2013

Evaluation Of Fc Receptor Genes In Bovine Neutrophils

Jamie S. Williams
North Carolina Agricultural and Technical State University

Evaluation of Fc Receptor Genes in Bovine Neutrophils

Jamie S. Williams

North Carolina A&T State University

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department: Animal Sciences

Major: Animal Health

Major Professor: Dr. Mulumebet Worku

Greensboro, North Carolina

2013

School of Graduate Studies
North Carolina Agricultural and Technical State University
This is to certify that the Master's Thesis of

Jamie S. Williams

has met the thesis requirements of
North Carolina Agricultural and Technical State University

Greensboro, North Carolina
2013

Approved by:

_____	_____
Dr. Mulumebet Worku Major Professor	Dr. Antoine Alston Interim Academic Associate Dean Committee Member
_____	_____
Dr. Tracy Hanner Committee Member	Dr. Ralph Noble Committee Member
_____	_____
Dr. Ralph Noble Department Chairperson	Dr. Sanjiv Sarin Dean, The Graduate School

Biographical Sketch

Jamie S. Williams was born on October 16, 1985 in Petersburg, Virginia. Jamie earned her Bachelor's of Science degree in Animal Science in 2007 at Virginia State University. Her studies and stipend were supported by the USDA, Evans Allen and the Agricultural Research Program in SAES at NCAT. She has presented her research work at national and international conferences which include: The 2011 Biennial Research Symposium of the Association of Research Directors, held in Atlanta, Georgia and the 2012 American Dairy Science Association (ADSA)- American Society of Animal Sciences (ASAS) Joint Annual meeting held in Phoenix, Arizona. She has collaborated with fellow MS students, PhD and postdoctoral scientists in lab research. Jamie has trained high school and undergraduate students in laboratory techniques along with participating in recruitment and peer mentoring. She also attended workshops hosted by the USDA, NC Biotechnology Center and Altech. Jamie has participated in many presentations held at North Carolina A & T State University. She is a member of the American Dairy Science Association (ADSA). Jamie is a candidate for the Master of Science degree in Animal Health Sciences.

Dedication

This thesis is dedicated to my late father, James A. Williams. Thank you for always reminding me that I can do anything and to never give up. I love you daddy.

"…to be absent from the body, is to be present with the Lord." II Corinthians 5:8

Acknowledgements

First and foremost, I would like to thank God for giving me strength and perseverance in successfully completing my graduate studies. Without his guidance this would not have been possible. I would also like to give honor to my mother and sister for the continued encouragement and confidence I needed to continue striving throughout this program. I am greatly thankful for my best friend, Derrick who has given me continued moral support and lending an ear in times of need. I am gratified in knowing each one of my circle of friends here. I will always cherish our time together; venting, problem-solving and laughing. We needed each other to get through the challenges we've endured.

Words cannot express the appreciation I have for my advisor, Dr. Worku. Her valuable guidance, patience, dedication and belief in me throughout the program has led me to a successful completion. She is truly a wonderful person. An extension of my gratitude is also given to Mr. Hamid Ismail who assisted me throughout my research work. I am grateful to my committee members for providing the necessary guidance, recommendations and constructive criticism needed for me to prevail. I would also like to recognize all the faculty, administrative, laboratory and dairy unit staff at North Carolina A & T State University for their assistance during my research endeavors. I would also like to acknowledge the USDA Evans Allen program and the Agricultural Research program at NC A&T for financial support towards completion of my studies.

Table of Contents

List of Figures

List of Tables

List of Symbols and Nomenclature

ACD	Acid Citrate Dextrose
APS	Ammonium Persulfate
bp	Base pair
CD	Cluster of differentiation
Cq	Quantification cycle
DEPC	Diethylpyrocarbonate
dH_2O	Deionized water
DHI	Dairy Herd Improvement
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
Fab	Fragment antigen binding
Fc	Fragment crytallizable
FCGR	Fc gamma receptor
FcR	Fragment crystallizable region
FcγR1	Fc gamma receptor I
FcγR2A	Fc gamma receptor II A
FcγR2B	Fc gamma receptor II B
FcγR2C	Fc gamma receptor II C
G	g-force
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
H_2O	Water

HRP	Horseradish Peroxidase
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IACUC	Institutional Animal Care and Use Committee
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KCl	Potassium Chloride
KH_2PO_4	Monobasic Potassium Phosphate
Min	minute
mm	millimeter
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium Chloride
NCA&T	North Carolina Agricultural and Technical State University
NK	Natural killer
NTC	No template control
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PMN	Polymorphonuclear
PMSF	Phenylmethylsulfonyl Fluoride

RBC	Red blood cell
Rpm	Revolutions per minute
s	second
SCC	Somatic Cell Count
SCS	Somatic Cell Score
SHIP	SH2-domain-containing inositol 5-phosphatase
SHP-1	SRC- homology phosphatase 1
SHP-2	SRC-homology phosphatase 2
SNP	Single nucleotide polymorphism
SSCP	Single strand conformational polymorphism
SYK	Spleen tyrosine kinase
TBE	Tris/Borate/EDTA
TLR4	Toll-like receptor 4
TMB	Tetramethylbenzidine
USDA	United States Department of Agriculture
V	Voltage
WBC	White Blood Cell

Abstract

Receptors for the Fc portion of IgG, Fc receptors (FcR) are found on immune cells and contribute to protective functions of the immune system. The FcγRs (I, II and sub isoforms) bind specifically to IgG molecules and are important due to stimulating effector cells to destroy pathogens by phagocytosis and intracellular killing. The SYK kinase is critical for FcγR-mediated phagocytosis and signal transduction in cells. Neutrophils contain a heterogeneous population of FcR. The objectives of this study were to evaluate genetic variation and the impact of IgG binding on transcription of FcγR and secreted levels of phosphorylated Syk between Holstein-Friesian and Jersey X Holstein cows. Blood was collected from Holstein-Friesian and Jersey X Holstein lactating cows at the North Carolina A & T State University dairy farm (n=6). The somatic cell count and packed cell volume were used to evaluate overall health. Genomic DNA was isolated and amplified products were separated on a 1% agarose gel following ethidium bromide staining. Polymorphisms were evaluated by SSCP analysis. Neutrophils treated with whole bovine IgG molecules were used for q-PCR. All genes were transcribed. An increase was observed in the transcription of FcγRII in Jersey X Holstein cows and a significant increase was observed in the Syk protein expression of Jersey X Holstein cows ($p<0.05$) when compared to Holstein-Friesian cows. Heterogeneity in FcγR gene expression may be related to breed differences in cows and impact downstream cellular function binding of IgG to Fc receptors. These results have implications for control of inflammatory diseases such as mastitis in cattle.

CHAPTER 1

Introduction

The study of natural resistance against diseases in different breeds of cattle has been increasingly important as an overall goal to identify breeds resistant to enzootic diseases (Paixao et al., 2006). Identifying breeds of cattle that are genetically less susceptible to pathogens will aid in the cost reduction of cattle management and breeding programs. Billions of dollars have been spent by the U.S and other countries on pathogen eradication programs and disease management (Bradley, 2002; Zhao and Lacasse, 2008). however; some diseases still linger despite up-to-date vaccination protocols, quarantines and slaughter methods. The lack of success in eradicating infectious diseases with these approaches indicate a need for new strategies such as genetically based natural resistance

Mastitis, in dairy cattle, is an inflammation of the mammary gland. It may result from injury or, more commonly, from bacterial or mycotic pathogens. It is the most costly disease in the dairy cattle industry. Mastitis is characterized by physical, chemical and usually bacteriological changes in the milk. Pathological changes in the mammary glandular tissue are also observed (Wellenberg, Van der Poel, Van Oirschot; 2002). Economic consequences of mastitis include reduced milk production (Cohen and Norins,1968) increased culling rates, increased veterinary costs, and higher somatic cell counts (SCC) in milk (Rupp and Boichard, 2000). Although sanitation management has been shown to be the most effective way to prevent intramammary infections, selection for mastitis resistance is an alternative to be considered since the risk of an infectious outbreak is still great. Recent years (2000s) have shown some decrease in clinical mastitis incidence but this has been accompanied by a change in the relative and absolute importance of different pathogens (Lucy, 2001). Studies are needed to

using genetic potential for the discovery of a more resistant breed. Genetics alongside modified antibiotic use will aid in combating diseases in the dairy industry.

High SCC are indicative of subclinical mastitis in cattle. Somatic cell counts are used to assess the inflammatory condition of the udders in dairy cattle. In general somatic cells in milk include epithelial cells and macrophages. However, infections cause an influx of neutrophils from the blood stream to the mammary gland causing increases in SCC. Dairy herd improvement reports are taken on a monthly basis to evaluate the SCCs of each cow in a herd. High somatic cell counts of 200,000 or more is symbolic of subclinical mastitis (Fox, 2009). There are some factors that can contribute to a high SCC without being an incidence of bacterial invasion. Milk yield can affect SCC as well as the stage of lactation. Early and late lactation stages could affect SCC. Nutritional management may also play a role if there are deficiencies in vitamin E or selenium as these vitamins help maintain a healthy internal milking environment.

Fc receptors (FcR) are proteins found on effector cells that govern a hosts' immunological response to microorganisms by activating defensive immune mechanisms. They are part of innate immunity. Changes or alterations in these receptors, known as polymorphisms, may help in understanding factors that impact susceptibility to invading pathogens, such as mastitis causing agents. This will aid in genetic improvement through selection of breeds and reduced antibiotic usage for food safety and security.

The objectives of this study were to evaluate genetic variation and the impact of IgG binding on transcription of FcγRs and secreted levels of phosphorylated Syk kinase between Holstein-Friesian and Jersey X Holstein cows.

CHAPTER 2

Review of Literature

2.1 Fc Receptors

Fc receptors (FcR) are proteins found on the surface of effector cells that link the humoral and cellular branches of the immune system. Fc receptors can be defined as "receptors present on a variety of cells for the Fc portion of an immunoglobulin" (Liu et al., 2011; Takai, 2005). They play a critical role in immunity and provide a defense against invading pathogens. They are responsible for initiating a cellular response to host defenses, determining the efficacy of therapeutic antibodies and in pathological autoimmune conditions (Firth, Chattha, Hodgins, Shewen, 2010; Ravetch and Bolland, 2001). These receptors are found on effector cells such as: neutrophils, eosinophils, macrophages and NK cells. The FcRs on these effector cells, in turn, will then bind to antibodies that are attached to microbes or microbe infected cells. This aids cells in identifying and eliminating microbial pathogens (McGaha, Karlsson, Ravetch, 2008; Selvaraj, Fifadara, Nagarajan, Cimino, Wang, 2004). The interaction between the bound antibody and the cell surface Fc receptor activates the immune cell to kill microbes by phagocytosis of an opsonized microbe and intracellular killing.

Fc receptors result in downstream signal transduction in immune cells. Phagocytes, such as neutrophils, begin to ingest and kill an IgG-coated pathogen by phagocytosis following engagement of their Fcγ receptors (Swanson, 2004). What is known of Fc receptors may lead to more advanced and personalized therapeutics for the treatment of different diseases. Studies of Fc receptors can further the understanding of the immune response and the genetic basis for Fc receptor activity. There is an expanding family of activation/inhibitory receptor pairs found in the immune system. The different classes of Fc receptors offer a paradigm for the biological

significance of balancing activation and inhibitory signaling. The hyper-responsive state that results, when receptors are activated, will lead to a significantly magnified effector response by cytotoxic antibodies and immune complexes. (Firth et al., 2010; Ravetch et al., 2001). A better understanding of Fc receptor expression coupled with immunoglobulin transport may help to develop new products which could be used not only for veterinary purposes but also for human therapy as well (Kacskovics, 2004).

2.2 Immunoglobulins and Fc Receptors

Receptors for immunoglobulins (FcR) play a central role during an immune response as they mediate the specific recognition of antigens, thereby linking the humoral and cellular components of immunity. Engagement of Fc receptors by immunoglobulins initiate a range of immuneoregulatory processes that also plays a role in disease pathogenesis (Bournazos, Woof, Hart, Dransfield, 2009). There are 5 main isotypes of circulating immunoglobulins (Igs) in the cow. These immunoglobulins are known to be: IgM, IgA, IgD, IgG and IgE (Schroeder and Cavacini, 2010). Immunoglobulins help address a variety of issues that may arise from allergic reactions to invading pathogens. There are different families of Fc receptors (i.e. fc *alpha* receptors, fc *epsilon* receptors and fc *gamma* receptors) specific Ig isotypes previously listed (Fridman, 1991). Immunoglobulin G provides the majority of antibody-based immunity against invading pathogens. IgG is known to be reactive with gram negative and gram positive bacteria (Cohen and Norins,1968) both of which are causative agents of mastitis. The binding of IgG and FcγR genes is shown to be responsible for protection against pathogen invasion.

Fc receptors associated specifically with IgG are referred to as Fc gamma receptors (FcγR) and are generally divided into three main classes: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (Gerber and Mosser, 2001; Yan, Li, Wang, Zhang, 2000). They transduce

signals through the immunoreceptor tyrosine-based activation motif (ITAM) in subunits or in the cytoplasmic domain. Inhibitory FcγR, such as FcγRIIB, are single chain receptors, transducing signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM) in cytoplasmic domains (Mishima et al., 2007; Pan and Pei, 2003). Both motifs are discussed briefly in the following section.

Each class of Fc gamma receptors possesses distinct structural and functional properties. FcγRI is a high-affinity receptor for monomeric IgG with three extracellular Ig-like domains expressed constitutively by monocytes and macrophages, as well as by many myeloid progenitor cells (Mina-osorio and Ortega, 2004). Three genes coding for FcγRI have been characterized: FCGRIA, FCGRIB and FCGRIC. However, it is generally accepted that only FcγRIA is capable of high affinity IgG binding, whereas FCGRIB and FCGRIC possibly represent truncated or soluble forms of the receptor, with poorly characterized function (Bournazos et al., 2009; Mishima et al., 2007; Pan et al., 2003).

2.3 Fc Receptors in Bovine Neutrophils

Phagocytosis plays an essential role in host defenses mediated by neutrophils and macrophages (Aderem and Underhill, 1999; Ganesan, 2003). The pathogen becomes engulfed by the phagocyte during an active process involving the binding and releasing of the Fc region/Fc receptor complex, until the cell membrane of the phagocyte completely encloses the pathogen (Underhill and Ozinsky, 2002).

Lymphocytes together with antigen-presenting cells function in the generation of an effective immune response in bovine (Paape, Shafer-Weaver, Capuco, Oostveldt, Burvenich, 2002). Migrations of neutrophils into the mammary gland of cows and the activation Fc receptors provide the first line of defense against invading mastitis pathogens. Bacteria release

potent toxins that activate white blood cells and epithelial cells in the mammary gland to secrete cytokines that recruit PMN that function as phagocytes at the site of infection (Paape, Bannerman, Zhao, Lee, 2003).

The analysis of FcγRs and neutrophils has allowed researchers to begin an exploration of some immunological characteristic of ruminants, particularly bovine. Research conducted by Worku, Paape, Filep, Miller, 1994 observed the binding of endogenous and exogenous homologous IgG2 and IgM to bovine neutrophils before and after in vitro migration through micropore filters, and in vivo migration through mammary tissues after intramammary injection of endotoxin. This research suggested that migration in vivo through cellular matrices induced receptor up-regulation for IgG and IgM which responds.

The studies of Worku et al. have shown that heterogeneous populations of FcR exist on bovine PMN, and that IgG1 and IgG2 share a common FcR. Further, bovine PMN are capable of gene activation and are responsive to changes in their environment, thus being amenable to modulation for effective pathogen destruction (Worku, Paape and Marquardt,1994)

2.4 Intracellular Signal Transduction of Fc Gamma Receptors

Fc gamma receptors generate signals within their cells through an important activation motif known as an immunoreceptor tyrosine-based activation motif (ITAM) (Pan et al., 2003). An ITAM is a specific sequence of amino acids occurring in the intracellular tail of the Fcγ chain in activation receptors (Isakov, 1997). Tyrosine residues within these motifs become phosphorylated following interaction of the receptor molecules with their ligands. This allows docking sites to be formed for other proteins involved in the signaling pathways of the cell and its phosphorylation induces phagocytosis in neutrophils. (Edberg, Moon, Chang, Kimberly, 1998; Ibarrola et al., 1997).

There is another motif responsible for preventing further up-regulated activity. This action is represented by a motif known as an immunoreceptor tyrosine-based inhibitory motif (ITIM). An ITIM is a conserved sequence of amino acids that is found in the cytoplasmic tails of many inhibitory receptors of the immune system (Vivier and Daeron, 1997) and does not induce phagocytosis. Instead, ITIMs performs inhibitory actions which are controlled by enzymes that remove phosphate groups from tyrosine residues. FcγRIIB has the ITIM sequence and is a known inhibitory Fc receptor (Bewarder et al., 1996; Cooney, Phee, Jacob, Coggeshall, 2001). After ITIM-possessing inhibitory receptors interact with their ligand, their ITIM motif becomes phosphorylated by enzymes of the Src kinases, allowing them to recruit other enzymes such as the phosphotyrosine phosphatases SHP-1 and SHP-2, or the inositol-phosphatase called SHIP. These phosphatases decrease the activation of molecules involved in cell signaling (Barrow and Trowsdale, 2006). Currently, not much is known about these motifs in bovine.

In literature, the clustering of FcγRs by immune complexes initiates a cascade of signaling events involving protein kinases. Protein kinases transfer the gamma (terminal) phosphate from nucleotide triphosphates to one or more amino acid residues in a protein resulting in a conformational change affecting protein function (Cooney et al., 2001; Ghazizadeh, Bolen, Fleit, 1994). Tyrosine kinases may catalyze the phosphorylation of tyrosine residues in proteins (Thomas and Brugge, 1997). Phosphorylation of ITAMs at tyrosine residues controls a range of properties in proteins such as enzyme activity, subcellular localization, and interaction between molecules (Radha, Nambirajan, Swarup, 1996).

Tyrosine kinases such as Syk have been well characterized. Members of these kinase families function downstream of antigen and Fc receptor binding in hematopoietic cells and transduce signals leading to calcium mobilization, altered gene expression, cytokine production,

and cell proliferation. (Bradshaw,2010). This kinase (Syk) is regulated by intramolecular interactions controlled by tyrosine phosphorylation, as mentioned previously, and play an important role in regulating signals from cell surface receptors (Bewarder et al., 1996). Several findings suggest that the tyrosine kinase Syk plays an essential role in IgG-dependent phagocytosis (Cooney et al., 2001; Ghazizadeh, Bolen, Fleit, 1994; Majeed, Caveggion, Lowell, Berton, 2001). The findings in research related to Syk kinases suggest that it plays a coordinated role in IgG-dependent phagocytosis once initial activation of the FcγR family has occurred.

2.5 Signaling of Fc receptors via Antibody Binding

The Fc receptors bind to antibodies at their Fc region or tail. Ligand binding activates the cell that possesses the Fc receptor (Raghavan and Bjorkman, 1996). When IgG molecules specific for a certain antigen or surface component bind to the pathogen with their Fab region, their Fc regions point outward, in direct reach of phagocytes (Beale and Feinstein, 1976). Inhibitory Fc receptors prevent binding of antibodies in the absence of antigens, and therefore reduce the chance of immune cell activation in the absence of an infection. Absences of inhibitory receptors or malfunctioning receptors oftentimes lead to autoimmune conditions (Smith and Clatworthy, 2010). Further, inhibitory receptors also prevent agglutination of phagocytes by antibody when there is no antigen. After a pathogen has been bound, interactions between the Fc region of the antibody and the Fc receptors of the phagocyte results in the initiation of phagocytosis. The interactions formed between receptor and antibody binding work together to tightly encase the antibody-coated microbe. These regions are depicted below in figure 2.5.1.where the “Fab” region is the antigen binding site and the “Fc” region is the FcR binding site. Phagocytes bind the Fc regions with their Fc receptors.

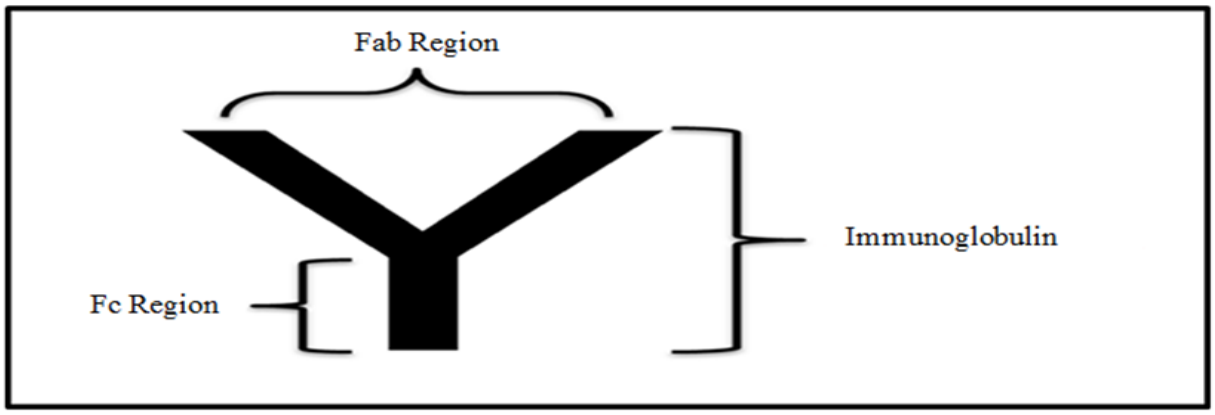


Figure 2.5.1. The binding site locations on an immunoglobulin molecule.

It is clear that Fc receptors are important to survey and identify pathogens, alert cytotoxic cells to react and ultimately initiate an immune response within the body. The way Fc receptors respond to antibodies, i.e. rate or aggression, can determine the effect on an individual, such as, if medications will be needed or if the body can respond fast enough to fend off the targeted microbe. It's worth noting that polymorphisms within the sequences coding for different Fc receptor genes can regulate the probability of susceptibility among animals of the same species but different breeds. Knowing and identifying these polymorphisms can aid in the discovery of a more resilient breed.

2.6 Effects of Polymorphisms on Disease in Bovine

A single nucleotide polymorphism (SNP) is a small genetic modification or variation that occurs within a DNA sequence (Vignal, Milan, SanCristobal, Eggen, 2002) such as an A (adenine) replacing one of the other three nucleotides: C (cytosine), T (thymine), and G (guanine) that generates genetic coding. The reason for the most current interest in SNP research is the hope that they could be used as markers to identify genes that predispose individuals to common, multifactorial disorders and disease (Syvänen, 2001). Polymorphisms play a huge role in susceptibility to diseases, infections, response to drug therapies and the

initiation of immune responses. Polymorphisms may be a key factor in controlling these diseases and/or altering the current remedy for certain conditions in the animal community. In literature, researchers have evaluated polymorphisms in different genes within the bovine species (Abatepaulo et al., 2008; Berkowicz et al., 2011; Paixao, 2006). These genes have proven to be associated with resistance to different ailments that may not necessarily be economical to cull but still quite expensive to treat. It is worth mentioning that genetic resistance, due to polymorphic factors has become a model for disease control.

For many years, breeding goals for dairy cattle had focused mainly on increasing productivity and ignored health traits such as disease resistance. Higher yielding cows tend to have higher health costs. For instance, mastitis is the most prevalent production disease in dairy herds world-wide and is responsible for several negative effects on production (Yuan et al., 2011). There are two types of mastitis in dairy cattle: subclinical mastitis, indicated by increased somatic cell counts in milk and clinical mastitis, which is visibly abnormal milk (Fox, 2009). Milk production losses, drugs, discarded milk, veterinarian services, labor, milk quality impairment and culling of cows are all economic damages caused by mastitis. As technology advances, mastitis related SNPs are continuously being characterized (Yuan et al., 2011)

A chance of developing an effective vaccine for mastitis prevention comes with many limitations. It would be similar to developing a vaccine for the common cold. There are varieties of microorganisms that cause mastitis. The importance of looking at different genes allows researchers to look at genetic factors that influence disease resistance. Knowing these factors, may give rise to select breeds for animal production and determining genetic markers related to resistance in different diseases that may be plaguing certain regions.

Sequencing of the bovine genome and availability of genomic information provide a window of opportunity to better understand disease resistance and immune responses. The study of genetic information corresponding to different ailments will be the new benefactor in prevention of infectious diseases worldwide.

CHAPTER 3

Materials and Methods

3.1 Animals Used

Holstein-Friesian and Jersey X Holstein cows at mid lactation (n=10) housed at North Carolina Agricultural and Technical State University dairy farm were used for blood collection and analysis. The cows used in the study were selected based on somatic cell counts reported on monthly DHI reports. Six animals were used for genetic analysis. One group consisted of 3 Jersey X Holstein cross cows and the second group was made up of 3 Holstein-Friesian cows. This study was approved by the Institutional Animal Care and Use Committee (IACUC).

3.2 Collection of Blood

Blood was collected by jugular venipuncture into 15 ml Becton Dickinson vacutainer tubes (Franklin Lakes, NJ) containing 1.5 ml of ACD as the anticoagulant and placed on ice for packed cell volume, blood smears and PMN isolation. Blood was collected on FTA cards (Whatman Ltd.) for isolation of genomic DNA. There were 6 tubes per animal.

3.3 Packed Cell Volume Determination

Heparinized micro hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) were filled with blood to the red indicating line and the tube was sealed using clay. Tubes were then placed in a micro hematocrit centrifuge (Damon/IEC Division, IEC MB Centrifuge) at a speed of 10,000 to 12,000 rpm for 5 minutes. The tubes were read using a micro-capillary reader using the manufacturer's instructions (Damon/IEC Division, micro-capillary reader, Needham Heights, MASS).

3.4 White Blood Cell Differential Counts

Blood smear were created using blood that was collected from the jugular vein of each animal. Dried blood smear was dipped in Wrights stain (Sure Stain WRIGHT, Fisher Scientific Company, Middletown, VA) for 10 seconds. The smear was dipped in water for 15 seconds to wash off excess staining. Each smear was then dipped a final time in fresh water for 15 seconds. The smears air dried overnight and was evaluated under oil immersion at 100X using and Olympus microscope. The total number of WBCs present out of 100 cells was counted in duplicate. The WBCs were identified based on morphology and staining characteristics.

3.5 DNA Isolation

A blood sample obtained directly from the jugular vein was spotted onto FTA® cards. Three cards per animal were used and allowed to air dry. This card preserved the sample until DNA was isolated. DNA isolation from FTA® cards was carried out following the manufacturer's protocol (Indicating FTA® card pack insert, July2007, Whatman, Piscataway, NJ). Three 3 mm discs were removed from the center of the FTA® card using a 3mm Harris Uni-Core device (Whatman, Piscataway, NJ) and were transferred into a 1.5 ml microfuge tube. Sterile water was added to the microfuge tube in the amount of 500 µl and immediately pulse vortexed three times for 5 seconds each time (Fisher Vortex Genie 2, Fisher Scientific, Bohemia, NY) to wash the sample disc and allow removal of any dirt or impurities. Using sterile pipette tips, the discs were squeezed against the side of the microfuge tube to remove excess liquid and then removed from the wash and transferred to another 1.5 ml microfuge tube that contained 100 µl of sterile H_2O. The discs were completely immersed in the H_2O and then centrifuged for 10 seconds. After centrifugation, tubes were incubated at 95°C for 15 minutes using a water bath. The tubes were then removed and pulse vortexed 15 times. The tubes were placed in the water bath again and for a final incubation of 15 minutes. After samples incubated for a total of 30

minutes, they were removed from the water bath and pulse vortexed 60 times per sample. Samples were centrifuged a final time for 30 seconds in order to separate the matrix from the eluate. The eluate now contained the purified DNA. Sterile pipette tips were used to gently remove the FTA® disc from the microfuge tubes and then discard. Purity and concentration of the isolated DNA sample was assessed using the Nanodrop Spectrophotometer 1000V 3.7.1 (Thermo Scientific Inc., Waltham, MA). The purity of each sample was approximately 2.0.

3.6 Polymerase Chain Reaction using Bovine Genomic DNA

DNA isolated from FTA cards was used for PCR amplification. Primers for FCGR1 and FCGR2C were selected based on previous studies conducted Klungland et al.,1997, while primers for FCGR2B were used by Jiang et al.,1999 and FCGR2A gene primers were used in studies lead by Flesch, Bauer, and Neppert (1998). All of the primers used in this study were synthesized by MWG Biotech (MWG Biotech, Inc., NC). A Qiagen Multiplex PCR kit (Qiagen, USA) was used for sample preparation. Briefly, 2.5 µl of forward primer and 2.5 µl of reverse primer was added to each tube along with 25 µl of the master mix which was provided with the kit. Each template DNA sample contained 250 ng in each PCR reaction tube. RNase- free water was added up to 50 µl in each PCR reaction tube. Total amount of volume in each tube was 50 µl. The tubes were mixed briefly, placed in a thermocycler (MWG-Biotech Inc., Primus 96, USA) and processed according to a program that was created for specific primers. All DNA was denatured at 95°C; however annealing temperatures were specific to each primer and varied between 57°C and 60°C. After amplification, analysis of amplicons was conducted using a 1% agarose gel for electrophoresis and viewed following Ethidium Bromide staining. The components of each PCR tube and amounts per the manufacturer's requirements in each reaction tube can be found in appendix C (table 3.6.1). Tables 3.6.2 and 3.6.3 show the program used for

the gene specific primers and can also be found in appendix C. The gene names, primer sequences and expected length (bp) of PCR products used to amplify bovine gDNA is shown below in table 3.6.4 found in appendix D.

3.7 Single Stand Conformational Polymorphism Gel Analysis of FCGR1and FCGR2C Genes

Single strand conformational polymorphism (SSCP) gels were generated following the modified manufacturer's protocol (Bio-rad, The DCode Universal Mutation Detection System handbook, USA). Briefly a 20 x 20 cm SSCP gel sandwich was assembled. After plate preparation, 6300 ml of dH_2O combined with 700 ml of 10X TBE was poured into the DCode System cooling tank. A cooling system (Rm 20, Lauda & Co., Germany) was used to maintain a steady environment of 4°C during the 14 hr electrophoresis run. The cooling tank was allowed time to reach 4°C. An 18% gel was cast by placing all of the related contents inside a conical tube and inverting 2-3 times to mix. To speed up polymerization, 0.1% APS was added lastly. Once APS was added to the conical tube it was gently inverted to mix a final time. An automatic pipette was used to add the contents of the gel solution into the sandwich until it was filled up to the arrowed lines located on the screw clamps. The 18% gel was allowed to polymerize for 60 minutes. Following polymerization a 14% stacking gel was added. A 14% stacking gel was produced and added atop the 18% running gel. The 14% stacking gel was created in a similar manner as the 18% gel. Briefly, reagents were added to a conical tube, inverted to mix and APS was added. An automatic pipette was used to insert the gel contents atop the solidified 18% gel. A comb was then placed immediately to ensure good well alignment and 60 minutes was allotted for the stacking gel to solidify. The amplicon samples generated from PCR were denatured by incubating for ~8 minutes at 95°C in a water bath and then immediately placed on ice. Each

sample was then placed in the desired well. Electrophoresis of the SSCP gel ran continuous for about 14 hours using a PS 500XT DC Power Supply at 280V (Hoefer Scientific Instruments, San Francisco, California). The gel was viewed using ethidium bromide staining.

3.8 Neutrophil Isolation

Neutrophils were isolated from whole blood using a modified protocol described by Carlson and Kaneko (Carlson and Kaneko, 1973). Blood from each animal was individually pooled from each vacutainer tube into separate 50 ml polypropylene conical tubes and centrifuged in a swinging bucket rotor (Eppendorf Centrifuge 5810 R, Eppendorf, Hamburg, Germany) for 20 minutes at 3500 x g and 4°C. Centrifugation leads to the separation of blood components into plasma, buffy coat and RBC layer. After centrifugation, disposable pipettes were used to aspirate off the plasma, buffy coat and 1/3 of the RBC layer from each sample. The bottom 1/3 of the RBC layer contained the pelleted PMN. Samples were gently rotated, not inverted, to re-suspend PMN and RBC layer. Next, 20 ml of ice cold DEPC treated deionized water was added and mixed by rotating the sample tubes for about 45 seconds to lyse red blood cells and re-suspend the pellet on the bottom of the conical tube. Next, 10 ml of ice cold 2.7% saline was added to each sample to restore isotonicity. The tubes were mixed again by gentle rotation for 45 seconds and returned to ice. The tubes were centrifuged again at 2000 x g for 2 minutes at 4°C and the supernatant was discarded. The cells were re-suspended in 10 ml of PBS with a pH of 7.4. PBS serves to provide an environment familiar to PMN cells as would a normal blood filled environment where they live. Lysing of cells was repeated once more in order to obtain a completely white pellet. After lysing, PBS was added one final time to the white pellet on the bottom of each conical tube for a dilution factor of 10 ml. Pellets were re-suspended by gentle rotation. Purity, concentration and viability were analyzed.

3.9 Analysis of Isolated Neutrophils

Concentration of Isolated Neutrophils was determined by transferring cells to a Hemacytometer (Bright-Line Hemacytometer, Hausser Scientific, Horsham, PA). Cells were diluted using Trypan blue (Sigma Cell Culture, Sigma Chemical Co., St. Louis, MO) at a 1:1 ratio. Using a coverslip over the Hemacytometer, cells were viewed at 40X resolution. Upon counting cells, the PMN concentration was calculated using the formula: average count per square x dilution factor x 10^4= cells/ml.

Neutrophil viability was determined by using Trypan blue staining. Isolated PMN products were mixed with Trypan blue (Sigma Cell Culture, Sigma Chemical Co., St. Louis, MO) at a ratio of 1:1. Samples were mixed properly. A cover slip was placed atop the Hemacytometer and both chambers were filled. Cells were viewed at 40X magnification. The cells that had not taken up the dye were counted as alive and expressed as the percentage of live, viable cells using the calculation: total viable cells/total cells counted.

The purity of the isolated cells was determined using Wrights stain. Smears were created in the same manner as mentioned previously. Briefly, smears of isolated PMN were created and air dried. The smears were stained using Wrights stain and viewed with oil immersion at 100X. The isolated PMN were identified based on granularity and staining characteristics. A total of 100 cells were counted and PMN content was expressed as a percentage.

3.10 Treatment of Isolated Neutrophils

Isolated viable PMN were re-suspended in PBS with a 7.4 pH at a concentration of 10^6cells/ml. Cells were maintained in flat bottom vials (Wheaton Omni-Vials, Wheaton Scientific, Milville, NJ) and centrifuged at 2000 RPM at 4°C. The supernatant was decanted. Cells were then treated in duplicate with 25 µg of Alexa Fluor® 647-conjugated ChromPure

Bovine IgG, whole molecule (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Untreated control samples were maintained in PBS. Tubes were wrapped with aluminum foil after treatment was applied to protect the fluorescent labeling. Samples were gently agitated once the antibody treatments were applied and incubated for 30 minutes on ice. After the incubation period, treated PMN were centrifuged at 2000 RPM for 5 minutes at 4°C. The supernatant was transferred to newly labeled 1.5 ml microfuge tubes and stored at 70°C for later use with the PathScan Sandwich ELISA and confocal microscopy imaging. The remaining pellet was washed two times by adding 1 ml of PBS to the pellet then centrifuging at 2000 RPM at 4°C for 2 minutes. The pellet was re-suspended a final time using 250 µl of PBS and viability was assessed using the TC10 automated cell counter (Bio-rad, Bio-Rad Laboratories, Inc., USA). Samples were centrifuged one final time and the remaining pellet was used for RNA isolation from PMN.

3.11 Confocal Microscopy Analysis of Alexa-Fluor labeled Neutrophils

Confocal microscopy enables the visualization and imaging of fixed as well as living cells and tissues that contain fluorescent probes (antibodies, green fluorescent proteins, dyes, substrates). This technique allows sharply defined optical sections to be collected. Following treatment of neutrophils, 3 randomly chosen treated and untreated samples prepared as above were sent to Dr. Mehrdad Tajkarimi at the Joint School of Nanoscience and Nanoengineering, University of North Carolina at Greensboro for further imaging. Neutrophils used for confocal microscopy were treated with the green fluorescent Alexa Fluor® 647-conjugated ChromPure Bovine IgG, whole molecule.

3.12 Detection of Phosphorylated SYK from Bovine Neutrophil Lysates

Cell lysates were prepared (Appendix B) and placed on ice and while preparing the PathScan® Phospho-Syk Sandwich ELISA kit (Cell Signaling Technology, Inc., Danvers, MA). The microwell strips were set out to reach room temperature and then the appropriate microwells for usage were broken off. The microwells were placed in a strip holder and the unused microwells were resealed and stored at 4°C immediately. Using a microfuge tube, 100 µl of sample diluent and 100 µl of the cell lysates from each sample were added. The tube was then briefly agitated by pulse vortex. Next, 100 µl of each diluted cell lysate was added to the appropriate wells. The wells were sealed with tape and incubated overnight at 4°C. The day following incubation, the sealing tape was removed from the plate, the plate contents were discarded and the plate was washed four times. After washing the plate, 100 µl of detection antibody was added to each well. The plate was then sealed with tape and incubated for 1 hour at 37°C. The washing procedure was repeated after the incubation period. Next, 100 µl of HRP-Linked secondary antibody was added to each well. The plate was sealed again and incubated for 30 minutes at 37°C. The washing procedure again followed this incubation. Lastly, 100 µl of TMB substrate was added to each well and sealed. The plate was incubated for 10 minutes at 37°C. There was no washing procedure after adding the TMB. Finally, 100 µl of STOP solution was added to each well. The plate was placed on The Belly Dancer (Stovall Life Sciences Inc., Greensboro, NC, USA) for a few seconds. The plate was read at absorbance determination of 450nm using a spectrophotometric plate reader (ELx800 Universal Microplate Reader, Bio-Tek instruments, Inc., Winooski, VT) within 30 minutes of adding the STOP solution.

3.13 Statistical Analysis of SYK ELISA and Transcription

The general linear model (GLM) procedure was performed using the statistical analysis software (SAS) (SAS Institute, Inc. NC) to analyze the effect of treatment (IgG molecules

binding to neutrophils) on transcription and SYK concentration. A significant difference was observed in SYK concentration between Jersey X Holstein and Holstein-Friesian cows after cells were treated ($p<0.05$).

3.14 Isolation of RNA From Neutrophils

Treated and control (PBS) PMN samples were preserved for RNA isolation using TRI Reagent (Molecular Research Center, Inc., USA). RNA isolations were done following a modified manufacturer's protocol (TRI Reagent, Molecular Research Center, Inc., USA). Samples were thawed out completely on ice and immediately centrifuged at 4°C for 10 minutes and 14000 RPM (Eppendorf Centrifuge 5417 R, Eppendorf, Hamburg, Germany). The supernatant was removed from each sample and placed into a 1.5 ml RNase-free microfuge tube (Ambion, The RNA Company, USA). The tubes with pellets on the bottom were discarded. Next, 200 µl of chloroform was added to each of the 1.5 RNase-free tubes and tubes were pulse vortexed individually. Samples were then left on ice for 2-3 minutes undisturbed. The tubes were centrifuged at 12000 RPM for 15 minutes at 4°C. The top, clear layer was transferred into a new RNase-free microfuge tube and 500 µl of isopropanol was added. Each tube was inverted, not shaken, to mix. The samples incubated on ice for 10 minutes. Samples were then centrifuged for 10 minutes at 12000 rpm at 4°C. RNA was seen as a white pellet on the bottom of the microfuge tube. The supernatant was removed and the pellet was washed with 100 µl of 75% ethanol, then centrifuged at 7500 rpm for 5 minutes at 4°C. The supernatant was poured off. The pellets were allowed to air dry by incubating under a hood on ice with open tops for about 10-12 minutes. The pellets were dissolved after air drying by adding 30 µl of DEPC treated water and heated for 10 minutes at 60°C to help dissolve the RNA pellet. Once the

incubation period ended, purity and concentration of RNA was checked using the Nanodrop Spectrophotometer 1000V 3.7.1 (Thermo Scientific Inc., Waltham, MA).

3.15 Reverse Transcription-PCR

To prepare RNA for Real-time PCR, it must first be converted into cDNA. Reverse transcription was carried out following the manufacturer's protocol (Ambion, Life Technologies Corporation, Grand Island, NY). In a PCR tube, 1-2 µg of isolated RNA was added to 2 µl of Oligo DT. Nuclease free water was added for a total volume of 12 µl in each reaction tube. The tubes were mixed briefly and centrifuged. Using a thermocycler, each tube was heated for 3 minutes at 80°C and placed on ice. The following components were then added to each tube: 2 µl of 10X RT buffer, 4 µl of dNTP mix, 1 µl of RNase inhibitor and 1 µl of MMLV-RT for a total volume of 20 µl in each tube. Each sample was mixed and briefly centrifuged. Using the thermocycler, each sample was incubated for 1 hour at 44°C and then 10 minutes at 92°C. This step inactivated the reverse transcriptase enzyme. The quality of the cDNA was measured using the Nanodrop Spectrophotometer. The samples were stored at -20°C until further use.

3.16 Real-Time PCR

RNA isolated from treated neutrophils was first converted to cDNA using Reverse Transcription PCR. SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, Ca.) were used for Real-time PCR. As recommended by the manufacturer (Bio-Rad Laboratories, Hercules, Ca.), 1.5 µl of a forward primer, 1.5 µl of a reverse primer and 100 ng of cDNA per sample was added to a 96-well tray along with10 µl of SsoAdvanced SYBR Green supermix. RNase- free water was added to each well for a total volume of 20 µl per well. The tray was centrifuged briefly. The components and amounts used are shown in Appendix E. Following centrifugation, the PCR tray was placed in the CFX Connect thermocycler (Bio-Rad

Laboratories, Hercules, Ca.) and the PCR cycling conditions were set using the manufacturers supplied software which is shown in table 3.16.2. The threshold cycle for each well was determined using the provided real-time cycler software.

CHAPTER 4

Results and Discussion

4.1 Health of animals

All cows used in the study were healthy female mid-stage lactating Holstein-Friesian and Jersey X Holstein crossbreeds as determined by the heard health management program at North Carolina A & T dairy unit. In addition, the subclinical health of the animals was confirmed by examination of the blood PCV, to measure anemia; milk SCC, as an indicator of mastitis; and whole blood cell differential count. All of the values were within the normal range (Table 4.1.1 and Figure 4.2.1). The normal values for packed cell volume in bovine range between 34%-46% (3) and the average PCV of the animals in the study was 39%, indicating no anemic conditions. The animals were clinically healthy (according to PCV and WBC values being in a healthy range per Merck manual). Some high somatic cell counts were reported from monthly DHI reports; however, no animals were being treated for mastitis at the time of the study.

Table 4.1.1.

General health results of each animal

Cow Number	Breed	SCC (10^3)	PCV (%)
1830	J X H	696	38
1854	J X H	400	34
1897	J X H	174	44
1862	J X H	230	35
2965	H	400	45
3001	H	107	39
3007	H	33	38
3012	H	87	38
4002	H	348	43
4003	H	22	37

4.2 White Blood Cell Differential Count

Lymphocytes, monocytes, eosinophils, basophils and neutrophils were observed in whole blood (figure 4.2.1) and are expressed as percentages. The mean WBC counts between the two groups (Holstein-Friesian and Jersey X Holstein cows) is shown in figure 4.2.1. All the WBC values were within the normal ranges for cattle indicating no negative health conditions of the animals. The normal values for WBC differential counts are as follows: lymphocytes, 45-75%; neutrophils, 35-75%; monocytes, 2-7%; eosinophils, 2-20% and basophils, 0-2% (3).

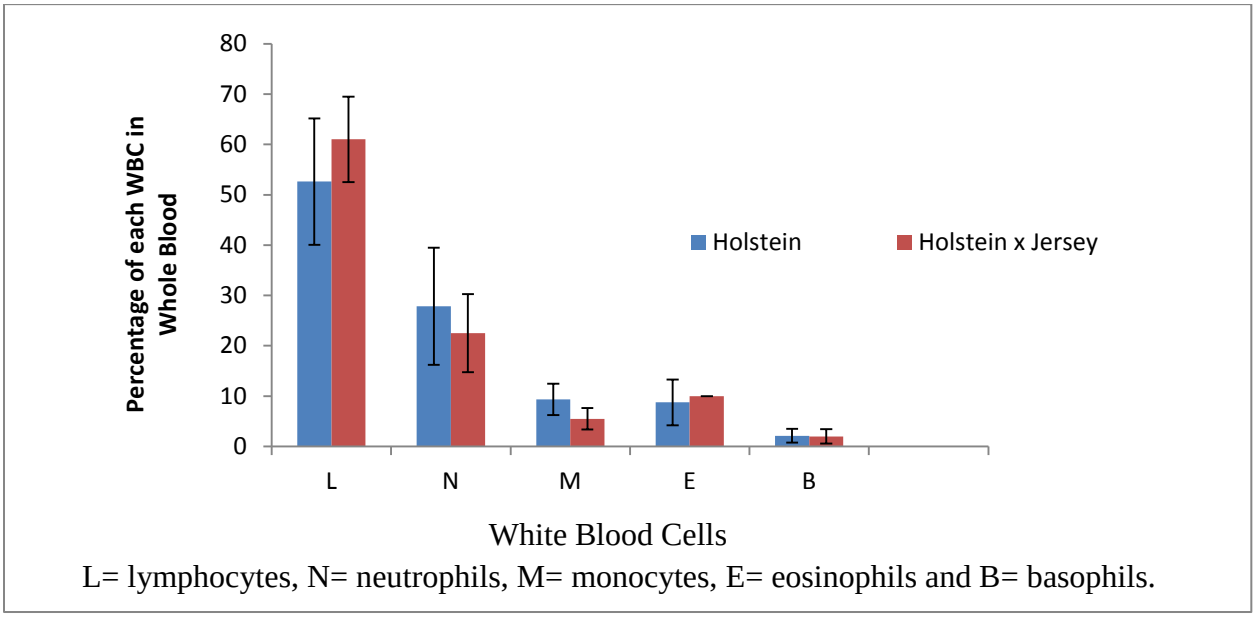


Figure 4.2.1. White Blood Cell differential counts in whole blood.

4.3 Purity and Viability of Isolated Neutrophils

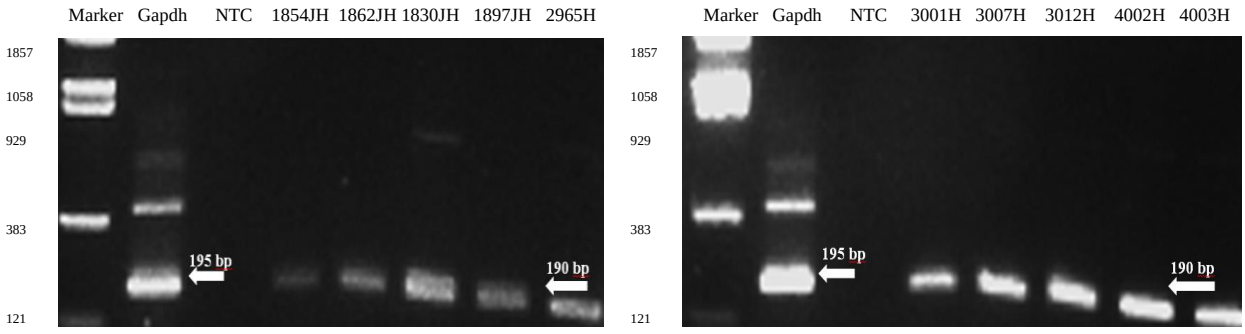
The purity of isolated neutrophils was 90% or higher in each sample. Viability of isolated neutrophils was more than 90% as determined by the Trypan Blue dye exclusion test.

4.4 Amplified FCGR1 and FCGR2C Genes in Genomic DNA

In this study, bovine gDNA was used successfully to amplify the genes of interest. According to literature, FCGR1 (CD 64) has a higher affinity for IgG compared to other FCGR gene family members. FCGR2A (CD32) has a lower affinity to CD32 as opposed to FCGR1

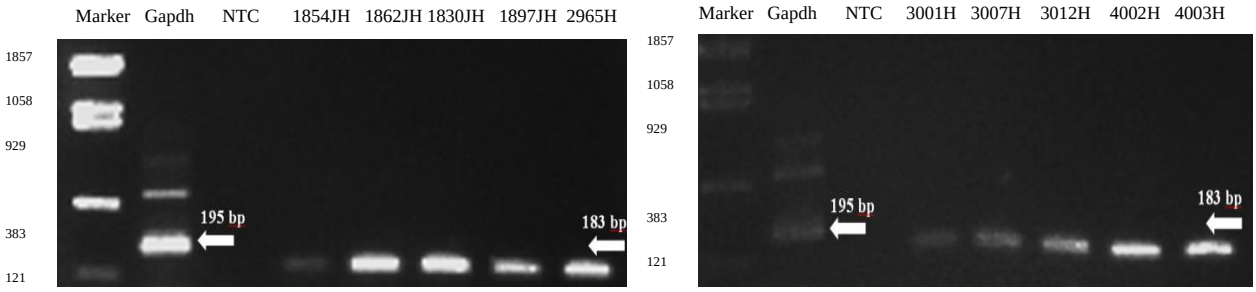
however both genes are needed (FCGR1 and FCGR2A) to activate the ITAM. Recent studies have shown, FCGR2C is the product of an unequal crossover of the FCGR2A and FCGR2B genes encoding the activating FcγR2A (CD32a) and inhibitory FcγR2B (CD32b), respectively. This means both genes are being expressed simultaneously FCGR2C has been shown to have no direct effect on the immune response that will help nor hinder the immune reaction effectiveness. DNA was isolated from FTA cards for gene amplifications in genomic DNA. Primers for FCGR1 and FCGR2C were selected based on previous studies conducted by Klungland et al., 1997. The results in this evaluation of FCGR2C on bovine neutrophils may be a novel finding in genetics as most studies of Fc receptors use human and/or mice samples for FCGR2C. Current research do not mention FCGR2C being found on neutrophils in the bovine species. The study surrounding FCGR2C conducted by Klungland et al. compares the location of the gene in human samples (blood) and bovine samples (sperm). This research evaluated information from the already highly developed human map to enable an establishment of precise genetic maps for functional genes in cattle. The linkage map of the bovine FCGR gene family, located on Chr 3 (BTA3), resembles the human map where all FCGR genes are located on Chr 1 (HSA1), in which they are positioned between q21-q24. Knowing the similarities of the map location demonstrates an evolutionary breakpoint between HSA1 and BTA3 within the human 1q24 region. The primers used recognized conserved regions and the relationship of these conserved FCGR genes have also been reported by (Konstantinos, Panagiotis, Antonios, Agelos, Argiris, 2008). The images below (figure 4.4.1 and 4.4.2) shows amplification of FCGR1 and FCGR2C following PCR. A housekeeping gene, Gapdh, was used throughout the experiment (expected bp is 195) (Yamamoto, Teramoto, Uetani, Igawa, Shimizu, 2001). The “no template control”

(NTC) contains all of the PCR required reagents except the DNA template and is used as a negative control.



Each well is labeled according to the cow number and breed with JH=Jersey X Holstein and H=Holstein-Friesian. The "no template control" is indicated by NTC and Gapdh was used as a reference control. The expected bp for FCGR1 is 190.

Figure 4.4.1. Product of the FCGR1 gene amplification in bovine genomic DNA (n=10).



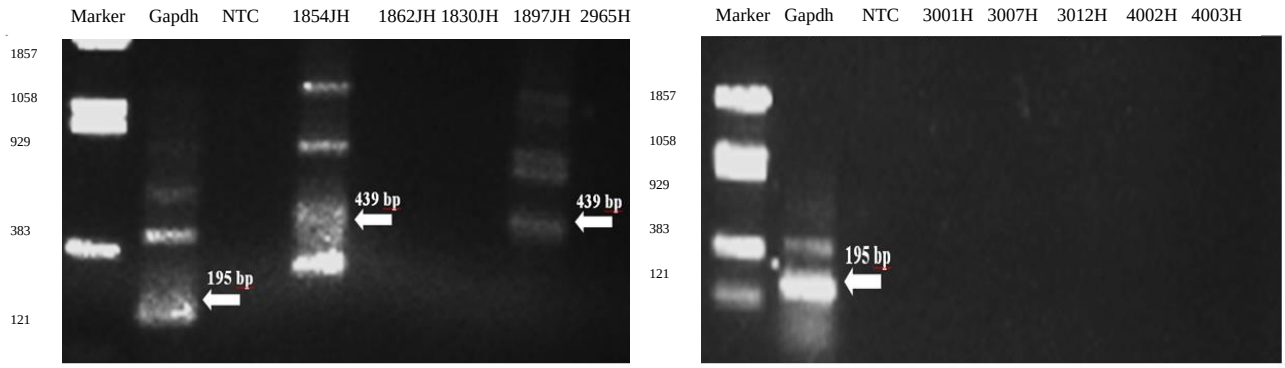
Each well is labeled with the cow number and breed as mentioned in the previous image. The expected bp of FCGR2C is 183.

Figure 4.4.2. Product of the FCGR2C gene amplification in bovine genomic DNA (n=10).

4.5 Amplified FCGR2A Gene in Genomic DNA

Primers for FCGR2A were selected based on studies conducted by Flesch et al. FCGR2A is the most widely distributed of the three classes of Fcγ receptors and is expressed on most types of white blood cells. FCGR2A is also known to be an activating receptor responsible for aiding in the initiation of an immune response (Edberg, Moon, Chang, Kimberly, 1998).

Presence of this gene will activate immune cells and recruit them to respond to invading pathogens. This research suggested that polymorphisms existing within this gene may cause different immunological reactions which can help or hinder the natural immune defenses to bacterial polysaccharides and encapsulated bacteria (Flesch, Bauer, and Neppert, 1998). This is especially important as most mastitis infections are caused by encapsulated bacteria. In figure 4.5.1 below, genes coding for FCGR2A were identified in the Jersey X Holstein crossbred cattle only and were not detected the Holstein-Friesian cattle. The wells are labeled accordingly.



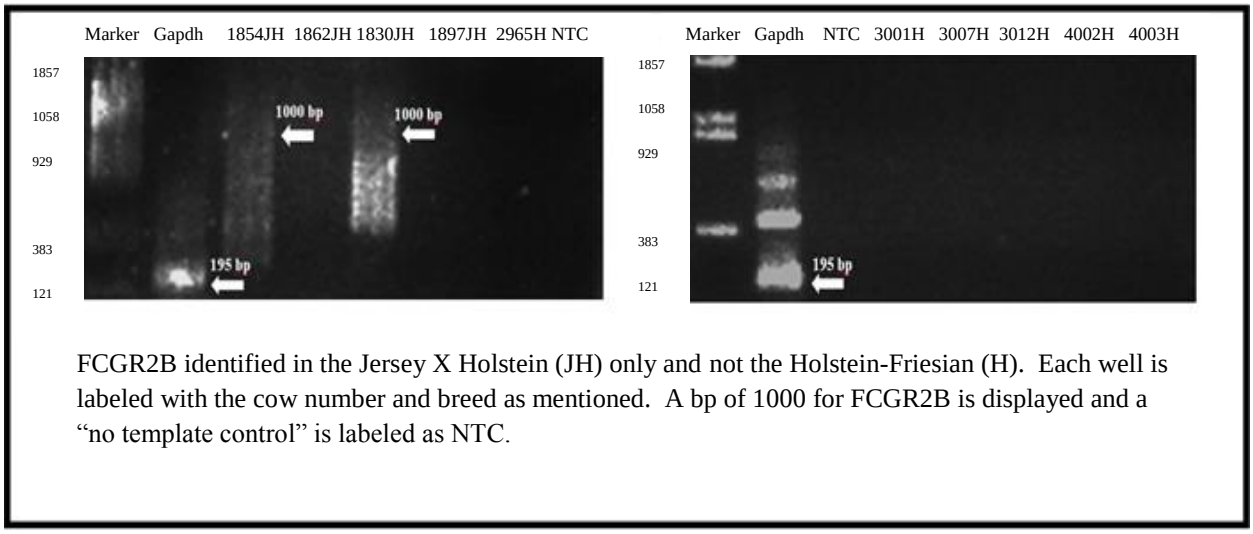
FCGR2A identified in the Jersey X Holstein (JH) crossbred cattle only and not detected in the Holstein-Friesian (H) cattle. The wells are labeled according to the cow number and breed. A bp of 439 was amplified in cows 1854 and 1897. The "no template control" is labeled as NTC.

Figure 4.5.1. Product of the FCGR2A gene amplification in bovine genomic DNA (n=10).

4.6 Amplified FCGR2B Gene in Genomic DNA

Primers for FCGR2B were selected based on studies conducted by Jiang et al. and are shown below. FCGR2B is an inhibitory receptor involved in ITAM but directly responsible for activating the inhibitory signal motif (ITIM). Ultimately, FCGR2B is responsible for normalizing the host's immune system after an invasion and preventing further growth of immune cells when there is no invasion. Because this gene turns off the immune response, polymorphisms within this gene have the potential to accelerate autoimmune diseases and

therefore increasing autoimmune susceptibility (Smith and Clatworthy, 2010). Figure 4.6.1.shown below, depicts FCGR2B gene present in the Jersey X Holstein crossbred cattle and not the Holstein-Friesian cows. The wells are labeled accordingly.



FCGR2B identified in the Jersey X Holstein (JH) only and not the Holstein-Friesian (H). Each well is labeled with the cow number and breed as mentioned. A bp of 1000 for FCGR2B is displayed and a "no template control" is labeled as NTC.

Figure 4.6.1. Product of the FCGR2B gene amplification in bovine genomic DNA (n=10).

4.7 Summary of FCGR Genes Detected in Cow Genomic DNA

In summary, FCGR1 and FCGR2C were detected in all cows. Variability was observed in detection of FCGR2A and FCGR2B, activating and inhibitory forms respectively. The presence differed by breed. Previous studies have shown their relevance to diseases, such as bacteria causing mastitis (Stefanescu, Olferiev, Liu, Pricop, 2004). FCGR2B receptors play a major role in controlling the amplitude of antibody- and immune complex-mediated reactions. Generally a deficiency in FCGR2B is associated with an increased susceptibility to disease and severity to organ-specific and systemic autoimmunity (McGaha, Karlsson, Ravetch,2008; Smith and Clatworthy, 2010)Thus, further studies with larger groups of animals may provide further evidence and limits to disease susceptibility. Below, a positive (+) sign indicates a band present on the gel and a negative (-) sign indicates no band present on the gel. Each breed is abbreviated

with H or JXH indicating: JXH= Jersey X Holstein and H= Holstein-Friesian. After PCR, cows were randomly selected to form two groups consisting of 3 cows each (Holstein-Friesian and Jersey X Holstein) the cows chosen for the remainder of the study are indicated by an asterisk beside the cow number.

Table 4.7.1.

Summary of PCR results

Cow Number	Breed	SCC (10^3)	Gapdh	FCGRI	FCGRIIA	FCGRIIB	FCGRIIC
1830	JXH	696	+	+	-	+	+
***1854**	JXH	400	+	+	+	+	+
***1862**	JXH	230	+	+	-	-	+
***1897**	JXH	174	+	+	+	-	+
2965	H	400	+	+	-	-	+
3001	H	107	+	+	-	-	+
***3007**	H	33	+	+	-	-	+
***3012**	H	87	+	+	-	-	+
***4002**	H	348	+	+	-	-	+
4003	H	22	+	+	-	-	+

4.8 Single Strand Conformational Polymorphism Gel Analysis

Single strand conformational polymorphism relies on denatured DNA amplicons migrating across a polyacrylamide gel in a nondenaturing fashion. This electrophoresis is based not only by size but also its sequence which dictates the conformational shape of one's DNA (Rodríguez, Cai, Teng, Spooner, 2011). The folded structure of DNA is determined by intramolecular interactions. Mutations are detected at mobility shifts of separated single strands. Any fragments differing on the gel may be a result of potential polymorphic genes (Konstantinos, Panagiotis, Antonios, Agelos, Argiris, 2008). Following PCR, the genomic DNA amplicons were used for SSCP gel analysis. The genes amplified by all animals (FCGRI and

FCGRIIC) were used for the SSCP analysis shown below in figure 4.8.1 and 4.8.2. The high IgG affinity receptor (FCGRI) shows apparent differences in the gene when comparing breeds and the mobility shifts are indicated by the red lines on the image. However, FCGR2C (figure 4.8.2) shows little difference in the samples and are also indicated by the red line.

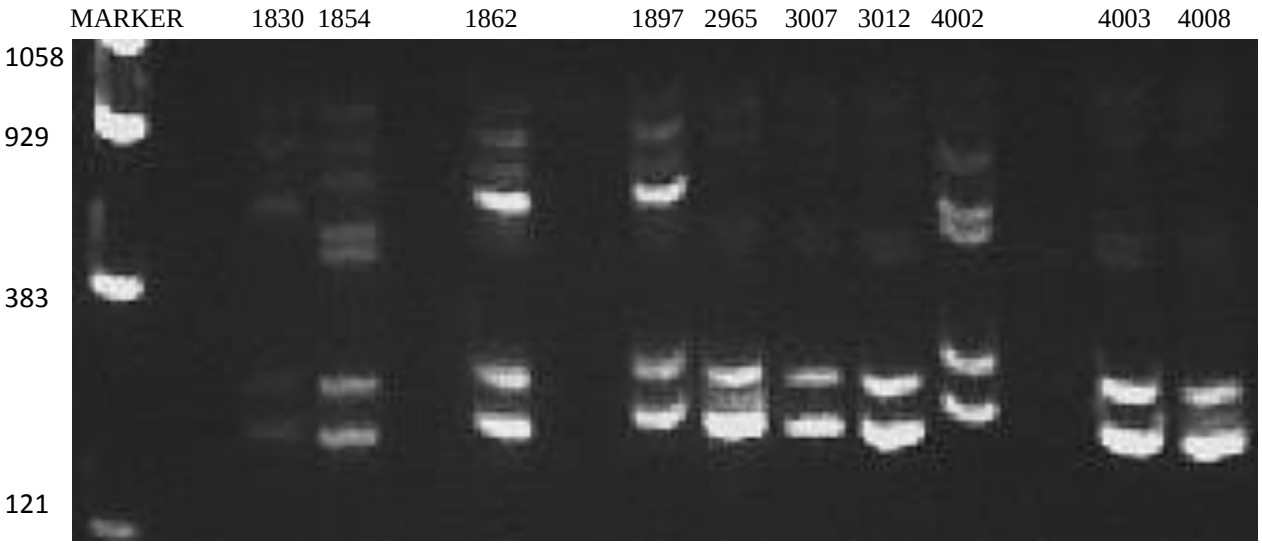


Figure 4.8.1. SSCP gel analysis of the FCGR1 gene.

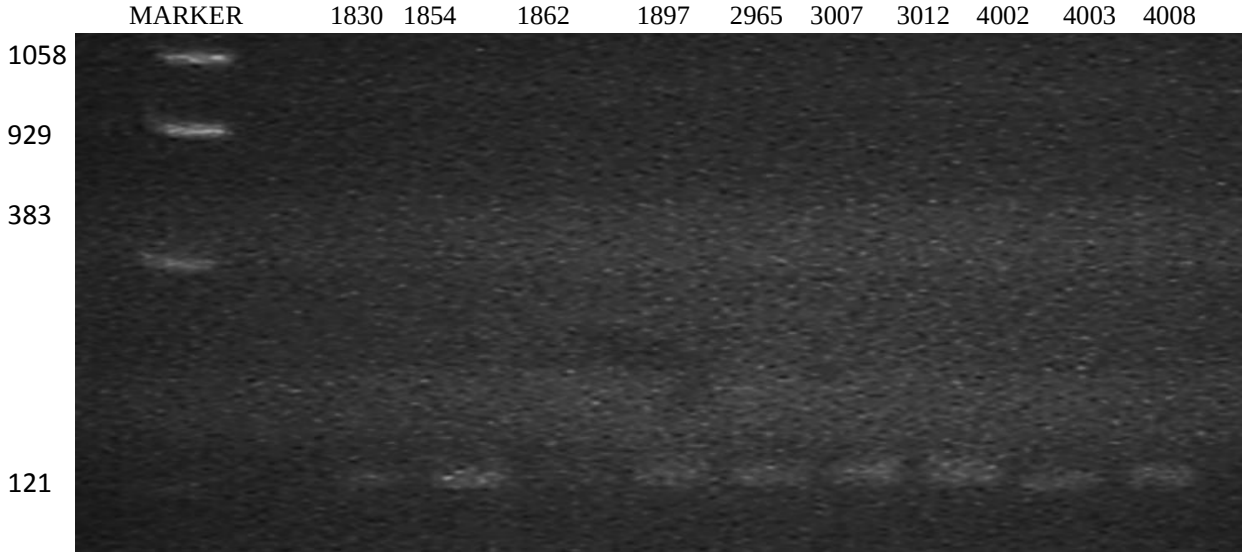
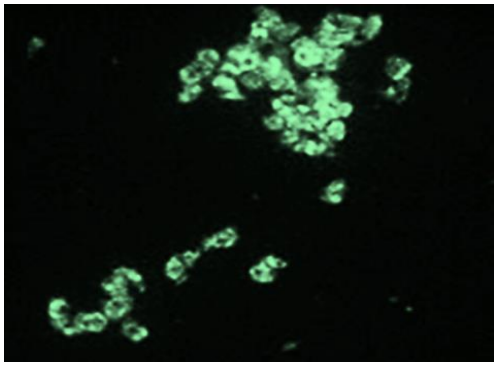


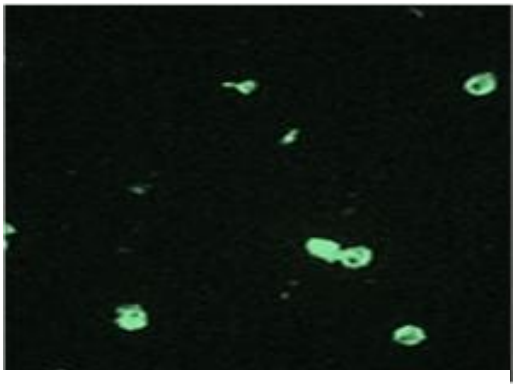
Figure 4.8.2. SSCP gel analysis of the FCGR2C gene.

4.9 Confocal Microscopy Analysis of Treated Neutrophils

In support of SYK expression with ELISA, cells were analyzed using confocal microscopy to confirm binding of IgG molecules to neutrophils by assessing fluorescence. Smears were created and sent to Dr. Mehrdad Tajkarimi at the Joint School of Nanoscience and Nanoengineering, University of North Carolina at Greensboro for imaging. Images from these samples are represented in figure 4.9.1. and show treated and untreated samples to substantiate IgG binding after treatment. Due to limited resources, 3 cows were selected for confocal microscopy analysis. One cow is represented in figure 4.9.1 below while the remaining samples can be found in figure 4.9.2. in Appendix F. The green fluorescence shown proves IgG binding to the neutrophils. There is some diffuse fluorescence associated with control (PBS) samples and may be due to autofluorescence, but patchy and more specific fluorescence can be seen in the treated samples. Each letter (A and B) provides a brief description of the image. Cow breeds are mentioned as JH= Jersey X Holstein and H= Holstein-Friesian.



A. Treated PMN sample from cow 1854 (JH).



B. Untreated (PBS) PMN sample from cow 1854 (JH).

Figure 4.9.1. Confocal microscopy images of a treated and untreated cow neutrophil sample.

4.10 Analysis of SYK Transcription Factors using ELISA

The SYK gene mediates signal transduction downstream of a variety of immunoreceptors and regulates the adaptive and innate immunity, this gene is important for providing immune protection and cellular activation as soon as cells are aware of invading pathogens. Elevations in SYK activity were evaluated using the PathScan® Phospho-Syk (panTyr) sandwich ELISA with treated PMN. In figure 4.10.1 below, stastical anaylsis is shown between and within the groups of cattle indicating Syk protein concentrations increasing with treated cells when compared to the control (PBS). Phosphorylated SYK concentrations per animal can be found in table 4.10.1 in appendix G. The indicated elevations show intracellular excitation after stimulation with IgG molecules. Upon evaluation of SYK gene concentrations there was a significant difference observed in Jersey X Holstein and Holstein-Friesian cows. Higher protein secretions were observed within the Jersey X Holstein group when compared to the Holstein-Friesian group ($p<0.05$). This may show breed genetic differences and further support the differences observed with SSCP gels.

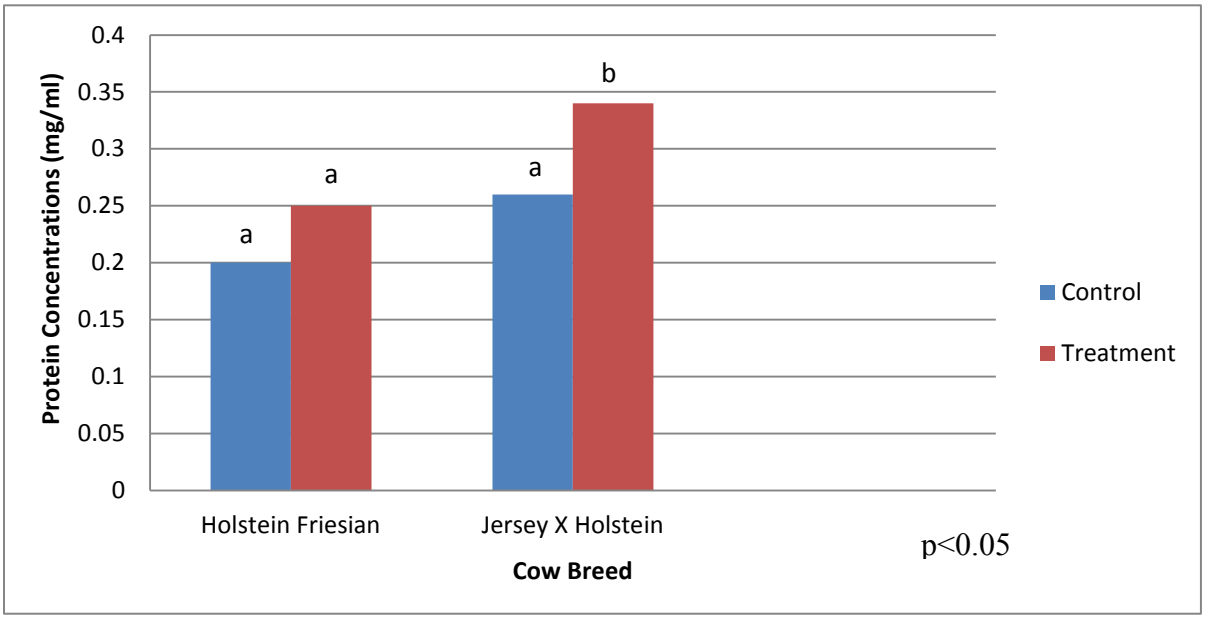


*Figure 4.10.1.*Concentration of phosphorylated SYK protein after treatment of cells.

4.11 Real-Time PCR Analysis of Gene Expression

Real time PCR (qPCR) was used to determine the expression of the FcγR genes using cDNA from treated and untreated samples. Treated samples were acquired from neutrophils treated with IgG antibodies. The Cq values obtained with the CFX Connect software provided evidence of gene expression and were further used to evaluate fold changes between each animal which can be seen below. Gapdh was used as a positive template control while the no template control served as a negative control. Fold changes were generated using the data collected from the CFX96 software for gene expression analysis and are presented in figure 4.11.1. The software used expression values and fold changes were calculated using the Livak method to create the bar graph showing gene expression among the samples provided. Each group is represented by the color scheme provided in the legend. Cycle of quantification values (Cq) for each animal can be found in table 4.11.1 located in Appendix H

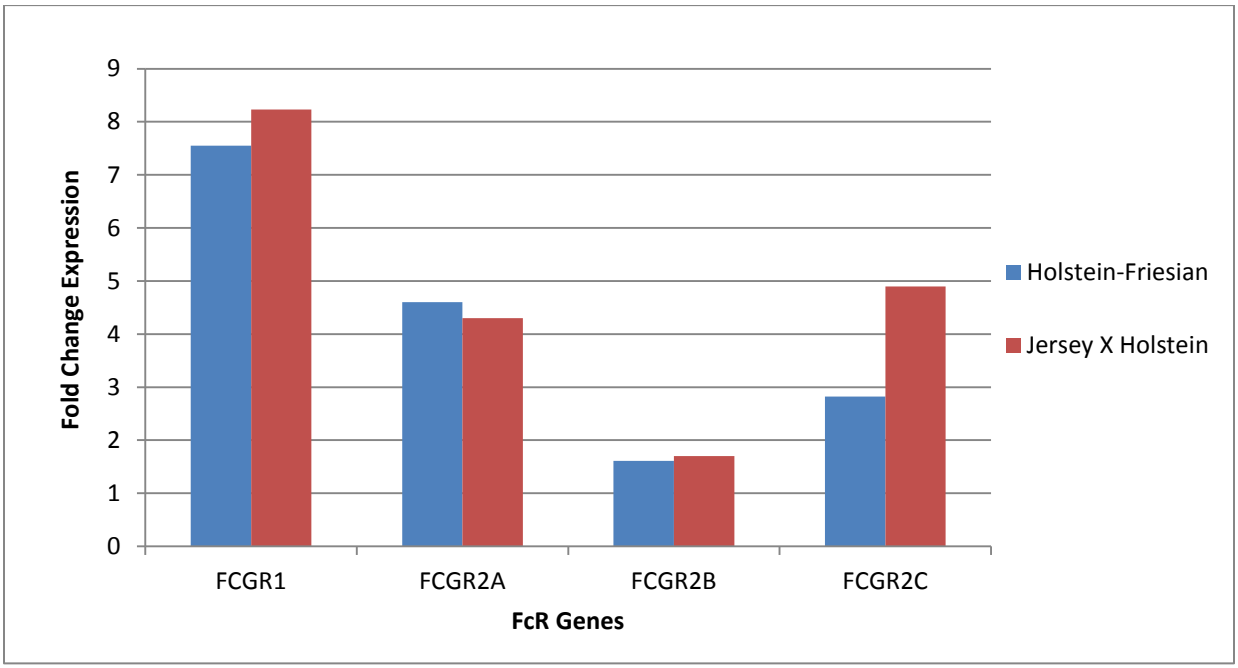


Figure 4.11.1. Fold changes of the FcR genes of interest Holstein-Friesian and Jersey X Holstein cattle.

4.12 Summary of Gene Expression and Fold Change

Holstein and Jersey cows are the first and second most popular and well-known dairy breeds of cattle in the U.S. In literature, Holstein-Friesian cows are more susceptible to mastitis infections due to possessing a trait for high milk production. Holstein-Friesian cows produce more milk than Jersey cows. Jersey cows are thought to be heartier and more robust when the risk of infection is involved, however, they do not produce as much milk and therefore minimizing the chance of an (mastitis) infection upon comparison of these two breeds. Jersey cows are known for the milk quality and taste more so than the amount they produce.

Jersey X Holstein cows showed a much higher fold expression of FCGR1 when compared to the Holstein-Friesian cows. It could be suggested that rapid transcription of FCGR1, which has a high affinity for the IgG molecules, allows for a speedy rate of aggression downstream of cellular activation may cause rapid immune response. The Jersey X Holstein crossbred group also displayed higher fold changes in FCGR2C when compared to the Jersey X Holstein group. These genes (FcRs) have not been fully characterized in the bovine. Studies suggest that FCGR2C is not expressed on neutrophils at all when indeed PMN samples were treated and used for gene analysis.

4.13 Electrophoresis of Real-Time PCR Amplicons

In order to support q-PCR transcription results, gel electrophoresis was used to view randomly selected samples in which Cq values were obtained along with a no template control sample, as labeled. When using reverse transcription to convert RNA to cDNA, deletions of introns and joining of exons occur, possibly leaving smaller or shorter amplified sequences. This will generally cause the base pairs of a product previously amplified with gDNA to be greatly

decreased when viewing the same product using cDNA. However, the bands present on the gel image below (figure 4.13.1) show amplification of these genes of interest.

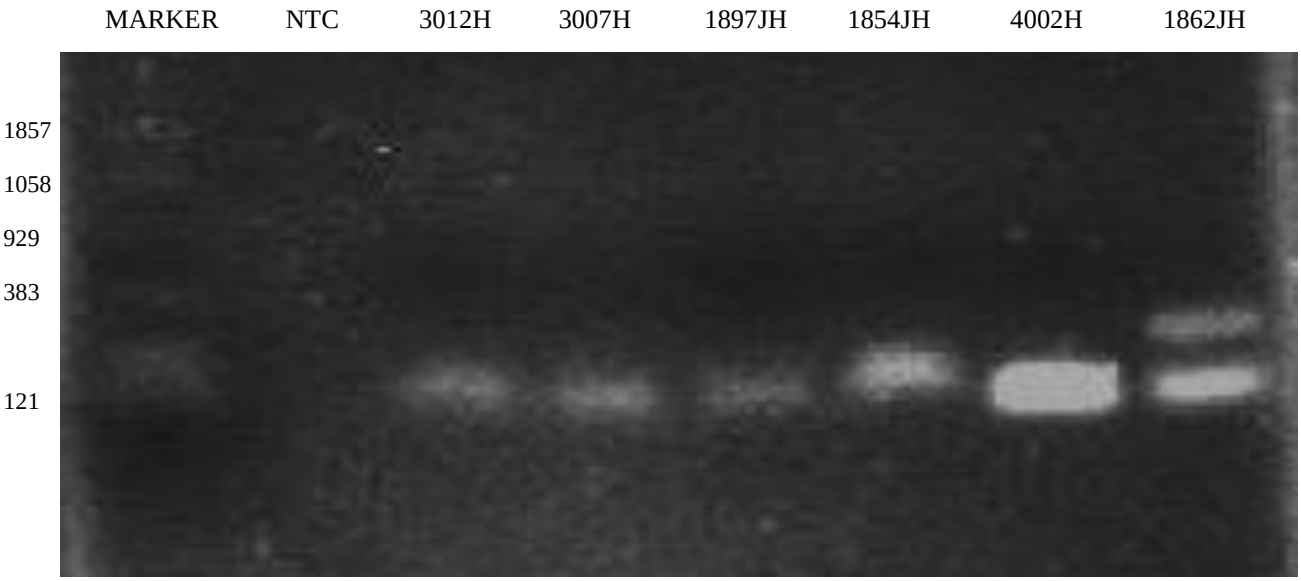


Figure 4.13.1. Amplicons of cDNA following Real-Time PCR (n=6).

CHAPTER 5

Summary and Conclusion

Variability in Fc receptor genes and signal transduction was observed throughout the study. Binding of IgG to Neutrophils results in cell activation. Through improved genetics, Holstein-Friesian cows have been able to adapt to their most common use, being a high milk producer. Evolution, nevertheless, has not changed the susceptibility to mastitis infection this breed carries. It is still the most costly disease in the dairy industry. Because Jersey cows do not produce as much milk this may also leave them (Jerseys) more vulnerable to infections relating to mastitis, although hearty, when over worked to meet the same milk producing capacity as a Holstein-Friesian cow. The results of this study justify this assumption as the crossbred group (Jersey X Holstein) maintained higher somatic cell counts when compared to the Holstein-Friesian cows. Contrary, Jersey X Holstein cows revealed elevated levels of gene transcription as well as higher Syk protein concentrations when compared to Holstein-Friesian cows. Producing a higher concentration of Syk may promote pathogen clearance. Intracellular activity downstream of Fc receptor activation drives immunity and may result in a more rapid response upon invading pathogens.

The Jersey X Holstein crossbreeds showed higher Syk protein levels upon treatment of neutrophils. This group had up regulation of FcRs genes observed using cDNA with real time. The Cq values for FCGRI and FCGR2C genes were higher. Although variability occurred within the group, gene expression analysis suggested an up regulation in FcR in the Jersey X Holstein crossbreeds when compared to the Holstein-Friesian cattle in the study. The genes studied here are responsible for a prompt immune response for preventing mastitis infections and appear to be suppressed or down regulated in the Holstein-Friesian cows. The breed of the

animal through gene expression may be used to predict whether or not the animal would succumb to sickness. Suppression or up-regulation of the genes of interest in these breeds may be linked to many factors including those that contribute to disease resistance.

In conclusion, the genetic differences observed may be related to susceptibility to pathogens. The differences in expression observed may help to locate genetic markers for select breeds of food animals.

Recommendations

Further studies will need to be conducted for the development of a more resistant breed of animal. Increasing the number of animals used will allow a larger pool of data. Other breeds of cattle should also be added in the study. In science a species' genetic sequence is ~99% identical however; the 1% change in a transcribed sequence will cause genomic differences within a species. Studying the same species but different breeds (ex. Holstein-Friesian vs. Jersey purebred) will help identify polymorphic genes that may cause one to be more resilient than the next. We know from research that the stage of lactation, nutritional management and milk yield can all affect somatic cell counts and therefore further analysis should include evaluation of these factors. Further investigation is needed on the FcR genes as they have not been completely characterized in the bovine genome and may play a role to immunity.

References

Abatepaulo, A. R., Caetano, A. R., Mendes Jr, C. T., Carvalho, W. A., Ferreira, B. R., & De Miranda Santos, I. F. (2008). Detection of SNPs in bovine immune-response genes that may mediate resistance to the cattle tick Rhipicephalus (Boophilus) microplus. *Animal Genetics*, *39*(3), 328-329.

Aderem, A., & Underhill, D. M. (1999). Mechanisms Of Phagocytosis In Macrophages. *Annual Review of Immunology, 17*(1), 593-623.

Aiello, S., & Mays, A. (1998). *The Merck veterinary manual* (8th ed., pp. 2190-2191). Whitehouse Station, NJ: Merck &, in cooperation with Merial.

Barrow, A., & Trowsdale, J. (2006). Correction: You say ITAM and I say ITIM, let's call the whole thing off: The ambiguity of immunoreceptor signalling. *European Journal of Immunology*, *36*(8), 2276-2276.

Beale, D., & Feinstein, A. (1976). Structure and function of the constant regions of immunoglobulins. *Quarterly Reviews of Biophysics*, *9*(02), 135.

Berkowicz, E. W., Magee, D. A., Berry, D. P., Sikora, K. M., Howard, D. J., Mullen, M. P., ... MacHugh, D. E. (2011). Single nucleotide polymorphisms in the imprinted bovine insulin-like growth factor 2 receptor gene (IGF2E) are associated with body size traits in irish holstein-friesian cattle. *Animal Genetics: Immunogenetics, Molecular Genetics and Fundamental Genomics*, 1-7.

Bewarder, N., Weinrich, V., Budde, P., Hartmann, D., Flaswinkel, H., Reth, M., & Frey, J. (1996). In vivo and in vitro specificity of protein tyrosine kinases for immunoglobulin G receptor phosphorylation. *Molecular and Cellular Biology*, *16*(9), 4735-4743.

Bournazos, S., Woof, J. M., Hart, S. P., & Dransfield, I. (2009). Functional and clinical consequences of Fc receptor polymorphic and copy number variants. *Clinical & Experimental Immunology*, *157*(2), 244-254.

Bradley, A. (2002). Bovine Mastitis: An Evolving Disease. *The Veterinary Journal*, *164*(2), 116-128.

Bradshaw, J. M. (2010). The Src, Syk, and Tec family kinases: Distinct types of molecular switches. *Cellular Signalling*, *22*(8), 1175-1184.

Carlson, G. P., & Kaneko, J. J. (1973). Isolation of leukocytes from bovine peripheral blood. *Experimental Biology and Medicine*, *142*(3), 853-856.

Cohen, I. R., & Norins, L. C. (1968). Antibodies of the IgG, IgM, and IgA classes in newborn and adult sera reactive with gram-negative bacteria. *Journal of Clinical Investigation*, *47*(5), 1053-1062.

Cooney, D. S., Phee, H., Jacob, A., & Coggeshall, K. (2001). Signal Transduction by Human-Restricted FcγRIIa Involves Three Distinct Cytoplasmic Kinase Families Leading to Phagocytosis. *The Journal of Immunology*, *167*(2), 844-854.

Edberg, J. C., Moon, J. J., Chang, D. J., & Kimberly, R. P. (1998). Differential regulation of human neutrophil Fc gamma receptor IIa (CD 32) and Fc gamma receptor IIIb (CD 16)-induced Ca Transients. *The Journal of Biological Chemistry*, *273*(14), 8071-8079.

Firth, M. A., Chattha, K. S., Hodgins, D. C., & Shewen, P. E. (2010). Multiple bovine FcγRIIb sub-isoforms generated by alternative splicing. *Veterinary Immunology and Immunopathology*, *135*(1-2), 43-51.

Flesch, B., Bauer, F., & Neppert, J. (1998). Rapid typing of the human Fc gamma receptor IIA polymorphism by polymerase chain reaction amplification with allele-specific primers. *Tranfusion*, *38*(2), 174-176.

Fox, L. (2009). Prevalence, incidence and risk factors of heifer mastitis. *Veterinary Microbiology*, *134*(1-2), 82-88.

Fridman, W. H. (1991). Fc receptors and Immunoglobulin binding factors. *The Federation of American Societies for Experimental Biology (FASEB), 5*, 2684-2690.

Ganesan, L. P. (2003). The protein-tyrosine phosphatase SHP-1 associates with the phosphorylated immunoreceptor tyrosine-based activation motif of FcγRIIa to modulate signaling events in myeloid cells. *The Journal of Biological Chemistry*, *278*(37), 35710-5717.

Gerber, J. S., & Mosser, D. M. (2001). Stimulatory and inhibitory signals originating from the macrophage Fc gamma receptors. *Microbes and Infection, 3*, 131-139.

Ghazizadeh, S. J., Bolen, B., & Fleit, H. B. (1994). Physical and functional association of Src-related protein tyrosine kinases with Fc gamma RII in monocytic THP-1 cells. *The Journal of Biological Chemistry*, *269*, 8878-8884.

Halushka, M. K., Fan, J., Bentley, K., Hsie, L., Shen, N., Weder, A., Chakravarti, A. (1999). *Nature America Inc.*, *22*, 239-247.

Huang, Z., Hunter, S., Kim, M., Indik, Z. K., & Schreiber, A. D. (2003). The effect of phosphatases SHP-1 and SHIP-1 on signaling by the ITIM- and ITAM-containing Fcγ receptors FcγRIIB and FcγRIIA. *Journal of Leukocyte Biology*, *73*(6), 823-829.

Ibarrola, I., Vossebeld, P., Homberg, C., Thelen, M., Ross, D., & Verhoeven, A. (1997). Influence of tyrosine phosphorylation on protein interaction with FcγRIIa. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research*, *1357*(3), 348-358.

Isakov, N. (1997). ITIMs and ITAMs. *Immunologic Research*, *16*(1), 85-100.

Jiang, Y., Hirose, S., Abe, M., Sanokawa-Akakura, R., Ohtsuji, M., Mi, X., Shirai, T. (2000). Polymorphisms in IgG Fc receptor IIB regulatory regions associated with autoimmune susceptibility. *Immunogenetics*, *51*(6), 429-435.

Kacskovics, I. (2004). Fc receptors in livestock species. *Veterinary Immunology and Immunopathology*, *102*(4), 351-362.

Klungland, H., Gomez-Raya, L., Howard, C. J., Collins, R. A., Rogne, S., & Lien, S. (1997). Mapping of bovine FcγR (FCGR) genes by sperm typing allows extended use of human map information. *Mammalian Genome*, *8*(8), 573-577.

Konstantinos, K. V., Panagiotis, P., Antonios, V. T., Agelos, P., & Argiris, N. V. (2008). PCR–SSCP: A Method for the Molecular Analysis of Genetic Diseases. *Molecular Biotechnology*, *38*(2), 155-163.

Liu, Y., Qiao, S., Wang, A., Chang, J., Chen, Y., Yang, S., Zhang, G. (2011). Cloning and characterization of ovine immunolglobulin G Fc receptor III. *Veterinary Immunology and Immunopathology*, *139*, 282-288.

Lucy, M. (2001). Reproductive Loss in High-Producing Dairy Cattle: Where Will It End? *Journal of Dairy Science*, *84*(6), 1277-1293.

Majeed, M., Caveggion, E., Lowell, C., & Berton, G. (2001). Role of Src kinases and Syk in Fcγ receptor-mediated phagocytosis and phagosome-lysosome fusion. *Journal of Leukocyte Biology*, *70*(5), 801-811.

McGaha, T. L., Karlsson, M. C., & Ravetch, J. V. (2008). FcγRIIB Deficiency Leads to Autoimmunity and a Defective Response to Apoptosis in Mrl-MpJ Mice. *The Journal of Immunology*, *180*(8), 5670-5679.

Mina-osorio, P., & Ortega, E. (2004). Signal regulators in FcR-mediated activation of leukocytes? *Trends in Immunology*, *25*(10), 529-535.

Mishima, T., Kurasawa, G., Ishikawa, G., Mori, M., Kawahigashi, Y., Ishikawa, T., ... Takizawa, T. (2007). Endothelial Expression of Fc Gamma Receptor IIb in the Full-term Human Placenta. *Placenta*, *28*(2-3), 170-174.

Morton, H. C., Pleass, R. J., Storset, A. K., Dissen, E., Williams, J. L., Brandtzaeg, P., & Woof, J. M. (2004). Cloning and characterization of an immunoglobulin A Fc receptor from cattle. *Immunology*, *111*(2), 204-211.

Niemialtowski, M., Nonnecke, B., & Targowski, S. (1988). Phagocytic Activity of Milk Leukocytes During Chronic Staphylococcal Mastitis. *Journal of Dairy Science*, *71*(3), 780-787.

Nimmerjahn, F., & Ravetch, J. V. (2008). Fcgamma receptors as regulators of immune responses. *Nature Reviews. Immunology*, *8*(1), 34-47.

Paape, M., Bannerman, D., Zhao, X., & Lee, J. (2003). The bovine neutrophil: Structure and function in blood and milk. *Veterinary Research*, *34*(5), 597-627.

Paape,, M., Shafer-Weaver, K., Capuco, A., Oostveldt, K., & Burvenich, C. (2002). Immune Surveillance of Mammary Tissue by Phagocytic Cells. *Advances in Experimental Medicine and Biology*, *480*, 259-277.

Paixao, T., Ferreira, C., Borges, A., Oliveira, D., Lage, A., & Santos, R. (2006). Frequency of bovine (Slc11a1) alleles in Holstein and Zebu breeds. *Veterinary Immunology and Immunopathology*, *109*(1-2), 37-42.

Pan, L., & Pei, P. (2003). Signaling transduction by IgG receptors. *Chinese Medical Journal*, *116*(4), 487-494.

Parsons, S. J., & Parsons, J. T. (2004). Src family kinases, key regulators of signal transduction. *Oncogene*, *23*(48), 7906-7909.

Radha, V., Nambirajan, S., & Swarup, G. (1996). Association of Lyn Tyrosine Kinase with the Nuclear Matrix and Cell-Cycle-Dependent Changes in Matrix-Associated Tyrosine Kinase Activity. *European Journal of Biochemistry*, *236*(2), 352-359.

Raghavan, M., & Bjorkman, P. J. (1996). Fc RECEPTORS AND THEIR INTERACTIONS WITH IMMUNOGLOBULINS. *Annual Review of Cell and Developmental Biology*, *12*(1), 181-220.

Ravetch, J. V., & Bolland, S. (2001). Igg Fc Receptors. *Annual Review of Immunology*, *19*(1), 275-290.

Rodríguez, F., Cai, D., Teng, Y., & Spooner, D. (2011). ASYMMETRIC SINGLE-STRAND CONFORMATION POLYMORPHISM:. *American Journal of Botany*, *98*(7), 1061-1067.

Rupp, R., & Boichard, D. (2000). Relationship of early first lactation somatic cell count with risk of subsequent first clinical mastitis. *Livestock Production Science*, *62*(2), 169-180.

Schroeder, H. W., Jr., & Cavacini, L. (2010). Structure and function of immunoglobulins. *Journal of Allergy and Clinical Immunology*, *125*(2), S348-S348.

Selvaraj, P., Fifadara, N., Nagarajan, S., Cimino, A., & Wang, G. (2004). Functional Regulation of Human Neutrophil Fc γ Receptors. *Immunologic Research*, *29*(1-3), 219-230.

Smith, K. C., & Clatworthy, M. R. (2010). FcγRIIB in autoimmunity and infection: Evolutionary and therapeutic implications. *Nature Reviews Immunology*, *10*(5), 328-343.

Stefanescu, R. N., Olferiev, M., Liu, Y., & Pricop, L. (2004). Inhibitory Fc Gamma Receptors: From Gene to Disease. *Journal of Clinical Immunology*, *24*(4), 315-326.

Swanson, J. A. (2004). The coordination of signaling during Fc receptor-mediated phagocytosis. *Journal of Leukocyte Biology*, *76*(6), 1093-1103.

Syvänen, A. (2001). ACCESSING GENETIC VARIATION: GENOTYPING SINGLE NUCLEOTIDE POLYMORPHISMS. *NATURE REVIEWS:GENETICS*, *2*, 930-942.

Takai, T. (2005). Fc Receptors and Their Role in Immune Regulation and Autoimmunity. *Journal of Clinical Immunology*, *25*(1), 1-18.

Thomas, S. M., & Brugge, J. S. (1997). Cellular Functions Regulated By Src Family Kinases. *Annual Review of Cell and Developmental Biology*, *13*(1), 513-609.

Underhill, D. M., & Ozinsky, A. (2002). PHAGOCYTOSIS OF MICROBES: Complexity in Action. *Annual Review of Immunology*, *20*(1), 825-852.

Vignal, A., Milan, D., SanCristobal, M., & Eggen, A. (2002). A review on SNP and other types of molecular markers and their use in animal genetics. *Genetics Selection Evolution*, *34*(3), 275-305.

Vivier, E., & Daeron, M. (1997). Immunoreceptor tyrosine-based inhibition motifs. *Immunology Today*, *18*(6), 286-291.

Wellenberg, G., Van der Poel, W., & Van Oirschot, J. (2002). Viral infections and bovine mastitis: A review. *Veterinary Microbiology*, *88*(1), 27-45.

Worku, M., Paape, M., & Marquardt, W. (1994). Modulation of Fc receptors for IgG on bovine polymorphonuclear neutrophils by interferon-gamma through de novo RNA transcription and protein synthesis. *American Journal of Veterinary Research, 55*(2), 234-328.

Worku, M., Paape, M., Filep, R., & Miller, R. (1994). Effect of in vitro and in vivo migration of bovine neutrophils on binding and expression of Fc receptors for IgG2 and IgM. *American Journal of Veterinary Medicine*, *55*(2), 221-226.

Yamamoto, H., Teramoto, H., Uetani, K., Igawa, K., & Shimizu, E. (2001). Stretch induces a growth factor in alveolar cells via protein kinase. *Respiration Physiology*, *127*(2-3), 105-111.

Yan, Y., Li, X., Wang, A., & Zhang, G. (2000). Molecular cloning and identification of full-length cDNA encoding high affinity Fc receptor for bovine IgG. *Veterinary Immunology and Immunopathology*, *75*, 151-159.

Yuan, Z., Li, J., Zhang, L., Zhang, L., Chen, C., Chen, X., Xu, S. (2011). Novel SNPs polymorphism of bovine CACNA2D1 gene and their association with somatic cell score. *African Journal of Biotechnology*, *10*(10), 1789-1793.

Zhao, X., & Lacasse, P. (2008). Mammary tissue damage during bovine mastitis: Causes and control. *Journal of Animal Science, 86*(13), 57-65.

Appendix A

Reagents

0.1% Ammonium Persulfate

1. Weigh out 1g Ammonium Persulfate.
2. Place powder inside a microfuge tube.
3. Add 1ml dH_20.

0.5M EDTA pH 8.0

1. Weigh out 18.6 g of EDTA.
2. Add the EDTA to 80 ml of dH_2O.
3. Slowly add 2.2 g of sodium hydroxide pellets.
4. Mix vigorously with a magnetic stirrer.
5. Add dH_2O to fill up to 100 ml.

0.5M EDTA must be stored at room temperature only.

1X TBE

1. Obtain 100 ml of 10X TBE buffer in a 1 liter bottle.
2. Fill bottle with dH_2O up to 1 liter.
3. Cap and shake vigorously.

7.4 pH PBS Solution

1. Weigh out 8.0 g of NaCl.
2. Weigh out 0.2g of KCl.
3. Weigh out 1.44g of Na2HPO4.
4. Weigh out 0.24g of KH2PO4.
5. Pour the contents into a 1000 ml graduated cylinder.

6. Add 800 ml dH_2O.
7. Use a magnetic stir bar and automatic stirrer to mix contents.
8. Once contents are dissolved, fill up to 1000 ml.
9. Autoclave the solution.

10X TBE Buffer

1. Obtain a 1000 ml graduated cylinder and place on a magnetic stirrer.
2. Weigh out 108g Tris base and add to the graduated cylinder.
3. Weigh out 55g Boric Acid and add to the graduated cylinder.
4. Add 40ml of 0.5M EDTA, pH 8.
5. Fill up to 1 liter with dH_2O.
6. Continue to stir until contents are fully dissolved

Transfer contents into a 1 liter bottle and store at room temperature.

Casting a 14% stacking gel

Add reagents in a conical tube in this order:

1. 7 ml of 30% acrylamide stock solution
2. 750 µl of 10X TBE
3. 750 µl of 5% glycerol
4. 6ml and 350µl of dH2O,
5. 15 µl of TEMED and
6. 150 µl of APS
7. Gently invert contents to mix.

APS is added last as this will speed up polymerization.

Casting an 18% running gel

1. Add reagents in a conical tube in this order:
2. 15 ml of 30% acrylamide/bis stock solution (Bio-Rad)
3. 1250 µl of 10X TBE
4. 1250 µl of 5% glycerol
5. 7ml of dH2O
6. 25 µl of TEMED (Fisher Bioreagents, Fisher Scientific, Fair Lawn, N.J.)
7. 220 µl of 10% APS
8. Gently invert contents to mix.

APS is added last as this will speed up polymerization.

75% Ethanol

1. Pour 75 ml of ethanol into a beaker or flask.
2. Fill flask with dH_2O up to 100 ml.
3. Swirl around to mix.

DEPC Treated Water

1. Add 1 ml of DEPC to a 1 liter glass bottle
2. Fill up with dH_2O to 1 liter.
3. Cap bottle and shake vigorously mix.
4. Autoclave contents.

DEPC treated water can be stored at room temperature or refrigerated.

Appendix B

Protocols

Assembling the SSCP Gel Sandwich

1. Clean and dry all plates and spacers before assembly.
2. On a clean surface, lay the large rectangular plate down first
3. Place the left and right spacers along the edges of the rectangular plate.
4. Place a shorter glass plate on top of the spacers so that it is flush with the bottom edge of the longer plate.
5. Loosen the single screw of each sandwich clamp by turning it counterclockwise.
6. Place each clamp by the appropriate side of the gel sandwich with the locating arrows facing up.
7. Guide the clamps onto the sandwich so that the long and short plates fit the appropriate notches in the clamp. Tighten the screws enough to hold the plates in place.
8. Place the sandwich assembly in the alignment slot of the casting stand with the short glass plate forward.
9. Align the plates and spacers by simultaneously pushing inward on both clamps at the locating arrows and at the same time, pushing down on the spacers with your thumbs.
10. Tighten both clamps just enough to hold the sandwich in place.
11. Once a good alignment and seal are obtained, tighten the clamp screws until it is finger-tight.

Casting 1% Agarose Gel

1. Weigh out 0.5 g of agarose powder and pour into a 200 ml beaker.
2. Pour 50 ml of 1X TBE buffer into the same beaker with the agarose powder.

3. Heat contents in a microwave for 2 minutes, stopping every 30 seconds and swirl around to prevent mixture from boiling over.
4. Allow contents to cool briefly before pouring the gel mix into the gel casting cube.
5. While the gel contents cool, use tape to seal the edges of the casting cube.
6. Pour gel mix into casting cube and add a comb for well alignment.
7. Allow mixture to completely solidify.
8. Once gel is solid, remove tape carefully. Removing the tape too abruptly can damage the gel.
9. Keeping the gel in its casting cube, carefully place the casting cube inside the electrophoresis tank.
10. Pour 1X TBE (running buffer) inside the tank to completely submerge the gel.
11. Remove the comb.

Pipette samples into each desired well and slide the top of the gel tank in place.

Checking the Purity and Concentration of DNA and RNA using the Nanodrop Spectrophotometer

1. Clean on and around the nanodrop by using RNase free water and Kim wipes.
2. Open the program ND-1000.
3. From pop-up menu select “nucleic acids”.
4. Place 1 µl of RNase free water on the eye of the spectrophotometer and close the lid.
5. Click “initialize” and wipe free when program is done.
6. Place 1 µl of RNase free water on the eye of the spectrophotometer again and click blank.
7. Wipe free once program has created the “blank”.
8. Type in the desired sample name and select DNA or RNA pending the sample type.

9. Place 1 µl of the sample on the spectrophotometer and close the lid.
10. Click “measure”
11. Record and save data.

Ethidium Bromide Staining of Agarose gel

1. After electrophoresis, obtain a plastic container to house the gel.
2. Remove the gel from the gel tank carefully and place it in the plastic container.
3. Pour Ethidium Bromide into the plastic container and allow gel to soak for 8-10 minutes.
4. After allotted time, decant EtBr back to its original container and rinse gel with water 2x.
5. Add a small amount of water to the plastic container to allow the gel to move easily until the gel can be viewed.

Store gel at room temperature until further analysis.

Obtaining Cell Lysates from Supernatant of Treated PMN

1. Spin down treated cells in microfuge tube to pellet and decant supernatant using a pipette.
2. Add 0.5 ml of ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to each microfuge tube and incubate on ice for 5 minutes.
3. Sonicate cell lysates briefly.
4. Centrifuge for 10 minutes at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

Washing Procedure for PathoScan Sandwich ELISA

1. Discarded plate contents and each well was washed four times with
2. Use 200 µl of 1X wash buffer for each well.
3. After each wash, strike the plate on fresh towels hard enough to remove the residual solution in each well but the wells should never be allowed to completely dry at any time.

4. Clean the underside of all wells using a lint-free tissue.

The washing procedure should be repeated a total of 4 times consecutively.

Appendix C

Table 3.6.1

Qiagen Multiplex PCR Components

Component	Volume/reaction	Final concentration
2X QIAGEN Multiplex PCR Master Mix	25 µl	1x
10x Primer mix	5 µl	0.2 µM
RNase-free water	Variable	–
Template DNA	Variable	≤1 µg DNA/50 µl
Total Volume	50 µl	-

Table 3.6.2

PCR Thermal Cycling conditions for Gap-dh, FcγRI, FcγR2B and FcγR2C

PCR Step	Temperature	Time	Cycles
Initial activation step	95°C	15 minutes	1
Denaturation	94°C	30 seconds	
Annealing	60°C	90 seconds	40
Extension	72°C	90 seconds	
Final extension	72°C	10 minutes	1

Table 3.6.3

PCR Thermal Cycling Conditions for FcγR2A gene

PCR Step	Temperature	Time	Cycles
Initial activation step	95°C	15 minutes	1
Denaturation	94°C	30 seconds	
Annealing	57°C	90 seconds	40
Extension	72°C	90 seconds	
Final extension	72°C	10 minutes	1

Appendix D

Table 3.6.4

Gene names, primer sequences, expected length (bp) of PCR products used to amplify bovine DNA and reference source of each primer.

Gene Name	Primer Sequence	Length of PCR product (bp)	Reference
Fc gamma receptor I	GGTCTTCATTGGTGTTTTCTCC GAGCTGCCCTAGATGAGGTG	190	Klungland et al., 1997
Fc gamma receptor II a	CTGGTCAAGGTCACATTCTTC CAATTTTGCTGCTATGGGC	439	Flesch, Bauer, and Neppert, 1998
Fc gamma receptor II b	GTGTGCGTTCTCACTTGCTGC GGTGGCGGCCATATTCTGGA	1000	Jiang et al., 2000
Fc gamma receptor II c	CCAGTGAACATCATGTCCA GCCGAGGTTTGCTACTATGG	183	Klungland et al., 1997
Glyceraldehyde-3-phosphate dehydrogenase (house-keeping gene)	AGGCAGAGAACGGGAAGCTC ATCGGCAGAAGGTGCAGAGA	195	Yamamoto, Teramoto, Uetani, Igawa, Shimizu, 2001

Appendix E

Table 3.16.1

Bio-Rad Reaction Set up protocol using SsoAdvanced SYBR Green Supermix.

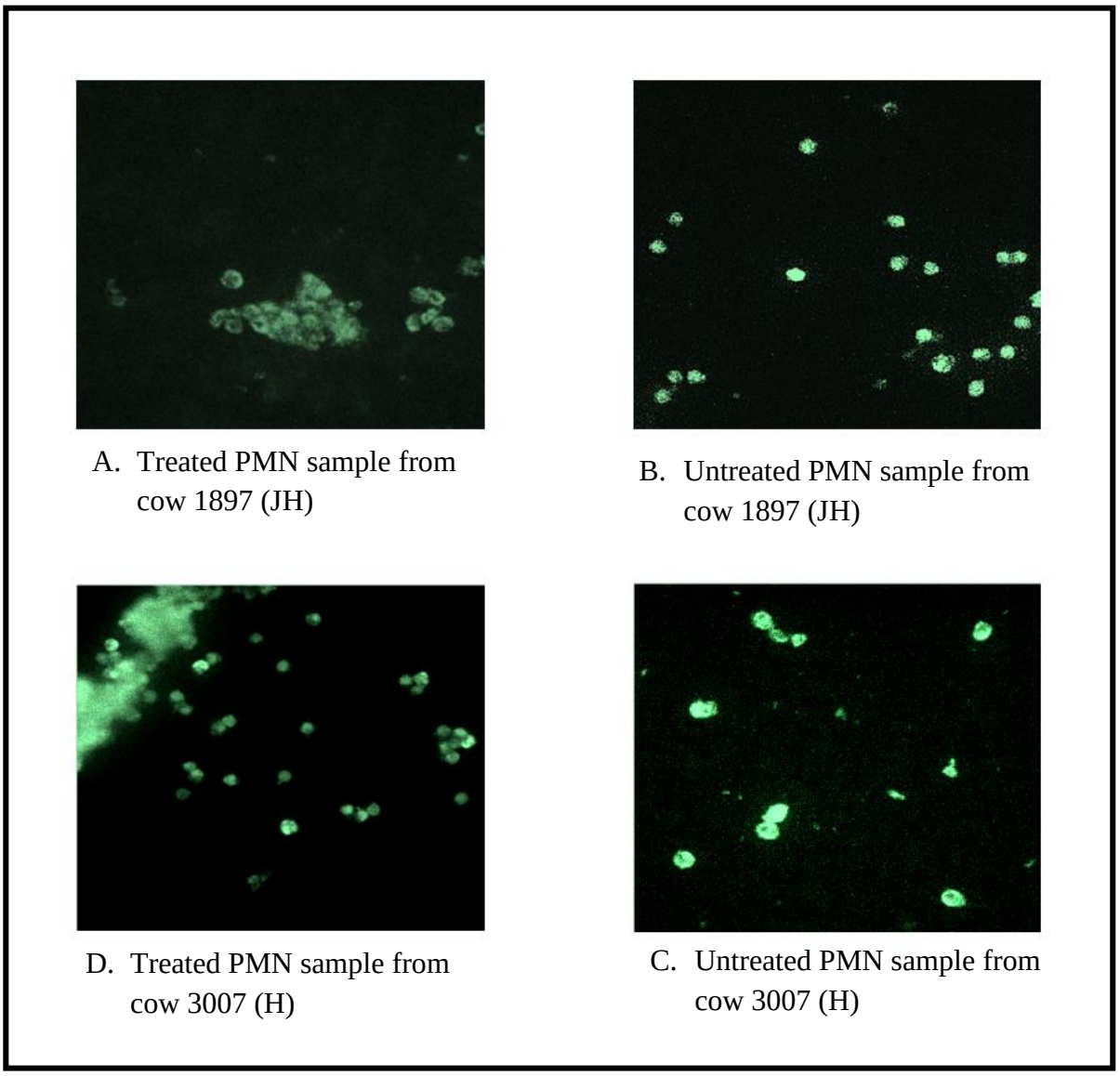
Component	Volume per Reaction	Final Concentraion
SsoAdvanced SYBR Green supermix	10 µl	1x
Forward Primer	1.5 µl	250-500 nM
Reverse Primer	1.5 µl	250-500 nM
RNase -free water	Variable	-
cDNA template	Variable	100 ng
Total Volume	20 µl	-

Table 3.16.2

Thermal Cycling conditions for Real-Time PCR using the Bio-Rad Real-time CFX Connect.

cDNA Standard Cycling Conditions			
Cycling step	**Temperature**	**Time**	**Cycles**
Enzyme Activation/Initial DNA denaturation	95°C	30 sec	1
Denaturation	95°C	5 sec	
Annealing	60°C	30 sec	40
Extension	65°C	5 sec	
Melt Curve	95°C	5 sec	1

Appendix F



A. Treated PMN sample from cow 1897 (JH)

B. Untreated PMN sample from cow 1897 (JH)

D. Treated PMN sample from cow 3007 (H)

C. Untreated PMN sample from cow 3007 (H)

Figure 4.9.2. Confocal microscopy images of a treated and untreated cow neutrophil samples.

Appendix G

Table 4.10.1

Phosphorylated SYK Concentrations per Animal in Neutrophils

Cow Number	Control (PBS)	Treated (IgG)
3007	0.146	0.209
3012	0.241	0.285
3017	0.199	0.256
1862	0.273	0.325
1854	0.249	0.386
1897	0.267	0.301

Appendix H

Table 4.11.1

Individual RNA Cq values used to calculate fold change

Cow Number	FCGR1	FCGR2A	FCGR2B	FCGR2C
1854	18.96	5.39	2.78	4.77
1862	3.56	4.84	0.34	7.73
1897	2.17	2.54	1.94	2.11
3007	7.21	7.11	1.53	5.31
3012	4.18	4.19	2.62	0.88
4002	11.27	2.46	0.69	2.27