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Investigating The Role Of Toll Like Receptor-4 (Tlr-4) In Goats

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INVESTIGATING THE ROLE OF TOLL-LIKE RECEPTOR-4 (TLR-4) IN GOATS

by

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BIOGRAPHICAL SKETCH

La'Toya D. Lane was born on September 6, 1984 in Philadelphia, Pennsylvania. La'Toya earned her Bachelor's of Science degree in Laboratory Animal Science in 2007. She received a scholarship through North Carolina Creating Opportunities through Education program, and was an inducted member into the Golden Key Honor society. She has presented her work at symposia which include: Ronald E. McNair Symposium, Biennial Research Symposium of the Association of Research Directors, Inc, Life & Sciences Research Symposium, N.C. Opt-Ed Alliance Day, and the Southern Section of the American Society of Animal Science conferences. She is a candidate for the Master of Science in Animal Sciences.

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LIST OF SYMBOLS AND NOMENCLATURE

ACD	Acid Citrate Dextrose
bp	Base pairs
C	Celsius
CD	Cluster differentiation
CO_2	Carbon dioxide
cDNA	Complementary DNA
DEPC	Diethyl Pyro carbonate
ELISA	Enzyme-linked immunosorbent assay
IL-8	Interleukin-8
LPS	Lipopolysaccharide
LBP	Lipopolysaccharide binding protein
mAb	Monoclonal antibody
mins	Minutes
mRNA	Messenger RNA
NK cells	Natural Killer cells
NF-κB	Nuclear factor kappa B
PMN	Polymorphonuclear neutrophils
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	ribonucleic acid
sCD-14	soluble CD-14
SCC	Somatic cell count
SSCP	Single Stranded Conformational Polymorphism
TNF-α	Tumor necrosis factor-alpha
TLR	Toll-like receptor
TLR2	Toll-like receptor 2

TLR-4	Toll-like receptor 4
TCR	T-cell receptor
TIR	Toll/Interleukin-1 receptor
Tm	Melting Temperature

ABSTRACT

Lane, La'Toya D. INVESTIGATING THE ROLE OF TOLL LIKE RECEPTOR-4 (TLR-4) IN GOATS. (**Major Advisor: Mulumebet Worku)**, North Carolina Agricultural and Technical State University.

An animal's ability to fight off infections with *Escherichia coli* can be impacted by polymorphisms in the TLR-4 gene. In goats, the effects of polymorphisms of the TLR-4 gene are not known and need to be researched to improve goat herd production. The objectives of this study were to evaluate gene polymorphism and transcription of TLR-4 and to assess the effects of LPS exposure on gene expression and signaling in neutrophils from Spanish and Boer goats. To study genetic polymorphisms, gene segments using primers specific for TLR-4 were amplified. PCR products were then examined on a 1% agarose gel to determine amplification. Single Stranded Conformational Polymorphism (SSCP) Gels of PCR products were used to determine any polymorphisms between samples. To evaluate TLR-4 and CD-14 expression and the effects of LPS in caprine neutrophils, blood neutrophils were isolated from Spanish and Boer goats. Neutrophils were treated with and without 0111 B4 serotype E. coli LPS. Cell surface expression of TLR-4 and CD-14 were determined by Flow Cytometry. Transcription of TLR-4, CD-14, and TNF-α after stimulation with LPS was determined by Reverse transcriptase PCR. Secretion of the cytokine IL-8 was determined by ELISA. A 494 base pair fragment of the TLR-4 gene was amplified from genomic DNA. Gene alleles run on SSCP Gels were found to be similar in all six goats used. Both TLR-4 and TNFα were transcribed in goat blood neutrophils. Treatment with LPS did not have an effect on transcription of TLR-4,

TNFα, CD-14, or GAPDH mRNA. Treatment with LPS resulted in increased secretion of IL-8. These studies provide evidence for polymorphisms in the TLR-4 gene and transcription and translation of TLR-4 associated genes in neutrophils from Boer and Spanish goats. Further studies are warranted.

CHAPTER 1

INTRODUCTION

The discovery of new strategies and drugs to help maintain the life of livestock is growing due to the increase in productivity. One of the techniques being investigated is selective breeding. By selecting specific traits of interest, an animal can be bred to withstand specific environmental factors. The exploration of using bio-techniques to study characteristics and predisposition to diseases in livestock has been growing. Thus bio-techniques can be used to identify genetic variation among different breeds as well as within breeds, to express desired characteristics in livestock.

Toll-like receptor 4 (TLR-4) expressed on the surface of many cells of the immune system recognizes lipopolysaccharide, one of the well characterized pathogen associated molecular patterns (PAMPS). Toll-Like Receptor-ligand binding results in cell signal transduction which leads to the production of pro-inflammatory cytokines such as IL-8 and TNF-α. This receptor plays an important role in the activation and the induction of innate immunity when exposed to certain infections (Medzhitov, 2001). Mutation in the gene or lack of TLR-4 can result in altered immune responses to pathogenic organisms that produce these PAMPs.

There is current evidence that associates TLR-4 polymorphism with both bacterial and parasitic infections. It has been implicated in signal transduction events induced by lipopolysaccharide (LPS) found in gram-negative bacteria. Some gram negative bacteria

that have LPS are Escherichia coli. Genetic variation of the TLR-4 has been reported in sheep, goats and cattle (Zhou et. al., 2008). However, there have been no reports of TLR-4 gene variation in Spanish and Boer goats. The specific objective of the present research is to establish flow cytometric methods for the evaluation of TLR-4 and CD-14. The second objective was to determine the effect of stimulation by LPS using ELISAs' specific for IL-8 cytokine and using reverse transcriptase PCR of mRNA from treated goat neutrophils to test transcription of TLR-4, CD-14, and IL-8.

CHAPTER 2

REVIEW OF LITERATURE

There has been a dramatic change in goat production in the United States. In the 1980s, goats were used primarily for the production of mohair. In 1987, it was estimated that 2.2 million angora goats were bred for their mohair. The breeding of Angora goats in the United States has changed due to the rising demand for goat meat, loss of government support for mohair production and the introduction of the Boer goat into the United States (Cameron et al., 2001). In 2008, the number of Angora goats in the United States decreased to 210,000 (NASS, 2010). The number of meat goats produced in the United States, however, increased dramatically from 415,000, in 1987 to 2.5 million in 2008. The amount of dairy goats also increased from the year 1987 to 2008 from 129,000 to 305,000 (NASS, 2010). In a census taken in 2010, Angora goats populations were estimated to be 150,000, meat goats populations were projected to be 2.54 million, and milk goats were estimated to be 355,000 (NASS, 2010).

Before the introduction of meat goats, meat was primarily taken from goats used in dairy production (Cameron, et al., 2001). The meat quality of the dairy goats were poor and was characterized by varying weights and traits (Cameron, et al., 2001). To produce a better goat meat product breeds that have been used for meat production in countries which include Africa and Spain were imported into the United States. The two primary breeds used in the United states in goat meat and dairy production are Spanish

and Boer goats (Cameron, et al., 2001). The Spanish goat originated in Spain. The goat breed then traveled from Spain to Mexico and lastly to the United States (Sahlu et al., 2009). The Spanish goat is a hardy goat with the ability to bred out of season and in harsh climates (Sahlu, et al., 2009). Spanish goats require limited management for care as a result they are growing in demand for their meat, milk and cashmere (Browning Richard, 2009). There are general groupings of Spanish goats which include those with pure blood lines as well as those that bred with other breeds of goats (Sahlu, et al., 2009). Some crossbreds include those with Angora and Boer. Until recently Spanish goats were used for clearing pastures for undesirable species of plants (Sahlu, et al., 2009).

The Boer goat breed originated in South Africa (Glimp, 1995). The Boer like the Spanish goat is bred for its meat (Sahlu, et al., 2009). The appearance of the standard Boer goat has good conformation, short white hair and red markings on the head and neck (Sahlu, et al., 2009). Boer goats are also noted to have high growth and fertility rates (Sahlu, et al., 2009). Boer goat is the only known goat breed routinely involved in performance and progeny tests for meat production (Sahlu, et al., 2009). Countries which include New Zealand, United States and Australian have imported the Boer goat for improving their own meat goat industries (Sahlu, et al., 2009).

Goats are susceptible to a number of bacterial and parasitic infections. The natural characteristics of goats allows for easy exposure to pathogens. Common practices of goat housing include housing them in a barn and letting them out to pasture during the day. Since goats live in large numbered herds within relatively small space, the contamination of living space due to farm dust and fecal matter in their litter is hard to control. Farm

dust and feces can contain many pathogenic or non pathogenic microbes that can be constantly spread by inhalation, consumption, and direct contact (W.C. Purdey et. al.,2007). This transfer of pathogens amongst the goat herd and to consumer raises many concerns (Burke et. al., 2009). Consumption of goat meat and milk is growing throughout the world and the control of infections is imperative in reducing transmissions of disease to the herd as well as to the consumer through consumption (Burke et al., 2007).

Mastitis, which is a major illness in dairy ruminants, is costly to many farmers due to the loss of livestock (Rainard & Riollet, 2005). Mastitis in goat herds is frequently associated with *Staphylococci* bacteria. *Staphylococci aureus,* a species of Staphylococcaceae is contagious and can contribute to chronic, subclinical and clinical, and gangrenous mastitis (Pisoni et al., 2010). *Staphylococcus* has also been studied in its effects on leukocytes in the immune responses of goats and cows. In a study conducted by Contreras and his colleagues, they found that *Staphylococcus* caused premature death of leukocytes due to its leukotoxin capabilities in both cows and goats. The effect of *Staphylococcus* however was more potent in cows compared to smaller ruminants (Contreras et al., 2007). Mastitis infections are usually detected by looking at the somatic cell count in the milk as well as taking samples to detect bacteria in the teats. Infections with bacteria that cause mastitis can lead to low quality milk production and loss of the milk glands of the goat (Pisoni, et al., 2010). There is also a concern in the transmission of the bacteria through infected goat milk since it can be distributed raw (White & Hinckley, 1999).

There are several bacteria that can interfere with the health of goats and hold great

economical problems for farmers. *Escherichia* coli (*E*. coli), a gram negative bacterium is prominent in mastitis and gastrointestinal infections of goats. Even though *E.* coli is found naturally occurring in the gastrointestinal floral of animals, there are several strains of *E. coli* which can cause harm to both animals and human (Beutin et. al., 1993). *Escherichia coli* has an integral component of the outer cell membrane, known as LPS also called lipopolysaccharide (LPS), which is responsible for elicitation of leukocytes and many other cells of the body that express receptors for recognition. There are approximately 2 million LPS molecules per *E. coli* cell (Gorbet & Sefton, 2005). The LPS from these bacteria are very heat stable and can shed during cell death and cell growth and division (Gorbet & Sefton, 2005). Some dangerous strains are *Shiga toxin* and *Verocytotoxin* producing *E. coli* (Cortés et al., 2005). The strain termed *Shiga toxin* producing *E. coli*, or STEC, has been shown to cause dysentery and diarrhea in weaning calves, edema disease in pigs, and hemorrhagic colitis and hemolytic uremic syndrome in humans with compromised immune systems (Cortés, et al., 2005). In a study conducted to asses LPS exposure in goats, it was found that LPS, lipopolysaccharide, from *E. coli* caused rectal temperature to rise and an increase in white blood cells to the site of infection (Purdy et. al., 2007). *Shiga* serotypes that cause major concerns in industrialized nations include, O157:H7, O26, 0111, 0103, and 0145 (Cortés, et al., 2005). These serotypes cause concern because they are the cause of enterohaemorrhagic conditions and are proven to be zoonotic in cows, sheep and goats. *Escherichia* coli can enter the body through several routes. Common routes for ruminants include oral routes through grazing practices and through the inhalation of bacteria in dust particles.

Infections with *E.* coli bacteria cause devastating effects which effect production of milk due to mastitis and has an effect on the body score of the goat. The invasion of the bacteria into the gastrointestinal tract can cause severe diarrhea leading to dehydration of the animal which can cause death. The main transfer of these bacteria from animals to humans is from lactating cows in their milk and contaminated produce. To obtain ways of combating the different strains of *E.* coli, many studies have been conducted to understand the structure and functions of the bacteria.

The fundamental component of *E.* coli outer layer is its' lipopolysaccharide coating, which is constructed of a core oligosaccharide which is a repeat of polysaccharides termed the O-specific chain, a 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) component, and a lipid A component which is responsible for the elicitation of proinflammatory response (Figure 1; Raetz & Whitfield, 2002) and (Dauphinee & Karsan, 2005) .

The lipopolysaccharide coating is a protective layer that aids in protection against degradation. The outer layer composed of polysaccharides can be variable while the lipid A layers generally is conserved. This conserved region is recognized by receptors of the innate immune system (Raetz & Whitfield, 2002). The conserved region along with the other components that comprise the outer layer of *E.* coli gram negative bacteria act together as an endotoxin that elicits an immune response within the host. An endotoxin is a toxin that is recognized by the immune system of the host it affects as being foreign. Endotosins are noted to be a part of many gram negative bacteria. The size of the LPS can be between 19-20kDa, in monomer structures and over 1000 kDa in vesicles.

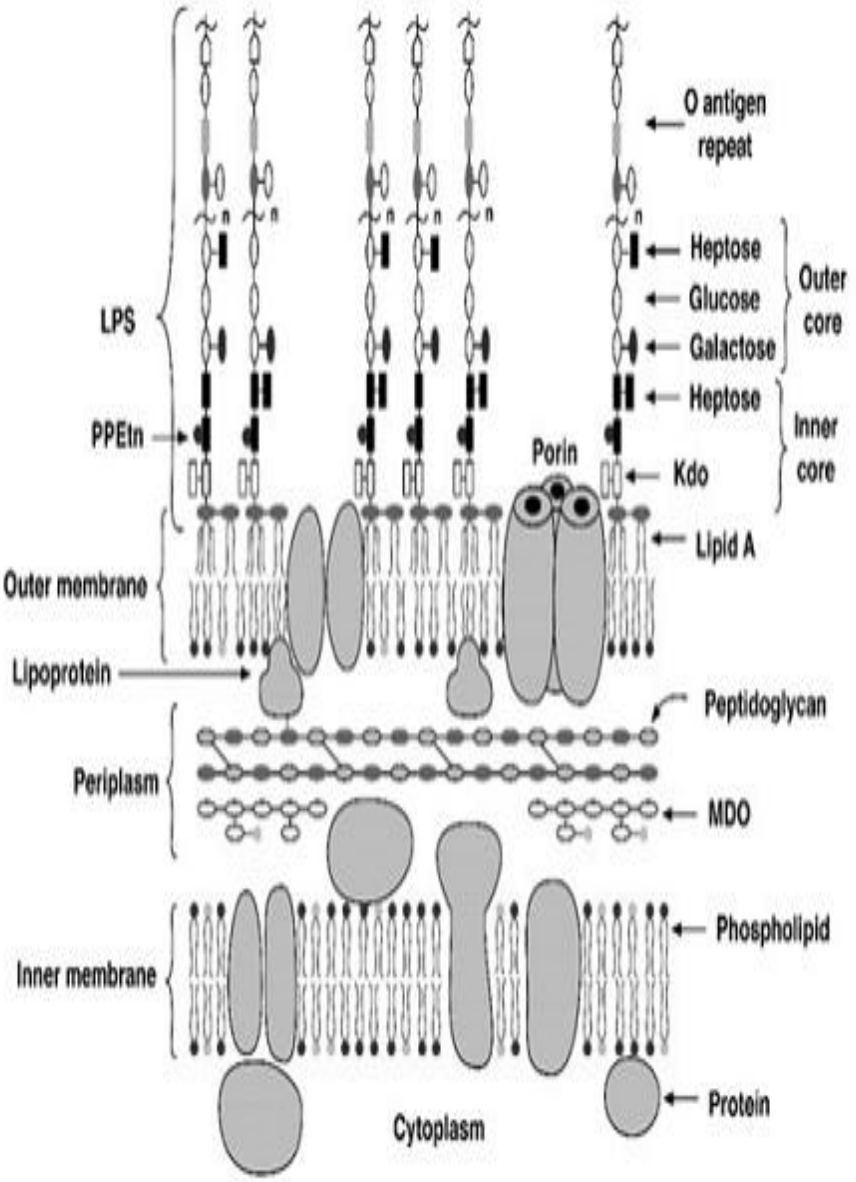


Figure 1. Schematic representation of the cell wall of gram negative bacteria. Molecular model of the inner and outer membranes of *E. coli* K-12. Geometric form: ovals and rectangles represent sugar residues, and circles represent polar head groups of different lipids. Abbreviation: PPEtn (ethanolamine pyrophosphate); LPS (lipopolysaccharide); Kdo (2-keto-3-deoxyoctonic acid) [Source: Raetz and Whitfield.Annual Review Biochemistry. 2002. 71:635-700]

Upon entering the body, LPS cause a vast range of symptoms which may include shivering, fever, or respiratory distress.The LPS of gram negative bacteria stimulates both a humoral and cell mediated response. The humoral components include complement and several cytokines such as IL-1β and TNFα. Cells that are involved in recognition of LPS include macrophages, monocytes, granulocytes, endothelial and epithelial cells (Gorbet & Sefton, 2005).

Coccidiosis is another condition that effect goat herds. It is caused by *Coccidia* a spore forming single-celled protozoan that lives in the small intestines. There are several species of *coccidia* that effect goats which include *EImeria ninakohlyakimovae, E. caprina, E. christenseni,* and *Eimeria arlongi* (Tafti & Mansourian, 2008). While grazing, goats ingest sporalated oocyts. These sporocysts then invade cells in the lining of the intestines. Within the cells the sporocyte becomes a shizont or meront, which grows many times its original size. Some of the merozoites are asexual and reinvade more host cells while others are shed in feces. These become spores and are ready to re-infect in 2 to 3 days. The entire life cycle is about 14 days. Symptoms of coccidiosis range from loss of appetite, diarrhea that ranges from short lived diarrhea to severe cases involving great amounts of dark and bloody diarrhea and death (Tafti & Mansourian, 2008).

Another big problem in goat herds is *Haemonchus Contortus*. *Haemonchus Contortus,* a parasitic nematode is ingested through grazing (Pralomkarn et al., 1997). Larvae eggs are passed through feces of animal. After two weeks, under optimal temperatures (75°F), *Haemonchus Contortus* reaches its infective stage. If environmental conditions are not suitable for development, they will stay in a dormant stage.

Haemonchus contortus feed on blood by attaching to the intestinal tract wall. *Haemonchus Contortus* can cause anemia low packed cell volume, diarrhea, dehydration, and internal fluid accumulation. Goats that are infected with *Haemonchus Contortus* have lower growth rates and reduced reproduction. In severe cases, illness and death may occur. *Haemonchus Contortus* and *Coccidia* have lead to greatly reduced profits for goat farmers (Correa, 1998). The ability to fight off parasitic infections differs amongst the immune system of goats and sheep (Newton, 1995).

There is a growing concern for learning why certain breeds are more susceptible to parasites while others are less likely to have severe problems with infection (Newton, 1995). One way of diagnosing *Haemonchus Contortus* infections in goats is by looking at the packed cell volume which can diagnose anemia and FAMACHA test (Burke, et al., 2007). FAMACHA was developed by Francois "Fafa" Malan and is a test that measures parasitic levels of *Haemonchus Contortus* by looking at the shade of redness in the eyelids of goats and sheep (Burke, et al., 2007).

The FAMACHA test consists of five stages which the eyelids can go from red to a very pale color, with the fifth stage being the most severe case of anemia. This test allows for treating only those animals suffering from high parasitic infection which will allow the farmer to be more economically efficient (Burke, et al., 2007). There is a growing problem with susceptibility of diseases in caprine which has been linked to the overuse of anthelminthics and antibiotics (Hounzangbe-Adote et. al., 2005). So the use of techniques that can alleviate the overuse of antibiotics has gained interest.The host defends itself from pathogenic organisms by its immune system. The immune system is

composed of cells, organs, and tissue that work together to protect the host (Cooper & Hausman, 2007). The steps that the host immune system takes in attacking foreign substances and organisms that cause disease, is known as the immune response. The immune system is composed of the innate immunity (natural immunity) and acquired immunity also known as the adaptive immune response. Innate and acquired immunity recognize invaders of the host by recognizing non-self organisms. Both humans and other animals have an innate immune response. The innate response provides multi-cellular organisms with immediate available defenses towards invading pathogens without requiring prior exposure (Cooper & Hausman, 2007).

The adaptive immune response is one that is specific and developed after the first exposure to foreign bodies. Adaptive immunity has a lag time between exposure and maximal response. The adaptive immune response is similar to the innate response in which both are composed of a cell -mediated components as well as humoral components. After exposure to non-self molecules, an adaptive response is developed that allows for an immunological memory to develop against the pathogen (Cooper & Hausman, 2007). Developing a memory to the antigen allows for fast responses to that specific antigen.

Hematopoietic stem cells (HSC) give rise to cells of innate and adaptive immune responses (Dzierzak & Speck, 2008). The cells of the immune response must be replaced due to the cells limited lifespan. Many cells die after exposure to pathogenic organisms. These cells are constantly replaced due to Hematopoietic stem cell production. The Hematopoietic stem cells produce cells that are capable of differentiating into different

cell types which are termed progenitor cells. The progenitor cells are multi-potent progenitor cells that give rise to myeloid progenitor cells (Nagai et al., 2006). The myeloid progenitor cells can produce either megakaryocytes and erythrocytes or granulocytes and macrophages. There are also lymphoid progenitor cells that give rise to T, B, NK cells of the adaptive immune response (Nagai, et al., 2006). The innate response is nonspecific, and responses by this system allows for recognition of pathogen associated molecular patterns that are present in microorganisms. The innate response initiates the activation of effecter mechanisms that will destroy microorganisms within an hour or more (Cooper & Hausman, 2007).

When pathogenic organisms invade the immune system, it causes an inflammatory reaction which leads to the influx of monocytes, neutrophils, and endothelial cells to the site of infection. A bacterial invasion of the host leads to the upregulation of adhesion molecules on vascular endothelial cells. As a result, this reaction contributes to the recruitment of leukocytes to the site of infection. Leukocytes migrate to the site of infection is by β_2 integrins. The activation of leukocytes stimulates the production of reactive oxygen intermediates which aid in clearance of bacteria from tissue. Also produce during a host response to infection are proinflammatory cytokines. Proinflammatory cytokines such as tumor necrosis factor and interleukin-1, are synthesized to intensify the response to bacterial infections (Cooper & Hausman, 2007). Cells of the innate system phagocytose foreign organisms and present them to cells of the adaptive immune response. The innate system also induces secretion of effecter cytokines, which include IL-12, TNFα, and IL-8 (Fitzgerald et al., 2007). Cytokines are

proteins that are known for their ability to carry signals between cells of the immune system. These effecter cytokines aid in recruitment of innate cells which include microphages and neutrophils to the site of infection (Fitzgerald, et al., 2007). The effecter cytokines also play a role in adaptive immunity by controlling CD4-T-cell differentiation, up-regulating co-stimulatory molecules on antigen-presenting cells that are necessary for T-cell activation, and signal B-cell proliferation (Cooper & Hausman, 2007).

During bacterial infections that cause mastitis, white blood cells travel into the mammary gland within four to six hours in dairy ruminants (McClenahan et al., 2006) .Neutrophils are Polymorphonuclear (PMN) cells, that are one of the primary granulocytes that arrive shortly after exposure to *Escherichia Coli* and *Staphylococci* bacteria in mastitis infections (McClenahan, et al., 2006).

Neutrophils play a key role in inflammation elicited in response to infections elicited by LPSs produced by *E.coli*. The ability of neutrophils to phagocytose bacteria such as *E.coli* is necessary in clearing infection. In cases of mastitis in goats, neutrophils are found in high concentration (Paape, et. al., 2004).

Many factors can inhibit the activity and movement of neutrophils. It has been shown that a neutrophils' ability to phagocytose Staphylococcus aureus is different in serum compared to whole milk due to fat globules. There was also variation among goats in the ability to fight off *Staphylococcus aureus* (Paape, et. al., 2004). Cytokines also contribute to the regulation and activation of neutrophils. Two main cytokines that are produced when exposed to bacteria having Lipopolysaccaride, and also contribute to the

activation and migration of neutrophils and other cells response are IL-8 and TNF-α (Hodgson, 2006).

Cytokines have also been noted in responses to LPS(Barber et. al., 1999). IL-8 has been detected in mammary secretions from glands after exposure to *Escherichia coli* in ruminants (McClenahan, et al., 2006). IL-8 is a chemokine that is responsible for recruiting and activating neutrophils (Barber et. al., 1999). Other immune properties of IL-8 are degranulation of neutrophils, and the aid in phagocytosis of particles by cells. Neutrophils, lymphocytes, monocytes/macrophages, and epithelial cells secrete IL-8. In caprine epithelial cells, IL-8 production was documented in infections with bacteria that cause mastitis (Barber et. al., 1999). Inhibition of IL-8 results in the decrease of neutrophils to an area of infection (Broaddus et al., 1994).

Tumor Necrosis Factor alpha (TNFα) is a known cytokine following LPS stimulation. Tumor Necrosis Factor alpha is also responsible for chemotaxis of neutrophils (Hodgson, 2006). Exposure of TNF-α to cells has also been characterized in an intracellular signal that causes the production of IL-8 and many other cytokines (Hodgson, 2006). In water buffalo suffering with hemorrhagic septicemia, there was an increase in concentrations of TNF-α after intravenous injection of LPS which indicated the importance of TNF-α in the innate immune response (Hodgson, 2006).

The Toll-like receptor family plays an essential role in the initiation of cellular innate immune responses (Akira et. al., 2006). These receptors are transmembrane molecules that link the extracellular compartment where contact and recognition of microbial pathogens occur to the intracellular compartment. Activation of Toll-like

receptors contribute to a signaling cascades leading to cellular responses to fight infection (Cooper & Hausman, 2007). Toll receptors consist of an ectodomain of leucine-rich repeats (LRRs) and two cysteine-rich regions. The intracellular domain consists of a Toll/interleukin-1 receptor (TIR) domain, based on homology of the region with a similar intracellular domain of the IL-1 receptor (IL-1R) (Cooper & Hausman, 2007). The intracellular domain provides the initial steps in the process of the signal cascade that takes place within the cell. There Toll/interleukin receptor interacts with myeloid differentiation primary response gene 88, (MyD88) (Schumann et.al., 2005). MyD88 is an adaptor protein that exhibits a TIR domain, which activates a signaling cascade that leads to the induction of proinflammatory cytokines (Schumann et. al. 2005). The first member of the Toll Receptor family deemed Toll was identified in *Drosophila melanogaster* embryos. Studies with Drosophila have shown that a mutation in Toll have dramatically reduced the ability to activate the expression of an antifungal peptide and also reduces the ability of Drosophila to survive fungal infection (Cooper & Hausman, 2007).Other mutations in Drosophila Toll-related receptor caused the larvae of Drosophila to be susceptible to bacterial infections (Cooper & Hausman, 2007). This finding help to conclude that Toll receptors play a key role in the host defense system. Since these findings, seven more Toll-related receptors have been found.

After the discovery of Toll in Drosophila, Medzhitov reported the cloning of a human Toll known as Toll like receptor 2 (Cooper & Hausman, 2007). Medzhitov demonstrated that the activation of Toll Like Receptor 2 caused human cells to produce NF-κB and the expression of genes controlled by NF-κB (Brikos & O'Neill, 2008).

Thirteen mammalian Toll-like receptors (TLRs) have been discovered since Medzhitov's discovery (Brikos & O'Neill, 2008). These receptors have been conserved through evolution in both plants and animals. There are two main groups into which TLRs can be placed. One group consist of TLR 1,2,4, and 6 that recognize PAMPs from lipids, and the other group consists of TLR 3, 7,8, and 9 that recognize PAMPS from nucleic acids (Brikos & O'Neill, 2008).

Toll-Like Receptor 4 is a well-known receptor that is expressed on the surface of neutrophils in response to *Escherichia coli* invasion. The initiation of TLR-4 is done by an interaction with a lipopolysaccharide (Schumann et al. 2005). Lipopolysaccharide is bound by lipopolysaccharide-binding protein (LBP) which is found in plasma (Elson et. al., 2007). After the binding of the lipopolysaccharide to LBP, it is transported to a receptor complex (Schumann et. al., 2005). The receptor complex is formed by a protein termed CD-14, TLR-4, and the adapter molecule MD2. Signal transduction is initiated by the interaction of the TIRAP (Toll/interleukin-1 receptor (TIR-domain containing adaptor protein) domain of TLR-4 with MyD88(Schumann et. al., 2005). A MyD88-independent pathway involves proteins named TIR-domain-containing adaptor protein (TRIF) inducing interferon beta, TRIF- related adapter molecule (TRAM), tumor-necrosis-factor-κB activator (TANK)- binding kinase 1 (TBK_1), and IκB kinase ε, which leads to the induction of type 1 interferon via the transcription factor interferon regulatory factor 3(IRF_3) (Dick et al., 2009) and (Schumann et. al., 2005). Interferon Regulatory Factor 3 is phosphorylated by TBK_1. Interferon Regulatory Factor 3 forms homodimers and is translocated to the nucleus to induce IFNβ gene and many other genes which include

CXC-chemokine ligand 10 (Dick, et al., 2009). CXC-chemokine is responsible for the production of inflammatory cytokines such as IL-8 (Dick, et al., 2009) (See figure 2). Activation of Toll-Like receptor 4 has been described in cases of mastitis caused by *Staphylococcus* and *Escherichia coli* in ruminants (Yang et al., 2008).

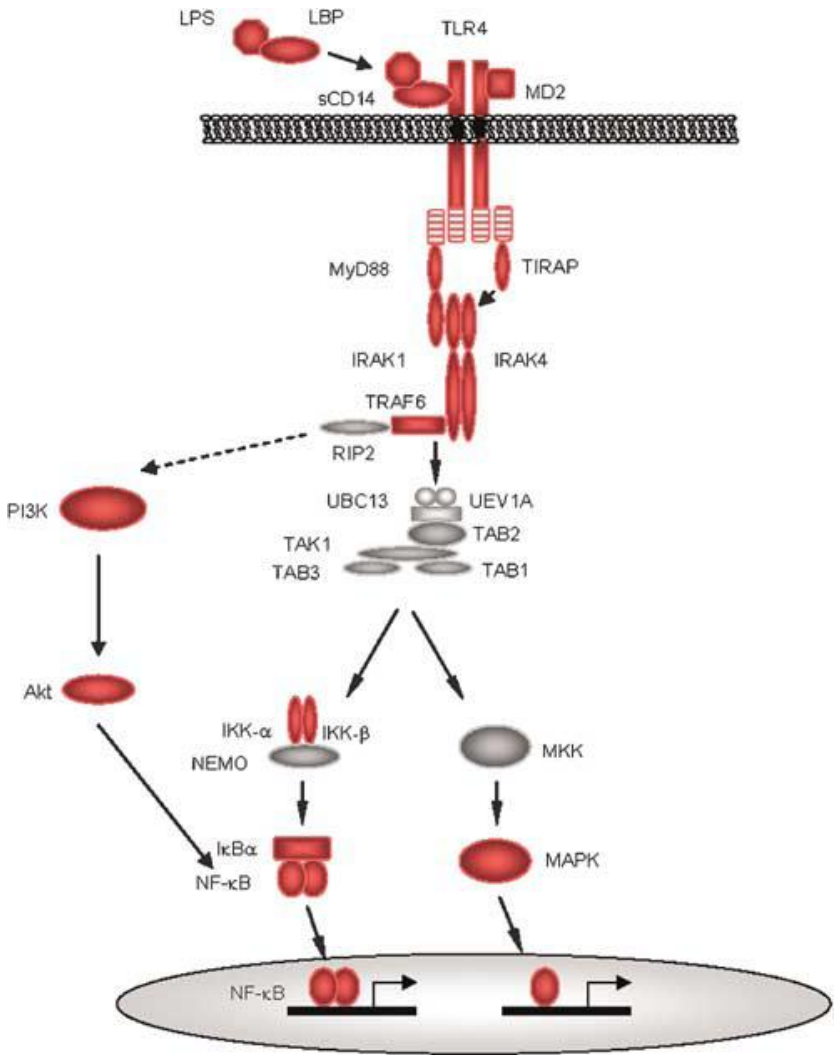


Figure 2. Schematic Representation of TLR-4 Pathway in Humans.
[Source: Dauphinee and Karsan.Lab Invest.2005.86: 9-22]

The activation of Toll-like receptor 4 in neutrophils during mastitis can lead to the activation of NF-κB. NF-κB is a transcription factor that consists of p50, p52, p65 (Rel A), c-Rel, and RelB. NF-κB activation leads to the activation of β-defensin encoding genes (Yang, et al., 2008). β-defensins are antimicrobial molecules that prohibit bacteria from colonizing on epithelial surfaces (Yang, et al., 2008).

By understanding the predisposition of the animal to infection by noting the genetic polymorphism of TLRs, susceptibility to disease and parasitic infection can be reduced. In one percent of human population, genetic polymorphisms are common variants (Schumann et. al, 2005). Most polymorphisms are single nucleotide polymorphisms (SNP) that can alter amino acid sequences, affect promoter characteristics, or may be completely silent. Mutations do not randomly occur within a genome, but rather depend on the particular genomic region, as well as selective pressure. One hypothesis developed stated that "genes encoding for proteins involved in immunity or disease resistance should exhibit a high number of polymorphisms." This hypothesis was strengthened by data obtained from genome-sequencing approaches (Schumann et. al., 2005). After the identification of the many techniques used to develop good genome-sequencing approaches, many genes involved in immune recognition have been described which were non-synonymous SNPs, including pattern recognition molecules and cytokines (Schumann et. al., 2005). In 2001, Arbour and colleagues described two polymorphisms of the human TLR-4 Gene Asp299Gly and Thr399Ile that were found at a higher proportion among individuals hyporesponsive to inhaled lipopolysaccharide, compared with a control population. Toll-Like Receptor 4 mutations are associated with

disease states in humans such as atherosclerosis, rheumatoid arthritis, type 2 diabetes, and acute allograft rejection. Rare SNPs in TLR-4 have been described in persons with the condition meningococcemia (Schumann et. al., 2005).

The detection of Single nucleotide polymorphism in livestock is a growing research interest. There have been several studies conducted to assess the expression of TLR 4 within the same breeds and amongst different breeds of ruminants to establish susceptibility to parasitic and bacterial infections (Zhou et. al., 2008). Allelic variations of different TLRs have been detected in bovine, ovine, porcine, and caprine genes (Zhou et. al., 2008) and (Palermo et al., 2009). In a study conducted by Zhou, Hickford and Gong, five nucleotide polymorphisms of TLR-4 were detected using 374 New Zealand goats mixed with different breeds. In other studies using sheep and cattle, 14 SNPs and 32 SNPS respectively, TLR-4 was found (Zhou et. al., 2008). There have been no conclusive studies found in the roles of the ability to fight off infections to gram negative bacteria in goats due to polymorphisms of their Toll-Like Receptor gene. Due to the high variability of expression of TLR-4 gene and the instance of infection of gram negative bacteria of goats, it is pertinent to see if there is correlation with the animals' ability to fight off infection.

By detecting variations of expression of TLRs, you can practice sustainable techniques such as selective breeding. This will allow the producers of livestock to produce environmentally friendly livestock while producing an economically beneficial animal (Rainard & Riollet, 2005). There are many techniques for detecting polymorphisms in TLRs. Some techniques include Polymerase Chain Reaction, SNP

array a type of Microarray analysis and Flow Cytometry (Cooper & Hausman, 2007). In such cases in which you would like to look at allelic variation of TLRs in organisms, the use of Polymerase Chain reaction (PCR) and gel electrophoresis is implemented.

Polymerase chain reaction is a technique used to amplify a gene of interest. Genes amplified using this technique can be compared from different samples to note genetic differences in the gene. For analysis of PCR amplified samples from genomic DNA, Single Stranded Conformational Polymorphism Gels (SSCP) are used to see the variation in expression depending on the DNA strands migration through the gel. Single Stranded Conformational Polymorphism Gels have been used to study nucleotide changes within a sequence. The components of the gel contain Sodium Dodecyl Sulfate (SDS), which binds to the DNA fragment causing it to become neutral. This allows the DNA strand to migrate based upon the molecular weight without charge having an effect. A study done by Zhou, Hickford et al., found 11 alleles in goats DQA gene, a glycogen encoding cellular protein, involved in antigen presentation to cells of the adaptive immune system using PCR and SSCP gels (Zhou et. al., 2005). The technique has been used to study differences in genes that contribute to components in immune responses of cows, pigs, mice, sheep, goats, and many other species. Microarray analysis, can be useful in studying the susceptibility of individuals to disease and help in the development of individualized drugs (Cooper & Hausman, 2007). Flow Cytometry is a technique that measures and analyzes characteristics of a particle, in most cases cells, as they flow through a beam of light. These technique uses side scatter and forward scatter to measure granularity and size of cells. This technique can be used to measure the amount of

expression of a particular TLR of interest on the cell surface by using antibodies tagged with a specific fluorescent marker (Becton, Dinkonson, & Company, 2000). After identifying polymorphism in a particular TLR sequence, many are put into databases were they can be shared by other researchers. Clustal W is a general purpose multiple sequence alignment program for DNA or proteins (Larkin et al., 2007). Availability of sequenced genomes databases analysis tools such as Basic Local Alignment Search Tool (BLAST) help in comparing genes between species to confirm any differences or similarities in nucleotides of a specific gene (Pruitt et. al., 2009) and (Larkin, et al., 2007). By using the Clustal W program after the discovery of SNPs in a given TLR sequence, you can align them with TLR sequences of other species to compare and contrast the importance of conserved and deletions of nucleotides. These databases will allow you to better understand the functions and expression of TLRs amongst different species (Pruitt et. al., 2009). Even though the impacts of polymorphisms of TLR-4 have been described in humans and rodents, little is known of their impact in ruminants. Since ruminants have been identified as primary carriers of *Escherichia Coli*, it is imperative to study the responses and variability in expression of TLR-4. There is little known on the expression and activation of TLR-4 in caprine neutrophils. The current study investigates TLR-4 expression in the genes and cell surface of Boer and Spanish goats' neutrophils. We also investigate the response of neutrophils to LPS stimulation by evaluating mRNA transcribed by the cells which included TLR-4, CD-14, and TNFα. We also used Enzyme Linked Immunosorbent Assay to study secretion of the IL-8 cytokine.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals Used for Collection of Blood

A total of nine clinically healthy meat goats of Boer and Spanish breeds (three were used for DNA isolation and six for gene expression studies in blood neutrophils) from the North Carolina Agricultural and Technical State University farm small ruminant unit were used . All animals were sheltered in a barn during the evening hours and let out to pasture during the day.

3.2 Collection of Blood and Fecal Samples

For neutrophil isolation ten ml of blood from six Spanish crosses, a Spanish goat and a Boer goat, were collected from the jugular vein into 15 ml Becton Dickinson (BD) vacutainer tubes containing 1 ml of Acid Citrate Dextrose mixed and placed in ice until use. For DNA analysis blood collected for neutrophil isolation was first spotted on FTA cards. Packed Cell Volume, blood smears and fecal egg counts were preformed to assess health of the animals. To assess parasite levels fecal egg counts using McMaster technique was used. Two grams of feces was used in each study. A magnification of 40X was used to look at the parasites in the glass slide chambers. To assess the number of eggs per gram of feces, the number of eggs counted in both chambers was multiplied by 100.

3.3 Neutrophil Isolation

In the current study, neutrophils, a primary cell in the innate response was used to look at responses to LPS infections in goats. All animals used in the study were first checked for any abnormal health conditions that may affect the results of the study. From the Becton Dickinson vacutainer tubes, Packed cell Volume, blood smears, and Polymorphonuclear (PMN) isolation was preformed to isolate neutrophils. Neutrophils were isolated from whole blood using the modified method described by Carlson and Kaneko (Carlson & Kaneko, 1973). Blood from each animal was transferred into separate 50 ml conical tube. Fifty ml conical tubes were transferred to a swinging bucket rotor (Eppendorf Centrifuge 5810R) (Eppendorf, Hamburg, Germany). The blood was centrifuged briefly (1500g for 10 minutes at 4ºC). Centrifugation allows for the separation of blood components into plasma, buffy coat, and red blood cell layer.

Using a glass pippetor, the plasma, buffy coat and 1/3 of the red blood cell layer were aspirated out leaving a 1/3 of the RBC layer containing the neutrophils. After RBC/PMN layer was mixed slowly and 20 ml of ice cold DEPC treated distilled water was added. The mixture was then mixed and the entire tube was rotated for 45 seconds to lyse the red blood cells. Ice cold 2.7% saline (NaCl) was immediately added to restore isotonicity and the contents were again mixed for 45 seconds. The tubes were then centrifuged (700g for 2 minutes at 4°C) and the supernatant discarded. The cells were then resuspended in 7 ml of ice cold PBS at pH 7.4. In order to obtain a clear white PMN pellet, the whole procedure of lysing was again repeated. After lyses, the clear white pellet containing PMN was resuspended in ice cold PBS.

3.4 Determining Neutrophil Concentration

Cells were counted in a Hemacytometer to find the concentration of isolated neutrophils. A small fraction of isolated PMN was diluted in PBS to facilitate individual counting of each cell. Ten µl of the diluted PMN was then loaded into one of the grooves of the hemacytometer and focused under a light microscope. PMN/µl concentration was calculated using a formula given below. Concentration of cells/ml=No of cells counted × 10^4. Neutrophils were then resuspended in an appropriate volume to obtain the desired concentration of cells.

3.5 Determination of the Viability of Neutrophils

PMN viability was determined by using the method of Trypan blue dye exclusion. Isolated PMN were mixed with Trypan blue at a ratio of 1:1. This was then mixed properly and placed in clean glass slide and covered with a cover slip. Cells were allowed to set for a few seconds before viewing. Cells were viewed at 40X magnification. A total of four chambers of cells were counted and the number of cells that had not taken up the dye was counted as alive and the results expressed as % of live (viable) cells. The viability of neutrophils was done in duplicate and the averaged.

3.6 Determination of Purity of Isolated Cells

Purity of neutrophils was determined by making smears of isolated PMN product. The smears were then stained using Wrights stain and viewed at 400X. Wrights stain allows for differentiation of blood cells.

3.7 Treatment of PMN

Isolated viable PMN were resuspended at a concentration of 10^7 cells/ml in PBS Ph 7.4. Cells were treated with LPS from *Escherichia* coli serotype 0111:B4 (Sigma Aldrich, St. Louis, MO) at concentrations of 100 ng for 30 minutes. Phosphate Buffer Saline treated PMN was used as a control. The PMN were incubated in an incubator at 37°C, 85% humidity and 5% CO_2. At the end of the incubation period, the tubes were kept on ice and then the cells were centrifuged at 700g for five minutes at 4°C. The cells were again washed in PBS and reconstituted in 1 ml of PBS.

3.8 Detection of TLR-4 Expression on Caprine Neutrophils by Flow Cytometry

Duplicate samples of isolated, viable caprine blood neutrophils from three Spanish/Boer cross goats were incubated (30 min, 37°C, 10% CO_2 and 95% humidity) with 100 ng of Escherichia coli serotype 0111:B4. Controls were maintained in PBS. Cells were washed by centrifugation (700g, 4°C, 5 minutes) and resuspended in 250 µl of PBS. Samples were then incubated with 3µg of anti-TLR-4 mAb (IMGENEX, San Diego, CA). Incubated cells were then washed by centrifugation (700g, 4°C, 5 minutes) and resuspended in 250 µl of PBS. Controls consisted of cells treated with just Allophycocyanin conjugated goat anti-mouse IgG. Samples and controls (treated and untreated) were then incubated with 1/150 dilution of Allophycocyanin conjugated goat anti-mouse IgG, (Jackson ImmunoResearch Laboratories INC., Baltimore, PA) was added to each tube and incubated (25 minutes, 4°C). Cells were washed by adding 250 µl of PBS by centrifugation (700g, 4°C, 5 minutes) and discarding the supernatants. Cells

were fixed in 2% paraformaldehyde using a 4% solution diluted in sterile deionized water.

3.9 Detection of Cell Surface Expression of CD-14 on Caprine Neutrophils by Flow Cytometry

Duplicate samples of isolated, viable caprine blood neutrophils from three Spanish/Boer cross goats were incubated (30 min, 37^0C, 10% CO_2 and 95% humidity) with 100 ng of 011 LPS. Controls were maintained in PBS. Cells were washed by centrifugation (700g, 4^0C, 5 minutes) and resuspended in 250 µl of PBS. These samples were then incubated (4°C, 25 minutes) with 5µg of anti-CD 14 mAB PE labeled (AbD Serotec, Raleigh, NC). Cells were washed by centrifugation (700g, 4°C, 5 minutes and resuspended in 250 µl of PBS. Controls were incubated with 5µg of anti-IgG conjugate mAB PE labeled (AbD Serotec, Raleigh, NC) samples and incubated (4°C, 25 minutes) briefly washed by centrifugation (700g, 4°C, 5 minutes). Samples were fixed in a 2% paraformaldehyde suspension. Flow Cytometry analysis helps in distinguishing cells using side scatter to measure granularity and forward scatter measure size. Samples which included those treated with antibodies to detect TLR-4, CD-14 and Controls were run at the Flow Cytometry laboratory at North Carolina State University using a Becton-Dickinson FACSCalibur machine. Each cell that passes through the detector was counted as an event. The histograms generated recorded the % cells binding monoclonal antibodies and florescence intensity of red (APC) and green (PE) fluorescence.

3.10 Polymerase Chain Reaction

Blood was collected on FTA cards via the jugular vein from six goats. Three 3.0 mm disc were collected from the FTA cards for each goat and put into individual 1.5 ml tubes as recommended by the manufacturer (Whatman, Piscataway, NJ). Five hundred microliters of sterile deionized H_2O was added to each 1.5 ml tube and immediately pulse vortex for five seconds (Whatman Inc, Piscataway, NJ). The discs were immediately transferred to six individual PCR tubes and 100µl of deionized H_2O was added to each PCR tube. The tubes were transferred to a heated water bath (95°C, 30 minutes). At the end of the incubation period the PCR tubes were removed from the heating water bath and pulse vortex 60 times. The PCR tubes were centrifuge for 30 seconds, to separate the matrix from the eluate and the FTA Elute matrix disc were removed using a sterile pipette leaving the purified DNA in the tubes. Concentration of DNA was assessed using a Nanodrop Spectrophotometer 1000 3.7.1 (Thermo Scientific Inc, Waltham, MA). DNA concentration was adjusted to 200ng and purity was approximately at 2.0.

3.11 Amplification of the Ligand Binding Region of Caprine TLR-4 Using the Polymerase Reaction Conditions

A twenty-five µl master mix was prepared which contained 1 µl of 10µM of each Primer (Primer 1 and Primer 2 (Table 2), 5µl of 10× PCR buffer, 2.5 µl of 300 µM dNTP Mix, 1 µl 5U Taq, and blood and deionized H_2O were prepared according to calculated DNA volume per reaction(Zhou et. al., 2008). Tubes used specifically to conduct polymerase chain reactions were immediately transferred to the PCR

thermocycler Primus 96 Plus (MWG Operon, Huntsville, Alabama). Polymerase chain reaction was carried out in three steps which consisted of an initial denaturation step, followed by an annealing step and a final extension or elongation step (Table 1).

PCR samples were visualized on a 1% agarose gel and a 14% SDS PAGE gel to note any polymorphisms (Zhou et. al., 2008) (look to appendix B for protocol). One percent agarose gels were run for thirty minutes and stained with ethidium bromide and observed under UV light to analyze the PCR product using the Gel-Doc documentation system (Bio-Rad, Hercules, CA). Precast fourteen percent Sodium Dodecyl Sulfate Polyacrylimide Gels (Jule Inc, Milford,CT) were run at 4^{o}C using electrophoresis at 300V for four hours and stained the following day using silver staining procedure described by Bio-Rad Company (Bio-Rad, Hercules, CA) (See Appendix B). To determine differences in TLR-4 expression between breeds of goats Polymerase Chain Reaction was carried out using blood obtained from (n=3) Spanish, Boer, and Spanish/Boer crosses.

Table 1. Specific Polymerase chain reaction conditions for amplification of TLR-4 gene from DNA.

PCR	Temperature	Time/Cycle
Pre-heating	94°C	2 mns./1 cycle
Denaturation	94°C	30 sec/35cycle
Annealing	54.5°C	30 sec
Final Extension/Elongation	72°C	30 sec

3.12 RNA Isolation Using Tri-Reagent

To study the effect of LPS on the transcription of TLR-4, TNF-α, CD-14 and GAPDH isolation of RNA was performed. Neutrophils at a concentration of 1×10^7 cells/ml were treated with 011 Lipopolysaccharide (Sigma- Aldrich Inc., St. Louis, MO) and washed with PBS by centrifugation as above (500g, 5 minutes, 4°C) and resuspended in 1 ml of Tri-reagent (Molecular Research Center, Inc, Cincinnati, OH) and stored overnight at -70°C. Briefly LPS treated Neutrophils and controls which were not treated were thawed at room temperature. Approximately 200 µl of chloroform was added and the tubes were shaken manually for 15 seconds. This was followed by centrifugation at 12,000 g in a centrifuge (Eppendorf Model 5810 R) (4° C for 15 minutes). The fraction containing the RNA was transferred to a RNAse free tube. Five hundred µl of isopropanol was added to the tube containing the RNA sample and incubated for 5 minutes at room temperature. Samples were then centrifuged (12000 g, 4°C, 10 minutes). The supernatants were transferred to new RNAse free tubes and RNA was precipitated by adding 450 µl of isopropanol. Samples were then mixed and incubated overnight at -20°C. Frozen samples were thawed and centrifuged (12000 g, 4°C, 8 minutes). The supernatant was discarded and the remaining RNA pellet was washed with 1 ml of 95% ethanol by centrifuging (7500g, 4°C, 5 minutes). The ethanol was poured off and the RNA pellet was air dried at room temperature for 15 minutes. The dried RNA pellet was then resuspended in 30 µl of DEPC treated water. Concentration and purity of RNA was assessed using the Nanodrop 1000 3.7.1 spectrophotometer (Thermo Scientific, Waltham, MA).

3.13 Complementary DNA (cDNA) Synthesis

Five micro liters of isolated RNA from cells previously treated with 100 ng of LPS at a concentration of 1µg was added to two µl of oligo DT primers (Ambion Inc, Austin, TX). Five µl of nuclease free water was then added to make a final volume to 12µl. The mixture was then mixed and centrifuged quickly. The samples were heated to 80°C for three minutes and then transferred to an ice bucket. To this reaction, 2µl of 10×RT buffer, 4µl dNTP mix, 1µl RNase inhibitor and 1µl of reverse transcriptase enzyme (Ambion Inc, Austin, TX) were added to make the final polymerase chain reaction volume to twenty µl. The samples were then mixed, centrifuged briefly and incubated for one hr at 42°C. The samples were then incubated at 92°C for ten minutes to inactivate reverse transcriptase enzyme. The samples containing the new synthesized cDNA were stored at -20°C until use.

3.14 Amplification of cDNA Using Specific Primers

Amplification of cDNA (200ng/µl, 2.0 purity) was performed in a thermocycler (MWG, Huntsville, AL). The reaction consisted of an initial denaturation step, followed by an annealing step and a final extension or elongation step consisting of specific temperatures and times for each gene under study (Table 1). Primers for TLR-4, CD-14, TNF-alpha and GAPDH were used (Table 2). All primers used in this study were synthesized by MWG Biotech Inc, NC. After cDNA copies were synthesized, products were added to a master mix for amplification of specific genes of interest. All samples were run in duplicate.

Table 2. Primer sequences and expected size of PCR products for the genes studied

Gene	Primer Sequence	Length of PCR Product (Base pairs or bp)
CD-14 forward	GACGACGATTTCCGGTTGTGT	600
CD-14 reverse	TGCGTAGCGCTAGATATTGGA	600
TLR-4 primer 1	GTATTCAAGGTCTGGCTGGTT	494
TLR-4 primer2	ATCATTGAAGCTCAGATCTAAAT	494
TNF-α forward	CGGTGGTGGGACTCGTATG	352
TNF-α reverse	CTGGTTGTCTTCCAGCTTCACA	352
GAPDH forward	GGCAAGTTCCATGCCACAGT	120
GAPDH reverse	GTCCTCCACGATGCCAAAG	120

3.15 Enzyme Linked Immunosorbent Assay to Detect IL-8 after LPS Stimulation

a) Treatment of Neutrophils

Neutrophils from three female Spanish and Boer Cross goats were stimulated with LPS (30 min, 37°C, 10% CO_2 and 95% humidity or maintained as control. Supernatant from neutrophils were collected after centrifugation (700g) and stored at -4°C until they were ready for use.

b) Enzyme Linked Immunosorbent Assay to Detect Interleukin-8

The Quantikine Immunoassay Human IL-8 Kit instructions (R&D systems, Minneapolis) was used to detect IL-8 from supernatants of caprine PMN treated with LPS or maintained in PBS as a control according to instructions (R&D systems, Minneapolis). Briefly a 100µl of assay diluents RDI-85 was added to each well of a precoated IL-8 ELISA plate. Fifty micro liters of IL-8 standards of different concentrations (3.9, 7.8,15.6,31.2, and 62.5 pg/µl) was prepared in calibrator diluents RD5P at 1X concentration was added to each well along with RD5P at 1X concentration serving as the zero standards (0pg/ml) to generate a standard curve. All standards and all test samples were run in duplicate. Along with standards, 50 µl of undiluted samples were added into respective wells. This was then covered securely with a plate sealer provided with the kit and incubated at room temperature for 2 hr with gentle taping of the plates to ensure proper mixing. After the 2 hr incubation, the plates were washed four times with was buffer and inverted and blotted against a clean paper towel. To each well on the plate, 100 µl of IL-8 conjugate was added. This was then incubated for a further period of 1 hr at room temperature. The plates were then washed in wash buffer four

times. After washing, the plates were inverted and blotted and 200 µl of substrate solution was added to each well and incubated for 30 minutes at room temperature in the dark. The reaction was stopped by the addition of 50 µl of stop solution to each well. The color change from blue to yellow was observed. The optical density was read at 450 nm with a background correction of 570 nm on a MR600 micro plate reader (Dynatech Laboratories Inc, Chantilly, VA) within 30 minutes with a back ground correction reading at 570 nm using the dual wave length mode of the ELISA plate reader.

3.16 Analysis of Enzyme Linked-Immunosorbent Assay for Interleukin-8 Detection

The optical density ranges were recorded and average determined for control and test groups. The Microsoft Excel program was used to summarize the data. Standard concentrations for analysis of test sample were plotted (Figure 3).

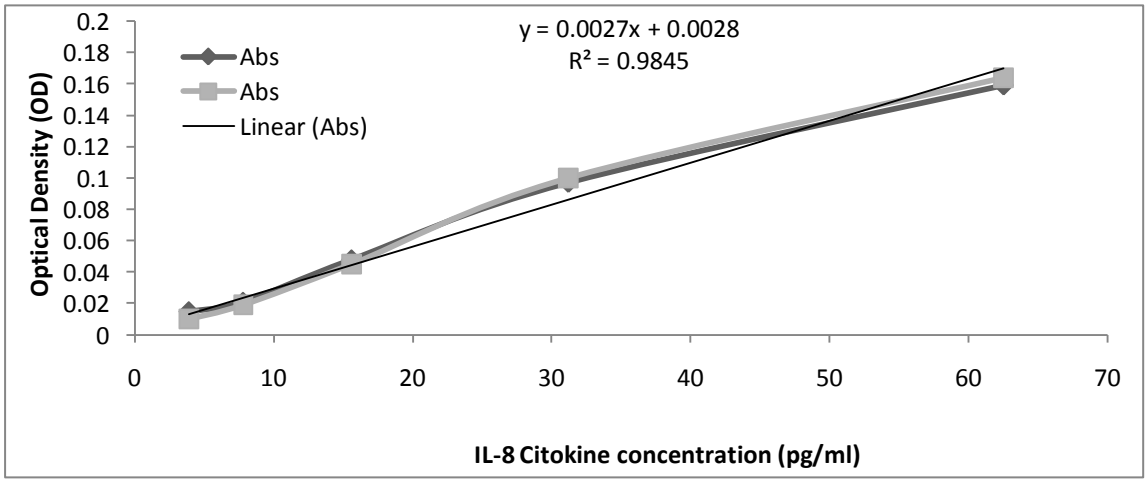


Figure 3. Standard curve for IL-8 cytokine (pg/ml). The X-axis is the concentration of IL-8 cytokines in pg/ml. The Y-axis is the optical density range. The two lines represent duplicate reading for absorbance (Abs).

ELISA concentrations were calculated using the best fit line obtained from the control run in duplicate. Dilutions of concentrations were made using the procedures outlined within the guide for detection of lower concentrations. The concentration of individual samples was obtained by using the liner equation obtained by the standard curve. Microsoft excel was used to calculate mean, standard deviation and the Standard Error of the mean.

CHAPTER 4

RESULTS AND DISCUSSION

The overall health of the animals in the study was obtained by looking at the Packed Cell Volume to measure anemia, the Whole Blood Count to look at leukocytes, and fecal egg count to measure parasite levels. All goats in the study were female Spanish, Boer, or Spanish and Boer mixed. These tests were used to verify if there were any anemic conditions (see Table 3). The average normal Packed Cell Volume for a goat is 28% and the normal Fecal Egg count is cut off at 1000 before treatment is necessary.

Table 3. Summary of health conditions of 6 goats based on Packed Cell Volume, and Fecal Egg Count per gram (epg) of feces

Goat Number/Breed	PCV %	Fecal Epg
9/ Spanish	32	800
13/ Spanish/Boer	23	3600
637/Spanish	30	300
701/Spanish/Boer	25	500
434/Spanish/Boer	23	2500
2022/Spanish/Boer	21.5	1200

The average PCV (n=6) was 32 indicating no anemic conditions. Fecal egg counts per gram of feces ranged from 300 to 36000 in the goats used in the study. The high fecal egg counts with normal PCV levels maybe indicative of a natural ability to withstand high parasite infections.

The purity of isolated neutrophils was always 90% or higher. The purity of neutrophils was determined using Wrights staining. Other cells that were observed after neutrophil isolation was lymphocytes and monocytes (See table 4). Viability of isolated neutrophils was always more than 90% as determined by the Trypan Blue dye exclusion. The animals in the study were clinically healthy; however some had high numbers internal parasites.

Table 4. Purity of isolated neutrophils from caprine peripheral blood

Goat Number	Neutrophils	Lymphocytes	Monocytes
13	93	N/C	7
434	92	3	5
2022	95	1	4
Average	93	2	4.5

4.1 Detection of the Gene Encoding TLR-4 in Genomic DNA

The TLR-4 gene was identified in peripheral blood using primers from a previous study conducted by Zhou, which investigated polymorphisms of TLR-4 in New Zealand goats (Zhou et. al., 2008). For a control, primer specific for GAPDH which is constitutively expressed and used as a loading control. A 494 bp fragment was observed on a 1% agarose gel in all goats. TLR-4 was detected in all six goats used in this study. In the study conducted by Zhou, five different polymorphisms were observed using a SDS-PAGE.

In the current study six Spanish crosses were investigated for polymorphisms proceeding with a study that investigated expression of TLR-4 between three goats which included Boer, Spanish, and Spanish crosses (Figure 5 and 6). To assess polymorphisms within the gene segment, a 14% polyacrylamide gel was ran and silver stained. Differences in expression of TLR-4 between Spanish goats and Spanish and Boer goats were not observed in the current study. However, similar band patterns were observed in the study reflecting those reported by Zhou (Zhou et. al., 2008).

Further studies are needed to evaluate the significance of these polymorphisms in goat immunity. Polymorphisms may be the result of location and environmental factors rather than a breed effect as seen in human SNPs (Lappalainen & Dermitzakis, 2010). Since goats are being considered as a new source of lean meat within several countries it is important to note the health characteristics of goat herds able to withstand harsh environments.

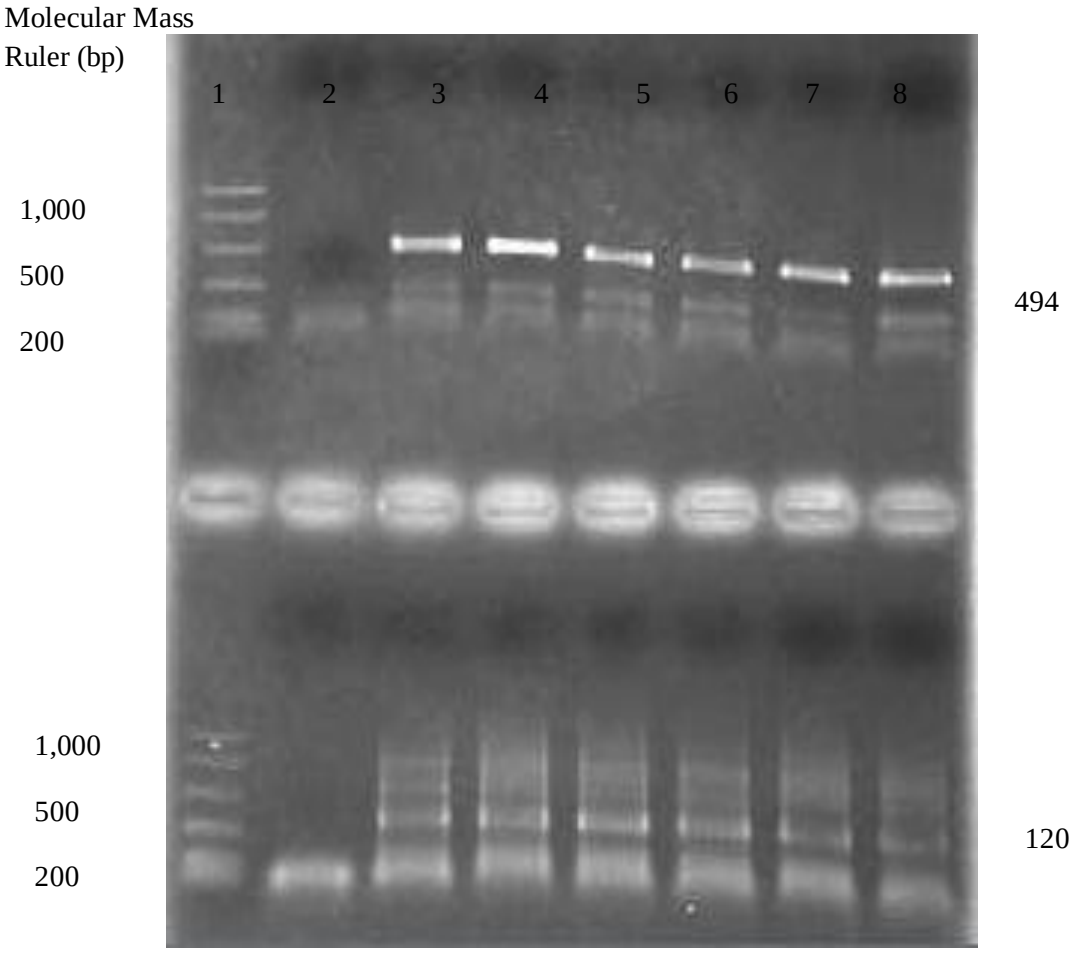


Figure 4. Image of the 494 bp Putative-ligand binding region of the gene encoding TLR-4 gene. Blood collected on FTA cards from goats (N=6) were used to amplify TLR-4 gene. Specific primers for TLR-4 were used to amplify a 494 bp putative-ligand region of TLR-4. Products were run in duplicate on 1% agarose gels. Upper gel l: Lane 1 is Molecular Mass Ruler (New England Bio-Labs, Ipswich, MA). Lane two is control (No DNA and Master Mix). Lane 3 - 8 are samples containing DNA from six goats in the following order (Lane 3 goat 9, Lane 4 goat 13, Lane 5 goat 637, Lane 6 goat 701, lane 7 goat 434, and lane 8 goat 2022. Lower gel: A 120bp fragment of GAPDH control- Lane 1 is Molecular Mass Ruler. Lane two is control (No DNA and Master Mix). Lane 3 -8 are samples containing DNA from six goats in the following order (Lane 3 goat 9, Lane 4 goat 13, Lane 5 goat 637, Lane 6 goat 701, lane 7 goat 434, and lane 8 goat 2022

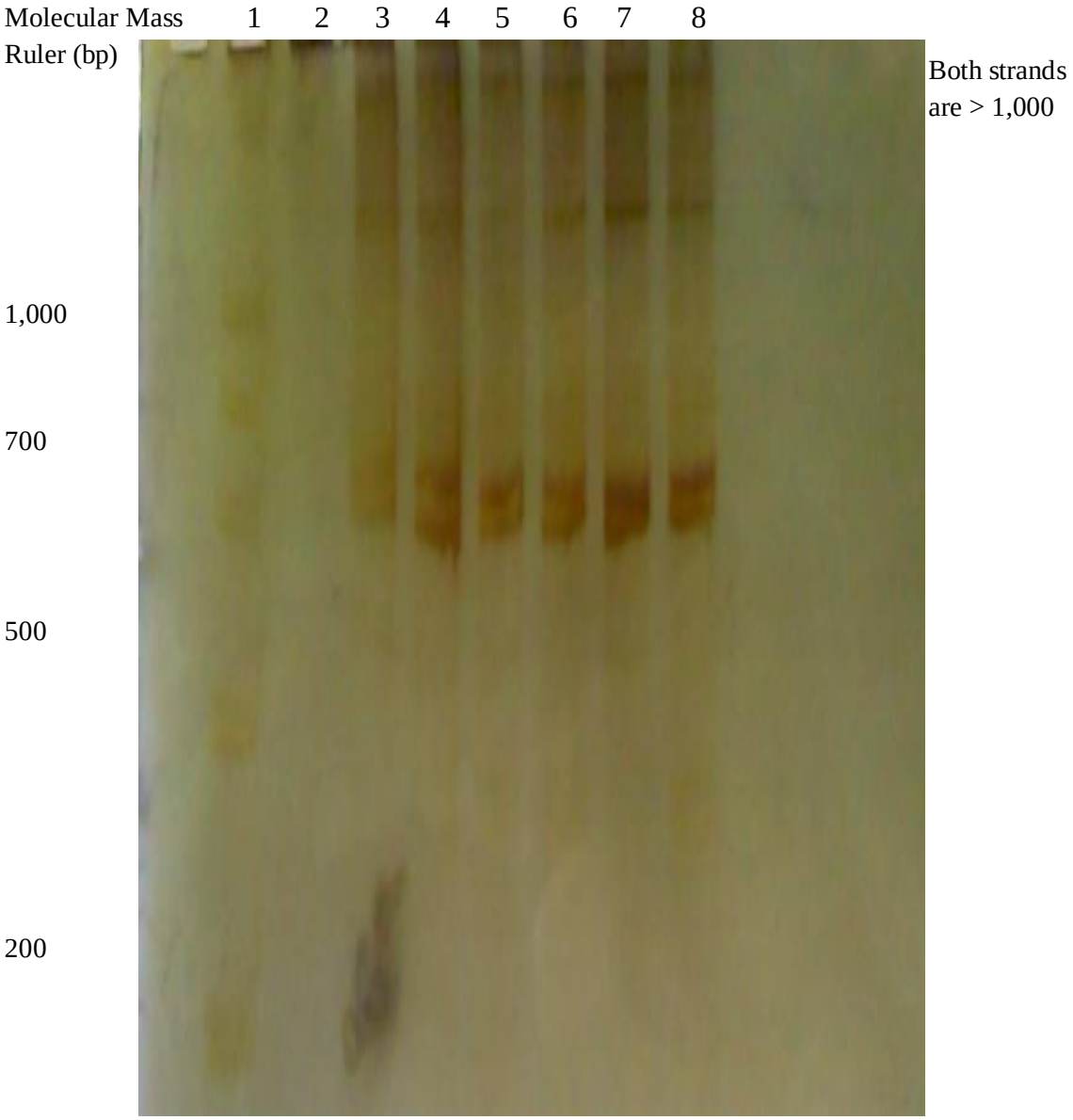


Figure 5. Representative image of silver stained single-stranded-conformational polymorphism gel analyzing the putative-ligand binding region of the gene encoding TLR-4 gene of Spanish/Boer crosses. Blood collected on FTA cards from goats (N=6) were used to amplify TLR-4 gene. Specific primers for TLR-4 were used to amplify a 494 bp putative-ligand region of TLR-4. Products were run in duplicate on Single Stranded Polyacrylimide Gels. Lane 1: Molecular Mass Ruler (New England Bio-Labs, Ipswich, MA). Lane 3-8 are single-stranded PCR products of Spanish cross goats. Lane 3 goat 9, Lane 4 goat 13, Lane 5 goat 637, Lane 6 goat 701, lane 7 goat 434, and lane 8 goat 2022. Fragments were greater than 1,000 bp.

To examine if any polymorphisms occurred between six female Spanish crosses, TLR-4 amplified products using specific primers for the putuative-ligand binding site were run on a 14% polyacrylimide gel (Jule, Milford, CT) to observe a single strand. Products obtained from polymerase chain reaction reactions were briefly heated in a denaturing dye for five minutes to allow for DNA double strands to separate.

Denatured DNA was run for 4 hours and silver stained using a protocol provided by Bio-Rad Laboratories (Bio-Rad, Hercules, CA). There were no differences observed between all six Spanish/Boer cross goats used in the study. Two distinctive bands were observed and were all bands were greater than a thousand. The bands observed had similar patterns to the DNA bands in the study conducted by Zhou (Zhou et. al, 2008) (Figure 5).

To detect differences in TLR-4 expression between three goats consisting of one Spanish, one Boer, and one Spanish and Boer cross; amplified DNA products from three goats of the different breeds were run and observed on a 14% acrylamide gel. The gel was run in duplicate for 4 hours and silver stained.

The DNA fragments of the three breeds Spanish, Spanish/Boer cross, and Boer observed on the 14% polyacrylamide gel were similar between all three breeds (Figure 6). There were two Bands observed greater than a thousand base pairs. The bands between the different breeds were also identified as being similar to those bands identified in the study conducted by Zhou (Zhou et. al, 2008). To conclusively determine if there were any polymormphic regions a sequence analysis of the amplified products need to be conducted.

Figure 6. Representative image of a silver stained single-stranded-conformational polymorphism gel of the putative-ligand binding region of TLR-4 gene in Spanish, Boer, and Spanish/Boer goats. Blood collected on FTA cards from goats (N=3) were used to amplify TLR-4 gene. Specific primers for TLR-4 were used to amplify a 494 bp putative-ligand region of TLR-4. Lane 1: Molecular mass ruler (New England Bio-labs, Ipswich, MA). Lane3-goat 13 Spanish X, Lane 5-goat 637 Spanish, and Lane 7 goat 2034 Boer.

4.2 Expression of TLR-4, CD-14, TNF-alpha in Caprine Blood

There has been an increase an interest on the effects of LPS on ruminants due to its cause of disease (Hodgson, 2006). Such diseases as mastitis and excessive diarrhea have caused great concerns for goat herders (La Ragione et al., 2005). There have been several studies on the effects of LPS on the host immune system. The responses of an individual's immune response can have differing effects(Werling et. al., 2009). Effects can be either beneficial to the host or detrimental (Werling et. al., 2009). Studies have been conducted that observe neutrophils response to gram negative bacteria. The expression of TLR-4 has been noted in drosophila, humans, mouse, and many ruminants (Schumann et. al., 2005) and (Zhou, Hickford, & Gong, 2008). There is limited information on the effects of LpS on TLR-4 expression in Boer and Spanish goat neutrophils.

To observe the transcription of TLR-4, CD-14, and TNFα neutrophils were isolated from whole blood of three Spanish and Boer cross goats. The total RNA was isolated from samples treated for 30 minutes with LPS and controls (those with PBS). The purity of isolated RNA was averaged 2.0 and concetration averaged 200ng/µl. The RNA collected from each sample reverse transcribed cDNA amplified using specific primers and observed on a 1% agarose gel. The results of this study illustrate TLR-4 gene expression, transcription and signaling in Spanish and Boer goats. Treatment with LPS did not have an effect on transcription, however the genes for TLR-4, TNF-α, and GAPDH were expressed in neutrophils. The transcription of CD-14 was not detected.

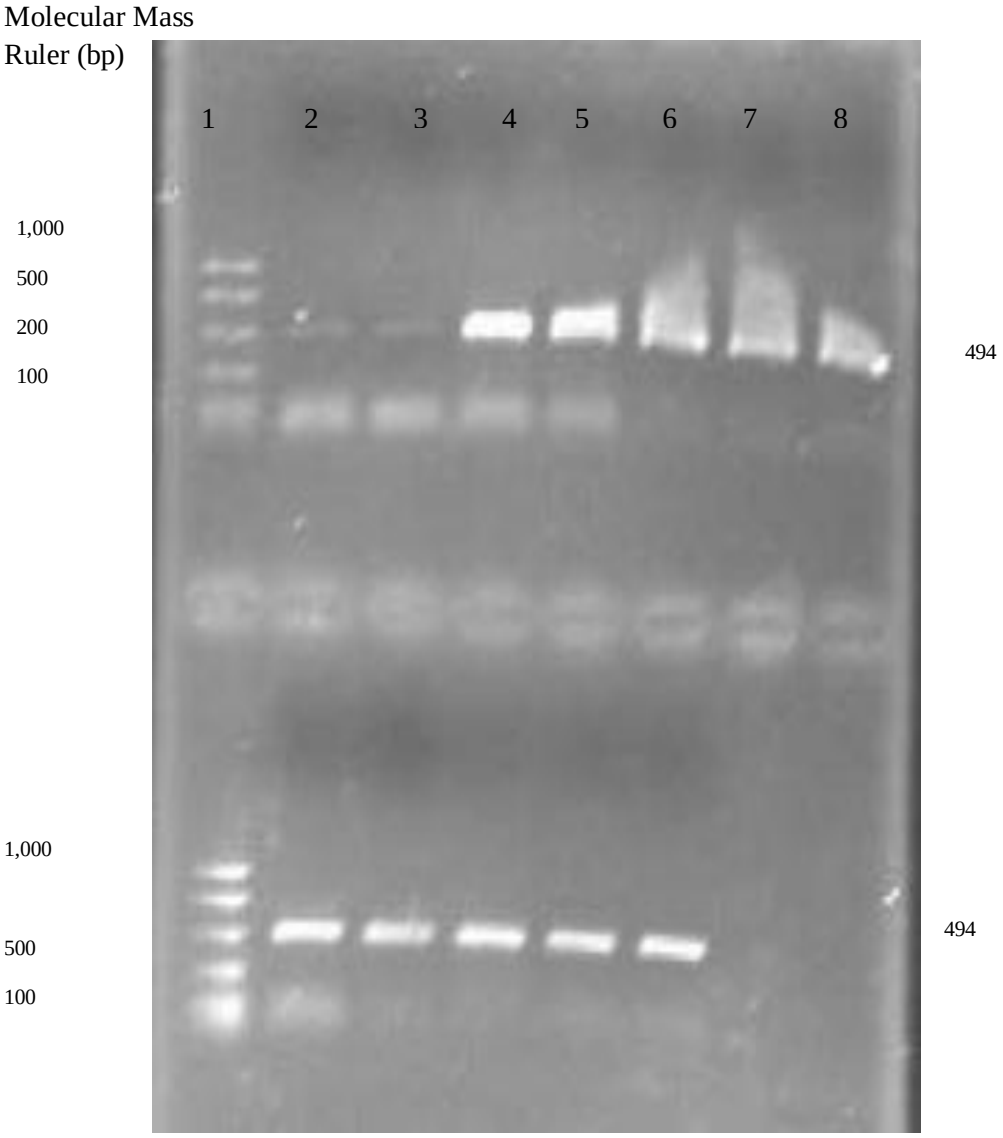


Figure 7. Image of Transcription of a 494 bp putative-ligand segment of the gene encoding TLR-4 gene. Isolated Peripheral blood neutrophils from goats (N=3) were treated with LPS or maintained in PBS as controls. Specific primers for TLR-4 putative-ligand segment were used to amplify a 494 bp segment. Upper gel lane: Lane 1: Molecular mass ruler (New England Biolabs, Ipswich, MA). Control samples: Lanes 2 and 3 (goat 706) and Lanes 6 and 7(goat 703). Treated samples: Lane 4 and 5 (goat 706) 8(goat 703). Lower gel lane: Lane 1 is Molecular mass ruler. Control Samples: Lane 3 and 4 (goat 701). Treated samples: Lane 2: (Goat 703) and Lane 5 and 6: (goat 701).

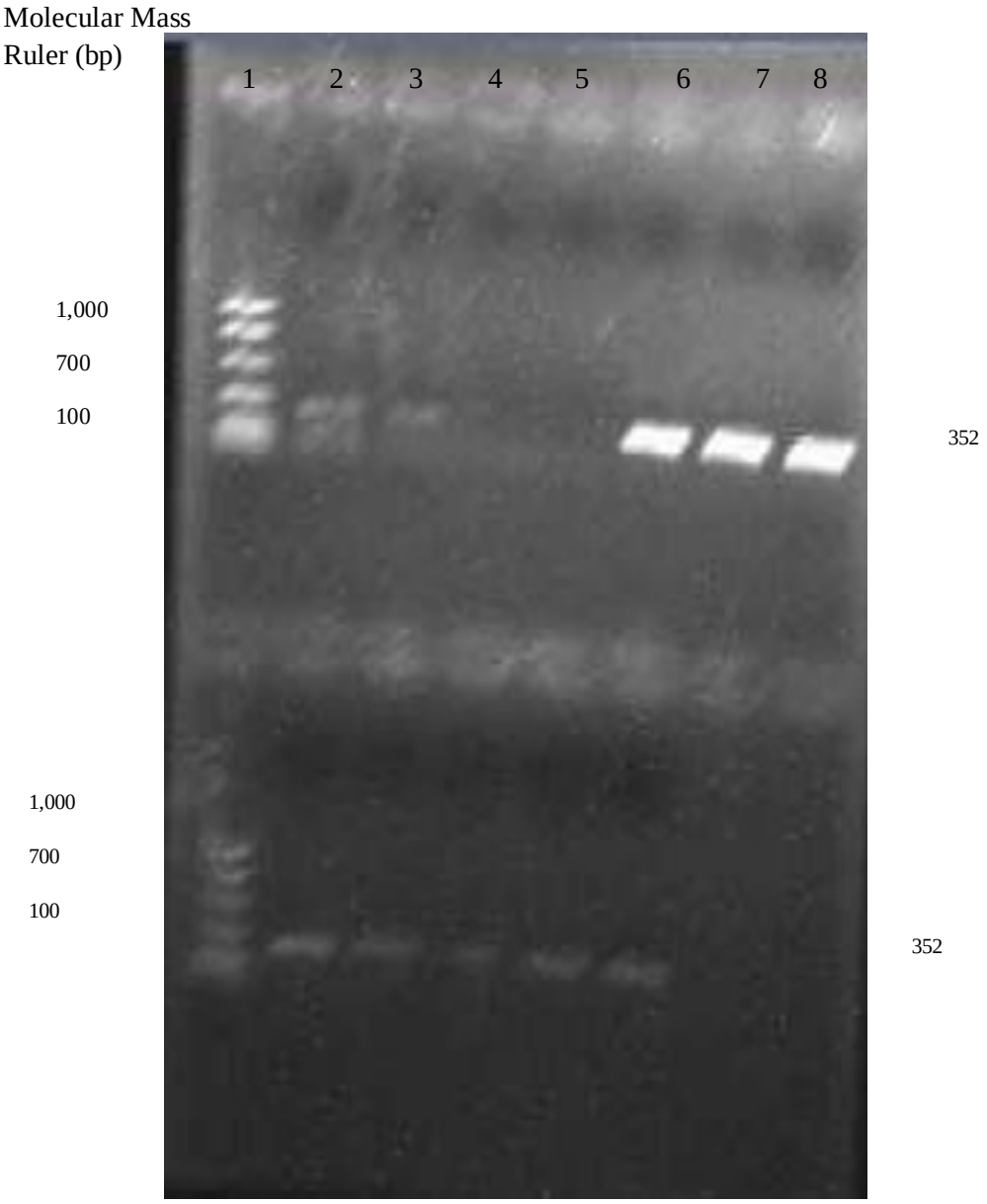


Figure 8. Image of Transcription of a 352 bp segment of the gene encoding TNF alpha gene. Isolated Peripheral blood neutrophils from goats (N=3) were treated with LPS or maintained in PBS as controls. Specific primers for TNF-α were used to amplify a 352 bp segment. Upper gel lane: Lane 1: Molecular mass ruler(New England Biolabs,Ipswich, MA). Control samples: Lanes 2 and 3 (goat 706)and Lanes 6 and 7(goat 703). Treated samples :Lane 8(goat 703). Lower gel lane: Lane 1 is Molecular mass ruler. Control Samples : Lane 3 and 4 (goat 701). Treated samples : Lane 2: (Goat 703) and Lane 5 and 6: (goat 701).

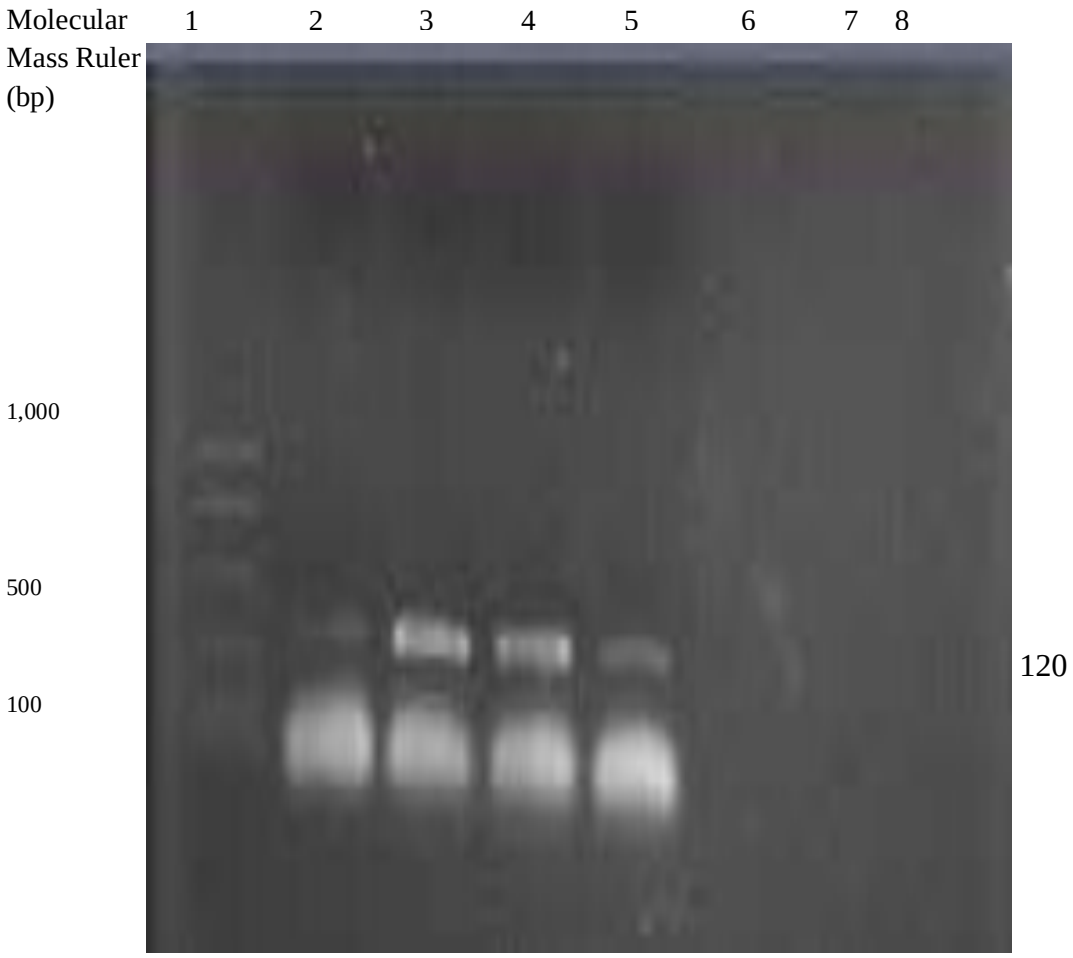


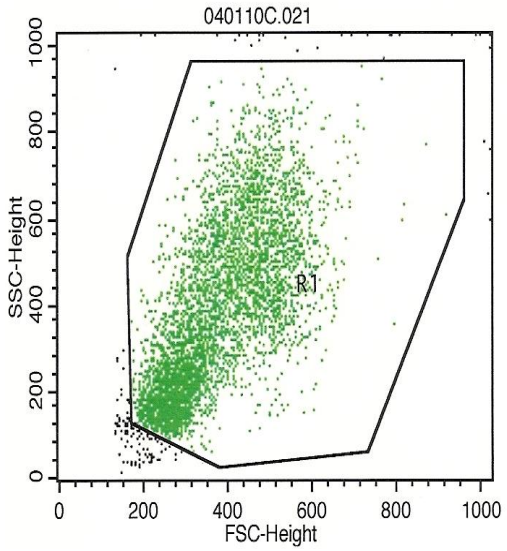
Figure 9. **Image of transcription of a 120 bp segment of the gene encoding GAPDH gene**. For a control GAPDH was used. Lane 1: Molecular mass ruler (1,000, 700, 500, 200, 100bp) (New England Bio-Labs, Ipswich, MA). Controls Lane: 2 and 3 goat 701 and 703. Treatment Lane: 4 and 5 goat 701 and 703.

4.3 Flow Cytometry Analysis of Expression of TLR-4 and CD-14 on Caprine Neutrophils.

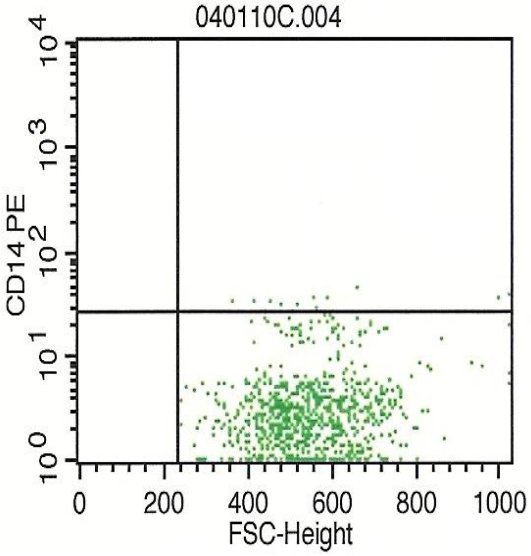
Flow Cytometry has been useful in studying toll-receptor expression on cells of the immune system. Using Flow cytometry samples containing isolated neutrophils labeled with APC, PE, or none were run to determine TLR-4 and CD-14 expression on the surface of neutrophils before and after treatment with LPS. Surface expression of TLR-4 and CD-14 were compared to isotype controls to determine significance of binding.

Samples for the study of TLR-4 and CD-14 contained two groups those treated with LPS and those without treatment with LPS. In the TLR-4 group study, samples were then divided into groups labeled with TLR-4 and APC 2º antibody, TLR-4 antibody, APC 2º antibody, and cells. In the CD-14 group study, samples were divided into groups labeled with CD-14 PE labeled antibody, Isotype IgG 2b PE labeled antibody, and cells only. Generated data obtained from the flow analysis included total amount of binding and fluorescent intensity.

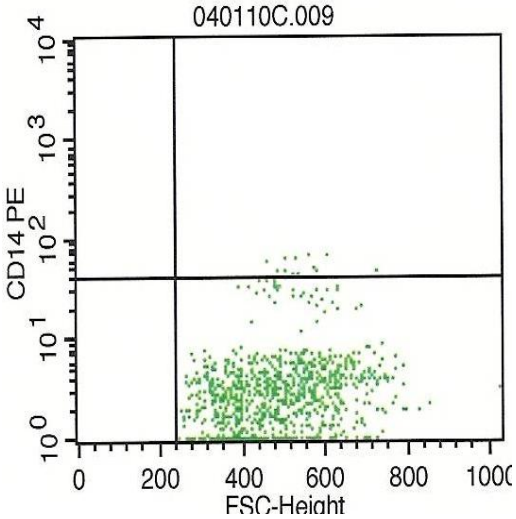
P-values for all samples run in the study were higher than 95% confidence interval. There was also no significant correlation between TLR-4 expression and CD-14 expression as determined by statistical analysis t-test. Statistical analysis of the data obtained proved to be not significant. To determine if there is any differences and significance amongst the herd that was studied a larger sample size maybe needed. Antibodies that are directed towards TLR-4 and CD-14 of goats could also be beneficial in getting a better result.



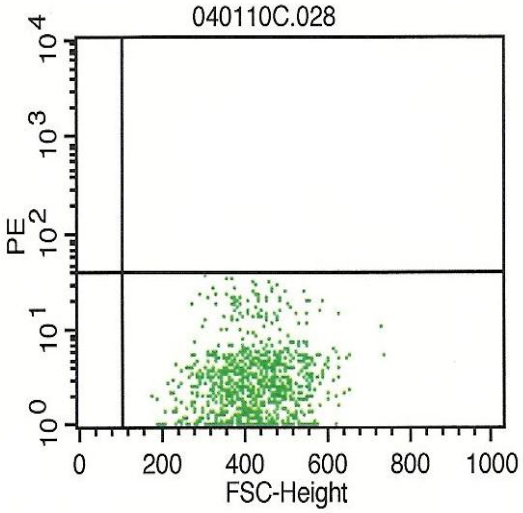
A.



B.



C.



D.

(Continued)

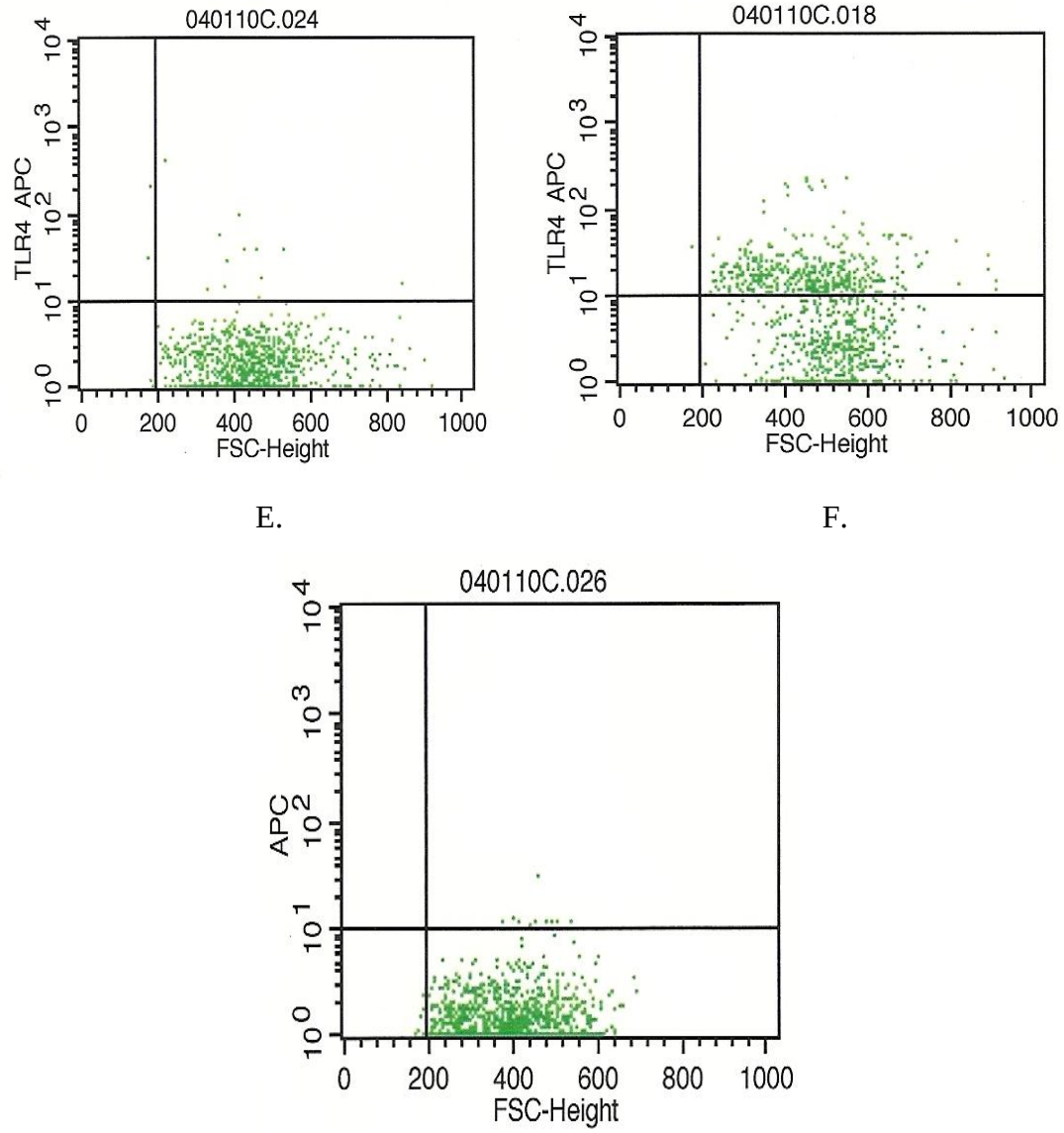


Figure 10. Representative histograms of flow cytometric analysis to detect TLR-4 and CD-14 on goat neutrophils. Grid A. Gated Cell population containing Neutrophils, Grid B. Goat 434 CD-14 after treatment with LPS, Grid C. Goat 13 CD-14 Not Treated with LPS, Grid D. Isotype Control of cells treated with LPS Goat 2022 TLR-4 without LPS, Grid E. Goat 13 TLR-4 after treatment with LPS, and Grid F. TLR-4 without treatment with LPS, and Grid G. Cells treated with LPS only labeled with APC 2° antibody as control

The effect of treatment in expression of CD-14 versus control samples were determined using PE conjugated monoclonal antibodies specific to the receptor (Figure 10, A and B). To assure binding of CD-14 (not shown) and TLR-4 isotype controls were used (Figure 10, E, G). Statistical analysis of the data run on the Flow Cytometry results did not show any significance in binding of antibodies in treated versus control samples of CD-14 and TLR-4.

The effect of treatment with LPS in expression of TLR-4 versus control samples were determined using APC conjugated monoclonal antibodies specific to the receptor (Figure 10, E, F and G).Three Spanish/Boer crosses were used to study the surface expression of TLR-4 and CD-14 expression on cells prior to and after treatment with LPS. Each goat had samples run in duplicate. To obtain if any samples had significant binding, values higher than the isotype control were considered positive. Neutrophils from one out of the three goats used in the study had values for % fluorescence and % Gated higher than the control (Figure 11). All other samples used in the study had lower percent binding than that of the controls.

To obtain Statistical Significance a larger sample group will be needed. The binding of monoclonal antibody for CD-14 was not observed in all samples used in the current study. To obtain statistical significance a larger sample size, optimization of antibody concentrations and animal to animal variability may need to be considered. The current findings observed show that goat neutrophils may express low levels of TLR-4 on their surface. Further studies are needed to evaluate the effect of LPS on expression of TLR-4 and CD-14 receptors in goats.

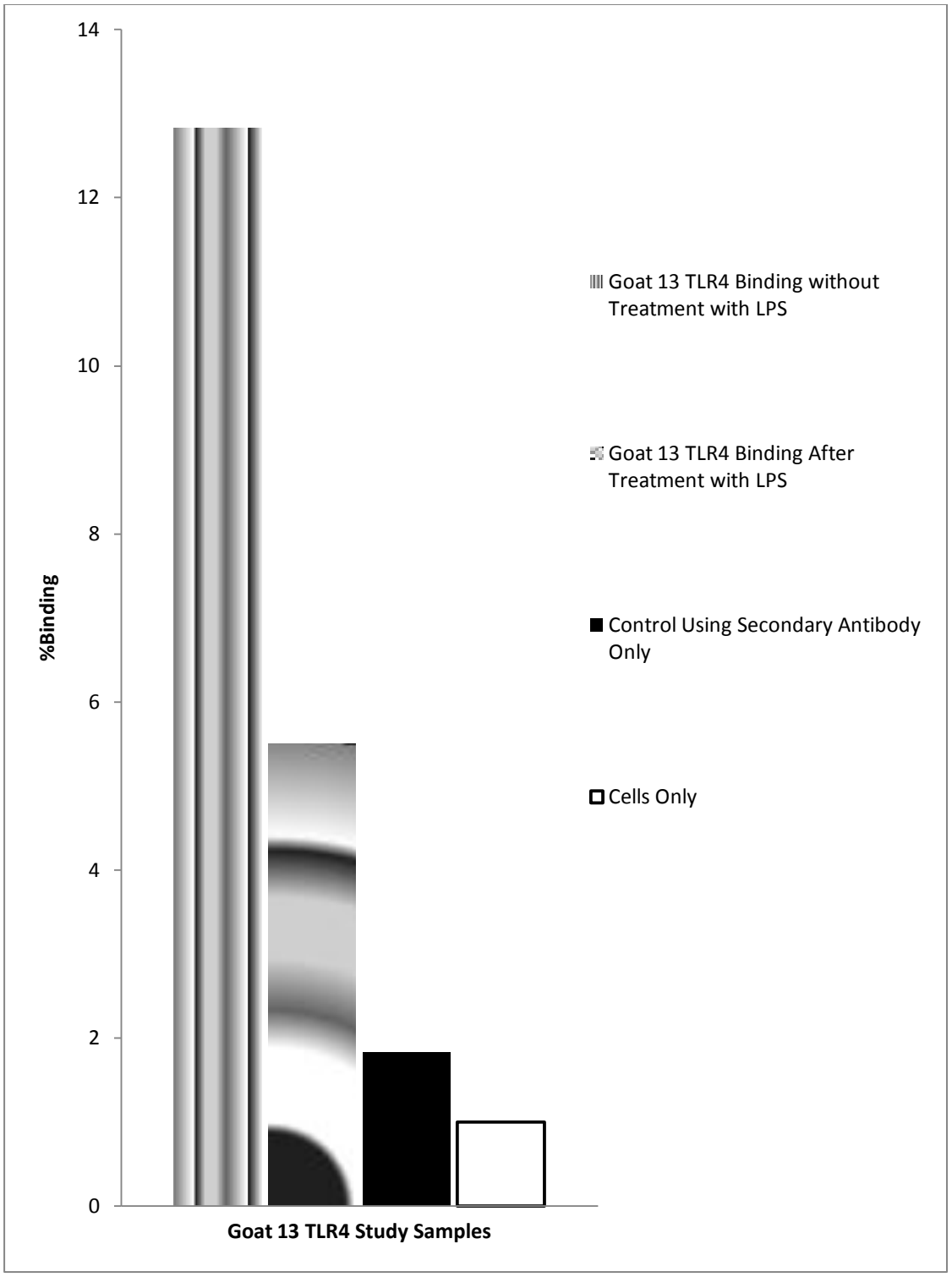


Figure 11. Binding of antibodies to TLR-4 to neutrophils of one goat

4.4 Secretion of IL-8 by Caprine Neutrophils

After the stimulation of the neutrophils isolated from three female Spanish/Boer crosses, TNF-α was determined to be transcribed within caprine neutrophils. One of the cytokines produced when TNF-α is transcribed is IL-8 cytokine a chemoattractant factor produced during an immune response (Hodgson, 2006). Neutrophils treated with and without LPS were spun down and their supernatant collected to test IL-8 presence using Enzyme-Linked Immunosorbent Assay specific to IL-8 cytokine. The first Assay ran was inconclusive due to low concentrations' of IL-8 produced by each individual sample.

To gain a better analysis of IL-8 production after stimulation with LPS samples from three Spanish/Boer crosses from treated groups were pooled together and the samples without treatment were pooled together to obtain higher concentrations. ELISA's of pooled treated and control samples showed that IL-8 production was increased after treatment. The study findings may be indicative that IL-8 is produced however it is produced in low quantities. Standards ranged from .009-.493 O.D.A standard curve was constructed and tested samples concentrations were documented by using the liner equation. The optical density ranges were recorded for all samples and an average was determined for both control and treated samples. Standard deviation was also calculated. The average measured pg/µl of treated serum samples from three Spanish/Boer crosses goats was 6.12 pg/µl with a standard deviation of 0.67 (Table 5). The average measured pg/µl for control samples from the Spanish X goats was 5.14 pg/µl with a standard deviation of 0.83 (Figure 12).

Table 5. Optical Density readings of IL-8 from control and Treated Samples.

	Optical Density
Goat Control	0.022 0.014 0.022
Control Average	0.019
Goat Treatment	0.022 0.012 0.016
Treatment Average	0.017
Standard Deviation of control	0.005
Standard Deviation of treatment	0.005

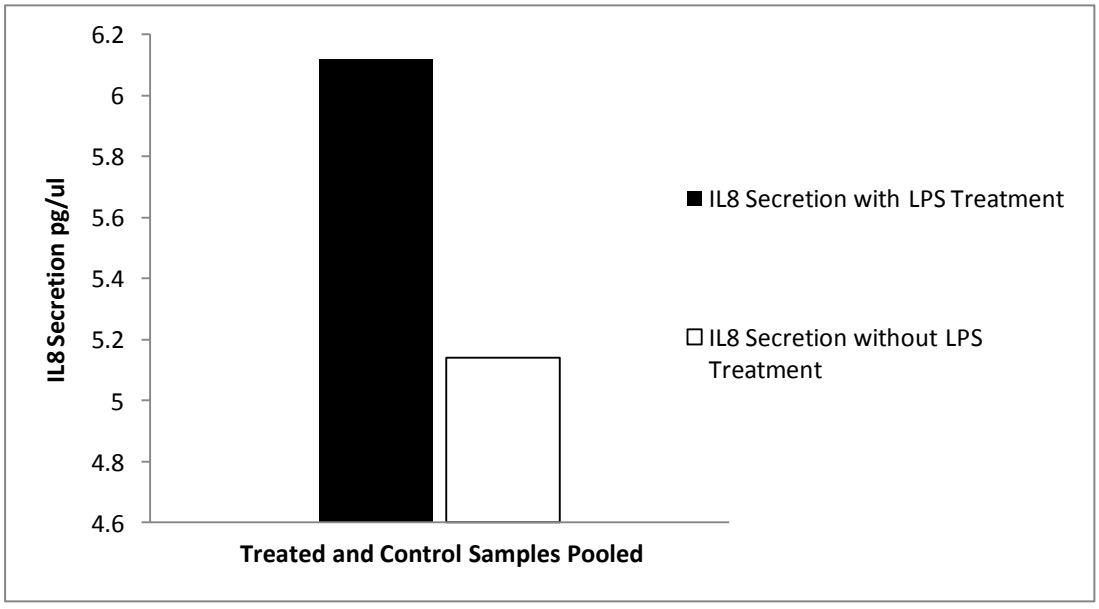


Figure 12. IL-8 cytokine secretion of samples pooled from supernatant of goat cells treated with LPS and control without LPS treatment

CHAPTER 5
CONCLUSION

Polymorphism in the gene encoding TLR-4 were observed in genomic DNA from Boer and Spanish goats Goat neutrophils are transcriptionally active cells that secrete IL-8 upon stimulation with LPS. Further studies are needed to conclusively determine the role of polymorphisms of TLR-4 gene in a goats' ability to fight off gram-negative bacterial infections, a larger sample size may be needed as well as specific reagents to goat TLR-4 and CD-14 receptors.

The techniques developed in this study to determine the impacts of gram negative bacteria on goat neutrophils may lead to valuable knowledge of the immune system of different breeds of goats. The effects of gram negative bacteria on goat herds can have great economical impacts on farmers. The growing resistance of bacteria to antibiotics currently in use raises many concerns. The importance of finding new ways of combating these pathogenic organisms is imperative in maintaining the growth in the goat industry. Many techniques are being studied that will eliminate the overuse of antibiotics in maintaining the health of farm animals. The ability to select a breed that can readily fight off infections and withstand harsh environment can help in farmers goals to obtain sustainability while being cost efficient. To determine if the Spanish and Boer goats have genetic resistance or better ability to fight off infections a broader sample group will need to be tested.

BIBLIOGRAPHY

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen Recognition and Innate Immunity. *Cell, 124*(4), 783-801.

Barber, M. R., Pantschenko, A. G., Hinckley, L. S., & Yang, T. J. (1999). Inducible and Constitutive In Vitro Neutrophil Chemokine Expression by Mammary Epithelial and Myoepithelial Cells. *Clin. Diagn. Lab. Immunol., 6*(6), 791-798.

Becton, Dinkonson, & Company. (2000, April). *Introduction to Flow Cytometry: A Learning Guide*. BD Biosciences.

Beutin, L., Geier, D., Steinruck, H., Zimmermann, S., & Scheutz, F. (1993). Prevalence and Some Properties of Verotoxin (Shiga-Like Toxin)-Producing Escherichia coli in Seven Different Species of Healthy Domestic Animals. *Journal of Clinical Microbiology, 31*(9), 2483-2488.

Brikos, L., & O'Neill, A. J. (2008). Signaling of Toll-like Receptors. *handbook of Experimental Pharmacology*, 1-40.

Broaddus, V., Boylan, A., Hoeffel, J., Kim, K., Sadick, M., Chuntharapai, A., & Hebert, C. (1994). Neutralization of IL-8 inhibits neutrophil influx in a rabbit model of LPS-induced pleurisy. *J Immunol, 152*(6), 2960-2967.

Browning Richard, J. (2009). Reproductive, Growth, And Fitness Traits Among Boer, Kiko, And Spanish Meat Goats Semi-Intensively Managed In The Southeastern Us. *Tropical and Subtropical Agroecosystems, 11*.

Burke, J. M., Kaplan, R. M., Miller, J. E., Terrill, T. H., Getz, W. R., Mobini, S., . . . Vatta, A. F. (2007). Accuracy of the FAMACHA system for on-farm use by sheep and goat producers in the southeastern United States. *Veterinary Parasitology, 147*(1-2), 89-95.

Burke, J. M., Wells, A., Casey, P., & Kaplan, R. M. (2009). Herbal dewormer fails to control gastrointestinal nematodes in goats. *Veterinary Parasitology, 160*(1-2), 168-170.

Cameron, M. R., Luo, J., Sahlu, T., Hart, S. P., Coleman, S. W., & Goetsch, A. L. (2001). Growth and slaughter traits of Boer x Spanish, Boer x Angora, and Spanish goats consuming a concentrate-based diet. *J. Anim. Sci., 79*(6), 1423-1430

.

Carlson, G., & Kaneko, J. (1973). Isolation of Leukocytes from Bovine Peripheral Blood. *Proceedings of the Society for Experimental Biology and Medicine, 142*(3), 853-856.

Contreras, A., Sierra, D., Sánchez, A., Corrales, J. C., Marco, J. C., Paape, M. J., & Gonzalo, C. (2007). Mastitis in small ruminants. *Small Ruminant Research, 68*(1-2), 145-153.

Cooper, M. G., & Hausman, E. R. (2007). *The Cell A Molecular Approach*. Washington: Sinauer Associates.

Correa, J. E. F., James G.; Kriese-Anderson, Lisa A. (1998). The Use of Sheep Breeds Resistant to Internal Parasites Retrieved June 14, 2010, 2010, from http://www.aces.edu/pubs/docs/U/UNP-0006/

Cortés, C., De la Fuente, R., Blanco, J., Blanco, M., Blanco, J. E., Dhabi, G., Orden, J. A. (2005). Serotypes, virulence genes and intimin types of verotoxin-producing Escherichia coli and enteropathogenic E. coli isolated from healthy dairy goats in Spain. *Veterinary Microbiology, 110*(1-2), 67-76.

Dauphinee, S. M., & Karsan, A. (2005). Lipopolysaccharide signaling in endothelial cells. *Lab Invest, 86*(1), 9-22.

Dick, E. P., Prince, L. R., Prestwich, E. C., Renshaw, S. A., Whyte, M. K. B., & Sabroe, I. (2009). Pathways regulating lipopolysaccharide-induced neutrophil survival revealed by lentiviral transduction of primary human neutrophils. *Immunology, 127*(2), 249-255.

Dzierzak, E., & Speck, N. A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nature Immunology, 9*(2), 129-136.

Elson, G., Dunn-Siegrist, I., Daubeuf, B., & Pugin, J. (2007). Contribution of Toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria. *Blood, 109*(4), 1574-1583.

Fitzgerald, D. C., Meade, K. G., McEvoy, A. N., Lillis, L., Murphy, E. P., MacHugh, D. E., & Baird, A. W. (2007). Tumour necrosis factor-[alpha] (TNF-[alpha]) increases nuclear factor [kappa]B (NF[kappa]B) activity in and interleukin-8 (IL-8) release from bovine mammary epithelial cells. *Veterinary Immunology and Immunopathology, 116*(1-2), 59-68.

Glimp, H. A. (1995). Meat goat production and marketing. *J. Anim Sci., 73*(1), 291-295.

Gorbet, M. B., & Sefton, M. V. (2005). LPS: The uninvited guest. *Biomaterials, 26*(34), 6811-6817.

Hodgson, J. C. (2006). LPS and Mammalian Host Responses During Experimental Disease. *Journal of Comparative Pathology, 135*(4), 157-175.

Hounzangbe-Adote, M. S., Paolini, V., Fouraste, I., Moutairou, K., & Hoste, H. (2005). In vitro effects of four tropical plants on three life-cycle stages of the parasitic nematode, Haemonchus contortus. *Research in Veterinary Science, 78*(2), 155-160.

La Ragione, R. M., Ahmed, N. M., Best, A., Clifford, D., Weyer, U., Cooley, W. A. Woodward, M. J. (2005). Colonization of 8-week-old conventionally reared goats by Escherichia coli O157 : H7 after oral inoculation. *J Med Microbiol, 54*(5), 485-492.

Lappalainen, T., & Dermitzakis, E. T. (2010). Evolutionary history of regulatory variation in human populations. *Human Molecular Genetics, 19*(R2), R197-R203.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics, 23*(21), 2947-2948.

McClenahan, D., Krueger, R., Lee, H.-Y., Thomas, C., Kehrli, J. M. E., & Czuprynski, C. (2006). Interleukin-8 expression by mammary gland endothelial and epithelial cells following experimental mastitis infection with E. coli. *Comparative Immunology, Microbiology and Infectious Diseases, 29*(2-3), 127-137.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat Rev Immunol, 1*(2), 135-145.

Nagai, Y., Garrett, K. P., Ohta, S., Bahrun, U., Kouro, T., Akira, S. Kincade, P. W. (2006). Toll-like Receptors on Hematopoietic Progenitor Cells Stimulate Innate Immune System Replenishment. *Immunity, 24*(6), 801-812.

NASS. (2010). *Sheep and Goats.* Washington, D.C.: United States Department of Agriculture Retrieved from http://usda.mannlib.cornell.edu/usda/nass/SheeGoat//2010s/2010/SheeGoat-01-29-2010.pdf.

Newton, S. E. (1995). Progress on vaccination against Haemonchus contortus. *International Journal for Parasitology, 25*(11), 1281-1289.

Paape, M. J., Contreras, A., & Ledbetter, T. K. (2004). Variation among goats in the ability of their polymorphonuclear neutrophil leukocytes and mammary secretions to support phagocytosis: inhibitory effects of milk fat globules. *Small Ruminant Research, 54*(3), 183-189.

Palermo, S., Capra, E., Torremorell, M., Dolzan, M., Davoli, R., Haley, C. S., & Giuffra, E. (2009). Toll-like receptor 4 genetic diversity among pig populations. *Animal Genetics, 40*(3), 289-299.

Pisoni, G., Moroni, P., Genini, S., Stella, A., Boettcher, P. J., Cremonesi, P., Castiglioni, B. (2010). Differentially expressed genes associated with Staphylococcus aureus mastitis in dairy goats. *Veterinary Immunology and Immunopathology, 135*(3-4), 208-217.

Pralomkarn, W., Pandey, V. S., Ngampongsai, W., Choldumrongkul, S., Saithanoo, S., Rattaanachon, L., & Verhulst, A. (1997). Genetic resistance of three genotypes of goats to experimental infection with Haemonchus contortus. *Veterinary Parasitology, 68*(1-2), 79-90.

Pruitt, K. D., Tatusova, T., Klimke, W., & Maglott, D. R. (2009). NCBI Reference Sequences: current status, policy and new initiatives. *Nucl. Acids Res., 37*(suppl_1), D32-36.

Purdy, C. W., Straus, D. C., & Hoover, M. D. (2007). Fever and leukocytosis responses in goats to inhaled LPS are dose-dependent. *Small Ruminant Research, 70*(2-3), 140-144.

Purdy, W. C., Straus, C. D., & Hoover, D. M. (2007). Fever and leukocytosis responses in goats to inhaled LPS are dose-dependent. *70*(2), 140-144.

Rainard, P., & Riollet, C. (2005). Innate immunity of the bovine mammary gland. *A Journal on Infection and Epidemology* 369-400.

Raetz, C., & Whitfield, C. (2002). Lipopolysaccharide Endotoxins. *Annual Review Biochemistry, 71*, 635-700.

Sahlu, T., Dawson, L. J., Gipson, T. A., Hart, S. P., Merkel, R. C., Puchala, R., Goetsch, A. L. (2009). ASAS Centennial Paper: Impact of animal science research on United States goat production and predictions for the future. *J. Anim Sci., 87*(1), 400-418.

Schumann, N., Schröder, W. J., & Ralf, R. (2005). Single nucleotide polymorphisms of Toll-like receptors and susceptability to disease. *Lancet Infect,* 1-9.

Tafti, A. K., & Mansourian, M. (2008). Pathologic lesions of naturally occurring coccidiosis in sheep and goats. *Comparative Clinical Pathology, 17*(2), 87-91.

Werling, D., Jann, O. C., Offord, V., Glass, E. J., & Coffey, T. J. (2009). Variation matters: TLR structure and species-specific pathogen recognition. *Trends in Immunology, 30*(3), 124-130.

White, E. C., & Hinckley, L. S. (1999). Prevalence of mastitis pathogens in goat milk. *Small Ruminant Research, 33*(2), 117-121.

Yang, W., Zerbe, H., Petzl, W., Brunner, R. M., Günther, J., Draing, C.Seyfert, H.-M. (2008). Bovine TLR2 and TLR-4 properly transduce signals from Staphylococcus aureus and E. coli, but S. aureus fails to both activate NF-[kappa]B in mammary epithelial cells and to quickly induce TNF[alpha] and interleukin-8 (CXCL8) expression in the udder. *Molecular Immunology, 45*(5), 1385-1397.

Zhou, H., Hickford, J., & Gong, H. (2008). Allelic variation of the caprine TLR-4 gene identified by PCR-SSCP *Molecular and Cellular Probes*, 65-66.

Zhou, H., Hickford, J. G. H., & Fang, Q. (2005). Polymorphism of the DQA2 gene in goats. *J. Anim Sci., 83*(5), 963-968.

APPENDIX A REAGENTS USED

Collection of Blood and Fecal samples

1. Acid Citrate Dextrose (ACD)

For 500 ml

22g trisodium citrate (anhydrous)

8 g citric Acid

25g dextrose

Add sterile water to bring to 500 ml, autoclave and store at 4°C. Use 1 ml of ACD/9 ml of blood

2. Fecal Egg Solution

For 1000 ml

400g Sodium Chloride

Add sterile water to bring to 1000ml. Stir thoroughly before use.

Analysis of DNA

3. Agarose Gels

1% Gel

.5g Agarose

50 ml water

Microwave contents to dissolve agarose, stopping every 30 seconds to avoid boiling.

4. 10XTBE buffer Stock solution

For 1000 ml

108g Tris base

55g Boric acid

40mls 0.5M EDTA (pH 8.0)

While stirring add ddH_2O to bring to final volume of 1000ml. To create 1X solution dilute 10X (1ml of 10XTBE added to 9ml of ddH_2O)

<u>14% Polyacrlylimide Gel SSCP PAGE</u>

5. 0.5X TBE buffer 4L

0.2ml 10X solution

Add sterile deionized water to 4L.

6. 10% Ammonium persulfate (APS)

100mg APS

1ml deionized water

7. 1.5M Tris-HCL pH 8.8 (100ml)

18.165g of Tris

100ml sterile deionized water

Adjust pH of Tris by adding HCL. Add half of water before adjusting pH then add the rest of the water to get a final concentration of 100 ml.

8. 10% Sodium Dodecyl Sulfate (SDS)

50g SDS

500ml of sterile deionized water

9. DEPC treated water

1ml DEPC

1L distilled water

Add DEPC to water and mix vigorously live for four hours then autoclave.

10. TEMED

50µl TEMED

PMN Isolation

11. 0.0132M Phosphate Buffer Saline (PBS) solution pH(7.4)

- Potassium Phosphate Monobasic,KH_2PO_4

Formula Weight=136.09

136.09X0.0132= 1.796 g/L

- Potassium Phosphate Dibasic, K_2HPO_4

Formula Weight=174.183

174.183X0.0132=2.299 g/L

Make 2 liters of K_2HPO_4 and 1 liter of KH_2PO_4 in 0.85% Saline to make 1500ml of buffer at a time. Use the KH_2PO_4 to bring pH down to 7.4.

For 16 Liters

36.784g K_2HPO_4

136.000g NaCl

Bring volume up to 16L with sterile water, autoclave and store at 4°C.

12. 2.7%Saline

For 1 Liter

27g NaCl

Add sterile water to bring up to 1000ml, autoclave and store at 4°C.

DEPC treated water will also be prepared look number 9 at preparation under 14% acrylamide reagents.

Silver Stain Reagents

13. Fixative (40% methanol)

For 400ml

160ml of methanol

240ml of deionized water

14. Fixative (10% ethanol)

For 800ml

80ml of ethanol

720ml of deionized water

15. Oxidizer (potassium dichrommate and nitric acid) Store at 4ºC

For 200 ml

20 ml of oxidizer

180 ml of water

16. Silver Reagent (silver nitrate) Store at 4ºC

For 200ml

20 ml of silver reagent

180 ml of water

17. Developer (sodium carbonate and paraformaldehyde)

For 700 ml

19.2 g

Add 19.2g to 600 ml of deionized water to dissolve than add up to 700ml

18. Stop solution (5% acetic acid)

For 400ml

20ml of Acetic acid

380ml of deionized water

APPENDIX B PROTOCOLS

The protocol of running SSCP gels for TLR-4 (Modified Method from John Hickford)

I. PCR Reaction

The following recipe is generally used:

Master Mix: 25 µL reaction x sample numbers

1x Reaction Buffer (Qiagen, Hilden, Germany)

Primer up (0.25 µM)

Primer dn (0.25 µM)

MgCl2 (1.5 mM)

dNTPs (150 µM)

dH2O

_ Taq DNA Polymerase (0.5 unit) __

The following parameters are used: 1 cycle of 94°C for 2 min., then 35 cycles of 94°C for 30 sec, 60˚C for 30 sec., 72 ˚C for 30 sec.

Examine your results on a 1 % agarose gel.

2. Setting PROTEAN II Xi cells

Take out combs carefully to avoid damaging top edge of gel.

Assemble two precast gels into chamber. Fill 0.5x 500 mL TBE buffer into upper buffer chamber and 2 L 0.5 x TBE buffer into lower buffer chamber (PROTEAN II Xi cells (Bio-Rad Laboratories))

Turn on the power supply for electrophoresis and water circulation

Pre-run for 150V, 30 min

To avoid the power supply stops, check if buffer leak occurs

3. Preparing Samples for Loading into SSCP gels

Make sure that the order of the samples and lanes on the gel are all written down on a data sheet**.** The left-right orientation of the gel can easily get confused in later gel handling steps.

1. Combine 30 μL of loading dye solution with 15 μL of the PCR products

2. Heat the reactions at 100°C for 5 min to denature the DNA.

3. Place the reactions immediately on ice until ready to load gel.

4. Carefully load 10 μL of each sample into the wells created by the comb.

One of the most important variables influencing how the PCR products will migrate in the gels is temperature. Therefore, it is important to use a power supply that monitors the gel temperature and allows for gels to be run at a constant temperature.

Run for 300V, 4 hours

4. Taking off, Staining, drying gels

Turn off power source and disconnect wires

Drain buffer and disassemble the glass plate

Staining using silver-staining method

Instrument and chemicals

1. Electrophoresis instrument

PROTEAN II Xi cells (Bio-Rad Laboratories)

14% PAGE Precast Gels (Jule Inc, Milford, CT)

2. Loading dye for PCR samples

98% formamide

10mM EDTA

0.025% bromophenol blue

0.025 xylene-cyanol

3. Preparation of samples: 15 µl PCR product + 50 µl loading dye needs to be high to insure there is no reannealing of separated DNA strands.

5 minutes denaturation then 7.5 µl for loading samples

4. Running condition for SSCP gels

Core water circulation: 4 ˚C (Julobo, FC 1600T water circulation)

Room temperature: 7.5 ˚C (Air-conditioned)

2 L of 0.5x TBE buffer (10x TBE 100 ml in 2L Distilled Water): Lower buffer chamber

500 ml of 0.5x TBE buffer: Top loaded buffer

5. Electrophoresis

Pre-run condition: 150V, 30 minutes

Running condition: 300V, 4hrs

Flow Cytometry

PMN Isolation Protocol

1. Blood was poured into one 50 ml conical tube and maintained on ice.
2. Centrifuge the blood for 10 minutes at 3500 RPM at 1800g at 4°C in a swinging bucket rotor.(Eppendorf Model 5810RCentrifuge)
3. Aspirate the plasma, buffy coat and 1/3 of the RBC layer off using a vacuum. The left over blood will contain the PMN.
4. Replace caps and gently invert tubes to resuspend PMN/red cell layer (10ml remaining) and immediately add 20 ml of ice cold dH_2O. Mix by inverting and rotating tube for 45 sec. to lyse red blood cells.
5. Add 10 ml of ice cold 2.7% saline to restore isotonicity, and mix gently as before. Keep cells cold by returning tubes to ice periodically.

6. Immediately centrifuge suspension for 2 min @ 2000 RPM (700g) @ 4°C and pour off supernatant.

7. Add 10 ml of ice cold PBS (pH 7.4) to cell pellet and gently resuspended cells by rotating tube manually. Repeat lysing with 20 ml of dH_2O (steps4-6) to clear pellet. More than two repeats may be needed to obtain a white (PMN) pellet.

8. After last addition of PBS, combine cells (if more than one tube), centrifuge as before and re-suspend in 5 ml or 10 ml of PBS depending on the size of the pellet.

    - Check viabilities by Trypan Blue Stain exclusion and make cell smears for Wright Stain differential cell counts to asses cell prep purity.

    - Cell Counts can be calculated by using a Hematocytometer or the Beckman Coulter Counter.

Hematocytometry

1. Obtain a uniform suspension of cells. Pipette the cell suspension up and down in the tube 5-7 times using a pipette with a small bore (5ml or 10ml pipette).

2. Prepare a 1:2 dilution of the cell suspension in trypan blue. Approximately 20 microliters of cell suspension will be required to charge the chambers of the hemacytometer. Prepare 30-50 microliters of trypan blue-diluted cell suspension. In a conical tube add 15 microliters of trypan blue solution. Gently swirl the cell suspension by finger vortexing the cell suspension and remove 15 microliters of trypan blue in the microfuge tube. Pipette up and down several times to ensure a uniform cell suspension using the same pipette tip.

3. Load Hemacytometer. Place the cover slip over the counting chambers. Load both counting chambers with 10 microliters of diluted cell suspension using a micropipette and tip.

4. Determine the number of cells (total and viable). View the cells under a microscope at 100x magnification.

5. Prepare another Blood Smear with the isolated PMN.

Calculate PMN for treatment with LPS

1. Treat isolated cells with RD and 011 forms of LPS at 100 ng/ml and PBS control for 30 minutes at 37°C in the presence of 5% CO_2 and 90% humidity.

2. Wash cells in PBS solution by centrifugation at 500X g. Resuspend in 250 µl of cell buffer

Treatment with CD-14 PE labeled

1. Neutrophils were incubated with anti- CD-14 antibody at 5 µl/ 10^6 cells made in cell buffer for 30 minutes at 4°C.
2. The cells were washed in 1 ml PBS solution at 500X g and then resuspended in 100ul of cell buffer.

Treatment with TLR-4 and secondary antibody IgG conjugate APC labeled

1. Neutrophils were then incubated with anti-TLR-4 monoclonal antibody at 2-3 µg/10^6 cells made in cell buffer for a further period of 25 minutes at 4°C with periodic shaking in between.
2. Cells are then washed and resuspended in 250 µl of cell buffer.
3. Then cells were incubated with 2-3 µg /10^6 cells Allophycocyanin conjugated goat anti-mouse IgG reconstituted in cell buffer for 25 minutes at 4°C.
4. The cells were washed at 500X g and then resuspended in 100µl of cell buffer.

Fix Cells

1. Cells were fixated in 2% paraformaldehyde using 4% paraformaldehyde diluted in PBS obtained from USB Corporation (USB Corporation, Ohio).

Silver Staining

1. Obtain a clean glass container that is wide enough to hold gel and liquid inside. Wash several times with deionized water.

2. Prepare a fixative of 40% methanol and add to glass container. Immediately after electrophoretic run is complete, transfer gel to the glass container and gently remove the glass on the gel. (The liquid solution should help and removing glass slides off of gel).This is a convenient stopping point. Gels can be stored indefinitely at this step but must at least sit for 60 minutes. Continue to step three when ready

3. Prepare all other reagents the day you plan to silver stain. Remove 40% methanol fixative solution from glass container, making sure not to touch the gel. Add 10% ethanol fixative solution to the gel and live for 30 minutes. (Make sure Gel is completely immersed in solution.)

4. Remove the 10% ethanol fixative solution and replace with new 10% ethanol fixative solution with minimal touching to the gel. Leave for 30 minutes.

5. Remove the 10% ethanol fixative solution and add 200ml Oxidizer solution to the gel. Leave on for 10 minutes.

6. Remove Oxidizer solution from gel and place into a waste bottle. Immediately wash with 400ml of deionized water for 10 minutes on a belly dancer. Repeat washing the gel until all yellow color is removed from the gel.

7. After final wash add 200ml of Silver reagent to the glass container. Leave on for 30 minutes.

8. Remove silver reagent in place in waste container. Wash gel with 400ml of deionized water for two minutes.

9. Immediately add 200 ml of Developer to gel glass container for 30 seconds. (A brown precipitate will form. Remove Developer from gel and replace with new developer.)Shift gel in glass container with developer liquid inside until brown bands start to appear replacing when smokey brown precipitate forms. (It may take a long time for bands to develop on gel.)

10. Add 5% acetic acid stop solution to gel to stop development and prevent unwanted darkening of gel.