, L.A. 1993. The relationship of milk somatic cell count to milk yield for Holstein heifers after first calving. Journal of Dairy Science 76, 728-733.

Mitchell, G.B., Albright, B.N., Caswell, J.L. 2003. Effect of interleukin-8 amd granulocyte colony-stimulating factor on priming and activation of bovine neutrophils. Infection and Immunity 71, 1643-1649.

Monardes, H.G., Cue, R.I., Hayes, J.F. 1990. Correlations between udder conformation traits and somatic cell count in Canadian Holstein cows. Journal of Dairy Science 73, 1337-1342.

Murphy, P. 1994. The molecular-iology of leukocyte chemoattractant receptors. Annual Review of Immunology 12, 593-633.

Nash, D.L., Rogers, G.W., Cooper, J.B., Hargrove, G.L., Keown, J.F. 2002. Relationships among severity and duration of clinical mastitis and sire transmitting abilities for somatic cell score, udder type traits, productive life, and protein yield. Journal of Dairy Science 85, 1273-1284.

National Mastitis Council. 2001. A practical look at contagious mastitis. National Masitis Council, Inc., Madison, WI.

Natzke, R.P., Everett, R.W., Guthrie, R.S., Keown, J.E., Meed, A.M., Merrill, W.G., Roberts, S.J., Schmidt, G.H. 1972. Mastitis control program: effect on milk production. Journal of Dairy Science 55, 1256-1260.

Neave, F.K., Dodd, F.H., Kingwill, R.G., Westgarth, D.R. 1969. Control of mastitis in the dairy herd by hygiene and management. Journal of Dairy Science 52, 696-707.

Notermans, S., Firstenberg-Eden, R., Van Schothorst, M. 1979. Attachment of bacteria to teats of cows. Journal of Food Protection 42, 228-232.

Odegard, J., Klemetsdal, G., Heringstad, B. 2003. Variance components and genetic trend for somatic cell count in Norwegian cattle. Livestock Production Science 79, 135-144.

Oliver, S.P., Mitchell, B.A. 1983. Susceptibility of bovine mammary gland to infections during the dry period. Journal of Dairy Science 66, 1162-1166.

Oliver, S.P., Mitchell, B.A. 1984. Prevalence of mastitis pathogens in herds participating in a mastitis control program. Journal of Dairy Science 67, 2436-2440.

Oliver, S.P., Sordillo, L.M. 1989. Approaches to the manipulation of mammary involution. Journal of Dairy Science 72, 1647-1664.

Oliver, S. P., Almeida, R.A., Calvinho, L.F. 1998. Virulence factors of Streptococcus uberis isolated from cows with mastitis. Journal of Veterinary Medical Science 45, 461-471.

Ostensson, K., Hageltorn, M., Astrom, G. 1988. Differential cell counting in fraction-collected milk from dairy cows. Acta Veterinaria Scandinavica 29, 493-500.

Ostergard, H., Kristensen, B., Andersen, S. 1989. Investigations in farm animals of associations between the MHC system and disease resistance and fertility. Livestock Production Science 22, 49-67.

Paape, M., Mehrzad, J., Zhao, X., Detilleux, J., Burvenich, C. 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. journal of the mammary gland biology and neoplasia 7, 109-121.

Paape, M.J., Lillius, E.M., Wiitanen, P.A., Kontio, M.P. 1996. Intramammary defense against infections induced by Eschericia coli in cows. American Journal of Veterinary Research 57, 477-482.

Paape, M.J., Pearson, R.E., Wergin, W.P., Guidry, A.J. 1977. Enhancement of chemotactic response of polymorphonuclear leukocytes into the mammary gland and isolation from milk. Journal of Dairy Science 60, 53-62.

Paape, M.J., Shafer-Weaver, K., Capuco, A.V., van Oostveldt, K., Burvenich, C. 2000. Immune survalence of mammary tissue by phagocytic cells. Advances in Experimental Medicine and Biology 480, 259-277.

Paape, M.J., Wergin, W.P., Guidry, A.J., Pearson, R.E. 1979. Leukocytes--second line of defense against invading mastitis pathogens. Journal of Dairy Science 62, 135-153.

Paape, M.J., Wergin, W.P., Guidry, A.J., Schultze, W.D. 1981. Phagocytic defense of the ruminant mammary gland. Advances in Experimental Medicine and Biology 137, 555-578.

Pankey, J.W., Dreschler, P.A. 1993. Evolution of udder hygiene. Premilking teat sanitation. Veterinary Clinics of North America: Food Animal Practice 9, 519-530.

Parham, P. 2000. The body's defenses against infection. In: The Immune System. Garland Publishing, New York, NY, pp. 201-240.

Peveri, P., Walz, A., Dewald, B., Baggiolini, M. 1988. A novel neutrophil-activating factor produced by human polymorphonuclear leukocytes. Journal of Experimental Medicine. 181, 1547-1559.

Pfutzner, H., Sachse, K. 1996. *Mycoplasma bovis* as an agent of mastitis, pneumonia, arthritis and genital disorders in cattle. Rev Sci Technol 15, 1477-1494.

Razin, S., Jacobs, E. 1992. Mycoplamsa adhesion. Journal of General Microbiology 138, 407-422.

Renzoni, E., Lympany, P., Sestini, P., Pantelidis, P., Wells. A., Black, C., Welsh, K., Bunn, C., Knight, C., Foley, P., du Bois, R. 2000. Distribution of novel polymorphisms of the interleukin-8 and CXC receptor 1 and 2 genes in systemic sclerosis and cryptogenic fibrosing alveolitis. Arthritis and Rheumatism 43, 1633-1640.

Riberio, M. G., Costa, E.O., Leite, D.S., Ferreira, A.J.P., Silva, A.S., Della Colleta, H.H.M. 2002. Cytotoxic necrotizing factor in Escherichia coli strain isolated from clinical bovine mastitis. Arquivo Brasileiro de Medicina Veterinaria E Zootecnia 54, 648-650.

Richardson, R.M., Marjoram, R.J., Barak, L.S., Snyderman, R. 2003. Role of cytoplasmic tails of CXCR1 and CXCR2 in mediating leukocyte migration, activation, and regulation. Journal of Immunology 170, 2904-2911.

Riollet, C., Rainard, P.,Poutrel, B. 2000. Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with *Staphylococcus aureus* a-toxin. Inflammation Research 49, 486-496.

Roberson, J.R. 2003. Establishing treatment protocols for clinical mastitis. In: Smith, R.A. (ed.), The Veterinary Clinics of North America. Food Animal Practice. W.B. Saunders Company, Philadelphia, PA, Vol. 19, pp. 223-234.

Rogers, G.W. 1993. Index selection using milk yield, somatic cell score, udder depth, teat placement, and foot angle. Journal of Dairy Science 76, 664-670.

Rogers, G.W., Banos, G., Sander-Nielsen, U., Philipsson, J. 1998. Genetic correlations among somatic cell scores, productive life, and type traits from the United States and udder health measures from Denmark and Sweeden. Journal of Dairy Science 81, 1445-1453.

Rogers, G.W., Hargrove, G.L., Cooper, J.B. 1995. Correlations among somatic cell scores of milk within and across lactations and linear type traits of Jerseys. Journal of Dairy Science 78, 914-920.

Rogers, G.W., Spencer, S.B. 1991. Relationships among udder and teat morphology and milking characteristics. Journal of Dairy Science 74, 4189-4194.

Ruegg, P.L. 2003. Investigation of mastitis problems on farms. Veterinary Clinics of North America: Food Animal Practice 19, 47-73.

Savill, J.S., Wyllie, A.H.,Henson, J.E., Walport, M.J., Henson, P.M., Haslett, C. 1989. Macrophage phagocytosis of aging neutrophils in inflammation. Journal of Clinical Investigation 83, 865-875.

Schroeder, J.-M., Mroweitz, U., Christophers, E. 1988. Purification and partial biochemical characterisation of a human lymphocyte-derived peptide with potent neutrophil-stimulating activity. Journal of Immunology 140, 3534-3540.

Schrooten, C., Bovenhuis, H., Coppieters, W., Van Arendonk, J.A.M. 2000. Whole genome scan to detect quantitative trait loci for conformation and functional traits in dairy cattle. Journal of Dairy Science 83, 795-806.

Schukken, Y.H., Leslie, K.E., Barnum, D.A., Mallard, B.A., Lumsden, J.H., Dick, P.C. 1999. Experimental *Staphylococcus aureus* intramammary challenge in late lactation diary cows. Quarter and cow effects determining the probability of infection. Journal of Dairy Science 82, 2393-2401.

Schutz, M.M., VanRaden, M.M., Wiggans, G.R., Norman, H.D. 1995. Standardization of lactation means of somatic cell scores for calculation of genetic evaluations. Journal of Dairy Science 78, 1843-1854.

Seykora, A.J., McDaniel, B.T. 1985. Heritabilities of teat traits and their relationships with milk yield, somatic cell count, and percent two-minute milk. Journal of Dairy Science 68, 2670.

Seykora, A.J., McDaniel, B.T. 1986. Genetic statistics and relationships of teat and udder traits, somatic cell counts, and milk production. Journal of Dairy Science 69, 2395.

Shanks, R.D., Freeman, A.E., Berger, P.J., Kelley, D.H. 1977. Consequences for selecting for high milk production. Journal of Dairy Science 60, 75.

Sharif, S., Mallard, B.A., Wilkie, B.N., Sargeant, J.M., Scott, H.M., Dekkers, J.C.M., Leslie, K.E. 1998. Associations of the bovine major histocompatability complex DRB3 (BoLA-DRB3) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. Animal Genetics 29, 185-196.

Shastry, B.S. 2002. SNP alleles in human diseases and evolution. Journal of Human Genetics 47, 561-566.

Shook, G.E. 1993. Genetic improvement of mastitis through selection on somatic cell count. Veterinary Clinics of North America: Food Animal Practice 9, 563-581.

Shook, G.E. 2001. Breeding, Selection, and Somatic Cell Counts: Where are we today? In: National Mastitis Council Annual Meeting Proceedings, pp. 113-127.

Shook, G.E. Schutz, M.M., 1994. Selection on somatic cell score to improve resistance to mastitis in the United States. Journal of Dairy Science 77, 648-658.

Short, T.H., Lawlor, T.J. 1992. Genetic parameters and conformation traits, milk yield and herd life in Holsteins. Journal of Dairy Science 75, 1987-1998.

Shuster, D.E., Kehrli, M.E.J., Stevens, M.G. 1993. Cytokine production during endotoxin-induced mastitis in lactating dairy cows. American Journal of Veterinary Research 1, 80-85.

Siite, K., Brinkworth, R., East, I.J., Jazwinska, E.C. 2002. A single amino acid deletion in the antigen binding site of BoLA-DRB3 is predicted to affect peptide binding. Veterinary Immunology and Immunopathology 85, 129-135.

Simensen, E. 1976. Milk somatic cells in dairy cows kept on pasture or confined indoors during the summer. Nordic Veterinary Medicine Dec. 28, 603-609.

Sischo, W.M., Heider, L.E., Miller, G.Y., Moore, D.A. 1993. Prevalence of contagious pathogens of bovine mastitis and use of mastitis control practices. Journal of the American Veterinary Medical Association 202, 595-600.

Skelton, N.J., Quan, C., Reilly, D., Lowman, H. 1999. Structure of a CXC chemokine-receptor fragment in complex with interleukin-8. Structure 7, 157-168.

Smith, K. 1983. Mastitis control: A discussion. Journal of Dairy Science 66, 1790-1794.

Smith, K.L., Hogan, J.S. 1993. Environmental mastitis. Veterinary Clinics of North America: Food Animal Practice 9, 489-498.

Smith, K.L., Todhunter, D.A., Schoenberger, P.S. 1985. Environmental pathogens and intramammary infection during the dry period. Journal of Dairy Science 68, 402-417.

Spooner, R.L., Leveziel, H., Grosclaude, F., Oliver, R.A., Vaiman, M. 1978. Evidence for a possible major histocompatability complex (BLA) in cattle. Journal of Immunogenetics 5, 335-346.

Strandberg, E., Shook, G.E. 1989. Genetic and economic responses to breeding programs that consider mastitis. Journal of Dairy Science 72, 2136-2142.

Sutra, L., Poutrel, B. 1994. Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Streptococcus aureus*. Journal of Medical Microbiology 40, 79-89.

Suzuki, H., Prado, G.N., Wilkinson, N., Navarro, J. 1994. The N terminus of interleukin-8 (IL-8) receptor confers high affinity binding to human IL-8. Journal of Biological Chemistry 269, 18263-18266.

Thelen, M., Peveri, P., Kernen, P., von Tscharner, V., Walz, A., Baggiolini, M. 1988. Mechanism of neutrophil activation by NAF, a novel monocytes-derived peptide agonist. FASEB Journal 2, 2702-2706.

Todhunter, D.A., Smith, K.L., Hogan, J.S. 1995. Environmental streptococcal intramammary infections of the bovine mammary gland. Journal of Dairy Science 78, 2366-2374.

Vage, D.I., Olsaker, I., Lingaas, F., Spooner, R.L., Van der Beek, S., Sorensen, A., Arnet, E.F., Lie, O. 1992. High levels of linkage disequilbria between serologically defined class I bovine lymphocyte antigens (BoLA-A) and class II DQB restriction fragment length polymorphism (RFLP) in Norwegian cows. Animal Genetics 23, 125-132.

Van Eijk, M.J.T., Stewart-Haynes, J.A., Lewin, H.A. 1992. Extensive polymorphism of the BoLA-DRB3 gene distinguished by PCR-RFLP. Animal Genetics 23, 482-496.

Van Tassell, C.P., Ashwell, M.S., Sonstegard, T.S. 2000. Detection of putative loci affecting milk, health, and conformation traits in U.S. Holstein population using 105 microsatellite markers. Journal of Dairy Science 83, 1865-1872.

Vidal, S.M., Malo, D., Vogan, K., Skamene, E., Gros, P. 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate gene for Bcg. Cell 73, 469-485.

Wang, K., Scheel-Toellner, D., Wong, S.H., Craddock, R., Caamano, J., Akbar, A.N., Salmon, M., Lord, J.M. 2003. Inhibition of neutrophil apoptosis by type 1 IFN depends on cross-talk between phosphoinositol 3-kinase, protein kinase C-g and NF-kB signalling pathways. Journal of Immunology, 1035-1041.

Ward, G.E., Schultz, L.H. 1972. Relationship of somatic cells in quarter milk to type bacteria and production. Journal of Dairy Science 55, 1428-1431.

Wattiaux, M.A. 1999 Mastitis: the disease and its transmission. Babcock Intstitute for International Dairy Research and Development; University of Wisconsin-Madison, Madison, WI, pp. 89-92.

Weigel, K.A., Freeman, A.E., Kehrli, M.E.J., Stear, M.J., Kelley, D.H. 1990. Association of class I bovine lymphocyte antigen complex alleles with health production traits in dairy cattle. Journal of Dairy Science 73, 2538-2546.

White, J.K., Shaw, M.A., Barton, C.H. 1994. Genetic and physical mapping of 2q35 in the region of the NRAMP and IL8R genes: Identification of a polymorphic repeat in exon2 of NRAMP. Genomics 24, 295-305.

White, L.J., Schukken, Y.H., Lam, T.J.G.M., Medley, G.F., Chappell, M.J. 2001. A multispecies model for the transmission and control of mastitis in dairy cows. Epidemiology and Infection 127, 567-576.

Whyte, M.K.B., Meagher, L.C., MacDermott, J., Haslett, C. 1993. Impairment of function in aging neutrophils is associated with apoptosis. Journal of Immunology 150, 5124-5134.

Wilson, D.J. 1995. Coagulase-negative Staphylococcus species mastitis: interpretation of diagnostic results and herd problem solving. In: Council, N.M. (ed.), 34th Annual Meeting of the National Mastitis Council, Fort Worth, TX, pp. 78-82.

Wilson, D.J. 1996. Factors influencing therapy decisions in clinical cases of gram-positive mastitis. The Bovine Proceedings, 171-173.

Wilson, D.J., Gonzalez,R.N. 1997. Evaluation of milk culture, SCC, and CMT for screening herd additions. In: 36th Annual Meeting of the National Mastitis Council, Albuquerque, NM.

Worku, M., Paape, M.J., Filep, R., Miller, R.H. 1994. Modulation of Fc receptors for IgG on bovine polymorphonuclear neutrophils on binding and expression of Fc receptors for IgG2 and IgM. American Journal of Veterinary Research 55, 234-238.

Wright, S.D. 1992. Receptors for complement and the biology of phagocytosis. In: Gallin, J.I., Goldstein, I.M., Snyderman, R. (ed.), Inflammation: Basic Principles and Clinical Correlates. Raven Press, Ltd., New York, NY, pp. 477-495.

Yagi, Y., Shiono, H., Shibahara, T., Chikayama, Y., Nakamura, I., Ohnuma, A. 2002. Increase in apoptotic polymorphonuclear neutrophils in peripheral blood after intramammary infusion of *Escherichia coli* lipopolysaccharide. Veterinary Immunology and Immunopathology 89, 115-121.

Zhelev, D.V., Alteraifi, A. 2002. Signalling in the motility responses of the human neutrophils. Annals of Biomedical Engineering 30, 356-370.

Zhong, B., Jiang, K., Gilvary, D.L., Epling-Burnette, P.K., Ritchey, C., Liu, J.H., Jackson, R.J., Hong-Geller, E., Wei, S. 2003. Human neutrophils utilize a Rac/Cdc42-dependent MAPK pathway to direct intracellular granule mobilization toward ingested microbial pathogens. Blood 101, 3240-3248.

Part III

IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS, HAPLOTYPES AND THEIR FREQUENCIES WITHIN THE BOVINE IL-8 RECEPTOR LOCUS IN JERSEY AND HOLSTEIN CATTLE

This chapter is a paper by the same name that will be submitted for publication in the *Animal Genetics,* in 2003, by Sara M. Youngerman, Arnold M. Saxton, Stephen P. Oliver, and Gina M. Pighetti.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) selection of cows for sequencing from sampled populations, (2) genomic DNA isolation and PCR analysis, (3) transformation, cloning, selection, and purification of plasmid DNA for sequencing, (4) DNA sequence analysis, (5) most of the statistical analysis, (6) most of the gathering and interpreting of literature, and (7) most of the writing of this paper.

I. INTRODUCTION

Mastitis, an inflammation of the mammary gland caused predominantly by bacterial infiltration of the teat, continues to be the most economically devastating disease affecting the dairy industry (National Mastitis Council, 1996). Progress towards a more mastitis resistant cattle population has been hampered due to low heritability and/or low genetic correlations of selected production traits with clinical mastitis (Hansen, 2000; Shook, 2001). Somatic cell counts (SCC) and clinical mastitis incidence have been associated with genetic markers such as quantitative trait loci (QTL) and single gene markers for immune function. Studies have associated several major histocompatability complex (MHC) class I and II alleles, including BoLA Class I CA42, W11, DQ1A alleles and DRB3.2 *3, *11, *16, *23 alleles, with SCC and clinical mastitis incidence (Dietz, 1997; Kelm, 1997; Sharif, 1998). Many QTL have been identified for SCC (chromosomes 2 and 12), and clinical mastitis (chromosomes 3, 4, 6, 14 and 27) (Ashwell, 1998; Ashwell, 1999; Kuhn, 2003; Schrooten, 2000; Van Tassell, 2000; Klungland,

2001). However, many of these MHC alleles and QTLs expressed across different breeds of cattle have had contradicting associations (Dietz, 1997; Heyen, 1999; Kelm, 1997; Klungland, 2001; Sharif, 1998). A genetic marker associated with the mammary gland's inflammatory response during mastitis would potentially enable producers to select for mastitis resistance and achieve more rapid gains in production.

Human and bovine neutrophils express several chemokine receptors, including interleukin-8 (IL-8) receptors CXCR1 and CXCR2, which are members of the seven-transmembrane G-protein coupled receptor family (Onuffer, 2002; Sprenger, 1994). Human CXCR1 and CXCR2 receptors express 77% sequence homology and differ in their binding affinities for $ELR^{+}CXC$ chemokines (Sprenger, 1994; White, 1994). CXCR1 expresses more binding selectivity as it binds only to IL-8 with high affinity, while CXCR2 binds to IL-8 and other $ELR^{+}CXC$ chemokines such as growth related oncogene-α and neutrophil activating peptide-2 (Ahuja, 1996a; Ahuja, 1996b; Murphy, 1994). Bovine IL-8 receptor loci (CXCR1 and CXCR2) have been mapped approximately 90.3 cM from the centromere of bovine chromosome (BTA) 2. These loci are approximately 1.3 cM from the natural resistance associated macrophage protein (NRAMP)-1, a polymorphic gene related to immune function in humans and mice, indicating that this region of BTA 2 may be associated with immune function and disease resistance (Blackwell, 2000; Grosse, 1999).

Four single nucleotide polymorphisms (SNPs) that corresponded with Mendelian inheritance patterns were identified within a 523 bp segment of the

bovine IL-8 receptor locus in a commercial beef cattle population (Grosse, 1999). This fragment's position is predicted to reside in the coding region for the bovine CXCR2 gene. Bison and several different breeds of beef cattle exhibited varying frequencies of these four IL-8 receptor SNPs and the resulting five haplotypes (Heaton, 2001). However, polymorphisms for the IL-8 receptor have not been identified previously in dairy cattle. Objectives of this experiment were to identify candidate SNPs and resulting haplotypes within a segment of the bovine IL-8 receptor locus and determine frequencies of both SNPs and haplotypes within two different breeds of dairy cattle. We hypothesize that both SNPs and haplotypes exist within the bovine IL-8 receptor locus in varying frequencies between breeds of dairy cattle. Identification of SNPs and resulting allelic haplotypes between distinct breeds of dairy cattle would enable the comparison of inflammatory disease phenotypes, including mastitis, with immune responses between both individual cows and across different breeds. The ability to select disease resistant cattle based on genetic markers associated with mastitis would allow dairy producers to minimize costly treatment regimens as well as provide a healthier and safer milk supply for consumers.

II. MATERIALS AND METHODS

A. Animal Selection and Blood Collection

Jersey cows (n=42) from the Lewisburg Dairy Experiment Station, Lewisburg, TN, and Holstein cows (n=37) from the Middle Tennessee

Experiment Station, Spring Hill, TN, that had completed at least two full lactations were selected. Blood was collected by puncture of the jugular vein in 10 ml Vacutainer (Becton-Dickinson, Franklin Lakes, NJ), tubes containing ethylenediamine tetracetic acid as anticoagulant. Blood was shipped overnight and upon arrival, 1.0 ml was aliquotted into 1.5 ml microcentrifuge tubes and stored at –80°C.

B. Genomic DNA Isolation

Aliquots of whole blood from each cow were removed from the freezer and thawed to room temperature (20°C). Genomic DNA (gDNA) was isolated from 300 μl of whole blood using the UltraClean DNA Isolation Kit (MoBio Labs, Inc., Solana Beach, CA). Following isolation, gDNA quantity and purity were determined by spectrophotometry and stored at –20°C.

C. PCR Amplification and Sequencing

Forward and reverse primers were designed from the bovine IL-8 Receptor B (CXCR2) (GenBank Accession No. U19947) sequence to amplify a 311 bp region that contained 4 SNPs as determined in a beef cattle population (Grosse, 1999). Each reaction was carried out in a final volume of 50 μl containing: 0.1 μM dNTP, 1.0 μM each of forward and reverse oligonucleotide primers IL8Rec-SSCPFor (5'-CTTCCGTGAGGCCTATCAAC-3') and IL8Rec-SSCPRev (5'-AGGTCTCAGCAATCACATGG-3'), 5 μl 10X magnesium-free

thermophilic buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100], 3 μl of 25 mM $MgCl_2$, 20.9 μl nuclease free water, 3 U *Taq* DNA Polymerase (Promega, Madison, WI), and 200 ng gDNA suspended in nuclease free water. Thermocycler (Eppendorf, Westbury, NY) parameters consisted of an initial denaturing step at 94°C for 5 min, 30 cycles of a denaturing step at 94°C for 2 min, an annealing step at 60°C for 30 sec, and an extension period of 1 min at 72°C. Following cycling, a 20 min extension step at 72°C was incorporated before a final hold at 4°C. Following each reaction, PCR products were checked by electrophoresis on a 1% agarose gel at 100v for 40 min and stored at –20°C.

D. DNA Sequencing

PCR products amplified from gDNA were purified using Wizard PCR Preps DNA Purification System (Promega). PCR products were transformed into plasmid vectors and cloned by utilizing TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Prior to sequencing, plasmid DNA was purified with Wizard Plus Minipreps DNA Purification System (Promega). Purified plasmid DNA was sequenced by an ABI 3100 Genetic Analyzer at The University of Tennessee Molecular Biology Resource Facility, Knoxville, TN. Sequence data were analyzed using Chromas 2.23 (Technelysium Pty Ltd, Australia) and ClustalW (European Bioinformatics Institute, UK). After polymorphic loci were identified, sequences were then compared to previously available bovine CXCR2 sequences (GenBank) for homology .

E. Statistics

All statistical calculations were carried out with SAS and SAS/Genetics version 9.0 (SAS Institute, Cary, NC). Observed allele and genotype frequencies were calculated using the ALLELE procedure. The HAPLOTYPE procedure used an expectation-maximization algorithm to obtain estimated haplotype frequencies. Independence between breed allelic, genotypic and haplotype frequencies were evaluated using chi-square. Tests for allelic and genotypic departures from Hardy-Weinberg equilibrium utilized chi-square goodness of fit. Tests for association between loci utilized chi square. All testing was done at P = 0.05 significance level.

III. RESULTS

A. Identification of Bovine CXCR2 Polymorphisms

Five single nucleotide polymorphisms were identified within a 311 bp segment of the bovine IL-8 receptor locus. Polymorphisms occurred at positions +612 (G→A), +684 (G→A), +777 (G→C), +858 (C→A) and +861 (A→G) when compared to an available bovine IL-8 receptor mRNA sequence (Accession No. U19947). Corresponding amino acid sequences determined a nonsynonymous substitution at +777 (G→C) results in a histidine replacement of glutamine at amino acid residue 245, which occurs in the receptor's third intracellular loop. The four other polymorphisms result in synonymous nucleotide changes.

B. Allele, Genotype and Haplotype Frequency Comparisons

Both Holstein and Jersey of cattle exhibited all five single nucleotide polymorphisms. Polymorphisms for both breeds did not depart from Hardy-Weinberg equilibrium ($P>0.05$). Allele and genotype frequencies are shown in Tables 2 and 3, respectively. Holsteins expressed all possible combinations of genotypes for each SNP, while none of the Jersey cows expressed the GG genotype for the polymorphism at +612. Breed had a significant effect on allele and genotype frequencies ($P<0.05$) for all polymorphisms except for SNP +684 ($P>0.05$). Furthermore, Holsteins expressed increased heterozygosity for each SNP, ranging from 0.46 - 0.65, while Jersey heterozygosity was lower and more variable, ranging from 0.09 -0.50 (data not shown). Haplotype frequencies are displayed in Table 4. Ten haplotypes were derived from the five polymorphisms. Both breeds expressed six common haplotypes, while two were unique to Jerseys and two were unique to Holsteins. In Jerseys, AGGCA and AAGCA haplotypes comprised 83% of the total expressed haplotypes. Four haplotypes (AGGCA, AAGCA, GGCAG, GGCCG) accounted for 95% of the total expressed haplotypes in Holsteins. Breed significantly affected haplotype frequencies ($P<0.0001$). Strong linkage disequilibrium was exhibited for both breeds of cattle among all five polymorphic loci ($P<0.001$) as was expected due to the small size of the amplified gene segment and the close proximity of SNPs. While all combinations of Holstein SNPs were in strong linkage disequilibrium ($P<0.001$), all combinations of Jersey loci expressed linkage ($P<0.01$) except between positions +684 and +858 ($P>0.05$).

Table 2. Estimated SNP allele frequencies within the CXCR2 locus.

SNP	Jersey allele frequency (n=84)	Holstein allele frequency (n=74)
(A612G)*		
A	0.92 (± 0.03)	0.58 (± 0.05)
G	0.08 (± 0.03)	0.42 (± 0.05)
(G684A)		
G	0.65 (± 0.05)	0.68 (± 0.05)
A	0.35 (± 0.05)	0.32 (± 0.05)
(G777C)*		
G	0.87 (± 0.04)	0.57 (± 0.05)
C	0.13 (± 0.04)	0.43 (± 0.05)
(C858A)*		
C	0.93 (± 0.03)	0.74 (± 0.05)
A	0.07 (± 0.03)	0.26 (± 0.05)
(A861G)*		
A	0.86 (±0 .04)	0.57 (± 0.05)
G	0.14 (± 0.04)	0.43 (± 0.05)

Standard error in parentheses

* Allele frequencies affected by breed ($P < 0.05$)

Table 3. SNP genotype frequencies within the bovine CXCR2 locus.

SNP genotype	Jersey frequency (n=42)	Holstein frequency (n=37)
(A612G)*		
AA	0.83	0.27
AG	0.17	0.62
GG	0.00	0.11
(G684A)		
GG	0.40	0.41
AG	0.50	0.54
AA	0.10	0.05
(G777C)*		
GG	0.76	0.24
GC	0.22	0.65
CC	0.02	0.11
(C858A)*		
CC	0.88	0.51
AC	0.10	0.46
AA	0.02	0.03
(A861G)*		
AA	0.74	0.24
AG	0.24	0.65
GG	0.02	0.11

* Genotype frequencies affected by breed ($P < 0.05$)

Table 4. Estimated CXCR2 haplotype frequencies for Holstein and Jersey cattle.

Haplotype label	612	684	777	858	861	Jersey haplotype frequency (n=84)	Holstein haplotype frequency (n=74)
1	A	G	G	C	A	0.48 (± 0.05)	0.24 (± 0.05)
2	A	A	G	C	A	0.35 (± 0.05)	0.30 (± 0.05)
3	G	G	C	A	G	0.05 (± 0.02)	0.23 (± 0.05)
4	A	G	C	A	G	0.01 (± 0.01)	0.01 (± 0.01)
5	A	G	G	A	G	0.01 (± 0.01)	0.01 (± 0.01)
6	A	G	C	C	G	0.06 (± 0.03)	---
7	G	G	G	C	A	0.03 (± 0.02)	---
8	G	G	C	C	G	0.01 (± 0.01)	0.18 (± 0.04)
9	A	A	C	C	A	---	0.01 (± 0.01)
10	G	A	G	C	A	---	0.01 (± 0.01)

All haplotype frequencies were affected by breed ($P < 0.0001$). Standard error in parentheses.

C. Distribution and Frequencies of CXCR2 Haplotype Genotypes

Frequencies of CXCR2 haplotype genotypes present in Holstein and Jersey cattle are in Table 5. Twelve different haplotype genotypes were identified in Jerseys, and thirteen were expressed in Holsteins. A total of 19 haplotype genotypes were observed; however, only six were identified in both breeds of dairy cattle. Two haplotype genotypes (1-1 and 1-2) were expressed by 57% of Jersey cows. Haplotype genotypes were more broadly distributed in Holsteins; four haplotype genotypes, 1-2, 1-3, 2-3 and 2-8, were expressed by 61% of the herd.

IV. DISCUSSION

In this study, a 311 bp fragment within the bovine IL-8 receptor locus in two different breeds of dairy cattle was sequenced. When compared with publicly available data, our sequences shared approximately 98% homology with a segment believed to lie within the coding region of the bovine CXCR2 gene. Analyses of the completed human CXCR2 genomic sequence indicate that at least seven variants of CXCR2 are created from a total of 3 exons, with the entire coding region residing on exon 3 (Sprenger, 1994). As suggested previously by Grosse et al. (1999), a completed sequence of the entire bovine IL-8 receptor locus is required to determine the exact number of exons and introns that comprise the gene, as well as to determine which exon contains the open reading frame. However, we speculate our sequence corresponds to the coding

Table 5. Bovine CXCR2 haplotype genotype frequencies by breed.

Haplotype combination (+612, +684, +777, +858, +861)	Haplotype abbreviation	Jersey combined haplotype frequency (n=42)	Holstein combined haplotype frequency (n=37)
AGGCA-AGGCA	1-1	0.19 (8)	0.05 (2)
AGGCA-AAGCA	1-2	0.38 (16)	0.14 (5)
AGGCA-GGCAG	1-3	0.02 (1)	0.14 (5)
AGGCA-AGGAG	1-5	0.02 (1)	0
AGGCA-AGCCG	1-6	0.05 (2)	0
AGGCA-GGGCA	1-7	0.05 (2)	0
AGGCA-GGCCG	1-8	0.02 (1)	0.11 (4)
AAGCA-AAGCA	2-2	0.10 (4)	0.05 (2)
AAGCA-GGCAG	2-3	0.02 (1)	0.19 (7)
AAGCA-AGCAG	2-4	0	0.03 (1)
AAGCA-AGCCG	2-6	0.05 (2)	0
AAGCA-GGGCA	2-7	0.02 (1)	0
AAGCA-GGCCG	2-8	0	0.14 (5)
GGCAG-AGCAG	3-4	0.02 (1)	0
GGCAGAGGAG	3-5	0	0.03 (1)
GGCAG-GGCCG	3-8	0	0.05 (2)
GGCAG-AACCA	3-9	0	0.03 (1)
GGCAG-GAGCA	3-10	0	0.03 (1)
GGCCG-GGCCG	8-8	0	0.03 (1)

Numbers in parentheses are the number of cows expressing the haplotype in each breed.

region of the bovine CXCR2 gene due to strong sequence homology shared by bovine IL-8 receptor mRNA (Accession No. U19947) and human CXCR2 mRNA (Accession No. NM001557), which is approximately 91%. Furthermore, studies have determined conserved synteny exists between regions of BSA2q and HSA2q, which contain the IL-8 receptor loci for both species (Hayes 1995; Solinas-Toldo, 1995; Chowdhary, 1996; Sonstegard, 1997).

Five single nucleotide polymorphisms that occurred in varying frequencies within two breeds of dairy cattle were identified. Four of these SNPs, +612 (A→G), +777 (G→C), +858 (C→A) and +861 (A→G), were reported previously in beef cattle. Further study is needed to determine if SNPs located at +684 (G→A) and +861 (A→G) conform to Mendelian inheritance models in dairy cattle as Grosse et al. (1999) determined only +612 (A→G), +777 (G→A), +858 (C→A) followed Mendelian inheritance patterns in beef cattle. Due to the strong linkage exhibited between all combinations of SNPs by Holsteins, and by all but one combination of Jersey SNPs, we predict that polymorphisms at +684 and +861 also correspond with Mendelian inheritance patterns. In support of this hypothesis, these SNPs also exhibited similar allelic frequencies when compared to the three previously identified SNPs within each respective breed.

Holstein cows exhibited increased heterozygosity for all polymorphic loci when compared to Jerseys, indicating that Holsteins are a more outbred population. This was expected as registered Holstein cattle outnumber registered Jersey cattle nearly 15:1 in the United States, and maintain a larger number of available sires contributing to the gene pool. Furthermore, Jersey

cattle exhibit a higher inbreeding trend than Holstein cattle, as well as have several active sires with inbreeding coefficients >10.0 (AIPL, 2003).

Differences in haplotype frequencies between breeds provides additional support that Holsteins are a more outbred population when compared to Jersey cattle. A total of ten haplotypes were derived from five single nucleotide polymorphisms; eight were expressed by Holstein cows and eight were expressed by Jersey cows. Both breeds exhibited the same two most common haplotypes. Four haplotypes expressed by Holstein cows comprised approximately 94% of all haplotypes present in the herd. Two haplotypes (AGGCA and AAGCA) accounted for 83% of the haplotypes in Jerseys, but only accounted for 54% in Holstein cows. Sharing of common haplotypes across similar breeds of cattle is not unexpected. Heaton et al. (2001) demonstrated that common haplotypes for cytokine and cytokine receptor genes occurred between distinct breeds of beef cattle. Although different breeds expressed common haplotypes, haplotype frequencies were variable between breeds (Heaton, 2001). Our results support findings of Heaton et al. (2001) as we observed haplotypes in distinct frequencies across two breeds of dairy cattle.

Holstein and Jersey cows commonly expressed six haplotype genotypes; six were expressed only within the Jersey breed and seven were unique to the Holstein breed. In Jersey cattle, two haplotype combinations (1-1 and 1-2) were the most common, while the predominant CXCR2 genotypes in Holstein cows were 1-2, 2-2, 2-3 and 2-8. This can be explained as haplotypes 1 and 2 accounted for approximately 83% of CXCR2 alleles in Jersey cows, while four

haplotypes (1, 2, 3, 8) accounted for approximately 94% of CXCR2 alleles in Holsteins. Similarly, Heaton et al. (2001) identified seven IL-8 SNPs that resulted in five haplotypes expressed by different breeds of beef cattle. These five haplotypes combined together to form eleven different haplotype genotypes, which were present in various frequencies for different breeds of cattle. However, different herds of cattle within the same breed showed varying frequency of both haplotypes and haplotype genotypes, although two IL-8 haplotypes predominated across all beef breeds. Thus, future studies may identify variable haplotype and haplotype genotype frequencies across different herds of Holstein and Jersey cattle, but most likely two to three alleles will predominate.

We identified five SNPs that occurred in two breeds of dairy cattle; however, only four of these were reported previously in breeds of beef cattle (Grosse, 1999; Heaton, 2001). Grosse et al. (1999) identified a SNP located at +783 that segregated according to Mendelian genetics that was not expressed by Holstein or Jersey cows in the present study. However, we identified a polymorphism at +684 that was expressed by both breeds of dairy cattle and not previously identified in beef cattle. Several different SNPs have been identified also within the human CXCR2 gene within different populations (Kato, 2000; Renzoni, 2000; Sprenger, 1994). Studies have identified a synonymous SNP expressed by both Japanese and British caucasians that occurred in the coding sequence at +785 (C→T) (Kato, 2000; Renzoni, 2000). Within the sampled Japanese population, two other polymorphisms were identified within the coding

region. One nonsynonymous SNP occurred at +238 (C→T), which resulted in a cysteine replacement of arginine at amino acid 80, and a synonymous nucleotide substitution at +768 (C→T) (Kato, 2000). No other SNPs were detected within the coding sequence in the British Caucasian sample group; however, two synonymous SNPs were identified in the 5' untranslated region at +1208 (C→T) and +1440 (G→A) (Renzoni, 2000).

The polymorphic nature of the human and bovine CXCR2 gene indicates that the CXCR2 gene may be an evolutionary hot spot for genetic mutation (Grosse, 1999; Heaton, 2001). Genetic variation may be accelerated in cattle when compared to humans due to increased selection pressure placed on specific traits such as milk production and carcass traits. Furthermore, the increased number of CXCR2 SNPs, haplotypes and haplotype genotypes expressed by both Holstein and Jersey cattle, when compared to beef cattle breeds, may be due to widespread use of artificial insemination in the dairy industry. As genotyping of different breeds of cattle continues for the CXCR2 locus, more SNPs located within both the coding and noncoding regions may potentially be detected. This may be a informative chromosomal region for association with disease phenotypes due to its highly polymorphic nature, as expressed across different populations in different species.

The ability to genotype cattle populations will enable future studies to identify important genetic markers associated with immune function and disease susceptibility and/or resistance. While no previous studies have associated bovine IL-8 receptor loci with immune responses during infection, two linked

SNPs (+785 and +1208) have been associated with susceptibility to fibrosing alveolitis and nonfibrosing alveolitis systemic sclerosis in humans (Renzoni, 2000). While the polymorphism located at +785 did not result in an amino acid substitution and most likely does not affect receptor function, the +1208 SNP was located within the 3' untranslated region of the CXCR2 gene and may control mRNA processing and translation (Renzoni, 2000). While four of the five SNPs (+612, +648, +858, and +861) identified in dairy cattle are synonymous substitutions and may have little affect on CXCR2 function, the non-synonymous +777 (G→C) polymorphism results in a histidine substitution for glutamine at amino acid residue 245. This amino acid lies within the third intracellular loop when compared to the publicly available protein sequence for the bovine IL-8 receptor (Genbank No. AAA84996.1) and may affect neutrophil activity during infection. Damaj et al. (1996) identified amino acid residues within the third intracellular loop of both human IL-8 receptors, CXCR1 and CXCR2, that were involved in mediating neutrophil calcium signaling and mobilization during IL-8 stimulation. Increased cytosolic free calcium is crucial for neutrophil respiratory burst and exocytosis during infection (Dewald, 1988; Lew, 1986). Thus, the amino acid substitution at residue 245 may affect CXCR2 signaling and neutrophil function in dairy cattle.

The bovine IL-8 receptor locus is an excellent candidate marker for immune function during infection. We identified five SNPs within the bovine CXCR2 locus in two breeds of dairy cattle. A non-synonymous SNP at nucleotide +777 results in an amino acid substitution that may affect neutrophil

response during infection. Studies are underway to analyze possible relationships between bovine IL-8 receptor polymorphisms and disease resistance in Holstein and Jersey dairy cattle.

REFERENCES

Ahuja, S.K., Lee, J.C., Murphy, P.M. 1996a. CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human Interleukin-8 receptor B. The Journal of Biological Chemistry 271, 225-232.

Ahuja, S.K., Murphy, P.M. 1996b. The CXC chemokines growth-related oncogene (GRO)-α, GRO-β, GRO-γ, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human Interleukin-8 receptor. The Journal of Biological Chemistry 271, 20545-20550.

Animal Improvement Programs Laboratory (AIPL). 2003 AIPL Inbreeding Trends. USDA Animal Improvement Programs Laboratory <http://www.aipl.arsusda.gov>.

Ashwell, M.S., Da, Y., Van Tassell, C.P., Vanraden, P.M., Miller, R.H., Rexroad, C.E.J. 1998. Detection of putative loci affecting milk production and composition, health, and type traits in a United States Holstein population. Journal of Dairy Science 81, 3309-3314.

Ashwell, M.S., Van Tassell, C.P. 1999. Detection of putative loci affecting milk, health, and type traits in a U.S. Holstein population using 70 microsatellite markers using a genome scan. Journal of Dairy Science 82, 2497-2502.

Blackwell, J.M., S. Searle, R. Goswami, E.N. Miller. 2000. Understanding the multiple functions of NRAMP1. Microbes and Infection 2, 317-321.

Chowdhary, B. P., Fronicke, L., Gustavsson, I., Scherthan, H. 1996. Comparative analysis of the cattle and human genomes: detection of ZOO-FISH and gene mapping-based chromosomal homologies. Mammalian Genome 7, 297-302.

Damaj, B.B., McColl, S.R., Neote, K., Na, S.Q., Ogborn, K.T., Hebert, C.A., Naccache, P.A. 1996. Identification of G-protein binding sites of the human interleukin-8 receptors by functional mapping of the intracellular loops. FASEB Journal 10, 1426-1434.

Dewald, B., Thelen, M., Baggiolini, M. 1988. Two transduction sequences are necessary for neutrophil activation by receptor agonists. Journal of Chemistry 263, 16179-16184.

Dietz, A.B., Detilleux, J.C., Freeman, A.E., Kelley, D.H., Stabel, J.R., Kehrli, M.E. Jr. 1997. Genetic association of bovine lymphocyte antigen DRB3 alleles with immunological traits of Holstein cattle. Journal of Dairy Science 80, 400-405.

Grosse, W.M., Kappes, S.W., Laegreid, W.W., Keele, J.W.. Chitko-McKown, C.G., Heaton, M.P. 1999. Single nucleotide polymorphism (SNP) discovery and linkage mapping of bovine cytokine genes. Mammalian Genome 10, 1062-1069.

Hansen, L.B. 2000. Consequences of selection for milk yield from a geneticist's viewpoint. Journal of Dairy Science 83, 1145-1150.

Hayes, H. 1995. Chromosome painting with human chromosome-specific DNA libraries reveals the extent and distribution of conserved segments in bovine chromosomes. Cytogenetics and Cellular Genetics 71, 168-174.

Heaton, M.P., Grosse, W.M., Kappes, S.M., Keele, J.W., Chitko-McKown, C.G., Cundiff, L.V., Braun, A., Little, D.P., Laegreid, W.W. 2001. Estimation of DNA sequence diversity in bovine cytokine genes. Mammalian Genome 12, 32-37.

Heyen, D.W., Weller, J.I., Ron, M., Band, M., Beever, J.E., Feldmesser, E., Da, Y., Wiggans, G.R., Van Raden, P.M., Lewin, H.A. 1999. A genome scan for QTL influencing milk production and health traits in dairy cattle. Phsyical Genomics 3, 165-176.

Kato, H., Tsuchiya, N., Tokunaga, K. 2000. Single nucleotide polymorphisms in the coding region of human CXC-chemokine receptors CXCR1, CXCR2 and CXCR3. Genes and Immunity 5, 330-337.

Kelm, S.C., Detilleux, J.C., Freeman, A.E., Kehrli, M.E. Jr., Dietz, A.B., Fox, L.K., Butler, J.E., Kasckovics, I., Kelley, D.H. 1997. Genetic association between parameters of innate immunity and measures of mastitis in periparturient Holstein cattle. Journal of Dairy Science 80, 1767-1775.

Klungland, H., Sabry, A., Heringstad, B., Olsen, H.G., Gomez-Raya, L., Vage, D.I., Olsaker, I., Odegard, J., Klemetsdal, G., Schulman, N., Vilkki, J., Ruane, J., Aasland, M., Ronningen, K., Lien, S. 2001. Quantitative trait loci affecting clinical mastitis and somatic cell count in dairy cattle. Mammalian Genome 12, 837-842.

Kuhn, C., Bennewitz, J., Reinsch, N., Xu, N., Thomsen, H., Looft, C., Brockmann, G.A., Schwerin, M., Weimann, C., Hiendleder, S., Erhardt, G., Medjugorac, I., Forster, M., Brenig, B., Reinhardt, F., Reents, R., Russ, I., Averdunk, G., Blumel, J., Kalm, E. 2003. Quantitative trait loci mapping of functional traits in the German Holstein cattle population. Journal of Dairy Science 86, 360-368.

Lew, P.D., Monod, A., Waldvogel, F.A., Dewald, B., Baggiolini, M., Pozzon, T. 1986. Quantitative analysis of the cytosolic free calcium dependency of exocytosis from three subcellular compartments in intact human neutrophils. Journal of Biological Chemistry 102, 2197-2204.

Murphy, P. 1994. The molecular-biology of leukocyte chemoattractant receptors. Annual Review of Immunology 12, 593-633.

National Mastitis Council. 1996 Current concepts of bovine mastitis. 1st ed. National Mastitis Council, Inc., Madison, WI.

Onuffer, J.J., Horuk, R. 2002. Chemokines, chemokine receptors and small-molecule antagonists: recent developments. TRENDS Pharma Sciences 23, 459-467.

Renzoni, E., Lympany, P., Sestini, P., Pantelidis, P., Wells. A. 2000. Distribution of novel polymorphisms of the interleukin-8 and CXC receptor 1 and 2 genes in systemic sclerosis and cryptogenic fibrosing alveolitis. Arthritis and Rheumatism 43, 1633-1640.

Schrooten, C., Bovenhuis, H., Coppieters, W., Van Arendonk, J.A.M. 2000. Whole genome scan to detect quantitative trait loci for conformation and functional traits in dairy cattle. Journal of Dairy Science 83, 795-806.

Sharif, S., Mallard, B.A., Wilkie, B.N., Sargeant, J.M., Scott, H.M., Dekkers, J.C.M., Leslie, K.E. 1998. Associations of the bovine major histocompatability complex DRB3 (BoLA-DRB3) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. Animal Genetics 29, 185-196.

Shook, G.E. 2001. Breeding, Selection, and Somatic Cell Counts: Where are We Today? In: National Mastitis Council Annual Meeting Proceedings, pp. 113-127.

Solinas-Toldo, S., Lengauer, C., Fries, R. 1995. Comparative genome map of human and cattle. Genomics 27, 489-496.

Sonstegard, T. S., Lopez-Corrales, N.L., Kappes, S.M., Beattie, C.W., Smith, T.P.L. 1997. Comparative mapping of human chromosome 2 identifies segments of conserved synteny near the bovine mh locus." Mammalian Genome 8: 751-755.

Sprenger, H., Lloyd, A.R., Lautens, L.L., Bonner, T.I., Kelvin, D.J. 1994. Structure, genomic organization, and expression of the human interleukin-8 receptor B gene. Journal of Biological Chemistry 269, 11065-11072.

Van Tassell, C.P., Ashwell, M.S., Sonstegard, T.S. 2000. Detection of putative loci affecting milk, health, and conformation traits in U.S. Holstein population using 105 microsatellite markers. Journal of Dairy Science 83, 1865-1872.

White, J.K., Shaw, M.A., Barton, C.H. 1994. Genetic and physical mapping of 2q35 in the region of the NRAMP1 and IL8R genes: Identification of a polymorphic repeat in exon 2 of NRAMP. Genomics 24, 295-305.

Part IV

ASSOCIATION OF BOVINE CXCR2 POLYMORPHISMS WITH SUBCLINICAL AND CLINICAL MASTITIS IN HOLSTEIN AND JERSEY CATTLE

This chapter is a paper by the same name that will be submitted for publication in the *Journal of Dairy Science*, in 2003, by Sara M. Youngerman, Arnold M. Saxton, Stephen P. Oliver, and Gina M. Pighetti.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) organization of bacteriological data for sample populations, (2) defined and identified subclinical and clinical mastitis cases, (3) combining previous contributions with bacteriological data, (4) most of the statistical analyses, (5) most of the gathering and interpretation of literature, and (6) most of the writing of this paper.

I. INTRODUCTION

Mastitis continues to be the most economically devastating disease affecting the dairy industry. Identification of a genetic marker associated with mastitis susceptibility and/or resistance would allow producers to decrease costs associated with mastitis by improving herd health through animal selection. Genes associated with immune responses of the mammary gland are potential markers due to their importance in mastitis. Several studies have identified polymorphic bovine genes responsible for encoding the major histocompatability complex (MHC), cytokine and cytokine receptors, and natural resistance associated macrophage protein (NRAMP)-1 (Ables, 2002; Dietz, 1997; Grosse, 1999; Heaton, 2001; Horin, 1999; Siite, 2002). However, only a few studies have associated MHC alleles DRB3.2 *3, *11, *16 and *23 with mastitis incidence or immune function. Furthermore, alleles DRB3.2 *16 and *23 have been associated with contradicting results (Dietz, 1997; Kelm, 1997; Lunden, 1993; Sharif, 1998; Siite, 2002).

Genes associated with neutrophil function are potential genetic markers for mastitis as neutrophil migration from blood to the site of infection is essential for resolution of most mastitis pathogens (Kehrli, 2001; Paape, 2000). The ability of neutrophils to migrate into infected tissues is dependent upon recognition of inflammatory mediators by neutrophil cytokine, chemokine and complement receptors (Burvenich, 1994; Walcheck, 1996). Two chemokine receptors present on neutrophil surfaces, CXCR1 and CXCR2, are required for maximum neutrophil function during infection (Holmes, 1991; Lindley, 1998; Murphy, 1991). Recognition of chemokines by CXCR1 and CXCR2 induces neutrophil activation, chemotaxis, and eventual phagocytosis of pathogens (Lindley, 1998; Peveri, 1988; Thelen, 1988).

Both CXCR1 and CXCR2 are potential candidate genes for mastitis resistance; however, only the sequence for bovine CXCR2 is publicly available (GenBank Accession No. U19947). The bovine CXCR2 gene has been mapped to BTA 2, approximately 90.3 cM from the centromere. This region on BTA 2 shares conserved synteny and gene order on both human HSA 2 and mouse chromosome 1 (Band, 2000; Grosse, 1999; Sonstegard, 1997). Single nucleotide polymorphisms (SNPs) have been identified within the human CXCR2 gene and different SNPs have been shown to occur in different geographical populations. Several of these SNPs have been associated with susceptibility to inflammatory diseases (Sprenger, 1994; Renzoni, 2000; Kato, 2000). While SNPs have been identified within the bovine CXCR2 gene in different breeds of

beef cattle and bison, none have been associated with disease resistance or susceptibility.

We identified five SNPs within a 311 bp segment of the coding region in exon 3 of the bovine CXCR2 gene in Holstein and Jersey dairy cattle (Youngerman et al., unpublished). These five SNPs created 10 total CXCR2 haplotypes; six haplotypes were common to both breeds, two were found only in Holsteins, and two haplotypes were unique to Jerseys. Four SNPs are synonymous polymorphisms and do not affect the amino acid sequence of CXCR2 (+612, +684, +858, +861). However, the nonsynonymous (G→C) SNP at +777 results in amino acid 245 glutamine→histidine substitution (Grosse, 1999; Youngerman, unpublished). Amino acid 245 is located within the third intracellular loop of CXCR2, an important region involved in mediating calcium signaling and mobilization, as well as G-protein binding when stimulated by IL-8 (Damaj, 1996). Therefore, the bovine CXCR2 gene is an excellent potential candidate marker for mastitis as CXCR2 is a critical component during neutrophil migration to the mammary gland during mastitis, CXCR2 polymorphisms have been associated with disease susceptibility in humans, and several polymorphisms have been identified in cattle. The objective of this experiment was to determine whether SNPs and/or haplotypes within the bovine CXCR2 gene were associated with subclinical and/or clinical mastitis in Holstein and Jersey cattle.

II. MATERIALS AND METHODS

A. Animal Selection

Forty-two Jersey cows from the Dairy Experiment Station, Lewisburg, TN, and 37 Holstein cows from the Middle Tennessee Experiment Station, Spring Hill, TN, that had completed at least two full lactations, were selected randomly. The majority of Jersey cows completed at least four lactations, while Holstein cows completed at least three lactations.

B. Experimental Herds

The Dairy Experiment Station (DES) research herd in Lewisburg, TN consists of approximately 170 lactating Jersey cows. Cows were milked twice daily in a 12-stall trigon milking parlor equipped with automatic milking machine takeoffs (Surge, Babson Bros., Oak Brook, IL). The Middle Tennessee Experiment Station (MTES) dairy research herd in Spring Hill, TN was composed of approximately 150 lactating Holstein cows. Lactating cows were milked twice a day in a double-eight parallel parlor equipped with a DeLaval milking machine system (DeLaval) with automated milking machine take-offs. Milking machines were backflushed (Surge Backflush II, Babson Bros.) with water following cow removal. Milking equipment was evaluated routinely and maintained by manufacturer's recommendation. Pre- and postmilking teat disinfection was performed. Cows were pastured 4 - 6 h/d, weather and pasture permitting. Cows were housed in free-stalls bedded with separated dairy waste solids from a

manure slurry (Alfa-Laval, Inc., Kansas City, MO) at the DES herd and with sawdust at the MTES herd. All cows were dried off approximately 6 to 8 weeks before expected calving. An antibiotic preparation approved for use in nonlactating cows was infused into all cows' mammary glands following the last milking of lactation.

C. Bacteriological Examination of Foremilk Samples

Quarter foremilk samples were collected from all lactating cows in both herds approximately every 3 mo, prior to dry off, two weeks prior to expected calving and when cows exhibited clinical mastitis and were evaluated microbacteriologically. Prior to sample collection, teats of cows were cleaned and dried with individual disposable paper towels, and teat ends were sanitized with swabs containing 70% isopropyl alcohol. After sample collection, samples were stored frozen at –20^{o}C until transported to the laboratory. Milk samples were examined following procedures recommended by the National Mastitis Council (Hogan, 1999). Samples of mammary secretion (10 μl) from each mammary gland were plated onto one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Becton Dickinson and Company, Franklin Lakes, NJ). Plates were incubated at 37^{o}C, and bacterial growth was observed and recorded at 24-hour intervals for three days. Bacteria on primary culture medium were identified tentatively according to colony morphologic features, hemolytic characteristics, Gram stain reaction, and catalase test. Isolates identified presumptively as staphylococci were tested for

coagulase production by the tube coagulase method. Isolates identified presumptively as streptococci were evaluated initially for growth in 6.5% NaCl, hydrolysis of esculin and sodium hippurate, and CAMP reaction. Streptococcal organisms were identified to the species level using the API 20 Strep system (bioMerieux Inc., Durham, NC) and a streptococcal agglutination system (Streptex, Remel, Lenexa, KS). Gram-negative isolates were identified to the species level using the following biochemical tests: triple sugar iron, urea, oxidase, motility, indole, and ornithine decarboxylase and by the API 20 E identification system (bioMerieux Inc.).

D. Determination of Clinical and Subclinical Mastitis

Clinical mastitis was defined as the presence of abnormal milk, and/or abnormal udder, and/or systemic signs of intramammary infection that warranted treatment with intramammary therapies. Milking personnel identified cows with clinical mastitis and treatment was administered following the collection of samples from the infected quarter(s). Subclinical mastitis was defined as the presence of the same pathogen in the same quarter in at least two out of three consecutive samples. Data for each cow were combined over time points and quarters to obtain percentages of subclinical and clinical mastitis cases. Final analyses were conducted using the percentages on a per cow basis. For example, at one collection time point a cow has four data points, one for each quarter. A cow with four lactations with five collection time points in each lactation had 80 total observations (4 quarters X 5 collection time points X 4

lactations = 80 total observations). If this cow had 2 subclinical events, she would have a 2.5% percentage for subclinical mastitis. If this cow had 2 clinical events she would also have a 2.5% percentage for clinical mastitis.

Subclinical mastitis due to *Corynebacterium bovis*, coagulase-negative staphylococci (CNS) and *Staphylococcus aureus* were observed in the Holstein herd, while the majority of clinical mastitis was due to *Staph. aureus* and *Streptococcus uberis*. The majority of subclinical mastitis in the Jersey herd was due to CNS, *Strep. uberis* and *Staph. aureus*, while *Strep. uberis* and *Escherichia coli* caused the majority of clinical mastitis.

E. Determination of Somatic Cell Scores and Milk Production Data

The number of somatic cells in milk was determined by Dairy Herd Improvement Association personnel at The University of Tennessee, Knoxville, TN with a Somacount 300 cell counter (Bentley Instruments, Chaska, MN). Somatic cell scores and projected 305-d mature equivalent milk yields were obtained from Dairy Records Management Systems (DRMS, Raleigh, NC).

F. Statistics

All statistical calculations were carried out with SAS version 9.0 (SAS Institute, Cary, NC). A randomized block design blocking on lactation with covariates on somatic cell score and milk yield was used to determine effects of CXCR2 SNPs and haplotype genotypes on the percent incidence of subclinical and clinical mastitis over lifetime for each breed. Analysis of variance was used

to detect significance differences between data. Data are presented as least squares means with associated standard error. Randomized block designs blocking on lactation were used to determine effects of CXCR2 SNPs on somatic cell scores and milk yield. Correlations between percentage of subclinical and/or clinical mastitis incidence, somatic cell scores and milk yield were determined. Statistical significance was declared at $P < 0.05$.

III. RESULTS

A. Analysis of Haplotype Genotypes with Subclinical and Clinical Mastitis

Significant differences ($P<0.01$) occurred among haplotype genotypes in Holsteins for both subclinical and clinical mastitis (Figure 1). Estimated means of percent subclinical mastitis incidence ranged from 12.4% to 54.4%. Cows that expressed haplotype genotypes 2-4 and 3-8 had significantly more subclinical mastitis than cows that expressed haplotype genotypes 1-1, 1-2, 1-3, 2-2, 2-3, 2-8 and 3-9. Estimated means of percent clinical mastitis for Holstein haplotype genotypes ranged from 0.12% to 6.97%. Significant differences were also observed among haplotype combinations with respect to clinical mastitis. Haplotype genotype 1-8 was significantly greater than all other haplotype genotypes except 1-1, 3-5 and 3-10.

In Jerseys, significant differences were observed between haplotype combinations and percentages of clinical mastitis ($P<0.0001$), but not subclinical mastitis ($P>0.05$) (Figure 2). Estimated percentages of subclinical mastitis

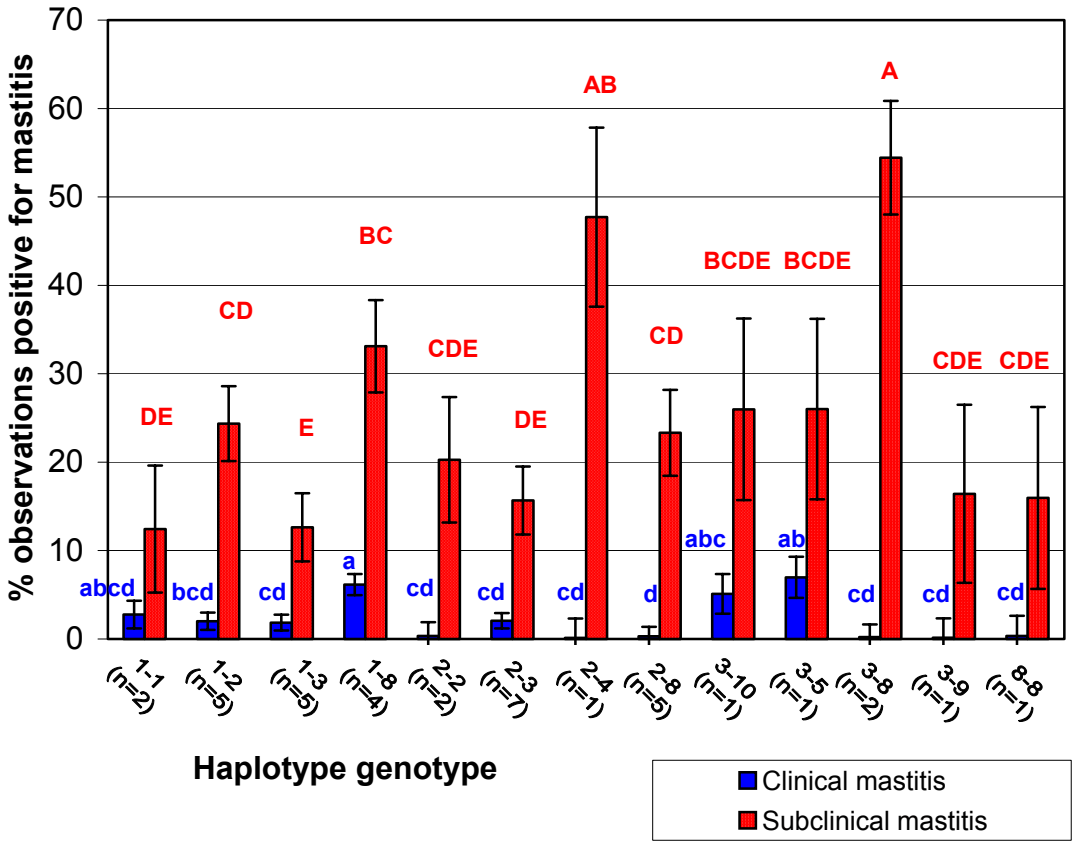

Figure 1. Estimated percent observations positive for subclinical and clinical mastitis by CXCR2 haplotype genotype in Holstein cattle. Data are means ± SEM. Small letters represent LSM separation for clinical mastitis (P<0.01). Letters in caps represent LSM separation for subclinical mastitis (P<0.001). "n" represents number of cows expressing haplotype genotype.

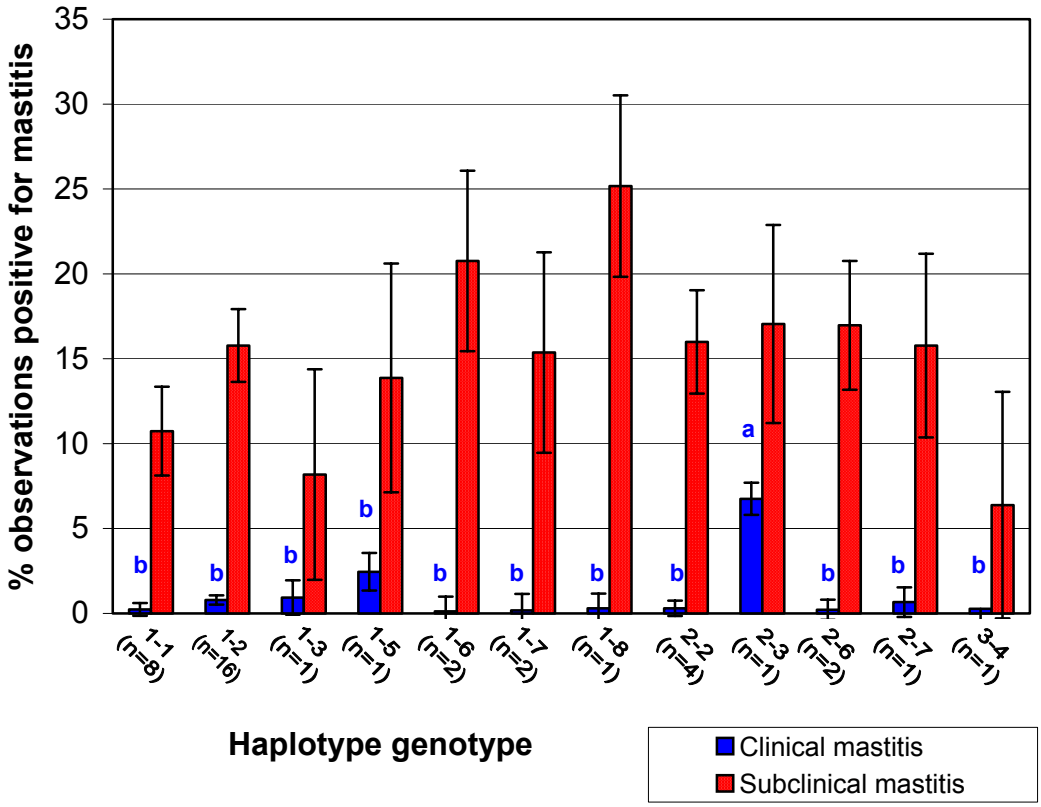

Figure 2. Estimated percent observations positive for subclinical and clinical mastitis by CXCR2 haplotype genotype in Jersey cattle. Data are means ± SEM. Small letters represent LSM separation for clinical mastitis ($P<0.0001$). "n" represents number of cows expressing the haplotype genotype.

means ranged from 8.2% to 25.2%. Percentages of clinical mastitis ranged from 0.1% to 6.8%. Haplotype genotype 2-3 was associated with a greater percentage of clinical mastitis than all other haplotype genotypes.

B. Analysis of SNP +777 Genotypes with Subclinical and Clinical Mastitis

Holstein cows exhibited higher estimated means of percent subclinical mastitis for all SNPs when compared to Jersey cows. SNPs +612, +777 and +861 showed a significant association with subclinical mastitis ($P<0.05$). Further attention focused on SNP +777, as SNPs +612 and +861 are nonsynonymous polymorphisms. SNP +777 (G→C) results in a Gln^{245}→His^{245} replacement, which may affect mastitis phenotype. Cows with genotype CC at SNP +777 expressed increased percentages of subclinical mastitis when compared to cows with genotype GG ($P<0.05$), 37% versus 22%, respectively (Figure 3). This difference was not observed in Jerseys ($P>0.05$). Significant differences were not detected in either breed between SNP +777 genotypes and percent of cows with clinical mastitis (Figure 4). However, Holstein cows tended to have lower incidence of clinical mastitis when the CC genotype was expressed compared to the GG phenotype.

C. Association of SNP +777 Genotypes with SCS and Milk Yield

The relationship between SNP +777 genotypes with SCS was evaluated. SNP +777 had a significant association with SCS in Holsteins ($P<0.0001$);

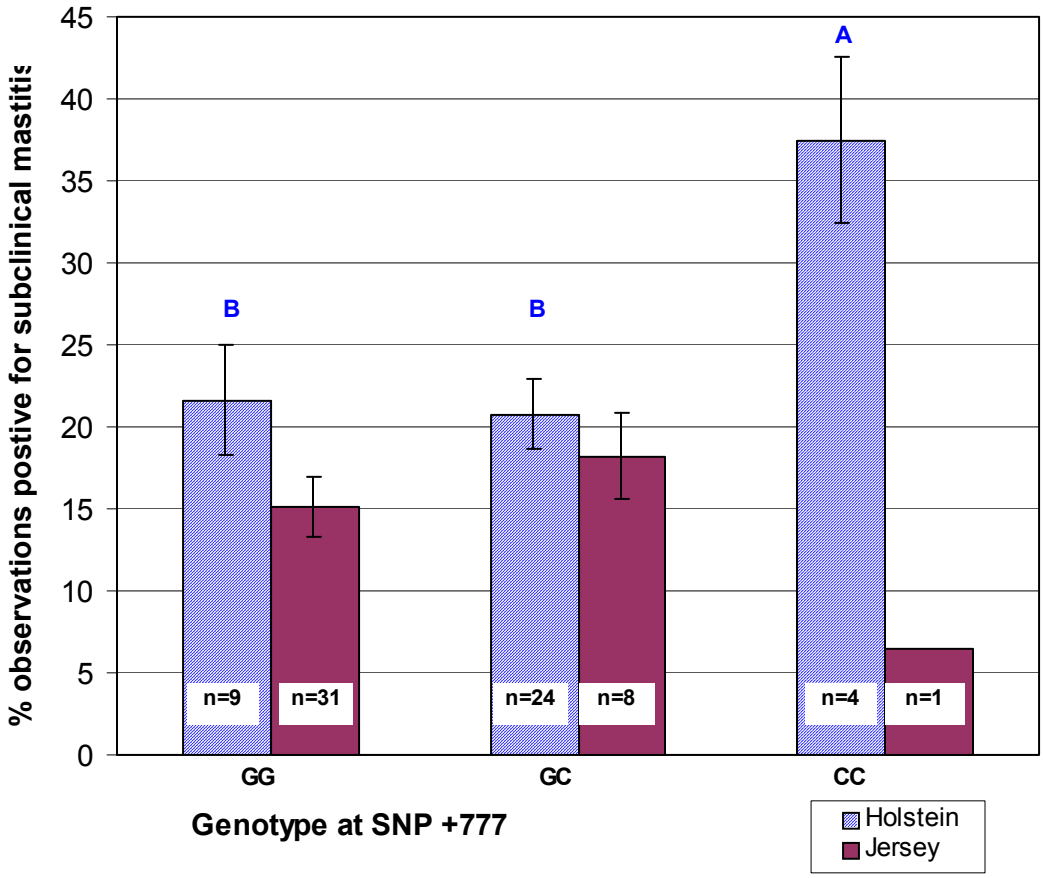

Figure 3. CXCR2 SNP +777 genotype by estimated percent observations positive for subclinical mastitis. Data are means ± SEM. Letters in caps represent LSM separation for Holstein cattle ($P<0.05$). "n" represents number of cows expressing CXCR2 SNP +777 genotype.

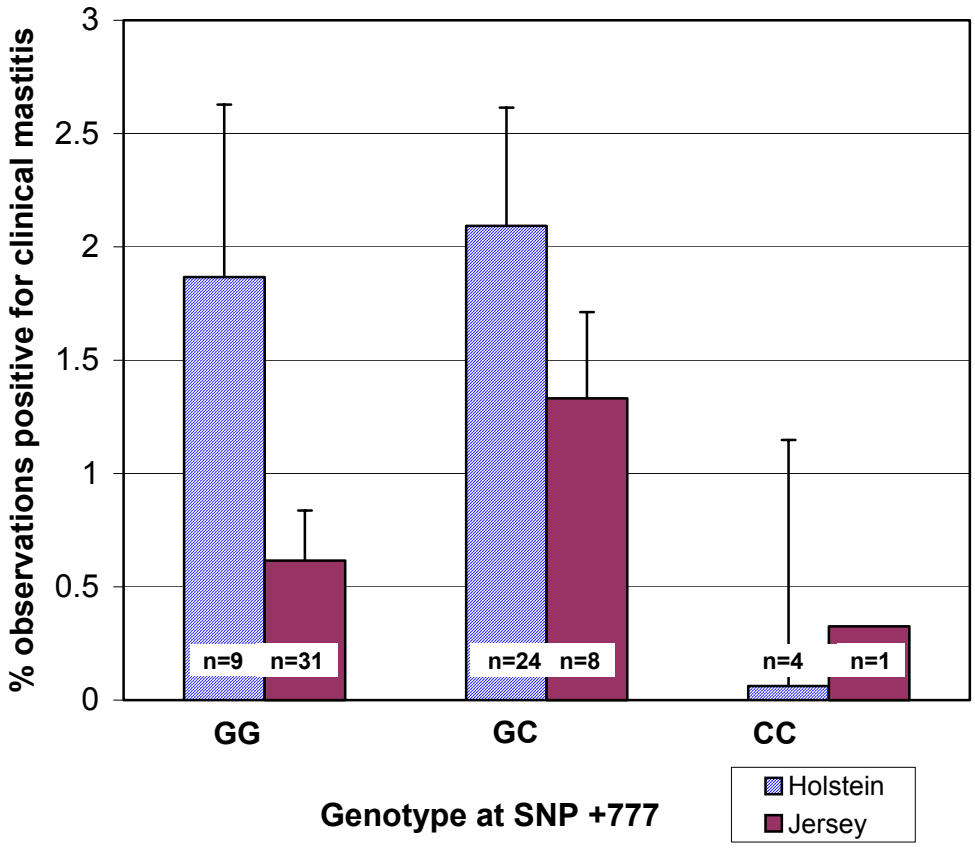

Figure 4. CXCR2 SNP+777 genotype by estimated percent observations positive for clinical mastitis. Data are means ± SEM. "n" represents the number of cows expressing SNP +777 genotype.

however, this polymorphism was not significant for Jersey SCS differences (Table 6). Cows expressing genotypes GG and GC had increased SCS relative to the CC genotype; however, only cows expressing the GC genotype had significantly greater SCS than those expressing genotype CC. No significant differences were observed for Jersey SNP +777 genotypes and SCS, which ranged from 3.09 to 3.40.

We observed no significant differences between SNP +777 genotype and 305-day milk yield in Jerseys, as estimated means ranged from 8419 to 8629 kg (Table 6). However, Holsteins expressing genotype GG had significantly lower 305-day milk yields (9412 kg) versus Holsteins expressing genotypes GC and CC (10231 and 10056 kg), respectively.

D. Correlation Coefficients

Weak positive correlations were observed between percentage of cows with clinical mastitis and SCS (0.11) and projected 305d milk yield (0.12) in Holsteins (Table 7). In contrast, little if any correlation was observed between subclinical mastitis with SCS and milk yield. As expected, lactation number was correlated with mastitis incidence, SCS and milk yield.

Percentage of subclinical mastitis and SCS were moderately correlated (0.40) in Jerseys (Table 8). Clinical mastitis incidence was also weakly correlated with SCS (0.20). In contrast to the Holstein population, a moderate negative correlation was observed in Jerseys for SCS and milk yield (-0.28). Similar to the Holstein population, a weak positive correlation was observed

Table 6. Bovine CXCR2 SNP +777 genotype by SCS and milk yield over all lactations.

Holstein SNP +777 genotype	Estimated SCS (P<0.0001)	Estimated 305d milk yield (kg) (P<0.0001)
GG	3.10 (± 0.15)[A]	9412 (± 151) [B]
GC	4.00 (± 0.11)[B]	10231 (± 112) [A]
CC	2.72 (± 0.20)[A]	10056 (± 220) [A]
Jersey SNP +777 genotype	**Estimated SCS (P>0.05)**	**Estimated 305d milk yield (kg) (P>0.05)**
GG	3.09 (± 0.07)	8419 (± 53)
GC	3.35 (± 0.13)	8585 (± 105)
CC	3.40 (± 0.40)	8629 (± 326)

[A,B] signify LSM separation within a herd and column (P<0.05)

Table 7. Correlation coefficients among Holstein SCS, milk yield, clinical and subclinical mastitis, and lactation.

	Correlation coefficients			
	% Subclinical mastitis	**Milk yield**	**SCS**	**Lactation**
% Clinical mastitis	-0.04	0.12[a]	0.11[a]	0.19[a]
% Subclinical mastitis		0.06	0.02	0.15[a]
Milk yield			-0.06	0.38[a]
SCS				0.17[a]

[a]P < 0.05

Table 8. Correlation coefficients among Jersey SCS, milk yield, clinical and subclinical mastitis, and lactation.

	Correlation coefficients			
	% Subclinical mastitis	**Milk yield**	**SCS**	**Lactation**
% Clinical mastitis	0.06	0.05	0.20[a]	0.00
% Subclinical mastitis		-0.01	0.40[a]	0.21[a]
Milk yield			-0.28[a]	-0.11[a]
SCS				0.20[a]

[a]P < 0.05

between lactation and percentage of subclinical mastitis (0.21) and SCS (0.20). However, a weak negative correlation (-0.11) was observed between lactation and milk yield.

IV. DISCUSSION

We previously identified ten CXCR2 haplotypes created by five SNPs located within the predicted coding region of exon three of the bovine CXCR2 gene (Youngerman, unpublished). The association of CXCR2 haplotype genotypes with percentages of subclinical and clinical mastitis incidences for each respective herd was examined. Several haplotype genotypes were associated with increased or decreased incidence of subclinical and clinical mastitis in Holsteins. In Jerseys, no haplotype genotypes were found to be significantly associated with subclinical mastitis incidence and only one haplotype genotype was associated with increased clinical mastitis incidence. However, due to the increased number of haplotype genotypes within a limited population, it is difficult to estimate mean incidences of mastitis for each haplotype genotypes. Consequently, the majority of our study focused upon individual SNPs.

CXCR2 SNPs +612, +777 and +861 were significantly ($P<0.05$) associated with incidence of subclinical mastitis. We focused our attention on SNP +777 as it resulted in an amino acid 245 change (His→Gln). As SNP +777 results in an amino acid change and resides within the coding region of the

CXCR2 gene, this nonsynonymous polymorphism may be subject to natural selection as it may affect mastitis phenotype (Cardon, 2003; Hannson, 2002; Shastry, 2002). Due to the tight linkage of these SNPs, as well as the fact that SNPs +612, +777, and +861 have near identical allele and genotype frequencies within the Holstein breed, it was not unusual to expect that synonymous SNPs +612 and +861 showed similar results as nonsynonymous SNP +777 (Youngerman, unpublished).

Due to potential effects caused by the nonsynonymous SNP +777 (G→C) on CXCR2 signal transduction and function, we examined the association of SNP +777 genotypes with percentages of subclinical and clinical mastitis incidences. A non-significant decrease of clinical mastitis incidence was observed in cows that expressed genotype CC. A significant difference ($P<0.05$) between subclinical mastitis incidence and SNP+777 genotype was identified also. Holsteins expressing the GG genotype had a lower incidence of subclinical mastitis than cows that expressed the CC genotype. While we did not detect significant differences between SNP+777 genotypes GG or CC and SCS, we did observe greater 305d milk yield in Holsteins that expressed genotype CC when compared to those expressing GG. This observation supports prior research that cows selected for greater milk yields also have increased mastitis infections (Hansen, 1979; Rogers, 1998; Shook, 1993).

One possible explanation for increased subclinical mastitis in Holstein cattle may be due to expression of other genes located near CXCR2 on BTA 2. NRAMP1 is a potential gene affecting mastitis and related immune responses as

it is located near CXCR2 (1.3 cM) and has been shown to be polymorphic and associated with immune function (Blackwell, 1989; Blackwell, 1996; Blackwell, 2000; Bellamy, 1999). Single nucleotide polymorphisms located within other regions of the bovine CXCR2 gene may also affect CXCR2 function during mastitis infections. Several single nucleotide polymorphisms have been detected within different regions of the human CXCR2 gene (Kato, 2000; Renzoni, 2000). While these SNPs have yet to be associated with disease resistance or susceptibility, White et al., (1994) associated an human CXCR2 RFLP with rheumatoid arthritis. As such, other nonsynonymous SNPs located within segments of the CXCR2 coding sequence may affect receptor binding and function. Additionally, either synonymous or nonsynonymous SNPs in the promoter and 3' untranslated regions may affect initiation and/or termination of transcription or translation. In order to determine if other SNPs are involved with changes in susceptibility or resistance, the complete sequence of the bovine CXCR2 gene should be determined.

Another possible explanation of increased subclinical mastitis in Holstein cattle that expressed the CC genotype for SNP+777 may be due to the amino acid change caused by the G→C substitution. During the onset of mastitis, different pathogens invade the mammary gland and elicit cytokine and chemokine release from macrophages and surrounding epithelial cells (Paape, 2002). Inflammatory mediators, such as interleukin-8, growth related oncogene-α, and neutrophil activating peptide-2, bind to CXCR2 receptors and induce CXCR2 internalization, initiating a signal transduction cascade resulting in the

release of intracellular Ca^{2+} and granular enzymes, as well as initiating chemotaxis (Ahuja, 1996; Baggiolini, 1992; Knall, 1996; Loetcher, 1994). While studies have identified binding sites in the N' terminus responsible for binding selectivity and in the C' terminus involved in receptor phosphorylation, few have focused on identifying functional roles of intracellular loops (Ahuja, 1996a; Ben-Baruch, 1995; Richardson, 2003). Damaj et al. (1996) determined that Met^{241} is important in G-protein binding and intracellular Ca^{2+} mobilization. Damaj et al. (1996) also determined other amino acids in the third intracellular loop were moderately involved in mediating intracellular signaling. Thus, Holsteins expressing SNP +777 CC genotype exhibited decreased SCS, indicating His^{245} may alter CXCR2 ability to mediate intracellular Ca^{2+} release relative to CXCR2 with Gln^{245} by affecting signal transduction through Ras, Raf or MEK (Knall, 1996). Disruption in CXCR2 G-protein binding and/or signaling processes can inhibit neutrophil chemotaxis and the ability of neutrophils to migrate to the site of infection. This hypothesis is supported by the lower SCS observed in cows expressing the CC genotype. If neutrophils do not migrate efficiently to the mammary gland in cows expressing the CC genotype, bacteria would be better able to proliferate. Moreover, there is evidence that the correlation between SCS and clinical mastitis ranges from 0.6 - 0.8, indicating that infection does not always increase SCS (Emanuelson, 1988; Rogers, 1993; Rogers, 1995; Shook, 1993). Research is currently underway to determine functional activities of neutrophils expressing CXCR2 SNP +777 genotype GG versus neutrophils expressing genotype CC during mastitis.

In conclusion, we were unable to identify one haplotype genotype responsible for either subclinical or clinical mastitis incidence, although we identified Holsteins expressing SNP +777 genotype CC had increased subclinical mastitis while Holsteins expressing genotype GG had decreased subclinical mastitis. This research is promising as this may represent an effective means of marker-assisted selection for mastitis resistance and potentially other inflammatory diseases involving neutrophils. The ability to genetically identify mastitis susceptible versus resistant animals may also identify other gene products that differ between the two, as well as open doors to novel preventative and therapeutic measures for mastitis.

REFERENCES

Ables, G.P., Nishibori, M., Kanemaki, M., Watanabe, T. 2002. Sequence analysis of the NRAMP1 gene from different bovine and buffalo breeds. Journal of Veterinary Medical Science 64, 1081-1083.

Ahuja, S.K., Murphy, P.M. 1996. The CXC chemokines growth-related oncogene (GRO)-α, GRO-β, GRO-γ, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. The Journal of Biological Chemistry 271, 20545-20550.

Baggiolini, M., Dewald, B., Walz, A. 1992. Interleukin-8 and related chemotactic cytokines. In: Gallin, J.I., Goldstein, I.M., Snyderman, R. (ed.), Inflammation: Basic Principles and Clinical Correlates. Raven Press, Ltd., New York, NY, pp. 247-263.

Band, M.R., Larson, J.H., Rebeiz, M., Green, C.A., Heyen, D.W., Donovan, J., Windish, R., Steining, C., Mahyuddin, P., Womack, J.E., Lewin, H.A., 2000. An ordered comparative map of the cattle and human genomes. Genome Research, 1359-1366.

Bellamy, R. 1999. The natural resistance-associated macrophage protein and susceptibility to intracelluar pathogens. Microbes and Infection 1, 23-27.

Ben-Baruch, A., Bengali, K.M., Biragyn, A., Johnson, J.J., Wang, J., Kim, J., Chuntharapai, A., Michiel, D.F., Oppenheim, J.J., Kelvin, D.J. 1995. Interleukin-8 receptor beta: the role of the carboxyl terminus in signal transduction. Journal of Biological Chemistry 270, 9121-9128.

Blackwell, J. M. 1989. The macrophage resistance gene Lsh/Ity/Bcg. Research in Immunology 140, 767-828.

Blackwell, J. M. 1996. Structure and function of the natural-resistance-associated macrophage protein (Nramp1), a candidate protein for infectious and autoimmune disease susceptibility. Molecular Medicine Today 1996, 201-204.

Blackwell, J. M., S. Searle, R. Goswami, E.N. Miller 2000. Understanding the multiple functions of NRAMP1. Microbes and Infection 2, 317-321.

Burvenich, C., Paape, M.J., Hill, A.W., Guidry, A.J., Miller, R.H., Heyneman, R., Kremer, W.D., Brand, A. 1994. Role of the neutrophil leucocyte in the local and systemic reactions during experimentally induced E. coli mastitis in cows immediately after calving. Veterinary Quarterly 16, 45-50.

Cardon, L. R., Palmer, L.J. 2003. Population stratification and spurious allele association. The Lancet 361, 598-604.

Damaj, B.B., McColl, S.R., Neote, K., Na, S.Q., Ogborn, K.T., Hebert, C.A., Naccache, P.A. 1996. Identification of G-protein binding sites of the human interleukin-8 receptors by functional mapping of the intracellular loops. FASEB Journal 10, 1426-1434.

Dietz, A.B., Detilleux, J.C., Freeman, A.E., Kelley, D.H., Stabel, J.R., Kehrli, M.E. Jr. 1997. Genetic association of bovine lymphocyte antigen DRB3 alleles with Immunological traits of Holstein cattle. Journal of Dairy Science 80, 400-405.

Emanuelson, U., Danell, B., Philipsson, J. 1988. Genetic parameters for clinical mastitis, somatic cell counts, and milk production estimated by multiple-trait restricted maximum likelihood. Journal of Dairy Science 71, 467-476.

Grosse, W.M., Kappes, S.M., Laegreid, W.W., Keele, J.W., Chitko-McKown, C.G., Heaton, M.P. 1999. Single nucleotide polymorphism (SNP) discovery and linkage mapping of bovine cytokine genes. Mammalian Genome 10, 1062-1069.

Hansen, L.B., Young, C.W., Miller, K.P., Touchberry, R.W. 1979. Health care requirements of dairy cattle. I. Response to milk yield selection. Journal of Dairy Science 62, 1922-1931.

Hannson, G., Westerberg, L. 2002. On the correlation between heterozygosity and fitness in natural populations. Molocular Ecology 11, 2467-2474.

Heaton, M.P., Grosse, W.M., Kappes, S.M., Keele, J.W., Chitko-McKown, C.G., Cundiff, L.V., Braun, A., Little, D.P., Laegreid, W.W. 2001. Estimation of DNA sequence diversity in bovine cytokine genes. Mammalian Genome 12, 32-37.

Hogan, J. S., Gonzalez, R., Harmon, R.J., Nickerson, S.C., Oliver, S.P., Pankey, J.W., Smith, K.L. 1999. Laboratory Handbook on Bovine Mastitis. 1st ed. National Mastitis Council, Inc., Madison, WI.

Holmes, W.E., Lee, J., Kuang, W.J., Rice, G.C., Wood, W.I. 1991. Structure and functional expression of a human IL-8 receptor. Science 253, 1278-1280.

Horin, P., Rychlik, I., Templeton, J.W., Adams, L.G. 1999. A complex pattern of microsatellite polymorphism within the bovine NRAMP1 gene. European Journal of Immunogenetics 26, 311-313.

Kato, H., Tsuchiya, N., Tokunaga, K. 2000. Single nucleotide polymorphisms in the coding region of human CXC-chemokine receptors CXCR1, CXCR2 and CXCR3. Genes and Immunity. 5, 330-337.

Kehrli, M.E., Jr., Harp, J.A. 2001. Immunity in the mammary gland. Veterinary Clinics of North America: Food Animal Practice 17, 495-516.

Kelm, S.C., Detilleux, J.C., Freeman, A.E., Kehrli, M.E. Jr., Dietz, A.B., Fox, L.K., Butler, J.E., Kasckovics, I., Kelley, D.H. 1997. Genetic association between parameters of innate immunity and measures of mastitis in periparturient Holstein cattle. Journal of Dairy Science 80, 1767-1775.

Knall, C., Young, S., Nick, J.A., Buhl, A.M., Worthen, G.S., Johnson, G.L. 1996. Interleukin-8 regulation of the ras/raf/mitogen-activated protein kinase pathway in human neutrophils. Journal of Biological Chemistry 271, 2832-2838.

Lindley, I.J.D. 1998. Interleukin-8. In: Cytokines. Academic Press, pp. 125-140.

Loetcher, P., Seitz, M., Clark-Lewis, I., Baggiolini, M., Moser, B. 1994. Both interleukin-8 receptors independently mediate chemotaxis. FEBS Letters 341, 187-192.

Lunden, L., Andersson-Eklund, L., Andersson, L. 1993. Lack of association between bovine major histocompatability class II polymorphism and production traits. Journal of Dairy Science 76, 843-852.

Murphy, P.M., Tiffany, H.L. 1991. Cloning of complementary DNA encoding a functional IL-8 receptor. Science 253, 1278-1280.

Paape, M.J., Mehrzad, J., Zhao, X., Detilleux, J., Burvenich, C. 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. Journal of the Mammary Gland Biology and Neoplasia 7, 109-121.

Paape, M.J., Shafer-Weaver, K., Capuco, A.V., van Oostveldt, K., Burvenich, C. 2000. Immune survalence of mammary tissue by phagocytic cells. Advances in Experimental Medicine and Biology 480, 259-277.

Peveri, P., Walz, A., Dewald, B., Baggiolini, M. 1988. A novel neutrophil-activating factor produced by human polymorphonuclear leukocytes. Journal of Experimental Medicine. 181, 1547-1559.

Renzoni, E., Lympany, P., Sestini, P., Pantelidis, P., Wells. A., Black, C., Welsh, K., Bunn, C., Knight, C., Foley, P., du Bois, R. 2000. Distribution of novel polymorphisms of the interleukin-8 and CXC receptor 1 and 2 genes in systemic sclerosis and cryptogenic fibrosing alveolitis. Arthritis and Rheumatism 43, 1633-1640.

Richardson, R.M., Marjoram, R.J., Barak, L.S., Snyderman, R. 2003. Role of cytoplasmic tails of CXCR1 and CXCR2 in mediating leukocyte migration, activation, and regulation. Journal of Immunology 170, 2904-2911.

Rogers, G.W. 1993. Index selection using milk yield, somatic cell score, udder depth, teat placement, and foot angle. Journal of Dairy Science 76, 664-670.

Rogers, G.W., Banos, G., Sander-Nielsen, U., Philipsson, J. 1998. Genetic Correlations Amoung Somatic Cell Scores, Productive Life, and Type Traits form the United States and Udder Health Measures from Denmark and Sweeden. Journal of Dairy Science 81, 1445-1453.

Rogers, G.W., Hargrove, G.L., Cooper, J.B. 1995. Correlations among somatic cell scores of milk within and across lactations and linear type traits of Jerseys. Journal of Dairy Science 78, 914-920.

Schutz, M.M. 1994. Genetic evaluation of somatic cell scores for United States dairy cattle. Journal of Dairy Science 77, 2113-2129.

Shastry, B. S. 2002. SNP alleles in human diseases and evolution. Journal of Human Genetics 47, 561-566.

Sharif, S., Mallard, B.A., Wilkie, B.N., Sargeant, J.M., Scott, H.M., Dekkers, J.C.M., Leslie, K.E. 1998. Associations of the bovine major histocompatability complex BRB3 (BoLA-DRB3) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. Animal Genetics 29, 185-196.

Shook, G.E. 1993. Genetic Improvement of mastitis through selection on somatic cell count. Veterinary Clinics of North America: Food Animal Practice 9, 563-581.

Siite, K., Brinkworth, R., East, I.J., Jazwinska, E.C. 2002. A single amino acid deletion in the antigen binding site of BoLA-DRB3 is predicted to affect peptide binding. Veterinary Immunology and Immunopathology 85, 129-135.

Sonstegard, T.S., Lopez-Corrales, N.L., Kappes, S.M., Beattie, C.W., Smith, T.P.L. 1997. Comparative mapping of human chromosome 2 identifies segments of conserved synteny near the bovine mh locus. Mammalian Genome 8, 751-755.

Speigel, A.M. 1992. Receptor-effector coupling by G-proteins: implications for normal and abnormal signal transduction. Current Opinions in Cellular Biology 4, 203-211.

Sprenger, H., Lloyd, A.R., Lautens, L.L., Bonner, T.I., Kelvin, D.J. 1994. Structure, genomic organization, and expression of the human interleukin-8 receptor B gene. Journal of Biological Chemistry 269, 11065-11072.

Thelen, M., Peveri, P., Kernen, P., von Tscharner, V., Walz, A., Baggiolini, M. 1988. Mechanism of neutrophil activation by NAF, a novel monocytes-derived peptide agonist. FASEB Journal 11, 2702-2706.

Walcheck, B., Kahn, J., Fisher, J.M., Wang, B.B., Fisk, R.S., Payan, D.G., Feehan, C., Betageri, R., Darlak, K., Spatola, A.F., Kishimoto, T.K. 1996. Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. Nature 380, 720-723.

White, J. K., Shaw, M.A., Barton, C.H. 1994. Genetic and physical mapping of 2q35 in the region of the NRAMP and IL8R genes: Identification of a polymorphic repeat in exon2 of NRAMP. Genomics 24, 295-305.

Part V

SUMMARY

Objectives of this project were to identify candidate SNPs and resulting haplotypes within a region of the bovine CXCR2 gene, determine their frequencies within Holstein and Jersey dairy cattle, and identify any associations between SNPs/haplotypes and susceptibility or resistance to clinical and subclinical mastitis. We identified five SNPs present in both Holstein and Jersey cattle. Four of these SNPs were identified previously in beef cattle (Grosse, 1999). Strong linkage disequilibrium was exhibited for both breeds of cattle among all five polymorphic loci ($P<0.001$). This was not an unexpected finding as these SNPs occurred within a 311 bp segment, which is a very small chromosomal segment and recombination within such a small region is highly unlikely, though not impossible. We hypothesize more polymorphisms exist within the bovine CXCR2 gene as SNPs have been located within the human CXCR2 gene in both the coding region, as well as in the 3' untranslated region (Kato, 2000; Renzoni, 2000). Furthermore, the bovine CXCR2 gene may be a evolutionary hot-spot for polymorphisms as CXCR2 SNPs have been identified in different human populations, different cattle breeds, as well as in bison. Future studies that examine the full length of the coding region for the receptor would determine if this hypothesis is true.

While the same SNPs occurred within both Holstein and Jersey breeds, they were exhibited in different frequencies. Holsteins exhibited more heterozygosity for each SNP than Jerseys. However, as the U.S. Jersey population appears more inbred than the U.S. Holstein population, this was not unexpected (AIPL, 2003). We identified ten CXCR2 haplotypes created by the five

SNPs. Six haplotypes were expressed in both Holstein and Jersey breeds, while two were unique to Holsteins and two were unique to Jersey cows. Nineteen different bovine CXCR2 haplotype genotypes were comprised from ten CXCR2 haplotypes. Both breeds commonly expressed six haplotype genotypes; six were expressed only within Jerseys and seven were unique to Holsteins. This is not unusual as different haplotype frequencies and genotypes have been identified across different breeds of cattle (Damaini, 2000; Heaton, 2001a; Heaton, 2001b; Konfortov, 1999). Furthermore, variable haplotype and SNP frequencies have been identified across several different human populations for cytokine and immune-related genes (Koss, 2001; Lazarus, 2003; Meenagh, 2002). Additionally, dairy cattle populations are not under natural selection processes due to intensive breeding for characteristics such as milk yield and conformational traits (Hansen, 2000; Shook, 1993). Selection for specific traits and limited sire numbers has caused dairy breeds to become increasingly inbred. Thus, differences in SNP heterozygosity, as well as allele and genotype frequencies between Jersey and Holstein breeds may be explained by differences in breeding selection and the extent of inbreeding within each respective breed.

We identified that Holsteins expressing CXCR2 SNP +777 genotype CC had significantly higher incidences of subclinical mastitis. One possible mechanism associated with mastitis susceptibility may be due to the Gln→His change at residue 245. This region within the CXCR2 third intracellular loop has been shown to be involved in G-protein binding and activation and intracellular Ca^{2+} release, affecting neutrophil activation and chemotaxis (Damaj, 1996).

Another possible explanation for increased subclinical mastitis in Holstein cattle may be due to expression of other genes and/or polymorphisms located near CXCR2 on BTA 2. The NRAMP1 gene is a potential candidate as it is within close proximity to CXCR2 on BTA 2. Furthermore, it is polymorphic and has been associated with immune responses during infection (Blackwell, 1989; Blackwell, 1996; Blackwell, 2000; Bellamy, 1999). Single nucleotide polymorphisms located within other regions of the CXCR2 gene may affect mastitis phenotypes as they may affect receptor binding and function, as well as transcription and translation.

In conclusion, we identified five SNPs within the bovine CXCR2 receptor. The bovine CXCR2 genotype CC at SNP +777 was associated with increased incidence of subclinical mastitis in Holsteins when compared to Holstein cows exhibiting SNP +777 genotype GG. Future studies are warranted to definitively link this genotype with mastitis susceptibility. Furthermore, as this chromosomal region seems to contain evolutionarily conserved polymorphic genes, sequencing this region of BTA 2 may identify other polymorphic genes associated with immunity, such as NRAMP1. This knowledge would further aid efforts to select for mastitis resistant and/or susceptible dairy cows, as well as identify potential genes associated with susceptibility or resistance to other inflammatory diseases.

REFERENCES

Animal Improvement Programs Laboratory, 2003 AIPL USDA Dairy Information. USDA Animal Improvement Programs Laboratory. http://www.aipl.arsusda.gov . access date: 8-01-03.

Bellamy, R. 1999. The natural resistance associated macrophage protein and susceptibility to intracellular pathogens. Microbes and Infection 1, 23-27.

Blackwell, J.M. 1989. The macrophage resistance gene Lsh/Ity/Bcg. Research in Immunology 140, 767-828.

Blackwell, J.M. 1996. Structure and function of the natural-resistance-associated macrophage protein (Nramp1), a candidate protein for infectious and autoimmune disease susceptibility. Molecular Medicine Today 5, 205-211.

Blackwell, J.M., Barton, C.H., White, J.K., Roach, T.I.A., Shaw, M.A., Whitehead, S.H., Mock, B.A., Searle, S., Williams, H., Baker, A.-M. 1994. Genetic regulation of leishmanial and mycobacterial infections: The Lsh/Ity/Bcg gene story continues. Immunology Letters 43, 99-107.

Damaini, G., Florio, S., Budelli, E., Polla, P., Caroli, A. 2000. Single nucleotide polymorphisms (SNPs) within Bov-A2 SINE in the second intron of bovine and buffalo k-casein (CSN3) gene. Animal Genetics 31, 277-279.

Damaj, B.B., McColl, S.R., Neote, K., Na, S.Q., Ogborn, K.T., Hebert, C.A., Naccache, P.A. 1996. Identification of G-protein binding sites of the human interleukin-8 receptors by functional mapping of the intracellular loops. FASEB Journal 10, 1426-1434.

Grosse, W.M., Kappes, S.M., Laegreid, W.W., Keele, J.W., Chitko-McKown, C.G., Heaton, M.P. 1999. Single nucleotide polymorphism (SNP) discovery and linkage mapping of bovine cytokine genes. Mammalian Genome 10, 1062-1069.

Hansen, L.B. 2000. Consequences of selection for milk yield from a geneticist's viewpoint. Journal of Dairy Science 83, 1145-1150.

Heaton, M. P., Chitko-McKown, C.G., Grosse, W.M., Keele, J.W., Keen, J.E., Laegreid, W.W. 2001. Interleukin-8 haplotype structure from nucleotide sequence variation in commerical populations of U.S. beef cattle. Mammalian Genome 12, 219-226.

Heaton, M.P., Grosse, W.M., Kappes, S.M., Keele, J.W., Chitko-McKown, C.G., Cundiff, L.V., Braun, A., Little, D.P., Laegreid, W.W. 2001. Estimation of DNA sequence diversity in bovine cytokine genes. Mammalian Genome 12, 32-37.

Kato, H., Tsuchiya, N., Tokunaga, K. 2000. Single nucleotide polymorphisms in the coding region of human CXC-chemokine receptors CXCR1, CXCR2 and CXCR3. Genes and Immunity. 5, 330-337.

Konfortov, B.A., Licence, V.E., Miller, J.R. 1999. Re-sequencing of DNA from a diverse panel of cattle reveals a high level of polymorphism in both intron and exon. Mammalian Genome 10, 1142-1145.

Koss, K., Fanning, G.C., Welsh, K.I., Jewell, D.P. 2001. Interleukin-10 gene promoter polymorphism in English and Polish healthy controls. Polymerase chain reaction haplotyping using 3' mismatches in forward and reverse primers. Genes and Immunity 1, 321-324.

Lazarus, R., Klimecki, W.T., Raby, B.A., Vercelli, D., Palmer, L.J., Kwiatkowski, D.J., Silverman, E.K., Martinez, F., Weiss, S.T. 2003. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics 81, 85-91.

Meenagh, A., Williams, F., Rodd, O.A., Patterson, C., Gorodezky, C., Hammond, M., Leheny, W.A., Middelton, D. 2002. Frequency of cytokine polymorphisms in populations from Western Europe, Africa, Asia, the Middle East and South America. Human Immunology 63, 1055-1061.

Renzoni, E., Lympany, P., Sestini, P., Pantelidis, P., Wells. A., Black, C., Welsh, K., Bunn, C., Knight, C., Foley, P., du Bois, R. 2000. Distribution of novel polymorphisms of the interleukin-8 and CXC receptor 1 and 2 genes in systemic sclerosis and cryptogenic fibrosing alveolitis. Arthritis and Rheumatism 43, 1633-1640.

Shook, G.E. 1993. Genetic Improvement of mastitis through selection on somatic cell count. Veterinary Clinics of North America: Food Animal Practice 9, 563-581.

APPENDICES

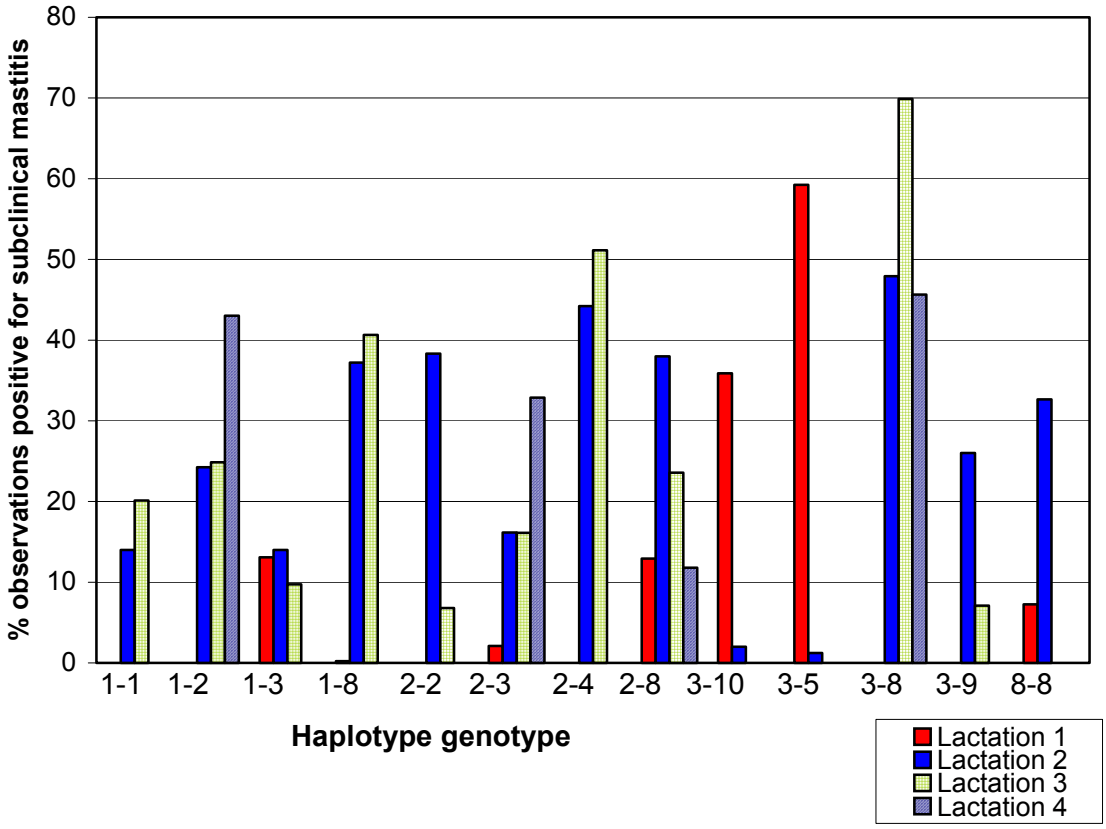

Figure 5. Estimated percentages of Holstein subclinical mastitis by lactation.

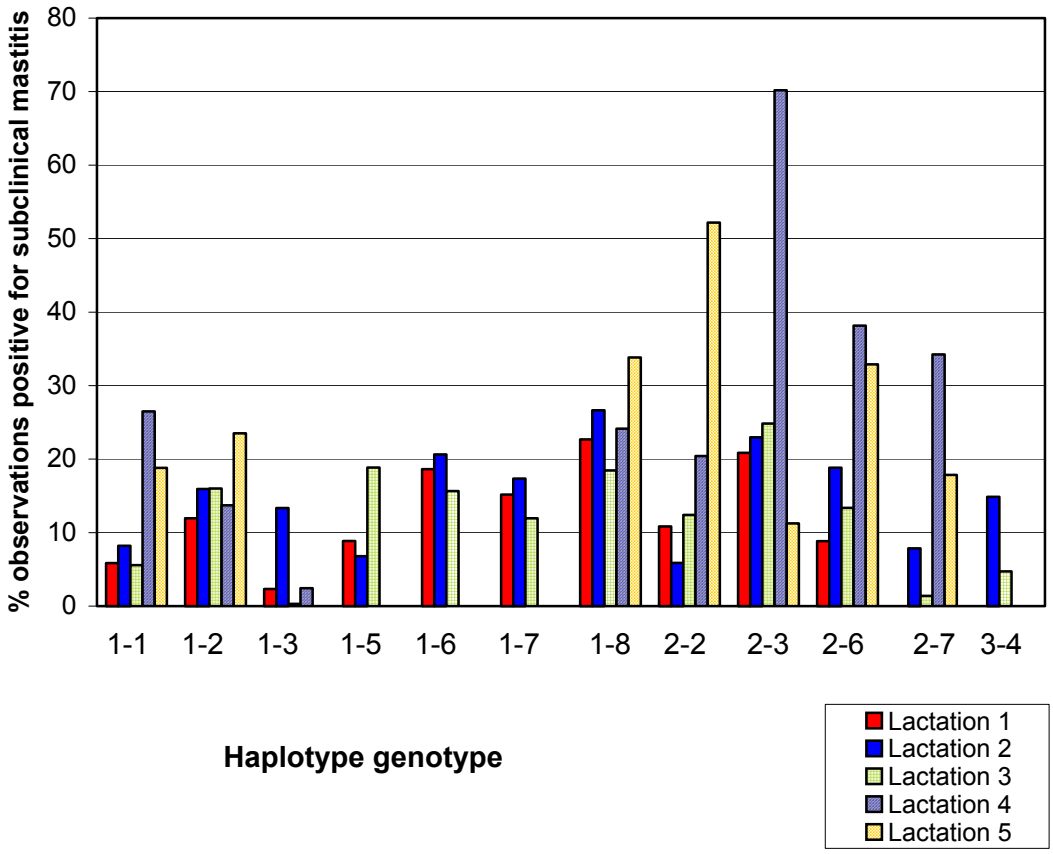

Figure 6. Estimated percentages of Jersey subclinical mastitis by lactation.

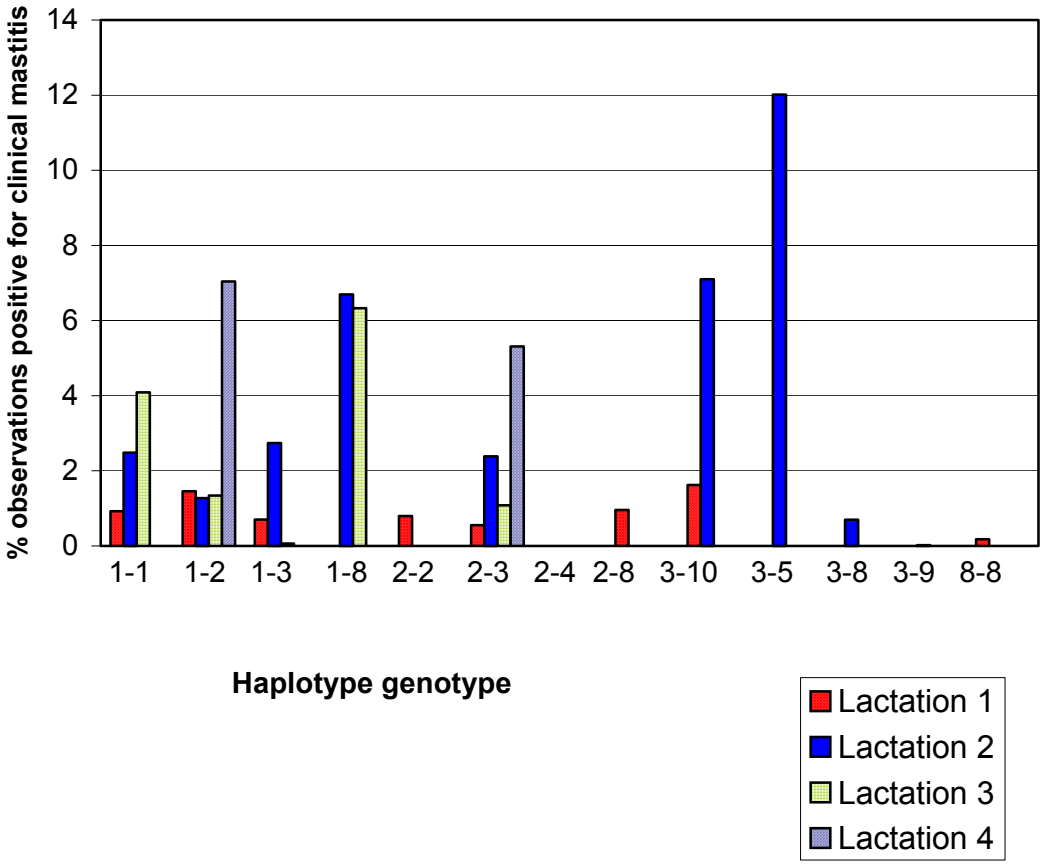

Figure 7. Estimated percentages of Holstein clinical mastitis by lactation.

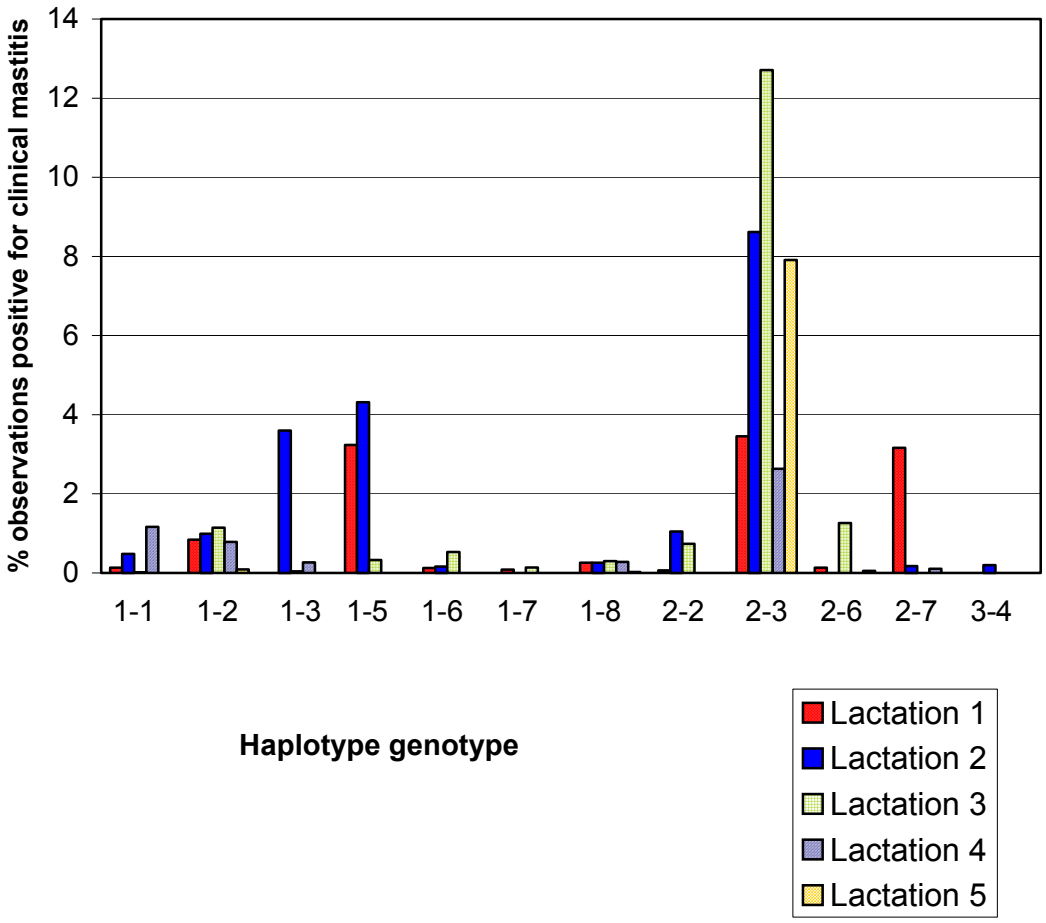

Figure 8. Estimated percentages of Jersey clinical mastitis by lactation.

Appendix 2: Partial Bovine CXCR2 Sequence, Accession No. U19947

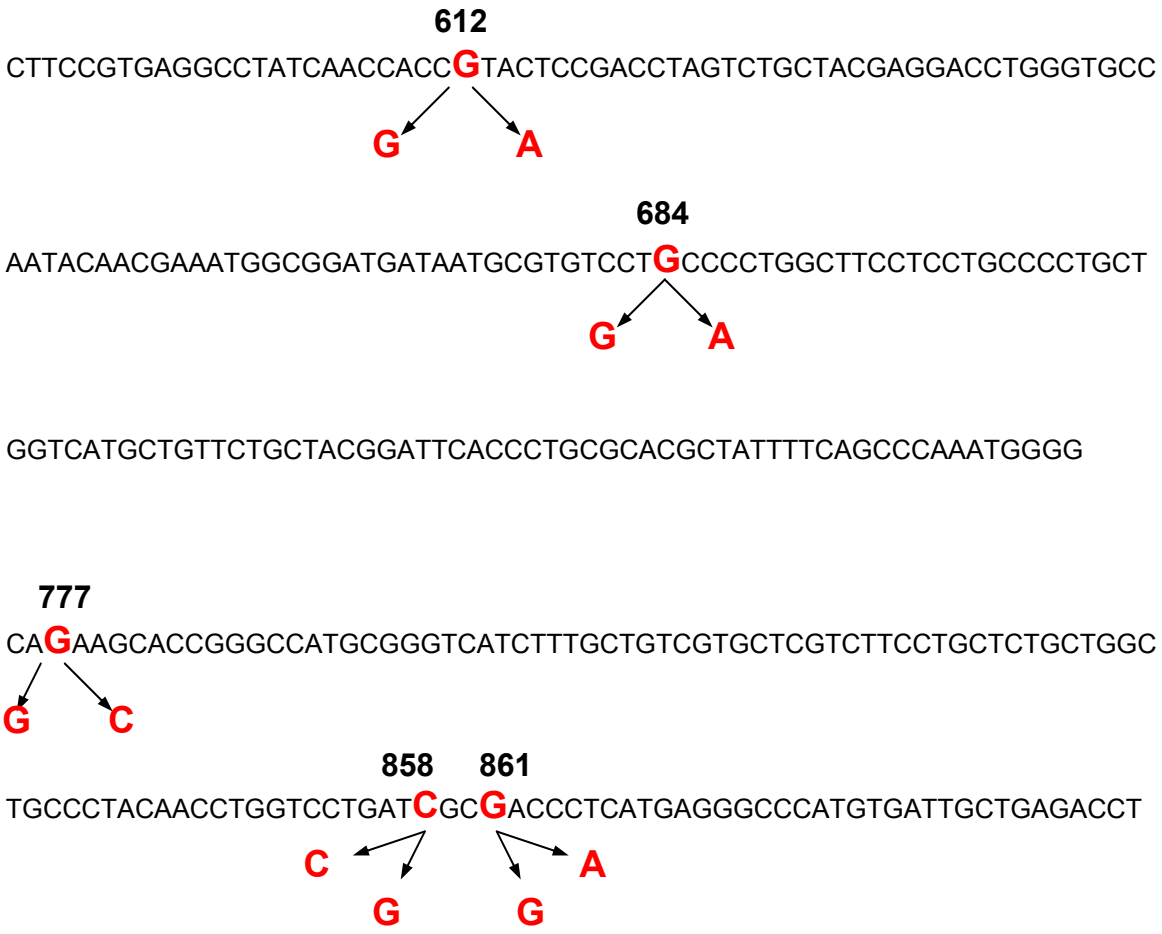

Figure 9. SNPs identified in Holstein and Jersey cattle in CXCR2 sequence Accession No. U19947. Nucleotide location is noted above each SNP, which are in bold. Nucleotide substitutions found in Holstein and Jersey cows are indicated by arrows below each SNP

VITA

Sara Marie Youngerman was born September 5, 1979, in Winfield, Illinois. She graduated from Ottawa Township High School in Ottawa, Illinois, in 1998. From there, she attended The University of Tennessee at Martin, TN, where she majored in animal science and received a Bachelor of Science degree in Agriculture in the fall of 2001. In the spring of 2004, she graduated from The University of Tennessee at Knoxville, TN, after receiving a Master of Science degree in Animal Science.