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Comparative Study Of Sheep And Goat Innate Immune Responses On Pasture

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List of Abbreviations

ACD	Acid Citrate Dextrose
ºC	Degrees Celsius
cDNA	Complementary DNA
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
epg	Eggs Per Gram
FEC	Fecal Egg Count
FECRT	Fecal Egg Count Reduction Test
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GIN	Gastrointestinal Nematodes
GCSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
Gt.	Goat
hr.	Hour
IFNr	Interferon Production Regulator
IL-1A	Interleukin 1 Alpha
IL-8	Interleukin 8
IP-10	Interferon gamma-induced protein 10
Kg	Kilogram
L1	First Stage Larvae
L2	Second Stage Larvae

L3	Third Stage Larvae
L4	Fourth Stage Larvae
µg	Microgram
µl	Microliter
min.	Minutes
ml	Milliliter
NCATSU	North Carolina Agricultural and Technical State University
NRAMP1	Natural Resistance Associated Macrophage Protein one
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PROC GLM	General Linear Model Procedure
RANTES	Regulated upon Activation Normal T-cell Expressed and Presumably Secreted
RNase	Ribonuclease
rpm	Rotations per minute
sec	Seconds
Shp.	Sheep
spp	Species
SSCP	Single Strand Conformational Polymorphism
TLR-2	Toll-Like Receptor 2
TLR-4	Toll-Like Receptor 4
TNF-α	Tumor Necrosis Factor-Alpha

Abstract

The objective of this study was to evaluate the innate immune response in goats co-grazed with St. Croix sheep. Boer goats (20) and St. Croix sheep (10) were co-grazed on the same pasture. A second group of Boer goats (10) were grazed on a separate pasture. On days 0 and 56 of the study body weights, FAMACHA© scores, blood (for packed cell volume and white blood cell differentials) and fecal samples (for fecal egg counts) were collected and the data analyzed using SAS PROC GLM. To evaluate the goat's infection status blood from sheep and goats with low infection (7) and goats with moderate infection (7) to measure cytokine and immunoglobulin concentrations in serum and to identify and measure the expression of genes (TLR-2, TLR-4 and NRAMP1) through PCR followed by single strand conformational polymorphism analysis. The extra-label use of Cydectin (Moxidectin) was assessed on goats resulting in a 90% efficacy in the reduction of *H.contortus* and 81% in coccidia. There were no differences in FAMACHA© scores or PCV's between the sheep and goats ($p>0.05$). Sheep had lower FEC's than the goats they were co-grazed with ($p<0.05$). Goats non co-grazed had higher levels of neutrophils ($p<0.05$). Sheep had higher levels of eosinophils than the goats ($p<0.05$). Proinflammatory cytokines and immunoglobulins were secreted in both the sheep and goats. All genes studied were detected in genomic DNA and expressed in blood. Single strand confirmation polymorphism analysis showed a different pattern of migration for the sheep TLR-2 compared to goat. The differences in eosinophil counts, GM-CSF production levels and the TLR-2 gene between the GIN resistant sheep and susceptible goats may offer markers for selection to improve host genetic resistance against GIN in goats.

CHAPTER 1

Introduction

Sustainable agriculture systems are more important now than ever, as the world population has reached an all-new high. This new dilemma will push many agriculture systems to their limits lowering the quality of their products and increasing potential for creating more hunger due to the shortages in supply (Goodland, 1997; Godfray et al., 2010). To ensure that food does not become a privilege-commodity, scientists are exploring new approaches with sustainable resources and innovations within these essential agricultural sectors to accommodate for and stabilize supply in this era of a rising population (Godfray et al., 2010). Ruminants such as cattle, sheep and goats can make significant contributions to sustainable production systems by utilizing products from non-arable land (Oltjen and Beckett, 1996).

Ruminants are valuable because they have the ability to breakdown cellulose consumed from pastures and by products and convert them (Oltjen and Beckett, 1996; Keys, Von Soest and Young, 1969). Although, these animals require low quality products for maintenance they are still subject to many varied ailments, diseases and parasites (Glimp, 1995). The rise in meat goat production in the United States can be attributed to practical production practices and goats are excellent meat converters of less preferred forages (Engle et al., 2000). According to the United States Department of Agriculture (USDA) National Agricultural Statistics Service (NASS) three million heads of goat were accounted for in the United States in the 2010 sheep and goat report. The recent increase in goat production also results from the rising demand by increase numbers of ethnic groups into the U.S. who favor goat meat and goat products (Engle et al., 2000). This new trend has brought about new opportunities to grow and sustain goat production in the U.S. (Nadarajah, 2010).

Gastro intestinal nematodes or parasites (GIN) are an important limiting factor in goat production systems globally, and especially in humid climates (Terrill et al., 2007). There are many parasites that cause an array of health problems in sheep and goat production, but none more significant than *Haemonchus contortus* and coccidia *Emiria spp* (Miller and Horohov, 2006). Due to overuse and incorrect application of anthelmintics to control parasitic infection, the results have been increases in anthelmintic resistance weakening many drug programs to control infection (Miller, 2000). This unfortunate occurrence has led to the search for alternative approaches including the use of copper oxide wire (Burke et al., 2009), tannins (Kimambo et al., 2002), improved farm management practices (Zajac, 2002), Introduction of breeding schemes for resistance (Mandonnet et al., 2001), and employing the FAMACHA© system (Zajac, 2002; Schoenian and Semler, 2006). Another growing threat is from studies highlighting resistance in nematodes and genetic variations in the host immune response to parasitic infection (Bishop et al., 1994, Terrill et al., 2001; Vanimisetti et al., 2003).

The innate immune system is the primary responder to non-specific pathogens and is governed by phagocytic dendritic cells and macrophages. The responsibilities of the innate immune system include the ability to differentiate between self (host) and foreign pathogens (Akira, 2006). St. Croix sheep have been reported to be naturally resistant to *Haemonchus contortus* (Zajac, 2002). Assessing the innate immune response of the St. Croix sheep to *Haemonchus contortus* permits the evaluation of certain immunological events which could aid in the identification of immune markers. Due to the lack of genetic studies performed on meat goats (Sahlu et al., 2009), this study could uncover possible immunological genetic polymorphisms, which could affect the performance and health of goats susceptible to gastrointestinal nematodes (Sahlu et al., 2009). This research study evaluates GIN infection and

the immune response experienced by Boer goats and the reported parasite resistant St. Croix sheep.

The findings of this study could possibly aid in the improvements of GIN control by identify the key immunological cellular components involved in the innate immune response between the two species of animals. Furthermore, this research could help strengthen GIN management practices in the United States meat goat industry as it continues to develop. The objectives of this study were to evaluate and compare the innate immune response of goats co-grazed and non-co-grazed with sheep on pasture. Further, an evaluation of the effectiveness of the extra label use of Cydectin® (Moxidectin) in small ruminants under co-grazed management practices in controlling gastrointestinal parasites was evaluated.

CHAPTER 2

Literature Review

2.1 Goat Production

For centuries man has depended upon goats for food, clothing, agricultural production, and environmental control (Solaiman, 2007). Goats are favored by small farmers because of their ability to adapt to almost any climate and convert forage into quality meat products (Solaiman, 2007). The attribute of goats being able to feed on otherwise non-production vegetation such as shrubs and rangeland makes them a very attractive animal over other livestock to start small farms with little capital investment (Morand and Fehr et al., 2004). In 2005, the Food and Agriculture Organization of the United Nations (FAOSTAT) reported that China, India, and Pakistan produced over 60% of the world's goat meat. In the same period, the U.S., China, and Italy were the top importers of goat meat with imports of over 31% of the 52,477 metric tons exported worldwide.

The recent rise in goat production and products in the U.S. can be directly attributed to the effects of an expanding market aligned with the on-going increase in foreign-born ethnic groups, migrating to the U.S. with a preference for goat meat and products (Solaiman, 2007). Additionally, goat meat is a wholesome and lean meat and it can be a beneficial meat-substitute for people seeking healthier diet alternatives (Solaiman, 2007). Due to the increased number of consumers of goat products, growth in demand can be projected to follow (Nadarajah, 2010). Although this new facet of agriculture is growing it faces many challenges due to the lack of organization and structure in the goat market (Nadarajah, 2010). This growth still brings many opportunities to improve and sustain the goat market as the demand for goat meat and other by-products increases. Improving selective genetic schemes and nutrition within marketed goat

breeds will be an important factor to the growth and future of the United States goat industry, as structure is gained (Nadarajah, 2010).

Historically, the goat industry evolved based on use. Goat production focused on specific goat products such as: meat, milk, hair, skins, fiber and by-products. Goats vary in sizes, colors and breeds, each with distinguishable characteristics. There are more than 300 distinct breeds of goats in the world. They fall into four categories: (1) Dairy, (2) Meat (3) Mohair and (4) fiber and other uses. The tolerance to prevailing environmental conditions, combined with consumer or market demand have dictated which goat breeds are produced. Meat goat breeds that were imported to the U.S. includes the African Boer goat which was developed in South Africa after the turn of the 20th century and imported into the U.S. during the 1990s (Nye et al., 2002). These animals were bred for superior weight gain and lean meat. They are also known for their humble disposition along with them being poly-estrus and short kidding cycles (Glimp, 1995).

The Kiko breed was developed in New Zealand from crossbreeding a combination of feral does with bucks with established breed lines. The Kiko meat goat was also imported into the U.S in the 1990s to improve the meat goat market (Browning, Leite-Browning and Byars, 2011)

Spanish goats were established by Spanish pioneers and Texas settlers (Nye et al., 2002). Spanish goats have been primarily used for brush control on pastures and they got their start are found mainly around Central Texas (Nye et al., 2002). The Spanish goat has not always been bred for meat production, but due to growing interest in goat meat and by-products has led to producers looking for ways to strengthen carcass production traits through cross breeding practices, especially with the South African Boer (Cameron et al., 2001; Nye et al., 2002).

2.2 Sheep Production

Sheep have also been used for food and clothing throughout the evolution of man and agricultural systems. Although, sheep for many years have been used for many purposes, there has been a global decrease in the demand for sheep products including their meat (Zygoyiannis, 2006). This decline has been caused by many factors such as advancements in artificial fibers reducing the significance of the main purpose of sheep production for wool. This decreased demand for wool directly led to the reduction in sheep meat production because it is considered a by-product after wool. The decline in sheep production is also attributable to many factors including, advancements in poultry production, low prices for sheep products, high prices of cultivated forages, and the large amounts that must be consumed by the animal for meat conversion (Boutonnet, 1999).

Since profit is the main objective of business, the lack of cheaper resources and feed slowly led to declining sheep production worldwide while furthering the loss of an alternative meat source (Morris, 2009). In the U.S. sheep production is predominantly practiced in the mountain and pacific regions as well as the southern plains (Jones, 2004). With the development and importation of certain breeds of sheep, there has been a new opportunity in sheep production in the southeastern US (Howell et al., 2008). This new growth is due to the availability of hair sheep that originated in the tropical regions of Africa, South America and the Caribbean. This tropical origin makes it easier for the hair sheep to adapt to southern climates and perform well in humid conditions (Wildeus, 1997). The St. Croix hair sheep originated from the Virgin Islands. They are poly-estrus and usually give birth to twins. They are useful for pasture management and are known for their capability to adapt to tropical conditions (The American Livestock Breeds Conservancy, n.d.). The St. Croix sheep have also been reported to be

parasite-resistant in many studies and are useful in co-grazing practices to control parasitic infections (Zajac, 2002).

2.3 Gastrointestinal Parasites

Internal parasitism is of great concern impacting goat and sheep production systems all over the world, including Africa (Faye et al., 2002), Australia (Olayemi et al., 2011) and Europe (Bauer, 2001). This is also a problem within the US, especially the southeastern region due to its warm and humid climate (Terrill et al., 2007). Parasitism causes economic loss, poor feed conversion, severe anemia and sometimes death (Kumba, 2002). Ailments due to parasitic infection directly impact the quality of the milk, skin and meat of the animals creating a decrease in profit (Knox, 2006). Susceptibility to parasitism is more common in younger sheep and goats due to their lack of a fully developed immune system (Patra, 2007). Challenges to production due to internal parasites are considered chronic when impacting reproduction, weight gain and causing depression in the animals (Miller and Horohov, 2006). The prevalence of internal parasites and their distribution depends on the seasonal climate and is more common in goats. Internal parasites usually affect young lambs and adults during periods when the animal is experiencing stress (Torina et al., 2004; Vlassoff and McKenna, 1994). The most common species of GIN parasites affecting sheep and goats are: nematodes (N) and protozoans (P) such as: (i) *Haemonchus contortus*, (N); (ii) *Telodorsagia circumcinta*, (N); (iii) *Trichostrongylus colubriformis*, (N); (iv) *Cooperia* spp., (N) and (v) *Eimeria coccidia*, (P) (Miller and Horohov, 2006). The species that cause the most severe damage and concern are the nematode *Haemonchus contortus* and protozoan *Eimeria spp* (Miller and Horohov, 2006). Although sheep and goats are infected with the same species of parasites most are usually host specific which

prevents cross contamination. However, pastures contaminated with droppings from infected animals have been cited as a key source for ruminant GIN infection (Zajac, 2006).

Haemonchus contortus (barber pole worm) is a nematode parasite that severely affects ruminants, especially goats. The parasite has the ability to consume 0.05 mL of blood daily from its host (Qamar et al., 2009). This process reduces blood volume resulting in anemia. The infected goat develops weakness, bottle-jaw, depression, loss of condition, and sometimes eventually death (Mir et al., 2008). Infection occurs when goats ingest third stage larvae produced by the adult female parasite. The goats come into contact with the larvae where fecal deposits occur on grazing grounds or from watering points contaminated with *Haemonchus contortus* or from other infected animals (Luginbuhl, 2000).

The prevalence and distribution of parasitism depends on rainfall and humidity (Qamar et al., 2009). The life cycle of *Haemonchus contortus* consists of four stages. The first larval stage (L1), eggs are released from female worms and are excreted by the host. Next, eggs germinate and molt into (L2) second stage larvae. During the third stage or the larvae stage 3 (L3), fecal pellets containing the eggs that, under the optimal environmental conditions such as high rainfall and humidity, lead to the emergence of the larvae from the pellet. The latter environmental conditions are most prevalent in the summer and early fall. During the infection phase, the larvae emerge from the pellets and rest on blades of grass to potentially be consumed by a host. Next, the third stage larvae shed the cuticle and enter the gastrointestinal tract, attach and penetrate the lining of the abomasum of the host (Huchens, 2004).

The fourth and final stage (L4) of the *Haemonchus contortus* life cycle is the reproduction stage. After the larvae penetrate the abomasum (true stomach) there is molting for a fourth and a fifth time to transition into the adult phase. Males and females feeds off of the

host blood in the abomasums and reproduce with the female laying 5,000 to 10,000 eggs per day passing through the feces to the external environment (Zajac, 2006). The total process of the *Haemonchus contortus* life cycle, from the L1 to the L4 stage, takes roughly three weeks (Luginbuhl, 2000).

Coccidiosis is a parasitic disease that affects the gastrointestinal tract of many animals. This disease is caused by *Eimeria* spp. that are protozoa (coccidia) naturally occurring in soils worldwide. Some species of the parasite have higher pathogenicity than others. Coccidia are unique to particular host and do not cross-infect different species of host (Schoenian, 2011). The symptoms of coccidiosis are dehydration, weakness, anorexia, fever, diarrhea, and death if not treated. The parasite causes harm to the host by destroying the mucosal lining of the abomasum depleting nutrient up-take, and leading to liver failure in dairy goats (Khan and Line, 2010). Goats are susceptible to *Eimeria* infection under conditions of stress, poor nutrition, and when poor farm management is being practiced on grazing grounds. Coccidia affect kids more than adults due to an immune system that is not fully developed. The coccidia that are more pathogenic in goats are *E. arloingi*, *E. christenseni*, and *E. ovinoidalis* (Kahn and Line, 2010).

In sheep coccidia infection is more prevalent in lambs than the adults also due to an immature immune system (Schoenian, 2011). The *Eimeria* species that are most pathogenic in sheep are *E. ahsata*, *E. ovinoidalis* and *E. bakuensis* (Levine, 1985). Symptoms include diarrhea, fever, weight loss, poor quality wool coat and death (Khan and Line, 2010). The life cycle of coccidia consists of several different stages. According to Dedrickson (2009), the cycle begins with the ingestion of a mature egg called an oocyst, which consists of four sporocysts and two sporozoites. The oocyst is consumed by the host and is opened with contact with enzymes of the digestive tract that leads to the expulsion of eight sporozoites. Next, the sporozoites

navigate to the small intestines and reproduce asexually individually producing up to 120,000 first generation merozoites that are expelled when the cell ruptures.

Another asexual division takes place in the upper large intestines and the lower small intestines. Then, the merozoites invade the large intestines where they are characterized as male (microgametes) and female (macrogametes) for the sexual phase of their life cycle. Zygotes are produced when the macrogametes and the microgametes mate and they develop a protective shell and become oocyst. This process fractures the host's intestinal cell and the oocyst is passed along with waste and other debris in the host feces during the non- infectious sporulated stage. Once the oocyst reaches the external environment and is exposed to oxygen, sporulation occurs whereby the oocyst becomes infectious if ingested by the appropriate host.

2.4 Diagnosis of Gastrointestinal Nematodes

The diagnosis of animals experiencing parasitic burdens depends heavily on the observation of clinical symptoms associated with parasitic infection, especially with coccidia and *Haemonchus.* The most common symptoms include weight loss, diarrhea, coarse hair and high fecal egg count (FEC). Sheep and goat infected with *Haemonchus* with a FEC greater than 1000 epg indicates heavy infection while 500 epg signifies mild infection (Taylor, 2010). Oocyte counts of 5000 oocytes per gram or more in feces of host suspected of coccidia infection is indicative of clinical infection (Schoenian, 2011).

Haemonchus contortus causes the only parasitic infection that can be determined by methods other than laboratory testing (Waller and Chandrawathani, 2005).

The current methods for the diagnosis of *Haemonchus* infection includes the utilization of packed cell volume and the FAMACHA© card system to assess anemia and the McMaster egg counting technique to determine the levels of parasitic burden. Since anemia is the most

detrimental effect of *Haemonchus* infection, it is very important to evaluate hematocrit levels periodically to diagnose parasitic infection (Miller, 2000). This method is effective in assessing anemia probably caused by parasitism in sheep and goats (Khan and Line, 2010). Another method for diagnosing the impact of *Haemonchus* infection is the use of the FAMACHA© card system. The system was developed in South Africa for the classification of animals into categories based on levels of anemia caused by gastrointestinal parasites (Vatta et al., 2000; Ejlertsen et al., 2006; Reynecke et al., 2011). This method is based on a chart using a scoring system for the animal's level of anemia based on the color of the animal's ocular membrane (1 to 5), with 1 and 2 being normal value and 3 through 5 indicating moderate to high FEC and therefore action should be taken to treat the animal. This system is widely used and is accepted by sheep and goat producers in the southern U.S. (Malan et al., 2001; Burke et. al., 2007).

Figure 1. FAMACHA© anemia guide. (Kaplan et al., 2004).

Fecal egg counts using the McMaster technique are used to evaluate and quantify parasitic burden. The McMaster technique is a rapid, simple, quantitative approach for counting parasite eggs in ruminant feces, based on flotation in a concentrated salt solution (Miller, 2007).

2.5 Treatments

Methods used to control parasites include chemical controls such as: anthelmintics or dewormers, which are drugs that expel parasites through killing or stunning actions.

Anthelmintics include three families of drugs for which there are several different commercial names, but are grouped by their chemical structure and target of disruption in nematodes (Schoenian, 2008). The three classes of drugs are (i) Benzimidazoles, (ii) Imidazothiaoles/Tetrahydropyridines and (iii) Macrolytic lactones. Benzimidazoles kill nematodes by binding to beta tubulin which disrupts energy metabolism in the parasites leading to starvation and death. Imidazothiaoles/Tetrahydropyrimidines kill parasites by causing paralysis, which contributes to the decrease in the ability of the parasite to attach to the host's intestinal wall.

Ultimately, this action prevents them from feeding resulting in the parasite's death (Schoenian, 2008). Macrolytic lactones effectuate death in parasites by disrupting their γ-aminobutyric acid (GABA)-mediated neurotransmission which is responsible for regulating the excitability in the nervous system leading to paralysis and eventually death. Macrolytic lactones are also effective in killing external parasites as well (Yates et al., 2003). However, several anthelmintics are not approved in the U.S. by the Food and Drug Administration (FDA) for sheep and goat use, but they are still effective and are administered through off label use (Mortensen et al., 2003; Howell et al., 2008).

Coccidiostats are feed additives that are used to control and prevent coccidiosis in susceptable animals (Mortier et al., 2003). Coccidiostats work by delaying or prohibiting reproduction decreasing coccidia numbers and passage through feces to pasture (Schoenian, 2011). There are several coccidiostats but only a few are approved in the U.S. by FDA for sheep and goat use. They include Bovatec® for sheep in confinement, Corid® if prescribed by a veterinarian, Deccox® for non-lactating goats and sheep and Rumensin® for goats in confinement (Schoenian, 2011).

The efficacies of anthelmintics and coccidiostats can be determined by the use of the fecal egg count reduction test (FECRT). This test is a common tool for assessing the effectiveness of anthelmintics after application in a treatment regimen (Kaplan, 2002). The test is not specific in the differentiation of variability in the fecal samples, but it is good for determining if the issued anthelmintic is effective or not (Denwood et al., 2009). Assessment of anthelmintic treatment involves performing fecal egg count reduction test. This method is endorsed by the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) which uses the empirical means for samples to calculate the efficacy of anthelmintics. The method is performed by subtracting the initial fecal egg count before treatment is administered (Xcg) from the second fecal egg count (Xtg) collected 14 days after administration and dividing by the initial egg count (Xcg) then multiplying by 100. This test aids in the determination of the usefulness of an applied anthelmintic and can indicate if adjustments are needed to be made with the application of the drug or if use should be discontinued (Cabaret and Berrag, 2004). The lack of selective deworming and the over use of anthelmintics have rendered most drug programs helpless (Miller, 2000).

Due to growing levels of resistance in these gastrointestinal nematodes to current drugs and the lack of interest in developing new drugs by companies, small ruminant producers are desperate for solutions (Zajac, 2002). Parasite drug resistance has resulted in the search for better treatment methods including the use of non-chemical alternatives including: production practices and the dissemination of genetics based on innate immunity into breeding schemes to contend with anthelmintic resistant GIN.

Alternative methods are being currently explored to reduce the burden of *Haemonchus contortus* and other parasites through non-chemical approaches. These alternatives include better

farm management practices, use of copper oxide wire, plant based anthelmintics, FAMACHA© card, tannins and genetic enhancement practices to confront the problems that are associated with gastrointestinal nematodes especially *Haemonchus Contortus.* Burke et al., (2009) explored an alternative use of copper oxide wire particles using a gel capsule and feed to control gastrointestinal nematodes in goats. The results indicated that the treatments caused a reduction in fecal eggs counts only when the predominant eggs were not from *Haemonchus contortus* parasites. Kiambo et al., (2002) investigated naturally occurring constituents of forage such as tannins from Wattle/Mimosa and the Quebraco tree to reduce the impact of parasitic infections.

Plant based anthelmintic have also been explored for use in the elimination of GIN with such extracts like: Garlic (*Allium sativum*), Neem (*Azadirachta india*,), Wormwood (*Artemisia absinthium*) and Tobacco (Nictiana tobacum) (Worku, Franco and Baldwin, 2009; Worku, Franco and Miller, 2009). Mixed species co-grazing methods have also been used to minimize gastrointestinal parasites (Hoste and Torres-Acosta, 2011). Management systems and methods that lessen parasitic burden have also been described. Zajac (2002) recommends that the farmers pay close attention to the goats, checking for anemia as well as allowing animals to browse other vegetation to avoid grasses infected with larvae to prevent the accumulation of the larvae and increase in parasitic load in the animals. Other measures include reduction of stock density and the maximization of pasture to reduce parasite numbers as alternative management practices. Finally, keeping animals on a high nutritional plan was described to aid in the development of an immune response, and allowing goats to develop some natural immunity against the parasites before using anthelmintics were recommended as best management practices (Zajac, 2002).

Physiological changes resulting in phenotypic effects such as changes in color of mucous membranes have also been used. The FAMACHA© card system provides an eye chart to assess

infection based on the color of the animal's ocular membrane indicating the need for deworming of individual sheep and goats or not (Schoenian and Semler, 2006). The FAMACHA© card prevents the over use of anthelmintics by enabling the farmer to practice selective deworming and decrease the frequency of parasites coming into contact with the drugs reducing the development of drug resistance (Zajac, 2002). The FAMACHA© card approach has been beneficial in providing the ability to quickly spot check goats and sheep for anemia in a large or smallholder settings (Ejlertsen et al., 2006).

2.6 Genetics and Gastrointestinal Nematodes

Variations within sheep and goat genes can influence susceptibility or resistance to multiple causative agents leading to disease (Bishop and Morris, 2007). Advances in research and studies identifying genetic differences within certain breeds of sheep and goats has led to better breeding schemes and programs to combat gastrointestinal parasites (Bishop and Morris, 2007). Evidence of host breeding schemes to improve immune responses to GIN in sheep has been presented (Baker et al., 2001), with emphasis being placed on the Florida Native and Caribbean hair sheep breeds such as: the St. Croix, Katahdin, and Barbados Blackbelly (Burke and Miller, 2002; Bishop and Morris, 2007). Many of these studies used FEC to determine if host resistance is a heritable trait (Bishop and Morris, 2007). The immune response to GIN has been evaluated through criteria such as antibodies (Coltman et al., 2001) quantity of eggs produced by the female and length of the adult worms (Stear et al.,1995) and the production of eosinophils against GIN (de la Chevrotière et al., 2012).

However, the FEC is still the golden standard in determining heritable parasite resistance (Bishop and Morris, 2007). Evaluating the immune response of *Haemonchus* resistant sheep breeds may provide opportunities to improve studies of the innate immune responses to

parasitism in the less studied goat. In an attempt to investigate genetic variation and heribility for resistance in goats to *Haemonchus contortus*, Mandonnet (2001) explored the effects of introducing traits into goats, through breeding schemes. The study used a highly resistant breed of goat (Creole), which are considered to be resistant to *Haemonchus contortus* to disseminate genes into a goat control population. The results suggested that resistance is a heritable trait that can be expressed in goats (Mondonnet et al., 2001). The study also indicated higher weight gain and a low mortality rate during the fattening stage due to good breeding practices and enhanced genetic variation.

2.7 Immune Response to Gastrointestinal Nematodes

The immune system is a highly complex system, which serves the purpose of preventing, as well as identifying self and non-self-elements and destroying foreign objects that may enter and/or can harm the body. The immune system is comprised of two defense mechanisms, the innate immune system and the adaptive immune system. The innate immune system serves as an important first response to foreign objects or antigens from pathogens and consists of many defense mechanisms such as the skin, saliva, mucus and inflammatory responses. In many cases the innate immune system is not equipped to effectively handle infection, so this gives rise to the adaptive immune system (Medzhitov and Janeway, 2002).

The adaptive immune system proceeds the innate immune system whenever it is overwhelmed by infection. Unlike the innate immune system the adaptive immune system has the ability to "memorize" infectious agents, allowing the adaptive immune system to serve as a rapid response system if pathological agents are encountered again (Medzhitov and Janeway, 2002). The innate immune system consists of natural killer cells, T-cell and B-cell, basophils eosinophils, monocytes, macrophages and polymorphonuclear neutrophils that act as phagocytic

cells to engulf foreign objects. These cells are called white blood cells or leukocytes and are also divided into two groups based on their morphology, granulocytes and agranulocytes.

Granulocytes include eosinophils, neutrophils and basophils and agranulocytes are lymphocytes (T and B cells) and macrophages. A differential white blood cell count is an important tool used to provide clinical diagnosis, monitoring of disease and blood disorders (Houwen, 2001). This system quantifies and differentiates white blood cells at one particular time giving insight to infection and if treatments are working (Houwen, 2001). White blood cell differentiation counts can be accomplished manually or by automated electronic systems (Krapp and Cengage, 2002). The white blood cell percentages are determined by the counting of the ratio and morphology differentiation of the first 100 white blood cells observed (Khan and Line, 2010) (see table 1).

Table 1.

White Blood Cell Range Percentages for sheep and goats. Adapted from Khan and Line, 2010.

Species	Neutrophils	Monocytes	Eosinophils	Basophils	Lymphocytes
Goat	30-48	0-4	1-8	0-1	50-70
Sheep	10-50	0-6	0-10	0-3	40-75

In the case of infection all leukocytes respond. T and B cells activation and migration depends on the presence and nature of the antigen (Luster, 2005). Cytokines are proteins produced by different cells of the immune response system. They are responsible for the chemical signaling between cells and regulate the inflammatory response (Calleja-Agius and Brincat, 2008). Helper-T cells (Th) can cause two different immune responses upon activation which initiates cytokine production (Raghupathy, 2001; Mosmann and Sad, 1996). In the Th1 response cytokines initiate cell mediated reactions defined as the activation of macrophages to

combat infectious pathogens by releasing IL-1, IL-2, IL-8, and IL-12 to activate inflammation (Raghupathy, 2001)(Mosmann and Sad, 1996). In the Th2 response T-helper cells activate B-cells,interleukins IL-4, IL-5, IL-6, IL-10 and IL-13 are released to counter infectious agents caused by extracellular organisms (Raghupathy, 2001; Mosmann and Sad, 1996).

Interleukin 8 is very important in the inflammation response directing neutrophils to the site of infection and viral pathogens. Interleukin -8 is minimally secreted from non-stimulated cells, but it can be quickly and massively produced if stimulated by proinflammatory cytokines and other viral agents (Hoffman et al., 2002; Mastronarde et al., 1998). Interleukin 10 is responsible for the regulation and termination of the inflammatory response (Asadullah et al., 2003) and the suppression of accessory interactions in initiating T-cells (Abbas et al., 1994). Interleukin 13 also serves as inflammatory mediators induced by the Th2 response (Kelly-Welch et al., 2003). In case of the activation of IL-13 it also activates immunoglobulin E (IgE), which is responsible for responding to allergens and lipopolysaccharides associated with gram positive bacteria and pathogen products (Burd et al., 1995). Corley and Jarmon (2012) examined the immune function of IL-13 and its relationship to resistance in parasite and non-parasite resistant goats to *Haemonchus contortus*. The results concluded that the parasite resistant goats secreted more IL-13 than the non-resistant goats.

The cells of the innate immune response perform phagocytosis of pathogens and foreign objects. These cells also have the ability to differentiate between pathogenic agents by the use of toll-like receptors (Akira and Takeda, 2004). This function is described as the specific recognition of motifs associated with different microorganisms, or pathogen associated molecular patterns (PAMPs), which leads to the production of cytokines (Akira and Takeda, 2004). The toll-like receptor (TLR) family consists of ten receptors: TLR-1 through TLR-10,

which are very important in the identification of microbes (Akira and Takeda, 2004; Medzhitov et al., 1997; Rock et al., 1998). The coding regions within the sheep and goats' TLR 1-10 genes have been sequenced and found to be conserved and highly similar in nucleotide composition (Raja et al., 2011). Tirumurugaan et al., (2010) used bovine specific primers to amplify goat TLR mRNA.. Toll-like receptor 4 (TLR-4) recognizes lipopolysaccharide, which is associated with the protein in gram-positive bacteria membranes and is bound; pathogen recognition activates the innate immune response (Akira and Takeda, 2004; Poltorak et al., 1998). Toll-like receptor 2 (TLR-2) identifies many bacteria, microbial agents and products which include peptidoglycan, *Staphylococcus aureus*, lipoproteins *Mycobacterium tuberculosis*, and *Trypanosoma cruzi*. (Akira et al., 2001; Medzhitov, 2001).

In sheep the immune response to GIN has been characterized as the recruitment of lymphocytes and mast cells (Shakya, Miller and Horohov, 2011), increases in the production of eosinophils (Gill et al., 2000) and increases in the production of IgG, IgE, and IgA (Pernthaner et al., 2006) at the sites of infection within the intestinal lumen (Miller and Horohov, 2006). These reactions indicate involvement off a Th2 immune response which are multiple and related to infections induced by GIN in non-resistant and resistant host (Gill et al., 2000; Miller and Horohov, 2006). Multiple studies have been performed on sheep to determine the host genetic variation and cellular immune response against GIN within resistant and non -resistant breeds (Bishop and Morris, 2007). To compare the difference between *Haemonchus* resistant St. Croix sheep and susceptible Dorset sheep Gamble and Zajac (1992) demonstrated that St. Croix sheep displayed resistance to *Haemonchus* two months after birth. Although there were no gross differences in the immune response between the breeds, yet the St. Croix sheep produced a considerable amount of abomasal mucosa globule leaukocytes compared to the Dorset breed.

Pernthaner et al., (2006) used *T. colubriformis* evaluated the differences in the cytokine gene expression with two genetic lines of Romey sheep that were susceptible and resistant to nematodes.

Both breed lines expressed selective proinflammatory cytokines, but the nematode resistant breed line displayed increased expressions in IL-5,IL-13and TNF-α representing a Th2 immune response. Eosinophils have been suggested to be involved in combating GIN with the capacity to eliminate larvae from the host tissue (Shakya, Miller and Horohov, 2011; Balic, Cunningham and Meeusen, 2006). Eosinophils and IgE have been reported to be involved in cytotoxicity activity induced by pathogens and the destruction of GIN larvae (Shakya et al., 2011). Hassan et al., (2011) examined the relationship of Th1/Th2/Treg related cytokine gene expression in GIN infected carrier lambs. Comparison of abomasal muscosal cytokine gene expression by quantitative real-time PCR showed varied response through the course of infection. The carrier animals had up-regulated expression of the Th1-related genes early but this was replaced by up = regulated expression of Th2 and Treg genes later. For non-carrier genes there was delayed expression of these genes. These researchers concluded that carrier animals showed an earlier interplay between Th1, Th2 and T regulatory immune response genes.

Using genetically resistant and random bred lambs (Gill et al., 2000) examined the production of Th1 and Th2 type cytokines during *Haemonchus contortus* infection. It was observed that the genetically resistant lambs experienced increased mucousal eosinophils compared to the random breed lambs. In the same study, an *in vitro* study was performed to measure IgG1 and IgE production in cultured abomasal and mesenteric lymph nodes after being induced with parasite antigen. The results revealed that the cells from the genetically resistant lambs produced higher concentrations of IgG1 and IgE compared to the cells from random bred

lambs. Studies conducted on the goat immune response to GIN are few compared to that of sheep (Bishop and Morris, 2007). Previous research suggests like the sheep there are correlations between animal breed and host resistance and susceptibility to GIN (Baker et al., 2001). Most studies performed with goats pertaining to genetic heritability in relation to GIN resistance focused mainly on breeding schemes that alter FEC, PCV and body weights of the animals to accommodate parasite infection.

The specific events in the initiation of the innate immune response are an area for increasing studies. Under natural conditions (on pasture) the response to infection in farm animals involves many complex interactions host-pathogen genomes and the environment (Stear, Bishop and Raadsma, 2001). Therefore, it is even more difficult to assess co-infection caused by multiple parasites and their effects to the host as well (Budischak, Jolles and Ezenwa, 2012). However, over time, it has also been noted that not all members of animal populations that have been exposed to pathogens succumb to diseases (Stear et al., 2001).

The development of genetic resources and technologies combined with classical genetics led to identification of several host resistance genes in laboratory mice including the gene that codes for natural resistance associated macrophage protein 1 (NRAMP1) (Vidal et.al., 1993). The identification of NRAMP1 and its function led to the research area of host resistance to intracellular pathogens causing significant diseases in important agricultural species. Two sequence variants within bovine NRAMP1 have been identified and associated with disease resistance (Capparelli et al., 2007). In addition, studies with mice have shown that the cation transporter role in NRAMP1 is associated with the regulation of macrophage activation as measured by production of nitric oxide, IL-1, INF-y and Major histocompatibility complex (MHC) call II expression and Th1/Th2 differentiation (Wang, Cao and Shi, 2008). Losses to

livestock from infection, despite traditional control measures cause economic impacts in the market. An alternative approach to enhancing animal health management systems is to increase the overall level of genetic resistance. Using comparative genomics as an effective tool with farm animals will generate new knowledge about genetic determinants involved in host resistance to infection.

This study was designed to generate new knowledge to enhance the goat industry by exploring and identifying genetic immune markers to improve host resistance against GIN. The overall objective of this study was to evaluate innate immune responses of goats co-grazed with sheep on pasture. Specific objectives were (i) to determine the efficacy of the anthelmintic Cydectin® (Moxidectin) and (ii) to determine immunoglobulin (IgG, IgA and IgE), and proinflammatory cytokine concentrations (iii) to detect genes associated with innate immunity and determine their expression and if these genes are polymorphic in sheep and goat genomic DNA.

CHAPTER 3

Material and Methods

3.1 Animals and Housing

South African Boer goats (30) and St. Croix sheep (10) from the North Carolina Agricultural and Technical State University farm, Small Ruminant Research Unit in Greensboro, NC, were used in this study. The animals were housed at the Small Ruminant Research unit in accordance with animal care and use guidelines. The animals were sheltered in a barn during the evening hours and were pastured during the day. Their age ranged from one to eight years. The animals were grazed on two separate pastures: Pasture 1 had co-grazed Boer goats (20) and St. Croix sheep (10) that were yearlings. Pasture 2 had Boer goats (10) with an age averaged of 3 years. Animals were maintained on pasture for 56 days from late summer (August) to early fall (October) and samples were collected on days 0 and 56. Body weights, FAMACHA® scores were recorded.

3.2 Sample Collection

Fecal and blood samples were collected on days 0, 14 and 56. The samples were processed for packed cell volume (PCV), differential white blood cell counts percentages, serum, fecal egg counts (FEC), DNA and cDNA. Blood (20 ml) was taken from the jugular vein from the sheep and goats by venipuncture and inserted into (2) 7 ml BD Vacutainer ® blood collection tubes(Becton, Dickinson and company, Franklin Lakes, NJ) containing 0.7 acid citrate dextrose to prevent coagulation for differential white blood cell counts and packed cell volumes.

Blood was processed to collect serum for detection of cytokines and immunoglobulins. Also blood was collected into (2) PAXgene tubes (Qiagen Inc., Valencia CA) 2.5 ml per tube for RNA isolation. Blood was also collected for extraction of DNA on FTA cards for genomic

studies. To evaluate the infection status of the animals the blood and serum samples for DNA, immunoglobulin and cytokine assays were pooled into the following categories: sheep (10) with a FEC average of 165 *Haemonchus* epg and 310 coccidia epg, goats (7) with low infection with a FEC average of 93 *Haemonchus* epg and 214 coccidia epg and goats (7) with moderate infection with a FEC average of 757 *Haemonchus* epg and 1185 coccidia epg. Fecal samples were obtained by inserting the index and middle fingers inside the animal's rectum removing feces with sanitized gloves with lubricant. Fecal samples were then placed in a plastic specimen bag.

3.3 Laboratory Analysis

Fecal egg counts were measured using a modified version of the McMaster's method to assess the numbers of Coccidia and *Haemonchus* eggs per gram in feces of each specimen. Two grams of feces were weighed out and crushed with a metal spatula. A saturated sodium chloride solution (30 ml) was added to the crushed feces and stirred to float the parasite's eggs. A portion of the mixture was removed using a 1 ml syringe and placed into both sides of a McMaster slide and counted with an Olympus B 201 microscope (Optical Elements Corporation, Dulles, VA) under 10x magnification. The numbers of Coccidia and *Haemonchus* eggs counts from both sides of the slide were averaged and multiplied by 50 to calculate the eggs per gram.

After the initial collection of specimens from the animals on day 0 of the study the animals were dewormed with Cydectin® (Moxidectin) (Fort Dodge) at the dose rate of 0.2mg/kg by oral drench. A second FEC was performed 14 days after the administration of Cydectin® to evaluate its efficacy. A fecal egg count reduction test was performed according to the World Association for the Advancement of Veterinary Parasitology by subtracting the initial FEC from the second FEC (14 days later) and dividing that number by the initial FEC and then multiplied by 100.

Heparinized micro-hematocrit capillary tubes (Fisher Scientific Inc., Pittsburgh, PA) were filled with whole blood samples from each animal and sealed (Fisher Scientific Inc., Pittsburgh, PA). The tubes were centrifuged at 14,000 rpm for 10 min. in an IEC MB Micro hematocrit centrifuge (Damon/IEC Division, Needham, MA) and the packed cell volume (PCV) read on a Damon microcapillary reader (Damon/IEC Division Needham, MA).

Blood smears were prepared in duplicates by placing 10 µl of whole blood and anticoagulant onto a slide and smeared with a spreader slide at a 45º angle. The slides were allowed to dry at room temperature and stained with Sure Stain Wright's CS-432 blood staining solution as recommended by the manufacturer (Fisher Scientific Inc. Pittsburgh, PA). Slides were dipped in Wright's stain for 5 seconds and then dipped into de-ionized water for 20 seconds to remove the stain. The slides were left to air dry at room temperature. Differential white blood cells were read on an Olympus B 201 microscope (Optical Elements Corporation, Dulles, VA) under oil immersion at 100x magnification. One hundred cells were counted in duplicates and recorded

3.4 DNA Isolation and Primer Selection

Samples from sheep, goats with low and moderate *Haemonchus* FEC animal group were selected for gene identification and evaluation of expression. Genomic DNA was extracted using FTA® Elute Cards (Whatman Inc. Piscataway, NJ). Each card had 1 ml of goat or sheep blood applied per animal and labeled with the identification number on the ear tag. The cards were allowed to air dry at room temperature. Using the Harris Uni-core device (Whatman Cat. No. Wb100039) a 3 mm disc was removed from the center of the FTA® cards. The discs were transferred to a 1.5 ml RNAse-free microcentrifuge tube. A 500 µl aliquot of RNAse/DNAse-free water was added to the tube and washed with 5-second vortexing 3 times. Using sterile

forceps the discs were collected, squeezed against the edge of the tube to remove excess fluid, and transferred to another 1.5 ml RNAse-free microcetrifuge tube containing 500µl of RNAse/DNAse free water. The tubes were placed into a 95ºC water bath for 30 min to elute the DNA from the disc. After 15 minutes, the tubes were vortexed and replaced in the 95ºC water bath for 30 min. At the completion of the 30-min incubation period the tubes were removed from the water bath and the disc were removed from the tubes. Finally DNA purities and concentrations were assessed using the Nanodrop spectrophotometer (Thermo Scientific Inc., Waltham, MA) and DNA samples stored at -20ºC.

Primers and amplification guidelines were followed as described (Table 2.) primers were purchased from MWG Inc., High Point, NC (see table 2).

Table 2.

Primer sequences and PCR amplicon sizes for the Genes studied.

Gene	Primer sequence	Expected Length of PCR Product Base pairs	Source
TLR-2 forward	GAGTGGTGCAAGTATGAACTGGA	260	Montes et al., (2006).
TLR-2 reverse	TCCCAACTAGACAAAGACTGGTCT		
TLR-4 forward	TGGCAACATTTAGAATTAGTTAAC	227	Montes et al., (2006).
TLR-4 reverse	CTCAGATCTAAATACTTAGGCCG		
NRAMP1forward	CATGAAGCCAACTGGCAAGG	433	Judy et al., (1998).
NRAMP1 reverse	GAAGCCTGCAAGATGACCAACA		
GAPDH forward	AGGCAGAGAACGGGAAGCTC	195	Olah et al., (2006)
GAPDH reverse	ATCGGCAGAAGGTGCAGAGA		

3.5 Polymerase Chain Reaction and Gel Analysis

For a 50 µl reaction for all DNA and primers the following reagents were mixed for PCR. First 250 ng 5 µl of DNA was added to a PCR reaction tube. Next 25 µl of QIAGEN Multiplex PCR Master Mix (QIAGEN Inc., Valencia, CA) was added to the reaction tube. Then 1.5 µl of forward and reverse primers were added to the reaction tube. Finally 15 µl of sterilized water was added to the reaction tube, vortexed and stored at -20º C.

Polymerase chain reaction (PCR) conditions for GAPDH (Olah et al., 2006), TLR-2 and TLR-4, (Montes et al., 2006) and NRAMP1 Judy et al., (1998) were used to amplify DNA and cDNA products in a thermocycler (MWG Biotech, Martinsried, Germany) (See Tables 3 and 4).

Table 3.

Polymerase chain reaction conditions for GAPDH, TLR2 TLR-4 and NRAMP1.

PCR Cycle	Temperature C	Time
Pre- heating	94º	5 min.
Denaturation	94º	30 sec.
Annealing GAPDH and TLR-4	55 º	30 sec.
Annealing TLR-2 and NRAMP1	60 º	30 sec.
Post-heating	72º	30 sec.
Final Extension	72º	5 min.

PCR amplicons were analyzed by mixing 5 µl of PCR amplicons (250ng) and 1 µl of 6x loading dye. The DNA products and EZ load™ molecular mass ruler (Bio-Rad Laboratories,

Hercules, CA) were loaded into individual lanes in a 1.5% agarose gel composed of 1X TBE 0.04 M Tris-acetate and 0.001 M EDTA (pH 8) buffer for the separation of DNA products by electrophoresis. The gels were run at 100 volts for 1 hr., stained with ethidium bromide for 20 min. and washed with deionized water to remove the ethidium bromide. The gels were visualized under ultra-violet light on the Bio-Rad gel documentation system (Bio-Rad Laboratories, Hercules, CA).

3.6 Single Strand Conformational Polymorphism Analysis

Single Strand Conformational Polymorphism gels were produced following a modified manufacturer's protocol (The DCode Universal Mutation Detection System Handbook, (Bio-Rad Laboratories, Hercules, CA). A 20 x 20 cm SSCP gel sandwich was assembled using glass plates provided with Bio-Rad's DCode Universal Mutation Detection System. The DCode System cooling tank (Lauda-Brinkman, Delran, NJ) was filled with 6300 ml of deionized water and 700 mL of 10X Tris base/Boric acid/ EDTA (TBE). An 18% gel was cast first into the sandwich and allowed to polymerize for 1 hr. After the 18% gel polymerized the 14% stacking gel was added atop of the 18% running gel. Next a comb was placed immediately into the solution to ensure a well alignment for the lanes and allotted 1hr. to solidify. After the solidification of the stacking gel the amplicons produced from PCR were denatured by incubation for 8 minutes at 95°C in a MWG thermocycler (MWG Biotech, Ebersberg, Germany). The amplicons were then placed into individual wells and electrophoresis was performed for 14 hrs using a PS 500XT DC Power Supply at 230Volts (Hoefer Scientific Instruments, San Francisco, CA). The gel was stained with ethidium bromide for 30 min. and washed with deionized water to remove the ethidium bromide. The gels were visualized under ultra-violet light with the Bio-Rad gel documentation system (Bio-Rad Laboratories, Hercules, CA).

3.7 RNA Isolation and cDNA Synthesis

Total RNA was extracted from the sheep (10), goats (7) with moderate infection and goats (7) with low infection. Blood from PaxGene RNA collection tubes (QIAGEN Inc., Valencia, CA) was used and RNA isolated using the ZR Whole-Blood total RNA kit (Zymo Research, Orange, CA) as recommended by the manufacturer. Briefly, Paxgene tubes were centrifuged at 3,600 rpm for 15 min. The supernatant was then removed and 5 ml of DEPC-treated water was added, vortexed and centrifuged at 3,600 rpm for 10 min. The supernatant was removed from the tubes and 3 ml of blood RNA buffer added, followed with vortexing and incubation for 10 min at room temp. Next, 600 µl of the mixture was transferred into a Zymo-spin III C column and a new collection tube and centrifuged at 14,000 rpm for 2 min.

The III C column was then transferred to a new collection column where 400 µl of RNA pre-wash buffer was added to the column and centrifuged at 12,000 g for 30 sec. A 400 µl aliquot of RNA wash buffer was added to the column then centrifuged at 12,000 g for 30 sec. Next the III C column was transferred into an RNase-free tube and 100 µl of RNA recovery buffer was added to the III C column, placed into an RNase-free tube and centrifuged at 12,000 g for 30 sec. Next 100 µl of ethanol (95-100%) was added to the flow-through in an RNase-free tube and mixed with a pipette. The mixture, in the I C column was added to a collection tube and centrifuged at 12,000 g for 30 sec. A 400 µl aliquot RNA prep buffer was added to the column and centrifuged at 12,000 g for 1 min. The flow-through was discarded. Next, 800 µl of RNA wash buffer was added to the column and centrifuged at 13,000 g for 30 seconds and the flow-through discarded. The wash step was repeated with 400 µL of RNA wash buffer. Afterward, the I C column was centrifuged in an empty collection tube for 2 min at 12,000 g, removed then transferred into an RNase-free tube. Next 10 µl of DNase-free water was added to

the RNase-free tube and centrifuged at10, 000 g for 30 sec. Finally, the samples were incubated at 65°C for 5 min. then chilled immediately. Purity and concentration was determined using the Nanodrop Spectrophotometer.

Complementary DNA (cDNA) was synthesized using a RETRO script Kit (Ambion Inc., Austin, TX) by taking 1-2 µg of total RNA from each sample and adding 2 µl of Oligo (dT) (Ambion Inc., Austin, TX)). The final volume was adjusted to 12 µl by the addition of nuclease free water in a PCR reaction tube. The tubes were vortexed, spun and placed into a thermocycler (MWG Biotech, Martinsried, Germany) then heated for 3 min at 85ºC, removed and placed on ice. Next, the remaining reverse transcription components were added to each sample, 2 µl 10X RT buffer, 4 µL dNTP mix, 1 µl RNase Inhibitor and 1 µl MMLV-RT (Ambion Inc., Austin, TX) totaling 20 µL in volume. The tubes were vortexed, spun down and placed into the thermocycler for incubation at 44ºC for 1 hr. to activate reverse transcription followed by incubation at 92ºC for 10 min to stop the action. Finally, the cDNA products were measured for purity and concentration using the Nanodrop spectrophotometer (Thermo Scientific Inc., Waltham, MA) and stored at -20ºC. PCR was performed using the reaction conditions for the specific gene primes in tables 3and 4.

3.8 Detecting Proinflammatory Cytokines

To evaluate the levels of proinflammatory cytokines that were secreted by the animals on days 0 and 56 of the study a BioSignal Capture Human Inflammation ELISA strip (Signosis Inc., Sunnyvale, CA) that profiles 8 different proinflammatory cytokines was use. Pooled serum samples collected for Days 0 and 56 from the sheep, goats with low infection and goats with moderate infection were used for this assay. Undiluted samples and standards (100µL) were applied, in duplicate, to the 8 different cytokines wells and incubated for 1 hr. at room

temperature gently mixed on a Belly Dancer platform shaker (Stovall Life Sciences Inc., Greensboro, NC).

At the end of the incubation period, the serum was aspirated from each well and washed 3 times with wash buffer to remove any non-bound proteins and inverted onto paper towels to remove any remaining wash buffer in the wells. Next, 100 µl of Biotin labeled antibody was added to each well to bind to the captured antibodies and incubated for 1 hr. with gentle mixing on the Belly Dancer platform shaker. Following incubation, the unbound Biotin was aspirated from each well and washed 3 times with wash buffer to remove all of the Biotin and inverted onto paper towels to remove any remaining wash buffer in the wells.

Next 100 µl of Streptavidin-Horse Radish Peroxidase was added to the wells to bind to the captured antibodies incubated for 45 min. at room temperature gently mixed on the Belly Dancer platform shaker. The plate was aspirated and washed 3 times after incubation and blotted onto paper towels to remove the remaining wash buffer. Then, 100 µl of substrate was added to each well to produce the color signal (blue) indicating the capture of the antibody and incubated for 30 min at room temperature. Finally 50 µl stop solution was added to the wells to stop the reaction and turning the wells yellow as described in the manufacture's protocol. The plate was read on a Bio-Tek Instruments universal microplate reader (Bio-Tek Instruments Inc., Winooski, VT) at 450 nm. Serial dilutions of ELISA standards were prepared as directed by the manufacture to generate standard curves for quantification. Standard curves were used to measure secreted cytokine concentration.

3.9 Immunoglobulin Detection

To detect immunoglobulins G and A in the pooled serum collected on Days 0 and 56 from the sheep, goats with low infection and goats with moderate. Both Goat IgG and IgA

ELISA Quantitation sets (Bethyl Laboratories Inc., Montgomery, TX) was used. The serum was serial diluted to 1:100, 1:1,000 and 1:10,000 for both assays. To fix the antibodies to the plate 100 µl of diluted coating antibody for IgG and IgA was added to the separate IgG and IgA predetermined wells on a NUNC™ 96 well microwell plates (Thermo Fisher Scientific Inc., Pittsburgh, PA) wells and incubated for 1 hr. at room temperature.

After incubation, the plates were washed 5 times with washing buffer to remove the coating buffer from the wells and aspirated. Next, 200 µl of blocking solution was applied to the wells to block unspecific binding and incubated for 1 hr. at room temperature and washed 5 times with washing buffer after incubation and aspirated. Then, the serial diluted serum and standards (100 µL) were applied to the pre-coated IgG and IgA antibody wells and incubated for 1 hr. at room temperature and then washed 5 times with washing buffer and aspirated. Next, 100 µl of Horse Radish Peroxidase detection antibody was applied to the wells to bind to the bound antibodies to produce a color signal (blue) when substrate is added and incubated for 1 hr. At the conclusion of the incubation period the plates were washed 5 times with washing buffer and aspirated. The TMB substrate (Bethyl Laboratories Inc., Montgomery, TX) was added to the wells and incubated at room temperature, in the dark, for 15 min to turn the wells blue signifying the presence of the antibody. Finally, stop solution was added to the wells stopping the reaction by turning the wells yellow as indicated by the manufacture's protocol. The plates were read with a Bio-Tek Instruments universal microplate reader (Bio-Tek Instruments Inc., Winooski, VT) at 450 nm. Serial dilutions of ELISA standards were prepared as per the manufacture recommendations to generate standard curves for quantification of secreted immunoglobulin concentration. To detect imunnoglobulin E in pooled serum from days 0 and 56 samples from: sheep, goats with low and moderate infection an ELISA kit was used (NovaTeinBio,Inc.,

Cambridge, MA). The pooled serum from each group (50µl) was applied to the wells of the pre-coated strip plate, which was provided with the kit, and diluted by 50µl of sample diluent. Then the plate was incubated for 30 min at 37ºC in an incubator (New Brunswick Scientific, Edison, NJ). After the incubation period the plate was washed 4 times by removing all of the liquid from the wells with pipetts, filling them with washing solution, oscillating the plate for 1 min. on a Belly Dancer platform shaker (Stovall Life Sciences Inc., Greensboro, NC) and blotting the plate on paper towels to remove excess fluid. Next 50 µl Horse Radish Peroxidase-conjugate antibody was added to each well and incubated 37 ºC for 30 min. and then washed. Then 50µl chromogenic substrate A and B was added to each well mixed with a pipette and incubated for 15 min. at 37ºC changing the wells blue. A stop solution was added to the wells following the incubation period to stop the reaction indicated by the blue wells turning to yellow. The plates were read at 450 nm with a Bio-Tek Instruments universal microplate reader (Bio-Tek Instruments Inc., Winooski, VT). Serial dilutions of ELISA standards were prepared as per the manufacture recommendations to generate standard curves for quantification.

3.10 Statistical Analysis

Statistical Analysis Software System (SAS) (SAS Institute, Cary, NC) general linear model was used to analyze FEC, BW, FAMACHA© scores, PCV and white blood cell differentials. Analysis of variance (ANOVA) was used to evaluate the difference between groups. Means were considered significant at the 5% level of probability. Concentrations for immunoglobulins and proinflammatory cytokines were determined using a standard curve and Microsoft Excel to generate means.

CHAPTER 4

Results and Discussion

4.1Evaluation of the Efficacy of Cydectin® (Moxidectin) in Goats

Haemonchus oocytes and coccidia oocysts were found in both the sheep and goats. The sheep had a mean of 165 *Haemonchus* oocytes epg and 310 coccidia oocyst epg at day 0 the goats had a mean of 353 *Haemonchus* oocytes epg and 976 coccidia oocysts epg at day 0. There were no significant differences between the Goats (6) with the highest *Haemonchus* FEC's that were selected to evaluate the efficacy of Cydectin® 14 days after its administration at its recommended dose. Results of the study showed a significant efficacy of 90.2% in the reduction in *Haemonchus contortus* oocytes and an 81.4% in the reduction of coccidia oocysts per gram of feces after using the fecal egg count reduction test (see Table 4).

Table 4.

Haemonchus contortus and coccidia fecal eggs per gram counts in goats before and after treatment with Cydectin® (Moxidectin).

EPG Day 0		EPG Day 14		Reduction Percentages %	
Haemonchus	Coccidia	*Haemonchus*	Coccidia	*Haemonchus*	Coccidia
1300	1050	0	750	100.0	28.6
750	1500	0	0	100.0	100.0
750	450	0	0	100.0	100.0
500	1595	0	0	100.0	100.0
650	3300	0	100	100.0	97.0
850	400	500	150	41.2	62.5

This finding supports a study performed by (Ragbetli et al., 2009) who reported that Cydectin® (Moxidectin) had a 100% efficacy against *Ostertagia*, *Haemonchus*, *Nematodirus* and *Trichostrongylus*. Although the efficacy of Cydectin® had an overall 90.2 % effect on the goats in this study, there were variations in the reduction of *Haemonchus* and coccidia epg in feces. It was observed that 1 out of the 6 goats had a 41.2% reduction in *Haemonchus* epg in feces and a 62.5% reduction in coccidia epg in feces, perhaps this was due to other health related or environmental parameters. There was 1 other goat out of the 6 that had a low reduction in coccdia epg after the application of Cydectin®. The mechanism of how Cydectin® affects coccidia is unknown but the observed reduction in coccidia epg s after the administration of Cydectin® in this study suggest potential utilization beyond just the control of *Haemonchus*. However, more studies and animals are needed to confirm these findings.

4.2 Infection Status

Packed cell volume (PCV) and FAMACHA© scores were similar between the individual grazed and co-grazed sheep and goats on pasture throughout the 56 day study ($p>0.05$). The sheep had lower FEC than the goats they were co-grazed with ($p<0.05$). This was expected of St. Croix sheep due to previous reports of low FEC and parasite resistance (Vanimisetti et al., 2004). There was no difference in the FEC between the sheep and the goats that were non co-grazed grazed. This suggest that possibly the goats on the separate pasture had a more robust immune response to *Haemonchus* or the pasture was less contaminated than the pasture the co-grazed sheep and goats were on. The body weights of the goats non co-grazed and goats co-grazed with the sheep were not significantly different ($p>0.05$). The goats had a higher body weight than the sheep ($p<0.05$). This suggests that species differences exist in body weight between the sheep and goats. This may also be due to the Boer goat's superior genetic ability to

convert forage into body weight (Glimp, 1995).

Differences in the percentages of neutrophils and eosinophils between the animal groups were observed. Goats and sheep co-grazed produced the same level of neutrophils on days 0 and 56 while goats on separate pasture produced higher levels of neutrophils ($p<0.05$). The goats that were co-grazed with the sheep had lower percentages of neutrophils in peripheral blood than goats non co-grazing on pasture ($p<0.05$). The increased level of neutrophils in goats grazed on separate pasture, which were older, may heightened immune response due to previous challenges by GIN as compared to the younger goats co-grazed with the sheep (see Figure 2).

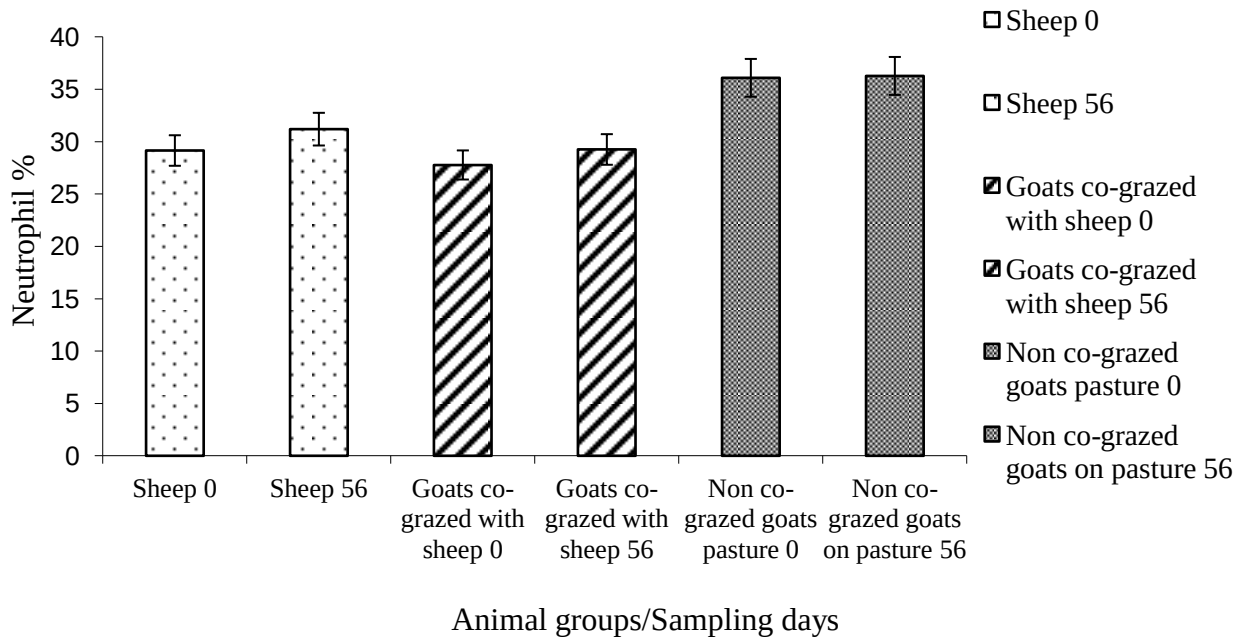


Figure 2. Comparison of day 0 and day 56 neutrophil percentages. (Mean±SD)

The sheep had a higher percentage of eosinophils in peripheral blood compared to the goats that they were co-grazed with and the goats that were grazed on separate pasture ($p<0.05$). A trend was also observed in the decreased production of eosinophils in the sheep and goats from days 0 to 56. The higher production of eosinophils observed in the sheep suggests a potential role in combating GIN infections (Balic et al., 2006) (see Figure 3).

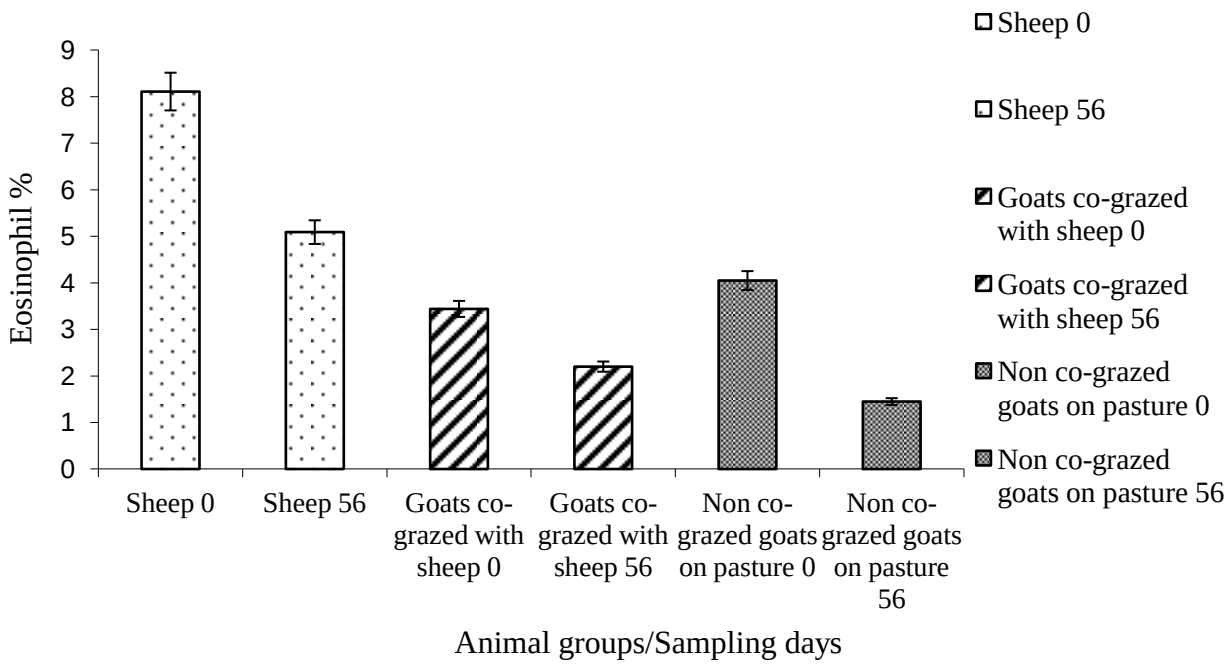


Figure 3. Comparison of day 0 and day 56 eosinophil percentages.(Mean±SD)

The goats were dived into groups based on low or moderate *Haemonchus* epg to evaluate their infection status compared to sheep. The sheep and the goats with moderate infection produced higher levels of monocytes and lymphocytes on day 0 compared to the goats with low infection, which were of normal hematological range for the sheep and goats. This observance later correlated with increased levels of the cytokine Granulocyte-macrophage colony-stimulating factor (GM-CSF) that was secreted by the sheep and goats with low infection on day 0 of the study (see figure 18). Although all hematological cell levels were within range, the goats with moderate infection, which were younger, had all lower white blood cell differentials compared to the goats with low infection, which were older. These suggest that the older goats had perhaps developed an immune response to *Haemonchus* over time (Miller and Horohov, 2006). It has previously been noted that the sheep produced higher levels of eosinophils on days 0 and 56 compared to the goats (see Table 5). The averages of the WBC on day 56 were similar to the values that were observed on day 0, but on day 56 there was a decrease in monocytes in

the sheep and goats with moderate infection. This was probably due to the reduction in infection status of the animals that was observed at the end of the study. However, the hematological values were still within range (see Table 6).

Table 5.

White blood cell differential cell counts of animals selected for the evaluation of infection status day 0.

Animal groups	Lymphocytes	Neutrophils	Monocytes	Eosinophils	Basophiles
Sheep	52.85	29.15	7.95	9.35	0.75
Goats with low infection	44.6	30.75	2.6	2.03	0.25
Goats with moderate infection	54.9	35.5	6.82	4.42	0.35

Table 6.

White blood cell differential cell counts of animals selected for the evaluation of infection status day 56.

Animal groups	Lymphocytes	Neutrophils	Monocytes	Eosinophils	Basophiles
Sheep	58.22	31.19	4.93	5.15	0.65
Goats with low infection	63.7	30.4	3.9	2.16	0.25
Goats with moderate infection	68.5	28.7	1.64	1.07	0.14

4.4 Detection of Genes Related to Innate Immunity

Polymerase chain reaction and electrophoresis were used to evaluate the presence and transcription of NRAMP1 (Judy et al, 1998), TLR-2 and TLR-4 (Montes et al., 2006) genes in pooled genomic DNA collected at Day 0 of the study from the above described animals groups used in previous assays in this study (10 sheep, 7 goats with low infection and 7 goats with moderate infection). The GAPDH gene (Olah et al., 2006) a house keeping gene was used as a loading control. At 195 bp GAPDH was present in all animal groups (see Figure 4).

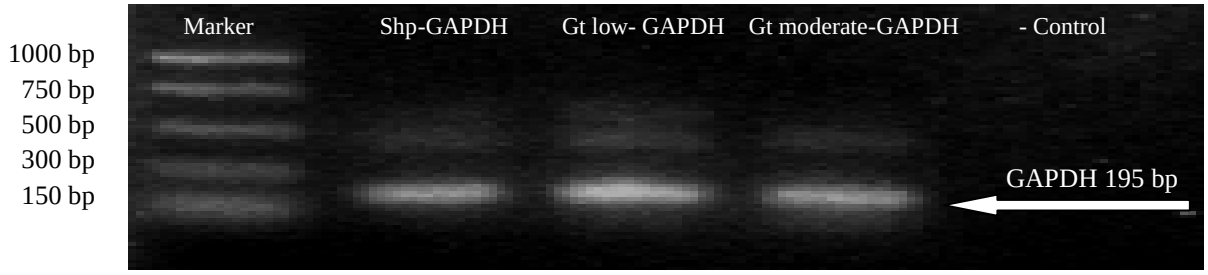


Figure 4. Detection of GAPDH in pooled genomic DNA. Lane 1 is PCR marker. Lanes 2, 3 and 4 were positive for GAPDH. Lane 5 is the negative control. This gel is a representation of 2 trials (Shp = sheep, Gt = goat).

Bands for NRAMP1 (Judy et al., 1998) in pooled genomic DNA were observed and appeared diffused for the sheep and goats with moderate infection. The goats with low infection did not appear to be positive for the NRAMP1 gene. However, as shown in (Figure 8) a positive band was observed for the expression of the gene with the same group's pooled cDNA products. The absence of the molecular band for the detection of the NRAMP1 gene or the appearance of diffused bands could possibly be a result of variations in the NRAMP1 gene size due to the use of pooled DNA samples (see Figure 5). The TLR-2 gene (Montes et al., 2006) gene was detected in the pooled sheep genomic DNA at 260 bp (see Figure 6). However, the TLR-2, gene was expressed in all of the animal groups. The TLR-4 gene (Montes et al., 2006) was detected in the pooled genomic DNA of each animal group at 227 bp (see Figure 7).

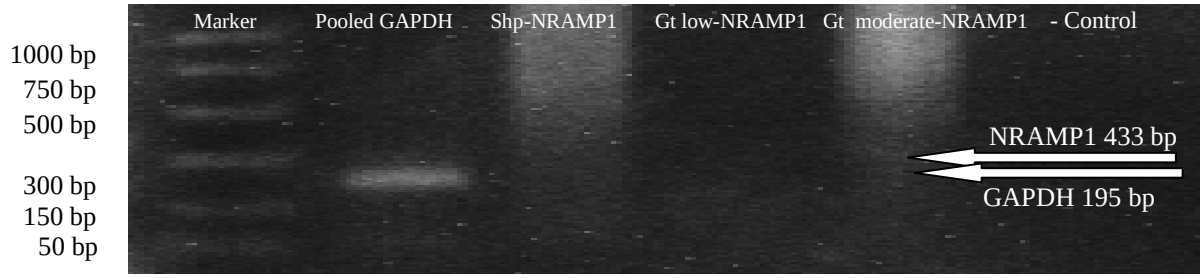


Figure 5. Detection of NRAMP1 in pooled genomic DNA. Lane 1 is PCR marker, lane 2 is the positive control GAPDH. Lanes 3, 4 and 5 indicates no detection of theNRAMP1 gene. Lane 5 is the negative control. This gel is a representation of 2 trials (Shp = sheep, Gt = goat).

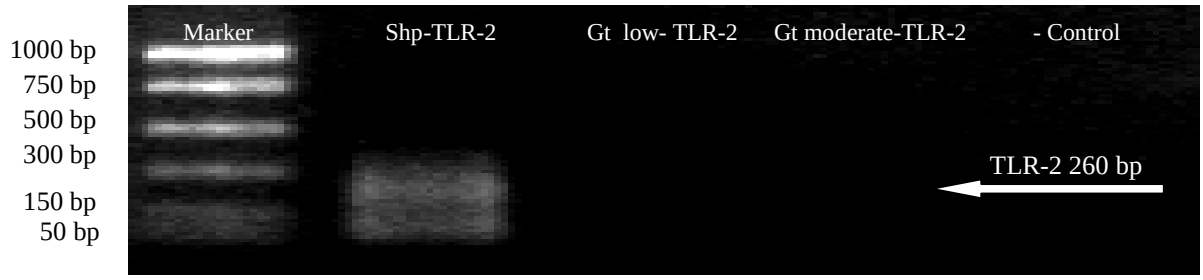


Figure 6. Detection of TLR-2 in pooled genomic DNA. Lane 1 is PCR marker; lane 2 indicates the presence of the TLR-2 gene. Lanes 3 and 4 indicates no detection of theTLR-2 gene. Lane 5 is the negative control. This gel is a representation of 2 trials (Shp= sheep, Gt= goat).

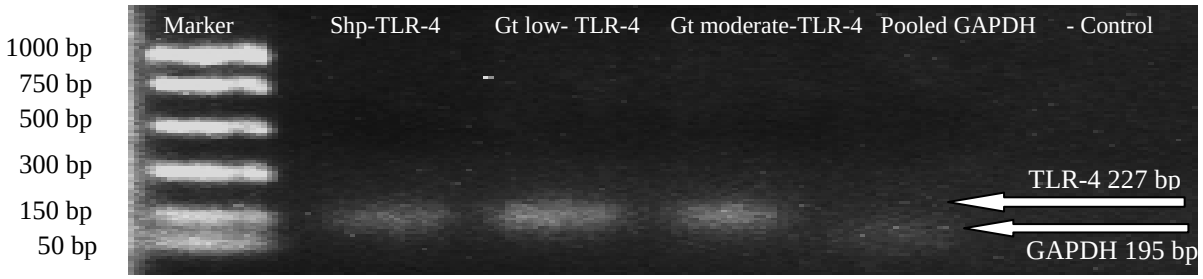


Figure 7. Detection of TLR-4 in pooled genomic DNA. Lane 1 is PCR marker, lanes 2, 3 and 4 indicates the presence of the TLR-4 gene. Lane 5 is a positive control pooled (GAPDH) and Lane 6 is the negative control. This gel is a representation of 2 trials (Shp= sheep, Gt= goat).

4.5 Expression of Genes Related to Innate Immunity

Although the goats with low infection appeared to be negative for the NRAMP1 gene (Judy et al, 1998), it was expressed at a lower molecular weight around 150 bp rather than its expected 433 bp. The lower molecular weight of the NRAMP1 gene could possibly be due to alternative splicing of the transcribed gene (see Figure 8).

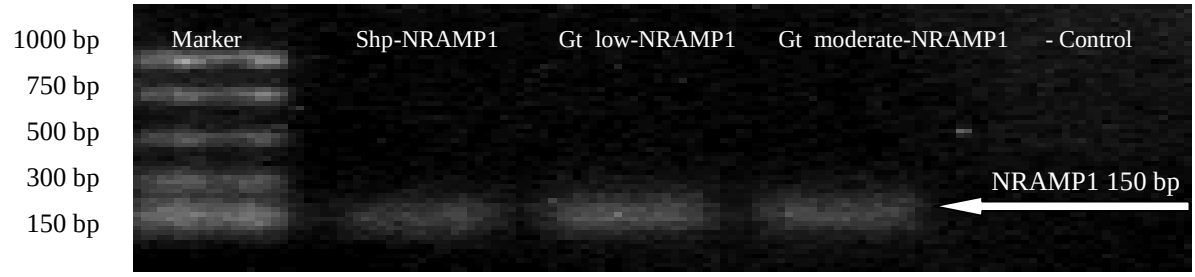


Figure 8. Expression of NRAMP1 in pooled cDNA. Lane 1 is PCR marker, lanes 2, 3 and 4 indicates the expression of the TLR-2 gene. Lane 5 is the negative control (Shp= sheep, Gt= goat).

The TLR-2 gene (Montes et al., 2006) in the sheep and goats with low and moderate infection was expressed at 100 bp or it's expected 260 bp or both. The diffused and double bands may reflect variations in the TLR-2 gene size within the pooled sample and its lower molecular weight could possibly be the result of alternative splicing during transcription (see Figure 9). The TLR-4 gene (Montes et al., 2006) was also expressed in each animal group at its expected molecular weight of 227 bp (Montes et al., 2006) (see Figure 10).

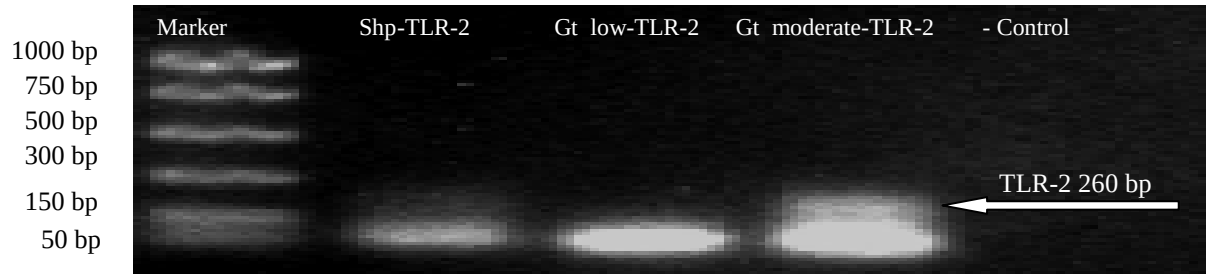


Figure 9. Expression of TLR-2 in pooled cDNA. Lane 1 is PCR marker, lanes 2, 3 and 4 indicates the expression of the TLR-2 gene. Lane 5 is the negative control (Shp= sheep,Gt=goat).

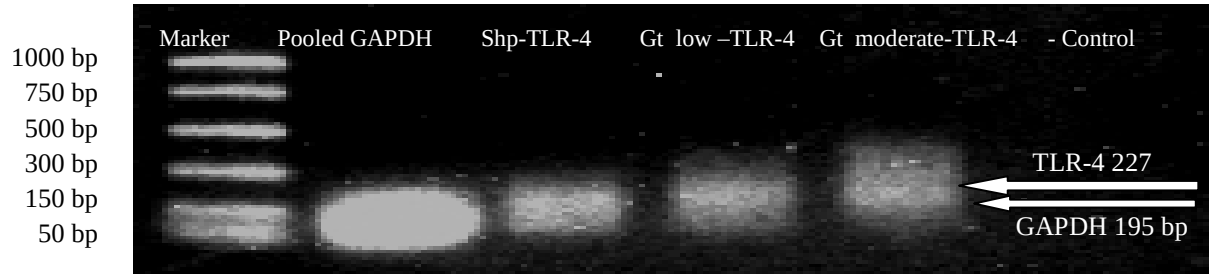


Figure 10. Expression of TLR-4 in pooled cDNA. Lane 1 is PCR marker, lane 2 pooled positive control GAPDH from each animal. Lanes 3, 4 and 5 indicates the expression of the TLR-2 gene. Lane 6 is the negative control (Shp = sheep, Gt = goat).

4.6 Detection of Genes Related to Innate Immunity in Individual Animals

A randomly selected single sheep, goat with low and moderate infection genomic DNA and cDNA were also evaluated for the detection and expression of NRAMP, TLR-2 and TLR-4 to determine if the genes were polymorphic through single stranded conformational analysis.

All animals were positive for GAPDH at the 195 bp and had other bands which could be variations of the gene in the animals' genome (see Figure 11).

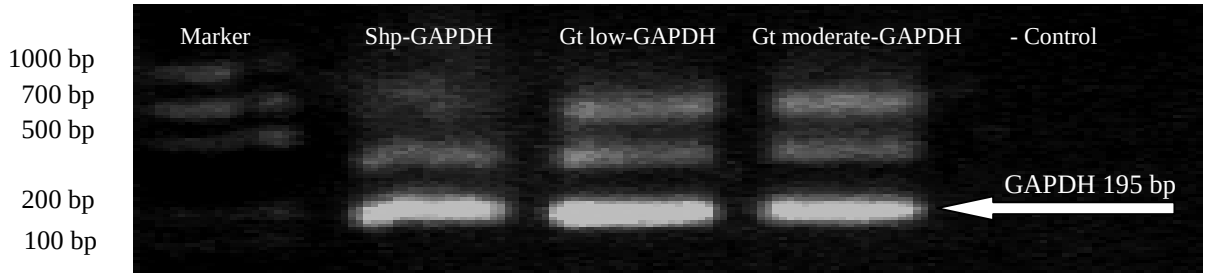


Figure 11. Detection of GAPDH in genomic DNA from individual animals representing the group. Lane 1 is PCR marker. Lanes 2, 3 and 4 were positive for GAPDH. Lane 5 is the negative control (Shp = sheep, Gt = goat).

The NRAMP1 gene was positive at the anticipated molecular weight for the goats with moderate infection at 433 bp and at different molecular weights in the sheep and the goats with

low infection (sees Figure 12). The multiple bands suggest possible variations in gene size, animal and species variations within the NRAMP1 gene.

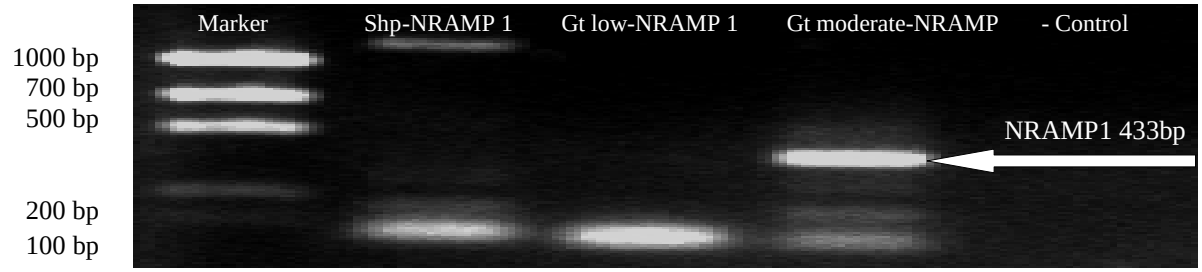


Figure 12. Detection of NRAMP1 in genomic DNA from individual animals Lane 1 is PCR marker; lanes2- 4 indicates the detection of the NRAMP1 gene. Lane 5 is the negative control (Shp = sheep, Gt = goat).

The TLR-2 gene (Montes et al, 2006) was detected in the sheep and goats with low and moderate infection. The sheep and goats had multiple bands that appeared for the gene. However the goats with low infection had a band that appeared at its anticipated molecular weight of 260bp (see Figure 13).

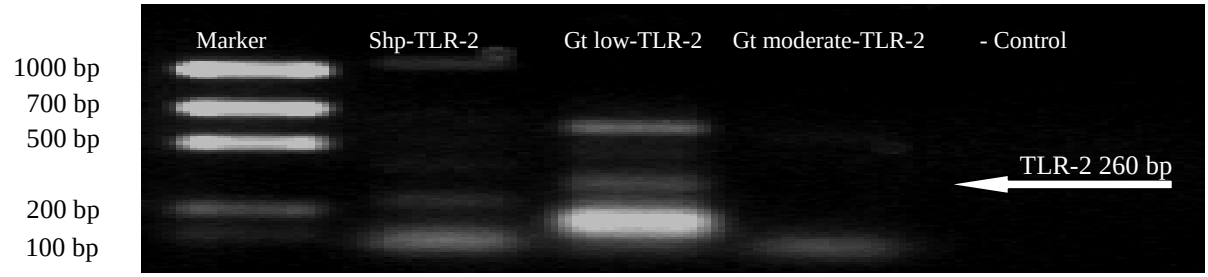


Figure 13. Detection of TLR-2 in genomic DNA from individual animals. Lane 1 is PCR marker; lanes 2- 4 indicates the detection of the TLR2 gene. Lane 5 is the negative control (Shp = sheep, Gt = goat). This gel is a representation of 2 trails.

As reported above the TLR-4 gene (Montes et al, 2006) was detected in the pooled animal samples at 227 bp. However, the gene was detected at a lower molecular weight in the individual sheep and goat with low infection at 120 bp and the goat with moderate infection

appeared to be negative for the gene. The lower molecular weight of the TLR-4 gene may reflect the individual genetic variation between the sheep and goats within the selected groups (see Figure 14).

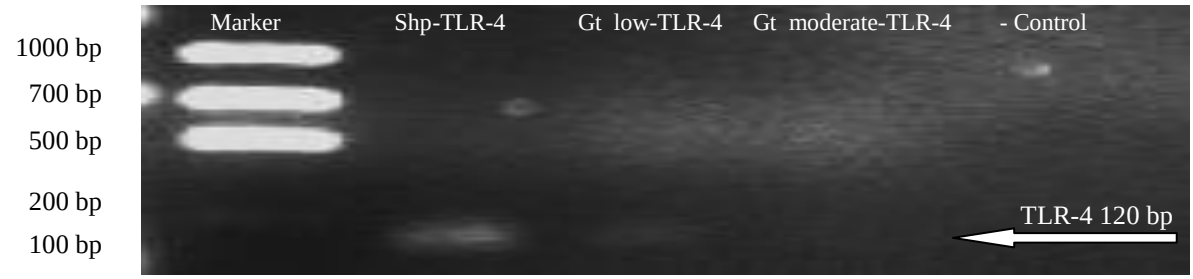


Figure 14. Detection of TLR-4 in genomic DNA from individual animals. Lane 1 is PCR marker; lane 2 and 3 indicates the presence of the TLR-4 gene. Lanes 4 indicates no detection of the TLR-4 gene. Lane 5 is the negative control (Shp = sheep, Gt = goat).

4.7 Evaluation of Gene Expression within Single Animals for Further Genetic Analysis

The NRAMP gene (Judy et al., 1998) was negative for expression in the sheep and goats with low and moderate infection. This could have been the result of the random selection of the single animals from their selected groups that were not expressing the NRAMP1 gene at the time of specimen collection for RNA extraction during day 0 of the study (see Figure 15). The gene TLR-2 (Montes et al., 2006) was expressed at its anticipated molecular weight of 260 bp and at a lower molecular weight of 100 bp in the sheep and goats with low and moderate infection (see Figure 16). The expression of the TLR-4 gene (Montes et al., 2006) appeared to be negative for expression in the sheep and the goats. However, the TLR-4 gene was detected in the sheep and goats with low infection genomic DNA. This may suggest also that the randomly selected single animals were not expressing the TLR-4 gene at the time frame that the blood specimens were collected for RNA extraction during day 0 of the study. Also the lack of the expression of the

NRAMP1gene by the animals could have been possibly due to low coccidia burden among other factors that may have impacted gene expression in the sheep and goats. (see Figure 17).

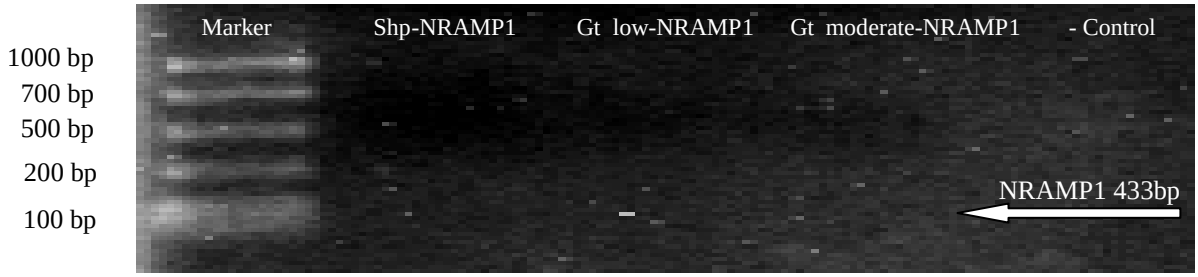


Figure 15. Expression of NRAMP1 in cDNA from individual animals. Lane 1 is PCR marker, lane 2, 3 and 4 indicates the non-expression of the NRAMP1 gene. Lane 5 is the negative control (Shp = sheep, Gt = goat).

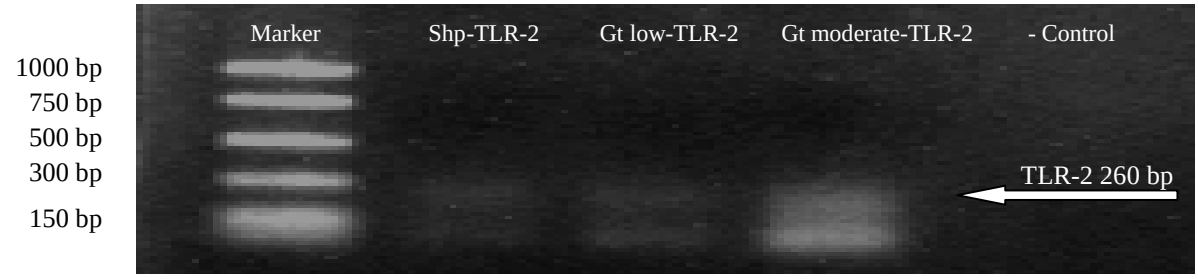


Figure 16. Expression of TLR-2 in cDNA from individual animals. Lane 1 is PCR marker, lane 2, 3 and 4 indicates the expression of the TLR-2 gene. Lane 5 is the negative control (Shp = sheep, Gt = goat).

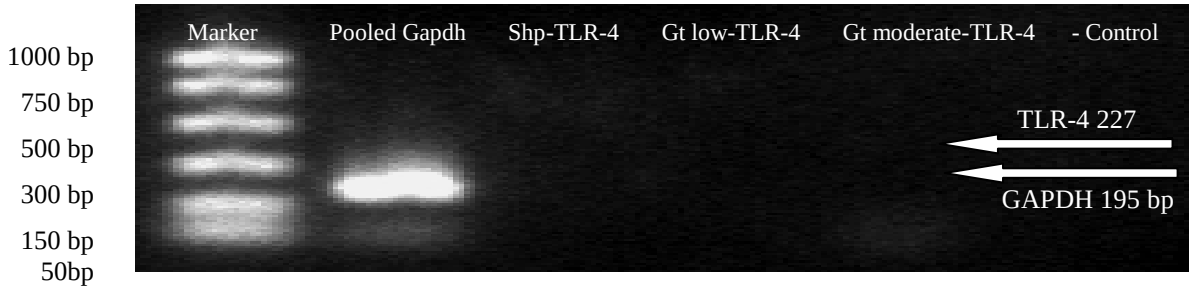


Figure 17. Expression of TLR-4 in cDNA from individual animals. Lane 1 is PCR marker, lane 2 pooled positive control GAPDH from all animals. Lanes 3, 4 and 5 indicates non-expression of the TLR-4 gene. Lane 5 is the negative control (Shp = sheep, Gt = goat).

Tables 8 and 9 summarize the results for the detection and expression of TLR-2, TLR-4 and NRAMP1 in the pooled and individual samples. Although TLR-2, TLR-4 and NRAMP1 were present in the sheep and the goats the level of expression and regulation of the genes under GIN infection conditions is not known. This opens the door for further studies to evaluate gene expression between susceptible and non-susceptible animals to GIN during infection.

Table 8**.**

Summary of genes detected in genomic DNA.

Gene DNA	Sheep	Goats low infection	Goats moderate
NRAMP1 Group	+	+	+
TLR-2 Group	+	+	_
TLR-4 Group	+	+	+
NRAMP1 Individual	+	_	+
TLR-2 Individual	+	+	+
TLR-4 Individual	+	+	_

+ indicates gene detection, - indicates no gene detection.

Table 9.

Summary of genes expressed from cDNA.

Gene cDNA	Sheep	Goats low infection	Goats moderate
NRAMP1Group	+	+	+
TLR-2 Group	+	+	+
TLR-4 Group	+	+	+
NRAMP1 Individual	_	_	_
TLR-2 Individual	+	+	+
TLR-4 Individual	_	_	_

+ indicates gene expression, - indicates no gene expression.

4.8 Single Strand Conformational Polymorphism Analysis.

Differences in gene sequences can possibly be determined by differential migration patterns through a gradient gel. The DNA amplicons from the individual animals were used to evaluate if such patterns could be detected. After performing single strand conformational polymorphism (SSCP) similar band patterns in the genes were observed for both the sheep and the goats for NRAMP1 (Judy et al., 1998) and TLR-4 (Montes et al., 2006). The migration band patterns for the TLR-2 (Montes et al., 2006) gene were observed to be similar for the goats but differed in the sheep. This suggests that there is a possible difference in the TLR-2 gene sequence between the goats and the sheep which could possibly be due to mutation or species variation in the gene. However, this is one sheep compared to two goats, many more studies with increased sample size and animal breeds are needed to confirm this finding (see Figure 18).

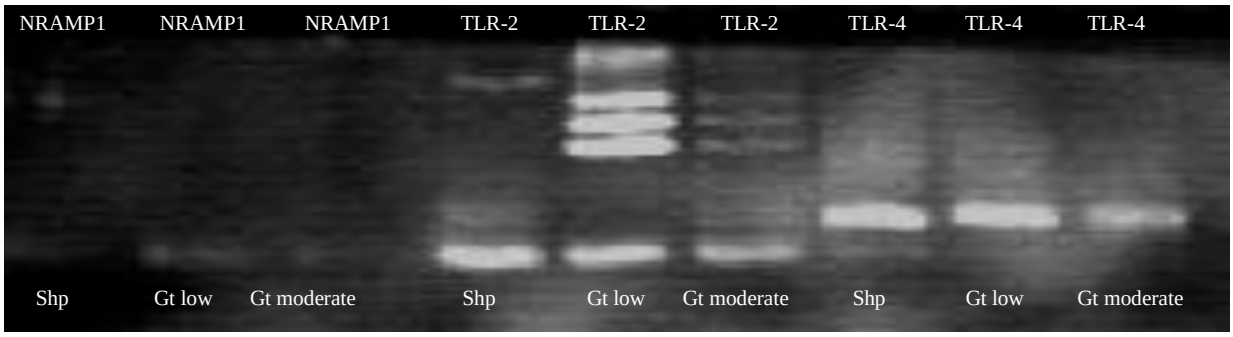


Figure 18. Single strand conformational polymorphism gel assay for NRAMP1, TLR-2 and TLR-4. This gel is a representation of 4 trials. (Shp = sheep, Gt = goat)

4.9 Immunoglobulin Concentrations

Immunogobulins (IgG, IgA and IgE) were measured in serum on days 0 and 56 from the sheep, goats with low infection and goats with moderate infection. Pooled serum samples were used from each animal group and were run in duplicates. The use of individual samples would have aided in the statistical validation of the sample variance but would have been costly.

However tendencies in the groups based on species (sheep and goat) and FECs (goats with low infection and goats with moderate infection) were still evaluated.

Secretion of IgG was observed at an average of 24 pg/ml for all of the animal groups and by day 56 decreased by 50% in sheep and goats with moderate infection at day 56. This suggests that the goats with moderate infection were possibly still responding to infection or had become re-infected before day 56 of the study. Also the goats that were selected for moderate infection were co-grazed with the reported GIN resistant sheep which subsequently could increase the parasitic loads of susceptible animals (Zajac, 2002) (see Table 12).

Table 12.

IgG concentrations in pooled serum.

IgG pg/ml concentrations			
Animal groups	Sheep	Goats low infection	Goats moderate
Day 0	23.64	29.48	21.34
Day 56	10.34	22.30	14.45

Comparable IgA secretion was also observed on days 0 and 56 serum from the sheep, goats with low infection and goats with moderate infection. The sheep had the highest levels of IgA on day 0 and decreased levels at day 56. However, the goats with moderate infection IgA levels increased at day 56 from day 0. This possibly correlates with the significant increase in eosinophils due to increased levels of IgA which has been observed in previous studies with sheep (Henderson and Stear, 2006). It was expected for the goats with moderate infection to have increased levels of IgA compare to the goats with low infection due to their infection status at day 0 of the study (see Table 13). All animals secreted IgE at day 0. The goats with low infection had the highest level of IgE secretion. All animals experienced a decrease in the

production of IgE at day 56. The increased levels of IgE produced on day 0 may possibly explain why the goats with low infection were experiencing low parasitic burdens compared to the goats with moderate infection. All animals experienced a decrease in the production of IgE at day 56. However, the goats with moderate infection IgE levels slightly decreased at day 56 which may also suggest that the animals were still coping with infection or had become re-infected at some point before day 56 of the study (see Table 14).

Table 13.

IgA concentrations in pooled serum.

IgA ng/ml concentrations			
Animal groups	Sheep	Goats low infection	Goats moderate
Day 0	419.34	311.11	284.91
Day 56	325.90	302.95	314.42

Table 14.

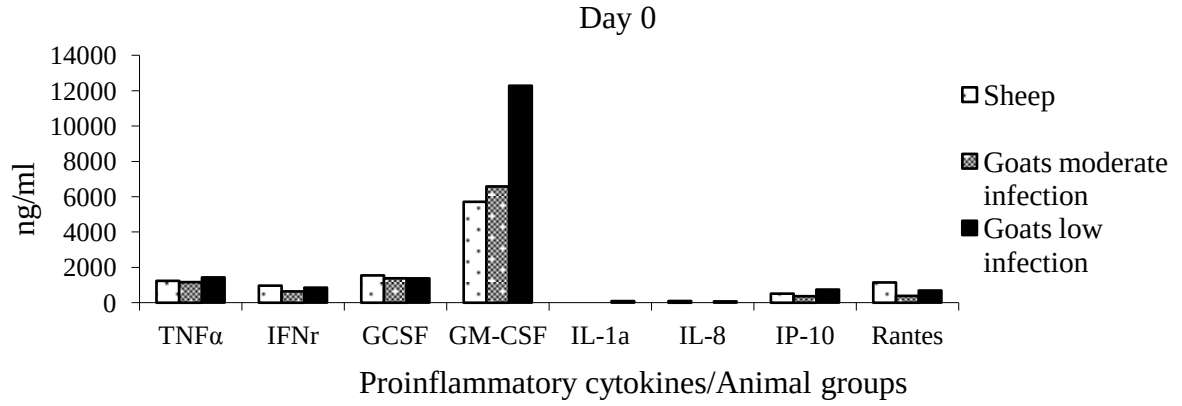
IgE concentrations in pooled serum.

IgE ng/ml concentrations			
Animal groups	Sheep	Goats low infection	Goats moderate
Day 0	338.75	581.25	313.75
Day 56	210.41	340.41	272.91

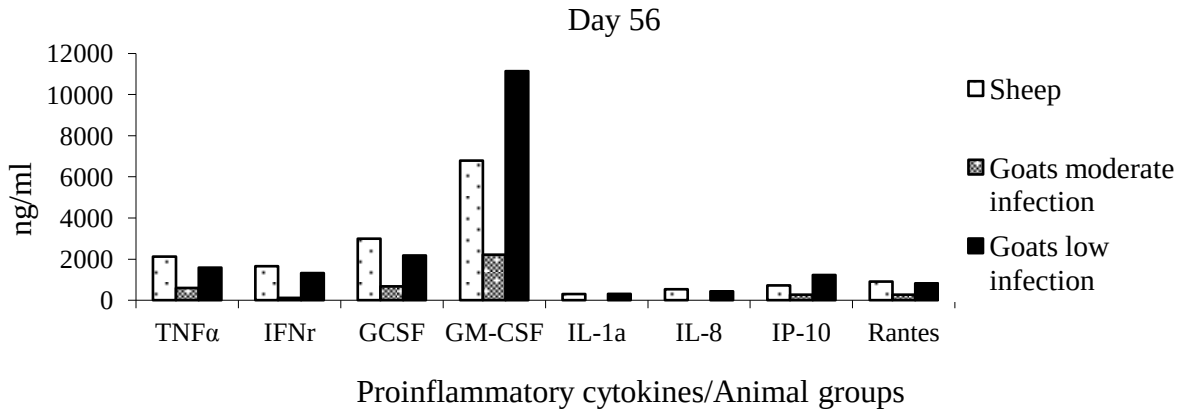
4.10 Detection of Proinflammatory Cytokines

Proinflammatory cytokine levels were also measured in serum from the respected animal groups at days 0 and 56 of the study. All animal groups were found to be secreting proinflammatory cytokines at low and elevated levels. Variations were observed between sheep

and goats in the pattern of cytokine secretion over time. The sheep tended to have a higher secretion of select cytokines. Over the course of the study the levels of some cytokines changed ,Tumor necrosis factor (TNF-α), Interferon receptor (IFNr), Granulocyte colony stimulating factor (GCSF) and Granulocyte macrophage-colony stimulating factor (GM-CSF) levels which increased from days 0 to 56 of the study in sheep (see Figures 19 (a) and (b).



(a)



(b)

Figure 19. (a) Concentration of proinflammatory cytokines at day 0. (b) Concentration of proinflammatory cytokines at day 56.

In contrast the goats with moderate infection levels with the same proinflammatory cytokines (TNF-α, IFNr, GCSF and GM-CSF) decreased from day 0 to 56 of the study. In the

goats with low infection proinflammatory cytokines levels of TNF-α, IFNr, GCSF and GM-CSF remained similar between days 0 and 56, yet their concentrations for GM-CSF were more elevated than the sheep and the goats with moderate infection on both days 0 and 56, which may suggest a potential role in reducing parasitism in susceptible animals if the elevated levels of GM-CSF were caused by gastrointestinal parasites.

CHAPTER 5

Summary and Conclusion

Sheep and goats are both ruminants and are susceptible to the same GIN on pasture. However, certain breeds within different species populations have been reported to be GIN resistant. This research evaluated the innate immune response of GIN susceptible goats co-grazed with the reported GIN resistant St. Croix sheep. The study evaluating Cydectin® (Moxidectin) efficacy with goats, which is approved for sheep, proved to be effective when administered at the recommended dose in reducing the production of *Haemonchus* oocytes and coccidia oocysts in goats. The sheep had lower parasite loads compared to the goats they were co-grazed with. The sheep had higher percentages of eosinophils than the goats. The goats that were non co-grazed grazed had higher levels of neutrophils. The sheep and goats were found to be secreting immunoglobulins IgG, IgA and IgE. At day 0 the sheep and goats with moderate infection had increased levels of IgA and the goats with low infection had increased levels of IgE. At day 56 the goats with low infection had increased levels of IgG and goats with moderate infection had increased levels of IgE. The sheep and goats s were also found to be secreting proinflammatory cytokines with variations over time and elevated GM-CSF levels at days 0 and 56. All genes (TLR-2, TLR-4 and NRAMP1) were present and expressed in the sheep and goat genomic DNA and cDNA. Differences were found in the migration pattern of the gene encoding for TLR-2 through single strand conformational polymorphism analysis. The differences in the innate immune responses of GIN resistant and susceptible animals under the same environmental conditions imply that host genetics may be responsible for GIN resistance or susceptibility. More genetic studies are needed to understand and elucidate genes associated with immunity against GIN. Also it is important to assess if polymorphisms exist within these genes and if so,

there effect on gene functionality may help to improve host genetics and breeding programs against GIN in goats.

Recommendations

The animals used in this study were not inoculated with *Haemonchus* and coccidia; perhaps if this study took place during a season favorable for the parasites could have increased the parasitic burden to evaluate the innate immune responses of the animals and comparison of the impact of parasite burden. Further studies with increased sample size are needed to understand the mode of action in which Cydectin® affects species of intestinal parasites other than Haemonchus. Additional studies with more sensitive and quantitative methods are needed to detect and assess genes that are related to GIN resistance. A larger sample size different animal breeds and species are needed to determine polymorphic genes and their expression levels during GIN challenges.

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Appendix A

Representative gels of Previous Trials of Gene Detection and Single Strand Conformational Polymorphism Analysis

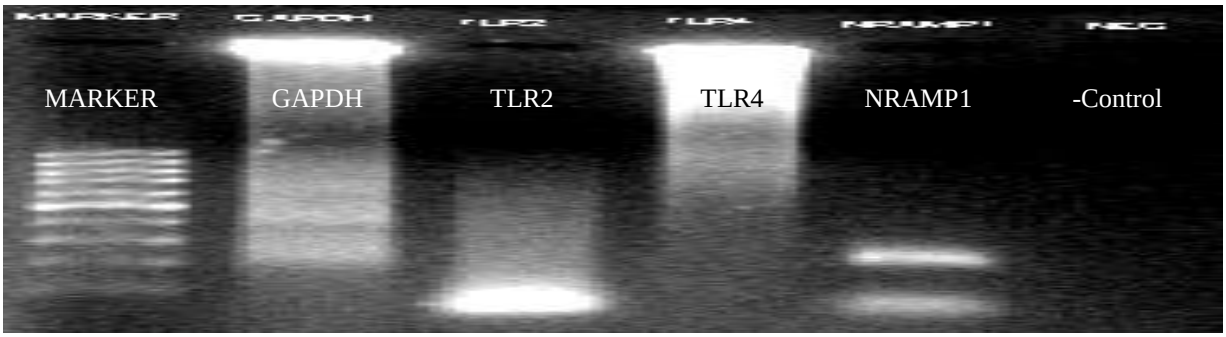

Figure A. Sheep DNA for the detection of TLR2,TLR4 and NRAMP1.

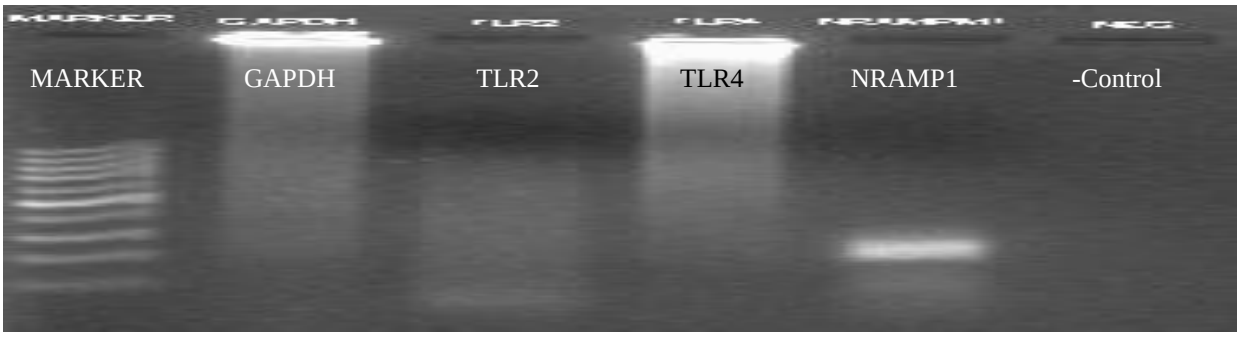

Figure B. Goats with moderate infection DNA for the detection of TLR2,TLR4 and NRAMP1.

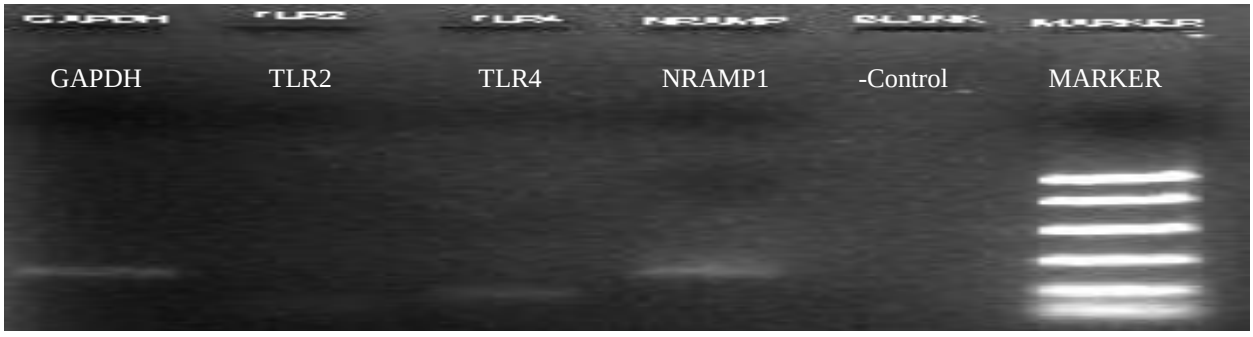

Figure C. Goat with low infection DNA for the detection of TLR2,TLR4 and NRAMP1.

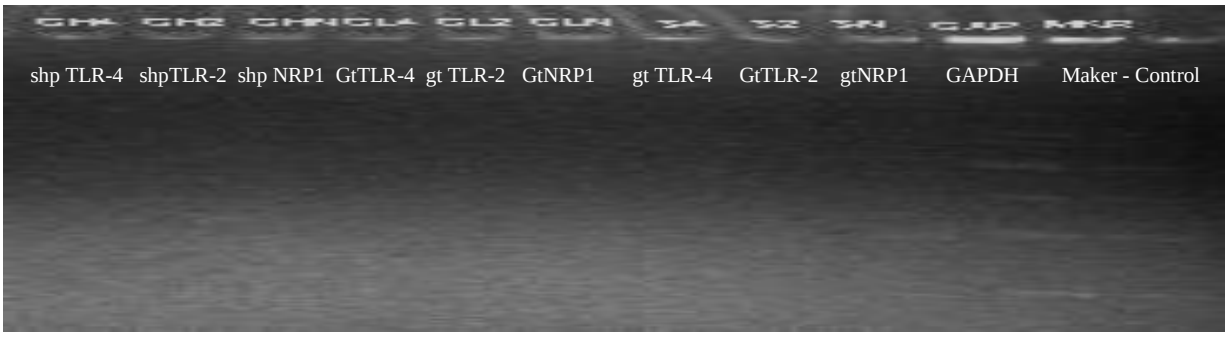

Figure D. Single strand conformational polymorphism gel assay first trial.

Figure E. Single strand conformational polymorphism gel assay second trial.

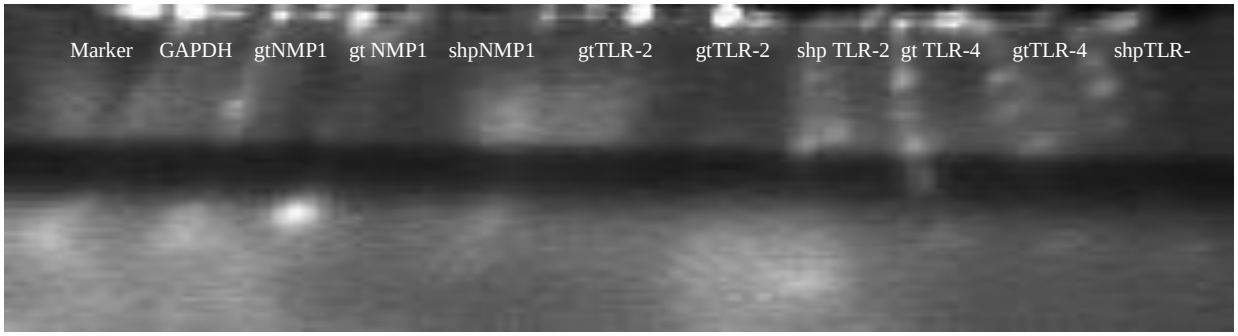

Figure E. Single strand conformational polymorphism gel assay third trial.

Appendix B

Protocol for Single Strand Conformational Polymorphism Assay

Source: Bio-Rad, DCode Universal Mutation Detection System Handbook.

Materials Needed:

30% acrylamide/bis stock solution

10X TBE

5% glycerol

deionized water

TEMED

0.1%Ammonium per sulfate (APS)

Single Strand Conformational Polymorphism Gel Sandwich Assembly:

1. Clean and dry all plates and spacers before assembly.
2. On a clean surface, lay the large rectangular plate down first
3. Place the left and right spacers along the edges of the rectangular plate.
4. Place a shorter glass plate on top of the spacers so that it is flush with the bottom edge of the longer plate.
5. Loosen the single screw of each sandwich clamp by turning it counterclockwise.
6. Place each clamp by the appropriate side of the gel sandwich with the locating arrows facing up.
7. Guide the clamps onto the sandwich so that the long and short plates fit the appropriate notches in the clamp. Tighten the screws enough to hold the plates in place.

8. Place the sandwich assembly in the alignment slot of the casting stand with the short glass plate forward.
9. Align the plates and spacers by simultaneously pushing inward on both clamps at the locating arrows and at the same time, pushing down on the spacers with your thumbs.
10. Tighten both clamps just enough to hold the sandwich in place.
11. Once an alignment is obtained, tighten the clamp screws until it is finger-tight.

Single Strand Conformational Polymorphism Gel Assay Procedure:

1. Fill the DCode System cooling tank with 6300 ml of deionized water and 700 mL of 10X Tris base/Boric acid/ EDTA (TBE). To maintain the constant temperature of 4°C throughout electrophoresis a cooling system (Lauda & Co., Germany).
2. Cast a 18% gel by adding in a conical tube (Thermo Fisher Scientific Inc., Pittsburgh, PA) 15 ml of 30% acrylamide/bis stock solution (Bio-Rad Laboratories, Hercules, CA)., 1250 µl of 10X TBE, 1250 µl of 5% glycerol, 7 ml of dH_2O, 25 µl of TEMED (Promega corp.,Madison,WI) and 220 µl of 0.1 % Ammonium per sulfate (APS) (Promega corp.,Madison,WI).
3. After the addition of APS, the conical tube should be gently mixed and applied to the sandwich followed by the addition of deionized water to make sure the gel is horizontally even at the top and allowed to polymerize for 1 hr.
4. After the 18% gel polymerize, pour off the deionized water and create a 14% stacking gel by combining: 7 ml of 30% acrylamide/bis stock solution, 750 µl of 10X TBE, 750 µl of 5% glycerol, 350 µl of deionized water, 15 µl of TEMED and 150 µl of APS then inverted to mix and poured atop of the 18% gel until it reaches the top of the.

5. Next place a comb into the top of the sandwich immediately to ensure a well alignment for the lanes and allow to 1hr. to solidify. After the solidification of the stacking gel the amplicons produced from PCR were denatured by incubation for 8 minutes at 95°C in a MWG thermocycler (MWG Biotech, Martinsried, Germany).
6. Determine the individual wells to place the DNA amplicons and perform electrophoresis for 10-14 hrs. at 230-250 volts using a DC Power Supply (Hoefer Scientific Instruments, San Francisco, CA).
7. Stain the gel with ethidium bromide for 30 min. and wash with deionized water by placing the gel into a plastic container with deionized water and slightly agitating the container by hand and pouring of the water to remove the ethidium bromide twice.
8. Image the gels under ultra-violet light with an illuminator apparatus.