Detection of anthelmintic resistance in nematodes infecting dairy heifers and evaluation of molecular markers presumably associated with nematode parasite resistance in dairy heifers

By

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Dedication

Everything I have, everything that I have accomplished, I owe to the All Mighty God who is the source of all intelligence and wisdom. I am eternally grateful for his Grace.

This thesis is dedicated to my parents, family and friends who have supported all of my dreams and inspired me to be a better person… I couldn't have done it without you.

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Detection of anthelmintic resistance in nematodes infecting dairy heifers in Puerto Rico

Abstract

The presence of anthelmintic resistance was evaluated in 21 dairy commercial farms in Puerto Rico. A total of 407 heifers, with an average age of 9 months, were used to determine efficacy of three anthelmintic treatments: albendazole **(ALB)**, doramectin **(DOR)** and ivermectin **(IVM)** in a fecal egg count reduction test **(FECRT)**. Of all anthelmintics used, ALB proved to be the most effective treatment with a 67.47% of efficacy, followed by DOR with a reduction of 25.50%. Fecal egg count reduction **(FECR)** for IVM was -8.13. Fecal cultures from 10 of the evaluated farms were used to determine the predominant species of gastrointestinal nematodes present in evaluated dairy heifers. *Cooperia spp.* was the most predominant specie observed (70.88%) followed by *Oesophagostomum radiatum* (20.00%) and *Haemonchus placei* (4.63%). FECRT results indicate that anthelmintic resistance is a widespread problem in Puerto Rico.

Capítulo I:

Detección de resistencia antihelmíntica en nemátodos de novillas lecheras en Puerto Rico

Resumen

Se evaluó la presencia de resistencia antihelmíntica en 21 fincas lecheras comerciales en Puerto Rico. Un total de 407 novillas, con una edad promedio de 9 meses, fueron utilizadas para determinar la eficacia de tres antihelmínticos: albendazol **(ALB)**, doramectina **(DOR)** e ivermectina **(IVM)** en un "Fecal egg count reduction test" **(FECRT)**. Albendazole resultó ser el tratamiento con mayor eficacia con un 67.47% de eficacia, seguido por DOR con una eficacia de 25.50%. La eficacia de IVM fue -8.13%, indicando un aumento en el número de huevos de nemátodos luego del tratamiento. Cultivos fecales de 10 de las fincas evaluadas fueron utilizados para determinar las especies predominantes de nemátodos gastrointestinales presentes. *Cooperia spp*. fué la especie más predominante observada (70.88%), seguido por *Oesophagostomum radiatum* (20.00%) y *Haemonchus placei* (4.63%). Los resultados obtenidos a partir de esta investigación demuestran la presencia de resistencia antihelmíntica en hatos lecheros en Puerto Rico.

Chapter I: Literature review

Introduction

The presence of gastrointestinal parasitism in ruminants usually presents itself as subclinical infections that can result in significant economic losses. Usually, infections with low parasitic burdens are asymptomatic, but high parasitic burdens can produce diarrhea, weight loss, stunted growth rates, and delays in puberty, breeding, and age at calving (Gasbarre et al., 2001). The dairy industry in Puerto Rico accounts for 29.93% of the agricultural production, representing a gross income of $237,107,000 in 2010-2011 (División de Estadísticas Agrícolas, 2012). In Puerto Rico, economic losses due to delayed calving age account for 33.3% of the expenses in dairy operations (Pantoja-López, 2008).

The variability in host susceptibility to parasites, the relationship between optimal conditions for parasite transmissions and grazing management, have made the eradication and control of gastrointestinal nematodes an arduous task in grazing cattle (Zarlenga et al., 2001). The control of gastrointestinal nematodes relies mostly in the use of broad-spectrum anthelmintics. The repeated use of these broad-spectrum anthelmintics has resulted in the development of resistant nematodes populations around the world (Sutherland and Leathwick, 2011). Because in the near future no new anthelmintics with different mechanisms of action are expected on the market, the preservation of the effectiveness of currently available anthelmintics is indispensable for continuing increments in dairy cattle productivity and efficiency (Coles et al., 2006).

Anthelmintic resistance develops when “there is a greater frequency of individuals within a population that can tolerate doses of an anthelmintic when compared to the normal population” (Prichard et al., 1980). Reported cases of anthelmintic resistance have been made in several countries

including New Zealand (Familton et al., 2001), Argentina (Mejia et al., 2003), Brazil (Anziani et al., 2004), the United Kingdom (Stafford and Coles, 1999) and the United States of America (Gasbarre et al., 2004).

Parasitic Nematodes of Cattle

Parasitism can affect performance parameters in livestock such as weight gain, feed consumption, forage utilization, reproductive potential, milk production and disease tolerance which in turn has an economic impact in cattle operations (Eysker and Ploeger, 2000). The measurement of the impact of parasitism is hindered by the fact that small populations of parasites are the cause of subclinical disease and unless parasite burdens are being screened, it remains unknown to the farmer. Also, the distribution of several species within the host population can cause a great variety of symptoms and it is possible to have a population where there are animals that are asymptomatic and animals with severe infections showing clinical signs (Bowman, 2009).

Gastrointestinal **(GI)** nematodes are classified under the Phylum Nematoda; Class Secernentea; Order Rhabditia; Suborder Strongylina; Family Trichostrongylidae. Members of the Trichostrongylidae family are parasites of the GI tract characterized by a direct life cycle, meaning that only a definitive host is needed for the life cycle to be completed (Schmidt et al., 2010). The general life cycle of Trichostrongyloids was described by (Schmidt et al., 2010). The adult female and male worms live in the GI tract where they mate. Eggs are shed into the pasture along with the feces. Once in the soil, the eggs hatch within 24 to 48 hours and the emerged L_1 larvae feeds on bacteria present in the feces and/or soil. Free living larvae molt into L_2 and then into the non-feeding infective stage larvae **(L_3)**. The L_3 is characterized due to the lack of a buccal cavity and a double cuticle which serves as a protective barrier against adverse environmental conditions (Wyk et al., 2004). The development from the moment the

egg hatches to the development of the L_3 takes about 7 to 10 days to complete and is affected by the environmental temperature, humidity and precipitation (Amaradasa et al., 2010). Cattle are infected through the oral-fecal route when they ingest the L_3 while grazing. Once in the rumen, the L_3 undergo exsheathment due to the acid pH in the rumen and loose the L_2 cuticle (Taylor, 2007). The L_3 will then migrate through the GI tract until they reach their predilection site where the larvae molt and become L_4. These newly molted L_4 burrow into the mucosa where they can remain for short or long periods of time depending on the parasite specie. Eventually, these larvae will emerge into the lumen where they will molt into the L_5 and then mature into sexually developed adults.

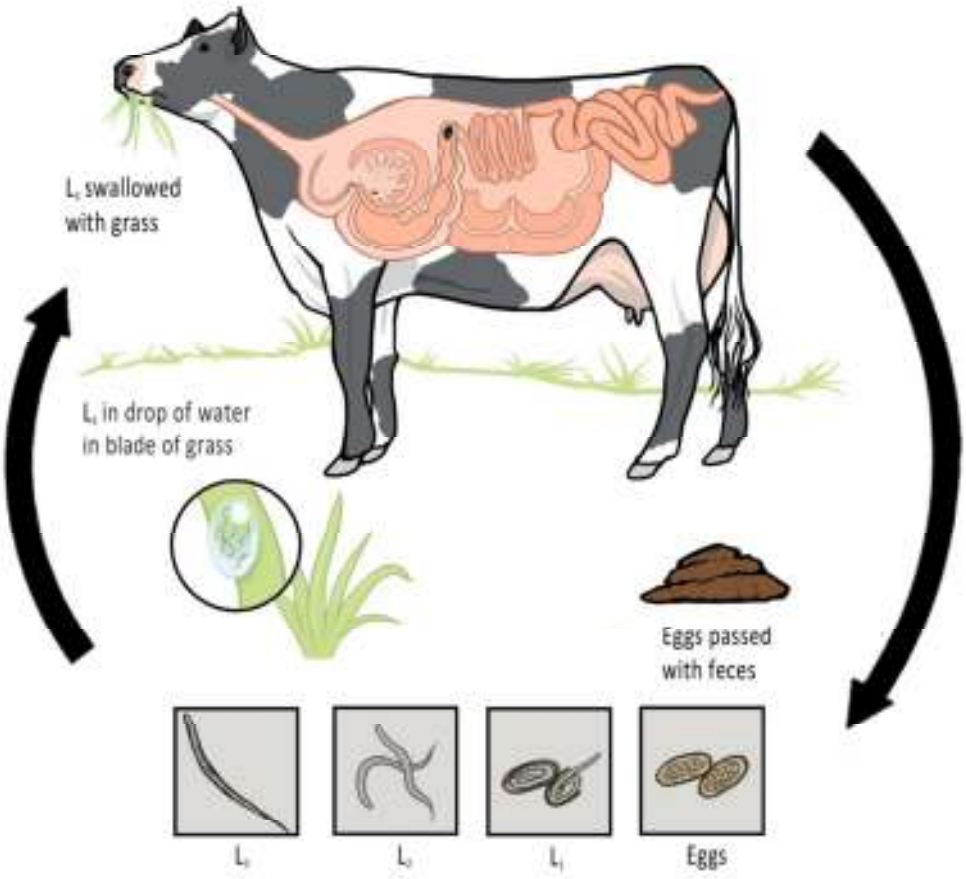

Figure 1. 1: Life cycle of gastrointestinal nematodes of cattle (adapted from Hansen, 1994).

Cooperia spp.

Cooperia spp. is a GI parasite of the small intestine of cattle, sheep, goats and deer (Taylor, 2007). As described by Taylor (2007), the adult *Cooperia spp.* worms live in the surface of the small intestine mucosa. Eggs are shed with the feces into the pasture where the eggs hatch and, under optimal conditions, the free living larvae develop and molt until the infective 3rd stage larvae within 2 weeks. When moist conditions prevail, the L_3 migrate from the feces to a blade of grass where they remain until they are ingested by the host. Once ingested, the L_3 exsheath in the rumen and migrate to the small intestine where the burrow into the intestinal crypts and undergo two molts. The adults develop in the surface of the intestinal mucosa. The prepatent period is 3 weeks. The survival and perpetuation of this specie in the host is possible due to its ability to undergo arrested development or hypobiosis. Larvae in arrested development do not feed and remain in a non-active metabolism state (Strube et al., 2007). In the tropical and subtropical areas, hypobiosis of the L_4 occurs during prolonged dry seasons (Taylor, 2007).

Cooperia spp. are considered to be the dose-limiting parasites for the macrocyclic lactone anthelmintics (Sutherland and Leathwick, 2011). Clinical signs of infections with *Cooperia spp.* include loss of appetite and poor weight gain (Stromberg et al., 2012). Moderate to heavy infections can induce catarrhal enteritis with localized villous atrophy and edema of the intestinal mucosa (Taylor, 2007). Infections with *Cooperia spp.* are typical in young calves since immunity to these species tend to develop late in the host's life (Sutherland and Leathwick, 2011).

Cooperia oncophora is a worldwide distributed gastrointestinal nematode that persists in temperate regions. In the tropics, it is more likely to find *Cooperia* species such as *C. pectinata* and *C. punctata*. There is a difference in the pathogenicity between *Cooperia* species. *C. pectinata* and *C. punctata* are presumed to have a greater impact in cattle productivity than *C. oncophora* (Stromberg et

al., 2012). All *Cooperia* species are pathogenic since they penetrate the epithelial surface of the small intestine, with a predilection site of the duodenum (Taylor, 2007). In a study made with dairy replacement heifers, parasite-free heifers reported an increased weight gain of 16 kg compared to parasite infected heifers over a 143 day period of time (Elsener et al., 2001). Another study made with a monoculture of *C. pectinata* resulted in a 12.73 kg weight gain advantage of parasite free calves over a 4 week period of time (Stromberg et al., 2012).

Haemonchus placei

Haemonchus species are blood sucking parasitic nematodes of the abomasum of ruminants (Taylor, 2007). Several species have been described worldwide. In cattle, *H. placei* and *H. similis* are the species most commonly found, although it is possible to also find mixed infections with *H. contortus*, the predominant specie found in sheep and goats (Hoberg et al., 2004). *Haemonchus* is commonly called "the barber pole worm" due to the appearance of adult female worms. In adult female worms, the blood-filled intestine is coiled with the uterus forming a red and white spiral which gives rise to this common name (Demeler, 2005).

Female worms are very prolific and are able to lay up to 10,000 eggs/day (Hoberg et al., 2004). Once in the soil, the eggs hatch and the L_1 develops and molts twice until becoming the infective 3rd stage larvae in as little as 5 days. Under cool conditions, larvae development can be completed in weeks or even months. Infective larvae are relatively resistant to desiccation and may survive for up to 3 months on the feces or pasture (Taylor, 2007). Cattle become infected while grazing in pastures that are contaminated with the infective larvae. Once the larvae reach the rumen, they lose the L_2 sheath and the L_3 migrate to the abomasum where they burrow into the gastric pits in the mucosa (White et al.,

2001). Within 1 to 2 days, the larvae molt again, becoming L_4 that then return to the surface of the mucosa and undergo a final molt becoming L_5 in the lumen of the abomasum after 2 to 3 days and mature into adult worms (Taylor, 2007). Just before this final molt, the larvae develop a structure similar to a lancet that they use to penetrate the mucosa and feed on blood from the mucosal blood vessels (Taylor, 2007). Eventually the sexually mature adult worms mate and egg production begins. The eggs are shed with the feces into the pasture for the life cycle to continue.

Haemonchus is able to undergo hypobiosis at the start of a dry season in tropical climates or at the start of autumn, when temperatures begin to drop in temperate areas. Hypobiosis ends in the beginning of spring, when rainfall propitiates favorable environmental conditions for the parasite's life cycle to continue (Bowman, 2009). The developed L_4 become arrested in their development and remain in the mucus membrane of the abomasum until a specific stimulus promotes their further development (Bowman, 2009). When the development of these hypobiotic larvae resumes, a great number of larvae will emerge leading to a massive production of eggs by the newly developed adult worms which accounts for high fecal egg counts often referred as "spring rise" (Bowman, 2009) and heavy contamination of pastures.

Severe infections with *Haemonchus* produce anemia due to blood loss and an insufficient amount of circulating erythrocytes (White et al., 2001). *Haemonchus contortus* is able to remove a fifth of the circulating erythrocyte volume daily at the peak of infection and may average one tenth of the circulating erythrocyte volume in non-fatal infections (Bowman, 2009). Clinical signs of haemonchosis are pale mucus membranes due to anemia, submandibular edema, commonly known as "bottle jaw" (Figure 1.2) due to hypoproteinanemia, lack of appetite, and weakness and feces may retain their normal consistency although diarrhea may occur during heavy or mixed infections (Taylor, 2007).

Figure 1. 2: Submandibular edema ("bottle jaw") characteristic of infections with Haemonchus.

Haemonchosis can be classified as hyperacute, acute or chronic. In acute haemonchosis, anemia can develop in as little as 2 weeks after infection (Taylor, 2007). In the tropical areas, chronic haemonchosis develops during a prolonged dry season when nutrients from pasture become scarce (Taylor, 2007). The combined nutrient deficiency and blood loss from light *Haemonchus* infections are enough to cause clinical signs such as weight loss, inappetence and lethargy (Bowman, 2009). Hyperacute haemonchosis is the result of extreme infections of over 30,000 worms and presents itself as the sudden death of an apparently healthy animal due to hemorrhagic gastritis (Bowman, 2009).

Oesophagostomum radiatum

Oesophagostomum radiatum is a parasitic GI nematode of cattle, that infects the distal small intestine, cecum and proximal colon (Gasbarre et al., 1985). Similar to the general life cycle of other GI

nematodes, the morulated eggs are shed to the pasture with the feces and within approximately 7 days the infective larvae develop (Lukešová et al., 2009). Infection occurs when grazing cattle ingest the L_3 while grazing (Taylor, 2007). Once ingested the L_3 exsheath in the rumen and migrate to the large intestine and penetrate the lamina propria of the distal ileum, resulting in the formation of fibrous nodules (Bremner et al., 1974). Inside the nodule, the L_3 molts into the L_4 and after two weeks, the larvae emerge into the mucosal surface where they molt into the L_5 and mature into adults after four weeks. The prepatent period is about 40 days (Taylor, 2007).

Pathogenesis due to infections with *Oesophagostomum* is the result of a vigorous inflammatory response due to the penetration of the larvae in the lamina propria of the intestine (Bremner et al., 1974). The immune response to *O. radiatum* creates granulomas in the intestinal wall which impair the intestinal function, particularly fluid absorption (Kahn et al., 2010). The presence of adult worms in moderate burdens is not sufficient to create clinical signs. Anemia and hypoalbuminaemia develops due to protein loss and blood leakage through the damaged mucosa (Bowman, 2009). Cattle develop immunity to *O. radiatum* due to age and previous exposures to this parasite (Bremner et al., 1976).

Anthelmintic use and the development of resistance

Anthelmintic resistance is defined as the presence of a greater frequency of individuals within a population able to tolerate higher doses of a drug (Prichard et al., 1980). This phenomenon is the result of genetic changes in the alleles conferring resistance to a drug that occurs slowly and increases with repeated drug selection over time (Vidyashankar et al., 2012), implicating that the offspring of such individuals are capable of inheriting drug resistance as well.

Anthelmintic resistance is the result several mechanisms that act in the parasite as well as in the host. In the parasite, differences in the metabolism of the drug, binding of the drug to the parasite's receptors and not enough exposure to the drug (underdosing), differences in the nematode's genome, and tolerance to drug treatment results in the ability of the parasitic nematode to tolerate doses that in a normal population should be deadly (Barragry, 1987). In the host, the route of drug administration, and improper animal management (which includes improper pasture management, lack of biosecurity measures when transferring infected animals between farms) are factors contributing to the development of anthelmintic resistance (Gasbarre et al., 2001). Repeated drug treatment accounts for the selection of resistant populations of gastrointestinal nematodes (Sutherland and Leathwick, 2011). Resistance develops more quickly when the same anthelmintic agent has been used intensively without any other measure for parasite control besides drug treatment (Martin et al., 1997).

The measurement of the economic impact of anthelmintic resistance is difficult. The variability in the parasitic worm species present in an infection, the magnitude of the worm burden, the genotype, and the proportion of resistant worms are factors to be considered when measuring the cost of anthelmintic resistance (Coles et al., 2006; Corwin, 1997; Prichard et al., 1980). Other factors such as the host's health and nutritional status are also important when measuring the economic impact of anthelmintic resistance.

Resistance to broad-spectrum anthelmintics in GI nematodes of ruminants is a world-wide described phenomenon. At present, there has been over 145 documented cases of anthelmintic resistance in parasitic nematodes of cattle involving all three classes of the broad-spectrum anthelmintics and at least ten species of nematodes (Sutherland and Leathwick, 2011). As a testimony of parasitic resistance, it has been found that resistance to the macrocyclic lactones has become common in *Cooperia spp.* infecting cattle in New Zealand (Familton et al., 2001) and in Argentina (Mejia et al.,

2003). Benzimidazoles resistance has been found in *Cooperia punctata*, *Ostertagia ostertagi* and *Haemonchus placei* in cattle in Argentina (Mejia et al., 2003) and in *C. oncophora* in New Zealand (Winterrowd et al., 2003). Other countries like Brazil (Anziani et al., 2001, 2004; Fiel et al., 2001) and the United Kingdom (Stafford and Coles, 1999) have reported resistance on *Cooperia* species and the United States of America has reported resistance on both *Cooperia* and *Haemonchus* species (Gasbarre et al., 2004). Most cases of anthelmintic resistance in cattle involve *Cooperia spp.*, (Stromberg et al., 2012).

Anthelmintics

Currently, only 3 broad-spectrum anthelmintic groups are available for the treatment of gastrointestinal parasitism in cattle: benzimidazoles, imidazothiazoles/hydropyrimidines and macrocyclic lactones (avermectins and milbemycins) (Coles et al., 1992). The mechanism of action of these anthelmintics in GI nematodes involves the selective binding of the drug to receptors in the parasite's nervous system or the disruption of important routes in the metabolism of the parasite (James et al., 2009; Köhler, 2001).

Macrocyclic lactones

Macrocyclic lactones **(ML)** were introduced in the early 1980's and ever since, have dominated the market of cattle anthelmintics (Barragry, 1987). Their effectiveness against developmental larval stages of parasitic nematodes and ectoparasites and availability in different routes of administration such as injectable, oral, and pour-on have influenced husbandry practices. The use of anthelmintics has become a common practice with the objective of maximizing profit by eliminating worm burdens that

compete for the host's nutrient availability instead of the treatment of individuals who are showing life-threatening clinical signs (Elsener et al., 2001).

Avermectins and milbemycins are macrocyclic lactones derivatives that have anthelmintic properties. These two chemical compounds are natural occurring fermentation products of several actinomycetes from the genus *Streptomyces* (Barragry, 1987). The chemical structure of avermectins and milbemycins are superimposable and differ in that the avermectins have a bisoleandrosyloxy substituent in the 13-position of the lactone ring, while milbemycins lack this substituent in that same position (Shoop et al., 1995). Abamectin, doramectin, eprinomectin, moxidectin, and ivermectin are avermectin analogues produced as a mixture of different products of the fermentation of *Streptomyces avermitilis* (Shoop et al., 1995). Of all avermectins, ivermectin was the first to be commercially available for the use in livestock with combined efficacy against endoparasites and ectoparasites, denominating it as an endectocide (Shoop et al., 1995).

Ivermectin is a gamma-amino-butyric acid **(GABA)** agonist (Barragry, 1987). GABA is a neurotransmitter mediating the transmission of inhibitory signals between interneurons and motor neurons in the ventral nerve chord of nematodes (Feng et al., 2002). Ivermectin stimulates the presynaptic release of GABA which keeps the chloride channels open when they should be closed which results in that the electrical impulse supposed to be received by the adjacent cell is blocked during synapsis (Barragry, 1987). Recent evidence suggests that all avermectins interact with high affinity in specific glutamate-gated chloride channels distinct from GABA-gated chloride **(Cl^-)** channels (Cheeseman et al., 2001; Njue, 2004; Wolstenholme et al., 2005). As a result of the non-reversible opening of the Cl^- channels and flow of Cl^- ions hyperpolarize the resting potential of the neuron, thus inhibiting the transmission of the electrical impulse from the pre-synaptic neuron to the post-synaptic neuron (Omura, 2008). The final effect of ML is paralysis of the pharynx, the somatic muscles and the

uterine muscle of the adult worm, resulting in the eventual death of the parasite (Glendinning et al., 2011). Evidence suggests that ML resistance arises from mutations in the extracellular domain of the glutamate-gated channels in *H. contortus* (Blackhall et al., 1998) and *C. oncophora* (Njue et al., 2004).

Benzimidazoles

Benzimidazoles were first introduced in the market in the 1960's as a fungicidal and ever since then, they have been proved to be effective against parasitic nematodes and some trematodes of different domestic animals including cattle, sheep, goats, dogs, and cats (Bowman, 2009; Kahn, 2010). Benzimidazoles act by disrupting the parasite's energy metabolism by selectively binding to nematode β-tubulin, a protein present in the microtubules, plasma, and mitochondrial membranes (Taylor, 2007). Two classes of tubulin, α and β-tubulin, are responsible for the formation of microtubules (Martin et al., 1997). The selective binding of benzimidazoles to β-tubulin inhibits the polymerization of β-tubulin to form the microtubules (Kahn et al., 2009). The result is parasite starvation due to inhibition of glucose uptake, protein secretion, and carbohydrate catabolism by the fumarate reductase system (Taylor, 2007). Some benzimidazoles available for use in livestock include thiabendazole, fenbendazole, oxbendazole, and albendazole, which is the newest and most potent benzimidazole currently available in the anthelmintic market (Taylor, 2007).

The molecular mechanism of anthelmintic resistance to benzimidazoles in trichostrongyles of small ruminants has been described by Elard et al., 1999. It involves a single nucleotide polymorphism **(SNP)** in the 200 residue of the isotype 1 β-tubulin gene. This SNP is a point mutation that involves a phenylalanine to tyrosine substitution in the β-tubulin gene. This same mutation has been found in *C. oncophora* in cattle (Njue and Prichard, 2003) and *Haemonchus contortus* in sheep (Ghisi et al., 2007).

Gastrointestinal nematode control methods

A common grazing system to control parasites is to limit the number of infective larvae in the pasture with either the use of an anthelmintic as a prophylaxis, the treatment and removal of individuals showing clinical signs of parasitism, and the rotation of animals in different grazing pastures (Coles et al., 2006). Strategic deworming programs are designed to administer anthelmintics during periods where the exposure to larvae contaminating the pasture is high (Elsener et al., 2001). However, the continuous use of anthelmintic treatments reduce the number of parasites *in refugia*, accelerating the selection for resistance (Coles, 2002). The free living developmental larval stages that have not been exposed to anthelmintics are described as being "*in refugia*". These larvae are important since they have not been selected by treatment, thus, providing an anthelmintic susceptibility gene pool in a parasite population (Soulsby, 2007).

Methods for the detection of anthelmintic resistance

The most common method used for the detection of anthelmintic resistance is the fecal egg count reduction test **(FECRT)** (Coles et al., 2006). This method can be used with all anthelmintic groups and is based on the comparison between the count of nematode eggs before and after the administration of a treatment and the percentage of reduction in the egg count after treatment (Coles et al., 1992). However, this test relies in the assumption that the number of counted eggs is a reflection of the parasite burden (Demeler et al., 2010a) and is only reliable once more than 25% of a population of gastrointestinal nematodes has developed resistance (Prichard et al., 1980). Anthelmintic resistance is confirmed if treatment efficacy is below 95% when calculated by comparing arithmetic means for the

percentage of reduction in a group with enough animals to yield statistically significant results (Coles et al., 2006).

Materials and methods

Animal selection

Dairy operations were selected based on good animal identification practices and general good dairy management. Groups of at least 20 heifers, predominantly Holstein or Holstein crossbreeds, between the ages of 3 to 12 months (9 months average) were selected to form part of the investigation. All heifers were required to have been grazing for a minimum of 3 months and have not been exposed to anthelmintic treatment for at least the 3 months prior to sampling.

Fecal Egg Count Reduction Test (FECRT)

A total of 407 dairy heifers from 25 different dairy farms were used for the evaluation of 3 anthelmintic treatments: doramectin (Dectomax®, Pfizer Animal Health), ivermectin (Ivomec®, Merial), and albendazole (Valbazen®, Pfizer Animal Health). Doramectin and ivermectin were administered via a subcutaneous injection at 0.2mg/kg of body weight and albendazole was administered orally at 7.5 mg/kg of body weight.

Fecal samples were collected 7 days prior to anthelmintic treatment, at the time of the treatment **(d0)** and 14 days after treatment **(d14)**. All heifers in the evaluated herd were randomly divided into 3 groups based on treatment administered: ivermectin **(IVM)**, doramectin **(DOR)**, or

albendazole **(ALB)** based on the treatment administered. For each treatment the observed drug efficacy was calculated by using the formula:

$$Eff = \left[\frac{(pre - post)}{pre}\right] X\ 100$$

pre is the average fecal egg count before treatment and

post is fecal egg count after treatment (d14)

Three grams (3g) of fecal samples from each heifer were collected and diluted in 40 mL of distilled water. The fecal solution was then filtrated through a piece of gauze to remove organic matter and centrifuged for 4 minutes at 4000g. The pellet was then suspended in 30 mL of a solution of sodium chloride (specific gravity = 1.2; 11.97M). Parasite eggs were counted through the modified McMaster technique as previously described by Coles, et al., 1992. Using this technique, the eggs per gram of feces **(EPG)** were calculated by counting the number of trichostrongyle eggs inside the McMaster slide frames and then multiplied by a factor of 33.

Figure 1. 3: Map of Puerto Rico with cities visited during the FECRT (in yellow). The number in each city states the number of farms visited in each city.

Statistical analysis of FECRT

For the FECRT, the arithmetic mean, percentage reduction of fecal egg count **(FEC)** and 95% confidence interval was calculated. Negative efficacies (FEC where the EPG increased after treatment) were reported as negative values. The arithmetic means of the percentage of FECR were analyzed by ANOVA (GLM) using the statistical analysis software InfoStat version 2012 (Di Rienzo et al., 2012). The statistical model used was to calculate the overall anthelmintic efficacy was:

$$Y_{hijk} = \mu_h + A_i + D_j + I_k$$

Y_{hijk} = observations pertaining the fecal egg count reduction after treatment

μ_h = overall mean

A_i = mean fecal egg count reduction after treatment with ALB

D_j = mean fecal egg count reduction after treatment with DOR

I_k = mean fecal egg count reduction after treatment with IVM

Identification of predominant species of GI nematodes in dairy heifers of Puerto Rico

From September 2012 to December 2012, fecal samples were collected from 10 of the evaluated farms in the FECRT and sent to the University of Georgia in Athens, GA. Pooled feces from selected farms were mixed with vermiculite (Sargent-Welch, Buffalo, NY) and incubated for 10 days at room temperature (25° C). The cultures were examined and stirred every day. To prevent larvae desiccation, distilled water was added as needed. After 10 days of incubation, the L_3 were recovered using the Baermann technique (Dinaburg, 1942) in which the pooled fecal matter is suspended over a

sieve inside a funnel with lukewarm water for 24 hours. Recovered larvae were stored at 10° C in deionized water. Using a 10% lugol iodine solution, larvae species were stained and identified using morphological characteristics of the anterior end, tail sheath extension, and overall body length (Wyk, et al., 2004).

Results

Fecal egg count reduction test (FECRT)

The objective of this investigation was to evaluate anthelmintic resistance of gastrointestinal nematodes of dairy heifers to three anthelmintics that are commonly used in Puerto Rico. A total of 25 farms from different cities in Puerto Rico were visited, but only 21 were used in the FECRT. Four of these farms were eliminated from the study because the average FEC before treatment was less than 100 EPG. Anthelmintic efficacy evaluation in 21 commercial dairy farms was carried out from November 2010 to May 2012.

The mean fecal egg count reduction **(FECR)** after the administration of anthelmintic treatment in all evaluated farms is presented in Figure 1.4. Overall, all treatments were found to differ statistically ($p=0.0004$). Of all anthelmintics used, ALB proved to be the most effective treatment with a 67.47% of FECR, followed by DOR with a reduction of 25.50%. The observed drug efficacy of IVM was -8.13% due to an increase in FEC after treatment.

Fecal egg count reduction test results from 21 farms evaluated demonstrate the presence of anthelmintic resistance in 19 farms (90% of all evaluated farms show anthelmintic resistance). Only farms 7 and 14 had a mean FECR over 95% for all evaluated treatments, indicating the presence of

susceptible populations of GI nematodes to all evaluated treatments (Table 1.2). FECRT results for each treatment on each individual farm are presented in Table 1.1. Resistance to IVM was detected in 17 farms (80.95% of all evaluated farms) of which 8 of farms showed negative efficacies for this drug (38.10% of all farms). Similarly, resistance to DOR was detected in 18 farms (85.71% of all evaluated farms) and 5 of these farms where characterized by negative drug efficacies. Based on a Fisher LSD comparison (α=0.05), the efficacy of both MLs, DOR and IVM, are not statistically different. However, there is a significant difference between MLs and the benzimidazole ALB ($p<0.05$). Anthelmintic resistance for this drug was detected in 12 farms (57.14% of all evaluated farms). ALB was effective in 9 farms, 6 of which had a FECR of 100.00%.

Fecal cultures

Overall, *Cooperia spp.* (Figure 1.6) was the most predominant specie observed (70.88%). *Oesophagostomum radiatum* (Figure 1.7) and *Haemonchus placei* (Figure 1.8) were the other most predominant species observed with 20.00% and 4.63% respectively. Other species such as *Srongyloides spp*., *Trychostrongylus spp.* and *Bonostomum spp.* were observed in a smaller proportion (Figure 1.5).

Discussion

Fecal egg count reduction test (FECRT)

Negative drug efficacies arise from the establishment of new worm populations after treatment eradicates the previous established population (Bowman, 2009). Drug treatment results in the death of

an established population and the vacant niche activates hypobiotic larvae that are dormant in the mucosa. Typically, these L_4 larvae develop into the adult worm in less than a week (Taylor, 2007), making it possible the establishment of adult worms that are able to produce eggs that are then found during the flotation procedure at d14. Some factors that are responsible for differences in a FECRT include that the egg output by female worms does not sufficiently correlate to the actual worm burden (Eysker and Ploeger, 2000), a non-uniform distribution of eggs in the feces, and the variable distribution of worm species in the host population (Vidyashankar et al., 2007).

The presence of anthelmintic resistance is detected if the observed efficacy in a trial is less than the cutoff value of 95% and the 95% confidence level is less than 90% (Coles et al., 1992). Based on the use of this cutoff value, evaluated anthelmintic treatments did not provide an effective reduction in FEC. Albendazole resulted in the highest FECR but it was still below the cutoff value.

The presence of anthelmintic resistance had been detected in a previous project in 2 commercial dairy farms (Rivera et al., 2009). At Farm A, ALB achieved a FECR of 79.00%, IVM had a FECR of 37.80%, and DOR of 30.97%. At Farm B, ALB achieved a FECR of 100%, IVM of 14.30% and DOR of 42.28%. These results concord with the results of this study in that ALB obtained the highest observed efficacy, followed by DOR and lastly IVM.

Anthelmintic resistance has been detected in several countries in the world. In Argentina, resistance to IVM was reported based on a FECRT with a 74.5% of efficacy (Mejía et al., 2003). In another evaluation of anthelmintic resistance in Argentina, a FECR of 13.00% for IVM was detected (Anziani et al., 2004). Fecal cultures obtained in this study revealed that the predominant specie was *Cooperia spp*. In Brazil, Soutello elt al., 2007 reported FECR of 28.6% for IVM and 94.36% for ALB. Both of these studies confirm the presence of anthelmintic resistance to IVM. Studies involving the efficacy of

ALB against GI nematodes in cattle are scarce, but in the evaluated cattle from Brazil, it was found that resistance to this drug has yet to develop in this country.

Fecal cultures

In Puerto Rico, the last published survey that evaluated the presence of parasitic helminthes in Puerto Rico was performed 26 years ago (Frame and Bendezú, 1987). In this survey, the presence of the trematodes *Fasciola hepatica* (64.7%) and *Cotylophoron cotylophorum* (13.8%) and the nematode *Haemonchus contortus* (21.1%) was documented. The results obtained from fecal samples from dairy farms form Puerto Rico indicate that the most predominant GI nematode in the evaluated cattle was *Cooperia spp.,* followed by *Oesophagostomum radiatum* and lastly, *Haemonchus placei*. It has been reported by several studies in the tropics that *Cooperia spp.* is the predominant GI nematode in cattle in the tropics. In Brazil, *Cooperia spp*. was found to be the most predominant specie, followed by *Haemonchus spp*. and *Oesophagostomum spp*. (Bricarello et al., 2007).

Conclusions

Using a cutoff value of 95% of drug efficacy, the presence of anthelmintic resistance was detected for ivermectin (-8.13%), doramectin (25.50%) and albendazole (67.47%) in 19 out of 21 evaluated dairy farms. The most abundant species of GI nematodes in cattle were *Cooperia spp*., followed by *Oesophagostomum radiatum* and *Haemonchus placei*. *Bonostomum phlebotumum, Strongyloides spp*. and *Trychostrongylus spp*. were also detected in smaller proportions with coprocultures. The presence of anthelmintic resistance in dairy heifers in Puerto Rico was measured

extensively for the first time and the findings of this investigation indicate that anthelmintic resistance is a widespread problem in Puerto Rico.

Table 1. 1: Mean fecal egg count (FEC) before treatment and the percentage of reduction on FEC obtained after treatment with injectable ivermectin and doramectin and oral albendazole.

Farm	Mean FEC pre Tx	% reduction in FEC		
		Ivermectin	Doramectin	Albendazole
1	139.50	51.79	11.54	46.73
2	111.69	-136.19	-38.69	88.64
3	286.79	48.63	-48.28	81.68
4	309.83	-101.25	54.58	40.12
5	385.00	65.94	45.54	97.03
6	370.22	-363.69	53.49	96.00
7	84.70	96.67	100.00	100.00
8	83.79	5.41	-10.97	30.24
9	218.97	59.93	79.23	87.02
10	36.06	63.00	76.30	100.00
11	31.25	-414.13	0.00	100.00
12	114.58	-20.86	-61.17	66.75
13	237.73	-50.94	15.46	79.06
14	79.20	100.00	100.00	92.86
15	50.47	100.00	47.33	-42.86
16	82.50	25.46	36.19	98.04
17	102.00	-133.33	15.08	100.00
18	269.50	85.50	100.00	-55.83
19	248.88	56.76	26.11	100.00
20	98.31	-40.52	-108.85	59.47
21	242.00	97.62	89.74	100.00
$\bar{Y}$	**170.62**	**-8.13**	**25.50**	**67.47**

Table 1. 2: Mean fecal egg counts (FEC) before and after treatment and the mean percentage of reduction on FEC obtained after anthelmintic treatment.

Farm	N	Mean FEC pre Tx	Mean FEC post Tx	FECR (%)
1	11	139.50	78.00	44.09
2	13	111.69	177.69	-59.09
3	21	286.79	69.14	75.89
4	18	309.83	515.17	-66.28
5	21	385.00	95.86	75.10
6	16	370.22	146.44	60.45
7	15	84.70	2.20	97.40
8	39	83.79	69.38	17.20
9	36	218.97	34.83	84.09
10	27	36.06	11.00	69.50
11	6	31.25	60.50	-93.60
12	18	114.58	67.83	40.80
13	49	237.73	124.59	47.59
14	10	79.20	3.30	95.83
15	17	50.47	27.18	46.15
16	19	82.50	27.18	67.05
17	11	102.00	51.00	50.00
18	15	269.50	61.60	77.14
19	12	248.88	57.75	76.80
20	24	98.31	94.88	3.49
21	9	242.00	22.00	90.91

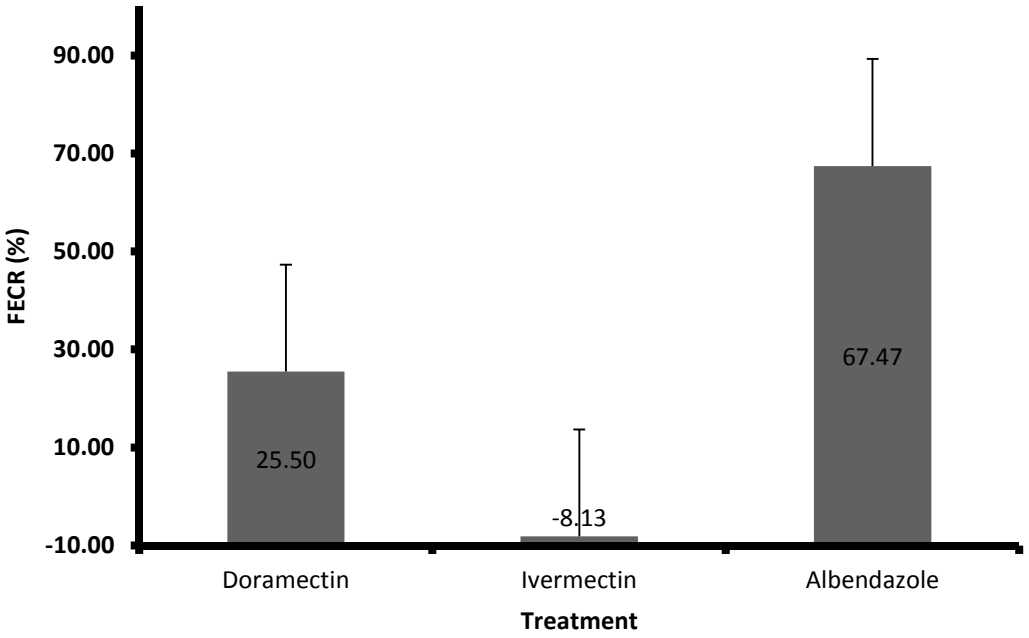

Figure 1. 4: Mean fecal egg count reduction (FECR) for doramectin, ivermectin, and albendazole for all 21 farms tested. Mean FECR was 25.50%, -8.13% and 67.47% for doramectin, ivermectin and albendazole respectively.

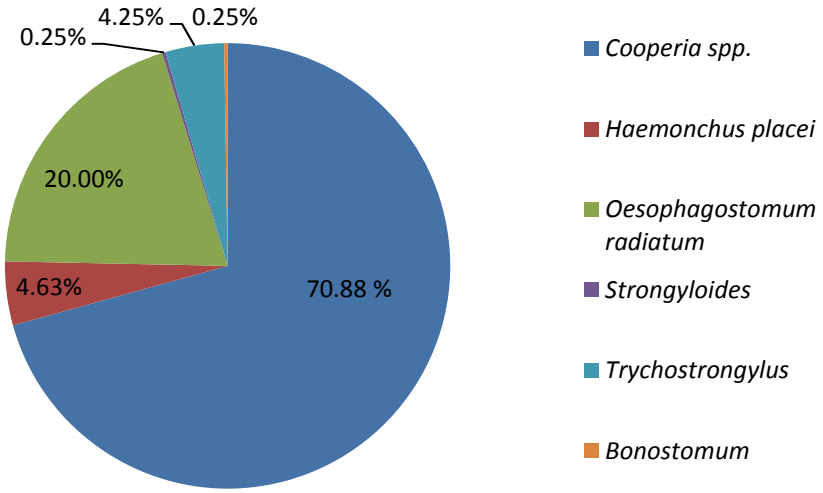

Figure 1. 5: Predominant GI nematode species found in coprocultures from 10 of the evaluated farms.

Figure 1. 6: *Cooperia spp.* L_3 larva

Figure 1. 7: *Oesophagostomum radiatum* L_3 larva

Figure 1. 8: *Haemonchus placei* L_3 larva

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Chapter II:

Evaluation of molecular markers presumably associated with nematode parasite resistance in dairy heifers

Abstract

Gastrointestinal parasitism is a serious affecting problem of the cattle industry. The use of molecular markers associated with resistance against gastrointestinal parasites has been contemplated as an alternative for the control of these parasites. Four candidate genes associated with the immune response against gastrointestinal parasites in cattle were evaluated: **CD3e** (CD3-epsilon), **CCL5** (chemokine (C-C motif) ligand 5), **IgHE** (Immunoglobulin E Heavy Chain Constant Region), and **ITGA4** (integrin alpha 4). Dairy heifers were grouped into 2 populations based on fecal egg count **(FEC)** and allelic and genotypic frequencies were evaluated using a Chi square **(X^2)** test. A total of 23 single nucleotide polymorphisms **(SNP)** were found segregating collectively in all evaluates genes. Of all these, a significant difference in allelic and genotypic frequencies between populations was detected in 4 SNPs in the CCL5 and one SNP in the CD3e gene.

Capítulo II:

Evaluación de marcadores moleculares probablemente asociados con la resistencia de nemátodos parasíticos en novillas lecheras

Resumen

El uso de marcadores moleculares asociados con la resistencia contra los parásitos gastrointestinales ha sido contemplado como una alternativa para el control de estos parásitos en el ganado vacuno. Se evaluaron cuatro genes candidatos asociados con la respuesta inmune contra los parásitos gastrointestinales en el ganado: **CD3e** (CD3-épsilon), **CCL5** (quimiocina (motivo CC) ligando 5), **IgHE** (región constante de la cadena pesada de inmunoglobulina E), y **ITGA4** (integrina alfa 4). Novillas lecheras se agruparon en 2 poblaciones basadas en el recuento fecal de huevos **(FEC)** y las frecuencias alélicas y genotípicas se evaluaron usando la prueba de Chi cuadrado **(X^2)**. Un total de 23 polimorfismos de nucleótido único **(SNP)** se encontraron segregando colectivamente en todos los genes evaluados. De todos ellos, se detectó una diferencia significativa en las frecuencias alélicas y genotípicas entre las poblaciones en 4 SNPs en el gen CCL5 y un SNP en el gen CD3e.

Chapter 2: Literature review

Introduction

The presence of gastrointestinal parasitism in ruminants usually presents itself as subclinical infections that can result in significant economic losses. In the United States, gastrointestinal nematode parasitism costs the cattle industry more than $2 billion per year (Li and Gasbarre, 2009). Clinical signs of parasitism include stunted growth, tissue edema and severe diarrhea (Gasbarre et al., 2001). In turn, these clinical signs translate into economic losses in cattle productivity such as reduced weight gain, weight loss, stunted growth rates, and delays in puberty, breeding, and age at calving (Stromberg et al., 2012) and decreased milk production in cows (Perri et al., 2011). The dairy industry in Puerto Rico accounts for 29.93% of the agricultural production, representing a gross income of $237,107,000 in 2010-2011 (División de Estadísticas Agrícolas, 2012). Calves and young heifers are the most susceptible to parasite infections due to a low resistance to gastrointestinal parasitic worm burdens. Usually, infections with low parasitic burdens are asymptomatic, but high parasitic burdens can produce clinical signs (Claerebout and Vercruysse, 2000). In Puerto Rico, economic losses due to delayed calving age accounts for 33.3% of the expenses in dairy operations (Pantoja-López, 2008).

The development of anthelmintic resistance, the public concern of chemical residues in food and the environment, and the high cost of drug treatment have made the use of alternative parasite control strategies imperative. The selection of cattle populations that show resistance to GI parasites based on genetic variations within the host's genome is one of these alternative methods for the control of infections with gastrointestinal **(GI)** parasites. Understanding the immune responses that contribute to the development of protective immunity, immunosuppression and resistance is a key component on the development of alternative parasite control strategies (Li and Gasbarre, 2010).

However, knowledge of the protective immune responses against GI nematodes is limited by the fact that parasites have evolved to evade the host's protective immune responses. The immune response against an infection will depend on the parasite species as well as age and health status of the host. Infections with *Oesophagostomum radiatum* produce protective immune response early in the host's life, while other parasites such as *Ostertagia ostertagi, Cooperia spp.* and *Haemonchus contortus* require longer periods of exposure to generate an immune response (Gasbarre et al., 2001).

Host Resistance

Age

As cattle age, there is an increase in resistance against parasite infections. The reasons for this phenomenon are not well understood, but it might be due to changes in the effectiveness of immune response as the host develops which in turn result in the host's ability to fight GI parasite infections (Taylor, 2007). The development of protective immunity against GI nematodes depends on the parasite specie. In cattle, infections with *Oesophagostomum radiatum* are associated with younger animals (Bowman, 2009). Adult cattle rarely have significant worm burdens with this specie. Similarly, *Strongyloides* infections are usually seen in ruminants at a very young age (Taylor, 2007). As another example of age resistance to GI nematodes, sheep become immune to infections with *Nematodirus battus* after 3 months of age. If the host becomes infected after immunity has developed, the adult parasites will fail to develop or the larval stages will become arrested in the tissues. Arrested larval stages can become active if there is immunosuppression due to sickness or pregnancy. *Strongyloides* larvae that are arrested in the mucosa will emerge due to unknown signaling in pregnant females and develop to the adult stage to infect the offspring *in utero* or when they nurse (Bowman, 2009).

Premune immunity

Premune immunity **(premunition)** is defined as "a state of resistance to infection which is established after an acute infection has become chronic and which lasts as long as the infective organism remains present in the body" (Dorland, 2011). Naïve calves grazing on infected pastures will become infected rapidly. Infections can become rapidly amplified due to the establishment of heavy worm burdens because of shedding of new eggs into the pastures, which in favorable weather conditions will result in further contamination of pasture with infective larvae. The presence of a stable population of adult worms in the GI tract tends to inhibit the chances of new infections to establish in the GI tract, preventing newly ingested larvae from maturating into the adult stage (Moreau and Chauvin, 2010). After several months of exposure to infection, worm burdens tend of stimulate an effective level of protective immunity (Li, et al., 2012). Reinfections with these parasites will result in a significant reduction in the number of worms that can become established in the GI tract.

Premunition also is manifest itself as a reduction in adult worm size and in female worm fertility (Moreau and Chauvin, 2010). In the case of infection with *C. oncophora*, the mechanisms of host protection that have been observed include reduced female worm fecundity and arrested parasite development (Li and Gasbarre, 2009). Reinfection with these parasites results in a reduction in the number of adult worms that can become established in the host. The removal of this established population due to anthelmintic treatment makes it possible for the arrested larvae to mature or the complete development of newly acquired infections (Bowman, 2009).

Important cells mediating the immune response to gastrointestinal parasites

Different cells of the immune system are able to detect specific parasite antigens that bind to receptors on their surface which results in the initiation of cell signaling. These cells are known as effector cells and are responsible for different immune responses during an infection with a pathogen such as GI nematodes.

B lymphocytes **(B-cells)** are lymphocytes that serve in the immune humural response. The main role of B-cells in the development of immunity relies on their ability to produce antibodies in the presence of an antigen and in the development of immunological memory (Murphy, 2012). B-cells are distinguished due to the presence of the B-cell receptor **(BCR)** on its surface. The binding of an antigen to the BCR on the cell surface propitiates the proliferation and differentiation of B-cells into plasma cells. Plasma cells are the effector form of B-cells and function in the production and secretion of antibodies (Kanobana et al., 2003). The function of the BCR is to recognize and bind an antigen in the variable region of the antibody which results in the activation of the cell, clonal expansion and antibody production (Liu et al., 2010).

T lymphocytes **(T-cells)** are responsible for the cell-mediated immune responses of the adaptive immunity (Murphy, 2012). After a T-cell encounters an antigen and becomes activated, it proliferates and differentiates into an effector T lymphocyte. Helper T-cells **(T_H)** are one of the types of effector T lymphocytes employed during infections with GI nematodes. T_H cells provide additional signals to antigen-stimulated B-cells, influencing the production of antibodies (Murphy, 2012). The response of T_H cells can be classified as T_H1 or T_H2 depending on the cytokines that it produces (Scripps and Jolla, 2001). Mature T_H cells can further differentiate by the expression of surface proteins CD4 or CD8 into $CD4^+$ or $CD8^+$ T_H cells respectively. $CD4^+$ T_H cells can further differentiate into different subsets based on the cytokines that they secrete. T_H2 cells are specialized in the promotion of immune responses to parasitic

infections characterized by an increased production of mucus at mucosal surfaces, eosinophilia, and the production of immunoglobulin E (Murphy, 2012).

Monocytes originate from stem cells in the bone marrow. After leaving the bone marrow, monocytes migrate into tissues, and differentiate into active phagocytic cells, becoming macrophages once they reach the spleen, lymph nodes and lungs (Schmidt and Roberts, 2010). Macrophages induce inflammation and secrete cytokines that activate and recruit other cells of the immune system. They also can initiate adaptive immune responses by acting as antigen presenting cells (Murphy, 2012). Antigen presenting cells **(APCs)** play an important role in the capture, processing and presentation of antigens to T-cells which on their own are not able to detect the presence of the antigen.

Dendritic cells originate from the bone marrow and circulate as immature dendritic cells in the blood. These cells serve as APCs capable of capturing antigens trough phagocytosis. Once a dendritic cell encounters an antigen, phagocytosis occurs which breaks down the antigen in smaller peptides that are able to be presented in the cell's surface (Wieder, 2003). This results in the maturation of the dendritic cell. Mature dendritic cells migrate from the site of infection to a secondary lymphoid organ where it presents the antigen on its surface to naïve T lymphocytes (Lebre et al., 2005). Antigen presentation is one of the many effector functions of dendritic cells and results in the activation of the T-cell receptor. Also, dendritic cells play an important role in directing the effector T-cell response to either a protective T helper type 1 **(T_H1)** or type 2 **(T_H2)** phenotype and are a major source of chemokines (Lebre et al., 2005).

Eosinophils are granulocytes rich in granules that contain a variety of toxic molecules that are released upon the cell's activation. These cells are thought to be important in the host's defense against parasites. They also contribute to the development of allergic inflammatory responses. During an infection, eosinophils are recruited from the blood into the site of infection where they can initiate and

modulate immune responses by acting as APCs (Rothenberg et al., 2001). Eosinophils are also a source of cytokines and chemokines, molecules involved in cell signaling. The main function of eosinophils during an infection with GI nematodes is based in the release of cytotoxic molecules contained inside granules inside the cell **(degranulation)**. Upon degranulation, eosinophils become an important source different molecules with cytotoxic potential like eosinophil peroxide, eosinophil-derived neurotoxin, and eosinophil cationic protein (Rothenberg et al., 2001) and leukotrienes, inflammatory molecules produced by leukocytes, which help in the mediation of vasodilation, smooth muscle contraction and the secretion of mucus (Behm and Ovington, 2000).

Basophils are polymorphonuclear leukocytes that contain cytoplasmic granules rich in inflammation mediators such as histamine, proteoglycans, proteolytic enzymes, lipid mediators and cytokines (Schmidt and Roberts, 2010). Along with eosinophils, basophils are recruited to the site of infection and draining lymph nodes early during an infection with GI nematodes (Balic et al., 2000). It has been postulated that both APCs contribute to the generation and maintenance of a $CD4^+$ T_H2 immune response due to the release of cytokines (Perrigoue et al., 2008). Infections with GI nematodes induce the proliferation of eosinophils and basophils in the bone marrow, produce eosinophilia and an increase in the number of circulating basophils in the blood, and the degranulation of these cells in the site of infection (Gasbarre, 1997).

Cytokines and chemokines

Cytokines are proteins secreted by some immune cells that have an effect on the behavior of other cells. These molecules are involved in cellular communication and are critical in the development and functionality of the innate and adaptive immune responses (Li et al., 2010). Cytokines regulate

inflammation processes and T and B cell growth and differentiation. They have a wide variety of effects of different cells, ranging from stimulatory to inhibitory actions (Gasbarre et al., 2001) and can affect the cell that secretes it (autocrine), adjacent cells (paracrine) and distant cells (endocrine) (Murphy, 2012).

Chemokines are small proteins that attract cells bearing chemokine receptors, such as neutrophils and monocytes, out of the bloodstream and into the site of infection and they are able to regulate leukocyte trafficking (Murphy, 2012). These molecules are secreted mainly by macrophages, endothelial cells, fibroblasts, T-cells and platelets (Schmidt and Roberts, 2010). Chemokines can be classified as homeostatic and inflammatory chemokines. Homeostatic chemokines are mainly produced in secondary lymphoid organs, while inflammatory chemokines are rapidly induced in peripheral tissues upon infection and regulate the income of inflammatory cells. Cytokines and chemokines that are released by activated macrophages initiate the inflammation process (Murphy, 2012).

Integrins

Cell adhesion molecules **(CAMs)** such as integrins, play an essential role in mediating cell adhesion and interactions, including cell proliferation and differentiation, tissue construction, wound repair, pathogen recognition and host defense (Li and Gasbarre, 2010). Integrins are a group of cell adhesion molecules involved in cell proliferation and differentiation, pathogen recognition, the recruitment of cells into tissues and intestinal immunity (Araujo et al., 2009; Li et al., 2010). Integrins are composed of a large α chain and a smaller β chain. During an inflammatory response, endothelial cells in blood vessels begin producing CAMs with the purpose of binding different cells that are circulating in the blood (Murphy, 2012). This process allows leukocytes to attach themselves to the endothelium and migrate from blood vessels into tissues. Similarly, integrins assist in the binding of lymphocytes to the

endothelium and migration into the site of inflammation (Murphy, 2012). Integrins also play an important role in the interaction of T-cells with APCs.

Immunoglobulins

An antigen is a foreign molecule, usually a peptide, that binds selectively to an immunoglobulin (antibody) or is recognized by a T-cell receptor (Murphy, 2012). Immunoglobulins are molecules that can be found as either membrane-bound receptors of B-cells or secreted by plasma cells derived from B-cells (Schmidt and Roberts, 2010). The main functions of antibodies are to bind selectively to a pathogen or its antigen and the recruitment of other cells for the destruction of the pathogen.

Immunoglobulins are composed of four polypeptide chains. Two identical light chains and two identical heavy chains, each of these divided in two regions: the variable region and the constant region are bound together by disulfide bonds and hydrogen bonds arranged in a Y form. The variable region of the heavy chain and light chain combine to form the antigen-binding site. The structure of the antibody's heavy chain constant region determines its effector function: how it will interact with various immune cells to dispose of an antigen (Clarke, et al., 2001).

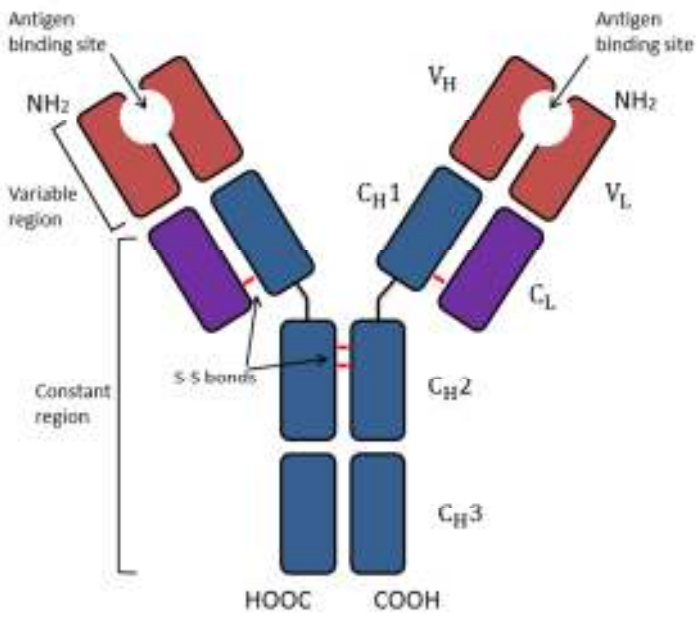


Figure 2. 1: The structure of an immunoglobulin molecule is composed of a heavy and light chains, each of these divided into a variable region and a constant region and joined by disulfide (S-S) bonds (adapted from Murphy, 2012).

The variable region of an immunoglobulin can be composed of an infinite variation in amino acid sequences which form different structures that allow antibodies to bind selectively to a specific antigen. There are two identical variable regions in a same antibody molecule, making it possible for two identical antigens to bind to the same antibody molecule.

The constant region of an antibody determines how the antibody will interact with various immune cells to dispose of an antigen once it has been bound into the antigen binding site. There are only five classes (isotypes) of immunoglobulins: IgA, IgG, IgM, IgD and IgE. Each antibody isotype is found in different organs of the body and has a particular function and utilizes different effector mechanisms to fight against an infection. In the case of infections with trychostrongyle parasites, genes encoding IgE chains have been observed in significantly higher levels in the small intestine mucosa of susceptible calves (Araujo et al., 2009).

T-cell receptors

T-cell receptors **(TCR)** are membrane bound receptors present in the surface of T-cells that are composed by a α- and β-chain. Each chain has a variable region and a constant region and the combination of both chains creates a single antigen-binding site (Murphy, 2012). The TCR does not bind to an antigen directly. Instead, it recognizes fragments of antigens bound on the surface of APCs. The function of this receptor is solely to activate the T-cell after an antigen has been presented to the T-cell. Dendritic cells are the major effector cells responsible for the activation of T-cells. This happens when an antigen has been found, captured, processed and finally presented in the surface of the dendritic cell along with a Major Histocompatibility Complex molecule **(MHC)** (Wieder, 2003). TCRs are able to recognize the combination of the processed antigen and the MHC molecule. The recognition of both molecules results in the activation of the T-cell.

The mechanism that confer immunity against GI Nematodes

The host's immune response to GI nematodes is species dependent and each parasitic larval stage has an unique antigen production profile (Meeusen et al., 2005). The immune response against adult parasites in the GI tract include an increase in the secretion of mucus by Goblet cells, changes in chemical properties of mucins (Li et al., 2009), increased contractility of the gut muscles and increased secretion of the gut (Claerebout and Vercruysse, 2000). All these mechanisms are produced with the purpose of adult worm or migrating larvae expulsion and results in the production of unfavorable conditions for the parasite's survival (Moreau and Chauvin, 2010).

During an infection, parasite larval stages and adult worms in different sites of the GI tract produce antigens, mostly peptides, that activate different mechanisms of the host's immune system

(Hein et al., 2010). In the first 3-4 days after infection, there is a significant increase in the size of the lymph nodes that drain into the site of infection compared to the lymph nodes of naïve calves, due to an increase in the number of lymphocytes that are either parasite-specific or are able to recognize antigens secreted by parasites.

The immune response of calves infected with *C. oncophora* was described by Li and Gasbarre, 2009. At 7 days post infection, the immune responses consist mainly in the rolling, recruitment, and chemotaxis of leukocytes and complement activation. By 14 days post infection, the immune response shifts to the priming and induction of lymphocytes, leukocyte trafficking, neutrophil release and migration of dendritic and natural killer **(NK)** cells. At 42 days post infection, proliferation of B-1 lymphocytes, rolling and accumulation of granulocytes and rolling of eosinophils characterized the major immune responses. At this point of infection the most dominant immune response involves the migration, trafficking and chemotaxis of effector cells and lymphocyte production. The development of nematode protective immunity is characterized by a decrease in female fecundity, stunting of worm growth and the removal of parasites in different stages of development (Balic et al., 2000). Adult worms can be eliminated from the host due to an increased gut motility and increased mucus production elicited by immune responses in the mucosa (Mulcahy, et al., 2004; Onah and Nawa, 2000).

Most infections with GI nematodes elicit a T_H2-like immune response as a result of the stimulation of different subsets of T-helper lymphocytes (Gasbarre et al., 2001). The result of the stimulation of this immune response is the production and secretion of different molecules known as cytokines which serve as cellular communicators. This strong immune response is generated by the presence of GI nematode infections or the presence of detectable parasite antigens, and is characterized by high levels of different cytokines and different antibodies such as IgE and an increase in the number of circulating effector cells (Li and Gasbarre, 2009).

Genes analyzed

Molecular biology tools have become available for the evaluation of different pathways that make an animal more resistant or immune to an infection with GI nematodes. Expression patterns of different genes in the small intestine mucosa and mesenteric lymph nodes of calves infected with trychostrongyle parasites demonstrate that genes related to IgE receptors, integrins, complement components, among others are expressed in high levels in resistant animals, while genes related to immunoglobulin chains and TCRs are highly expressed in susceptible animals.

Chemokine (C-C motif) ligand 5 (CCL5)

CCL5 forms part of the CC chemokine group which promotes the migration of different cells of the immune response. This chemokine is produced by T-cells, platelets endothelial cells and serves in the recruitment of monocytes, T-cells, NK cells, basophils, eosinophils and dendritic cells into the site of infection. Its major effects are the degranulation of basophils, activation of T-cells and chronic inflammation (Murphy, 2012). Basophils and mast cells are granulocytes. These cells contain cytoplasmic granules that are rich in biological mediators such as histamine, proteoglycans and proteases. Degranulation of these cells can be induced within minutes after stimulation (Balic et al., 2000). CCL5 serves as a secondary mediator for the degranulation of basophils and mast cells. The effects of mast cell derived mediators include an increase in peristalsis, increased vascular permeability, increased mucus secretion, and the degradation of blood vessels and epithelial cell basement membranes. Degranulation of eosinophils also releases CCL5 as part of an inflammatory response (Rothenberg et al., 2001). The encounter of an antigen with a dendritic cell propitiates its maturation which results the

mass production of inflammatory chemokines such as CCL5 which help sustain the recruitment of more dendritic cells and T-cells into the site of infection (Lebre et al., 2005).

The precise role of CCL5 during GI nematode infections remains unknown and no correlation between polymorphisms in this gene and disease has been made. However, CCL5 has been intensely studied in humans due to its involvement in inflammatory reactions mostly involved during pulmonary diseases (Dorfmüller et al., 2002; Levy, 2009; Schall et at., 1990) involving the recruitment of T-cells and monocytes that bear CD4 antigens in their membrane (Schall et al., 1990), eosinophils, and basophils (Appay and Rowland-Jones, 2001). Human and bovine CCL5 share a 80.6% similarity in amino acid sequence (Aust et al., 1999).

CD3-epsilon (CD3e)

The CD3e gene codifies for the ε chain of the CD3 TCR complex. The TCR CD3 complex **(TCR-CD3)** is composed of six different chains paired as dimers: the α:β chains which serve as the antigen-binding site and CD3 proteins: CD3γ, CD3δ, CD3ε and CD3ζ chains. These CD3 proteins are associated with the α:β TCR (Murphy, 2012). Two CD3e chains are present in the TCR-CD3 complex each one associated with CD3γ and CD3δ respectively. It is thought that the CD3δ:CD3ε dimer interacts with the TCR α-chain and the CD3γ:CD3ε dimer interacts with the TCR β-chain (Call et al., 2002).

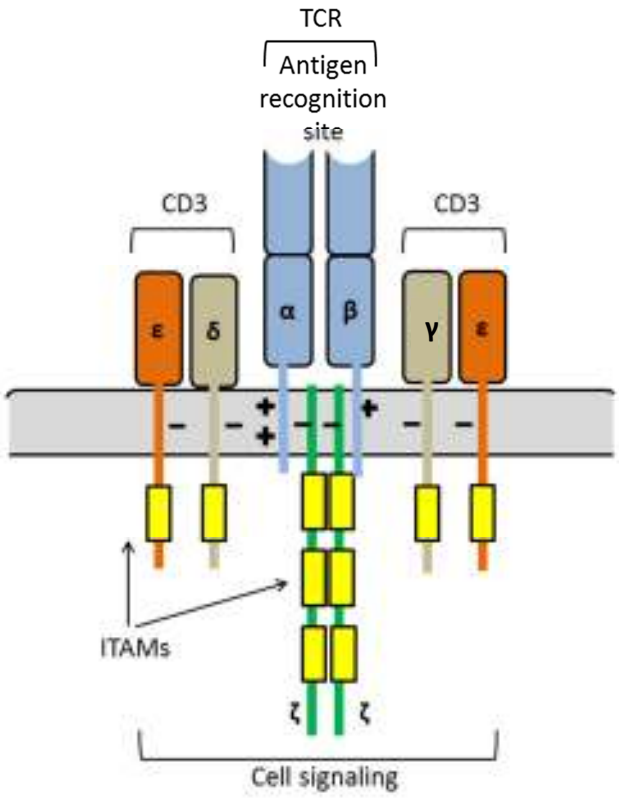


Figure 2. 2: The CD3 T-cell receptor complex is composed of an α:β TCR associated with a CD3γ:CD3ε dimer and a CD3δ:CD3ε dimer (adapted from Murphy, 2012).

Once the TCR recognizes an APC ligated with its corresponding MHC molecule, signaling is produced due to the phosphorylation of tyrosine residues present in the CD3γ, CD3δ, CD3ε and CD3ζ chains in a region known as the receptor tyrosine-based activation motifs **(ITAMs)**. The phosphorylation of ITAMs results in the further phosphorylation of several other molecules that produce several co-stimulatory signals that result in the activation, proliferation and differentiation of the T lymphocyte (Brattsand et al., 1990).

During a natural infection with trichostrongyle parasites, calves have shown increased levels in the regulation of CD3e in the small intestine mucosa of susceptible animals (Araujo et al., 2009). During infections with GI nematodes, effector cells are continuously being recruited into the site of infection.

Upon an encounter with a parasite antigen, effector cells become APCs that in turn will migrate to draining lymph nodes to encounter naïve T-cells with the purpose of activating the immune response these cells. The activation of T-cells is essential for the immune response of the host against GI nematodes, which will result in changes in the mucosa secretion and an increase in contractions of the gut with the purpose of the expulsion of adult worms that are established (Onah and Nawa, 2000).

Although the precise function of CD3e in the immune response of cattle against GI nematodes remains unknown, it has been reported that deficient expression in the membrane of T-cells or structural changes in CD3e gene are linked to severe immunodeficiency characterized by an absence of T-cell proliferation and activation (Basile et al., 2004; Le Deist et al., 1991; Roberts et al., 2007; Soudais et al., 1993; Thoenes et al., 1992). The study of SNPs in this gene has been centered only in reporting the presence of polymorphisms in different cattle breeds (Barris et al., 2012; Kawahara-Miki et al., 2011; Stothard et al., 2011). So far, no relationship between SNPs in the CD3e gene and resistance or susceptibility of cattle against parasitic nematodes has been established.

Integrin alpha 4 (ITGA4)

Integrins are adhesion molecules responsible for the proliferation, differentiation, migration and recruitment of cells, contributing in the intestinal immunity. ITGA4 is an integrin heterodimer composed of an alpha 4 and a beta 1 unit, which form the Very Late Activation Antigen-4 **(VLA-4)**. During the early stages of the development of B-cells, the common lymphoid progenitor binds to adhesion molecules on stromal cells through the integrin VLA-4 receptor and other CAMs which results in the proliferation of B-cell progenitors (Murphy, 2012). This integrin serves as a receptor on lymphocytes, monocytes, eosinophils, NK cells and thymocytes. It is involved cell adhesion and the promotion of inflammation,

recruitment of hematopoietic cells, and immune response (National Library of Medicine, 2011). In order to migrate into lymphoid tissues, lymphocytes must bind to high endothelial venules through CAMs.

VLA-4 is highly expressed after T-cell activation occurs. The α4β1 subunits bind to VCAM-1, an adhesion molecule found in activated vessels, lymphocytes and dendritic cells, on the activated endothelium (Elices et al., 1990). This results in the homing of effector T-cells into the tissue where inflammation is happening. The activity of integrins in T-cell membranes can be increased after chemokine stimulation (Balic et al., 2000). This produces a stronger binding between the T-cell and the endothelium which propitiates diapedesis, the migration of a lymphocyte through a blood vessel into a tissue.

ITGA4 is involved in inflammatory responses and is partially responsible for eosinophil recruitment (Rothenberg et al., 2001) and a strong up-regulation of several integrins, including ITGA4, has been observed at 42 days post infection with *Cooperia oncophora* (Li and Gasbarre, 2009). In a study of microarray of genes associated with cattle immune system or immunologically-mediated responses, genes encoding integrins such as ITGA4, had higher expression levels in the mesenteric lymph nodes of resistant animals. The presence of ITGA4 is also necessary for the diapedesis of leukocytes through the endothelium (Murphy, 2012). Up to this date, the effects of polymorphisms in the ITGA4 gene in the immune response of cattle have not been reported. However, in humans this gene has been extensively studied as a candidate gene for the treatment of autoimmune diseases such as the HIV-virus (Glatigny et al., 2011) and multiple sclerosis (O'Doherty et al., 2007) and several polymorphisms have been associated with the delayed development of these diseases. The interaction between VLA-4 and its endothelial ligand is responsible for the diapedesis of leukocytes into tissues (Ransohoff, 1999). Any polymorphism that results in changes in the amino acid sequence codified by the ITGA4 gene will result

in the blockage of ITGA4-mediated migration of immune cells into the tissues (Darc et al., 2011; Glatigny et al., 2011; O'Doherty et al., 2007; Ransohoff, 1999).

Immunoglobulin E Heavy Chain Constant Region (IgHE)

The function of IgE in the protection against GI nematodes in ruminants is not well understood. IgE serves as an important mediator in the cellular cytotoxicity of eosinophils and macrophages during infections with GI nematodes (Thatcher et al., 1989). During infections with *Cooperia*, parasite-specific IgE titres in blood serum increase rapidly over a period of 21 days and remain elevated during the period of infection (Kanobana et al., 2003). However, conflicting results have been reported. Thatcher et al., 1989 reported that calves with low infections with *Ostertagia ostertagi* show a significant increase in IgE levels in blood serum but calves with moderate to severe infections only show a mild increase. This difference may be due to the fact that *Ostertagia* and *Cooperia* reside in different niches; *Ostertagia* adult worms are present in the abomasum while *Cooperia* worms are found in the small intestine. This reinforces the theory that the immune response to GI nematode infection depends on the nematode specie and the site of infection.

IgE levels in serum in a continuous *Haemonchus contortus* larval challenge in the GI epithelium propitiates the degranulation of mast cells due to the binding of parasite antigens to IgE receptors (Meeusen et al., 2005). Mast cell degranulation and activation contributes to the recruitment of cells to the site of infection and is responsible for the secretion of mucus, electrolytes and fluids, smooth muscle contractility, and increased vascular and epithelial permeability (Farthing, 2003). IgE is associated with a T_H1 type response. The clinical manifestations of this type of immune response include eosinophilia and the degranulation of several effector cells including basophils, eosinophils, and mast cells (Balic et al.,

2000). The heavy constant region of IgE has high affinity receptors on mast cells, basophils and activated eosinophils which are effector cells important in the activation of immune responses (Murphy, 2012). The constant region can either bind to receptors present in immune effector cells, bind to the C1q protein of the complement, initiating the complement cascade, or deliver antibodies to different sites through active transport (Murphy, 2012).

The relationship between a 36 bp deletion in a 87 bp repeating sequence in the untranslated region of the IgHE gene has been recently associated with resistance to the intestinal parasite *Trichostrongylus colubriformis* of sheep (Clarke et al., 2001). This same deletion was found in cattle, but no association between the polymorphism and GI nematode resistance was made in this study. Besides this study, no other evaluation of polymorphisms in the IgHE gene and resistance or susceptibility to GI nematodes has been made.

Materials and methods

Animal Selection

Dairy operations were selected based on good animal identification practices and general good dairy management. Groups of at least 20 heifers between the ages of 3 to 12 months (average 9 months) were selected to form part of the investigation. All heifers were required to have been grazing for a minimum of 3 months and have not been exposed to anthelmintic treatment for at least the 3 months prior to sampling. Heifers were placed in two groups based on fecal egg count **(FEC)** prior to the administration of anthelmintic treatment. The "Negative" group (n=149) was composed of heifers whose FEC was <33 eggs per grams of feces **(EPG)**. The "Positive" group (n=76) was composed of heifers whose FEC was >100 EPG.

Blood collection and DNA isolation

Blood samples were collected from heifers selected for the FECRT by jugular venipuncture. 15 mL of blood were collected for DNA extraction using the protocol established by Sambrook et al., 1989. Using this method, red blood cells are lysed and eliminated through centrifugation. DNA was extracted from the buffy coat of blood which is composed of leukocytes and platelets which are then treated with different buffers to lyse the cell's membrane and extract the DNA within. Quantification of DNA samples was performed by spectrophotometry at 260 nm. Aliquots of 120 ng of DNA were used as a template for polymerase chain reaction.

Single nucleotide polymorphisms (SNP) genotyping

Polymerase chain reaction **(PCR)** was performed using an Eppendorf thermocycler (Model No. 950040015, Hauppauge, NY). Single nucleotide polymorphism **(SNP)** detection was performed using primers at a final concentration of 0.3 µM for the genes:

CD3e (CD3-epsilon),

CCL5 (chemokine (C-C motif) ligand 5),

IgHE (Immunoglobulin E Heavy Chain Constant Region), and

ITGA4 (integrin alpha 4).

The amplified PCR product was sequenced at MacroGen Inc. Laboratory at Seoul, Republic of Korea.

Statistical Analysis

Differences in genotypic and allelic frequencies for the two populations were determined and analyzed using contingency tables using InfoStat statistical analysis software (Di Rienzo et al., 2012). Additionally, genotype and allele frequencies were analyzed following the Hardy-Weinberg **(H-W)** equilibrium (Rodriguez, 2009). Haplotypes for all genes was also analyzed using contingency tables with InfoStat.

Results

Frequencies and single nucleotide polymorphisms (SNP) in the CCL5 gene

The genotypic frequencies and allelic frequencies of each of these SNPs can be found in Table 2.3 and Table 2.4 respectively. Five different SNPs were found in the exon 3 of the CCL5 gene. For each of these SNPs, Chi-square test **(X^2)** was performed to evaluate the distribution of each genotype in the two evaluated populations. Rs109200822, a transition of cytosine to thymine and 3 transitions of adenine to guanine (Rs136242974, Rs109769870, and Rs110457788) were found segregating in both evaluated populations (Table 2.3). Using contingency tables, a significant difference was detected in the genotypic frequencies between both evaluated populations. Results of the H-W equilibrium test show that there is no significant difference in the genotypic and allelic frequencies in population P, meaning that SNPs found in this population are in equilibrium (Table 2.5). The segregation of Rs109200822 in the population N was found to be in H-W disequilibrium. This is the result of a higher segregation of the heterozygote allele in the in this population compared to the P population.

Additionally to previously published polymorphisms, a new SNP was found in the evaluated cattle populations in this study. SNP CCL5-187 (Figure 2.4) is a transition of guanine to adenine in the base 187 of the amplified fragment of the exon 3 of the CCL5 gene. The genotypic distribution of this SNP is dominated by the segregation of the heterozygous allele followed by the homozygous GG and finally AA. No significant difference was detected in the distribution of this polymorphism between populations (p=0.241). Frequency of the allele A was 0.337 and 0.301 for groups N and P respectively. For the guanine allele, the allelic frequencies were 0.663 and 0.699 for the N and P groups respectively (Table 2.4). H-W equilibrium test for both populations suggests that group N is in H-W equilibrium (p=0.024), while group P is not (p=0.980).

Frequencies and single nucleotide polymorphisms (SNP) in the CD3e gene

Three different regions of the CD3e gene were amplified with different primer sets. Primer set CD3e_1 was used to amplify exon1, intron 1, exon 2 and part of intron 2. After the analysis of the DNA sequence of the PCR product of this primer set from 126 heifers, no further analyses were made with this primer set due to the absence of polymorphisms in the amplified product. The summary of all SNPs found in all amplified fragments of the CD3e gene can be found in Table 2.7. Genotypic and allelic frequencies for all SNPs detected in the CD3e gene can be found in Tables 2.8 and 2.9 respectively. The results of H-W equilibrium test are in Table 2.10 and the analysis of haplotype distribution is in Table 2.21. No significant difference was found in the distribution of haplotypes (p=0.204).

Primer set CD3e_3 was used to amplify part of Intron 6, exon 7, intron 7, and exon 8 of this gene. Two published SNPs are in this fragment, both of them transversions of thymine to guanine. Rs13576648 is located in exon 7. This transversion causes a silent mutation since both genotypes code for the amino acid glycine. Rs132883476 is another transversion of thymine to guanine that is found in intron 7. All evaluated heifers of both groups (n=216) were only segregating the TT genotype of these two SNPs. A new polymorphism was found in intron 7 of the CD3e gene (Figure 2.6). This SNP, CD3e-258, consists of a transition of guanine to adenine. Only the homozygous allele GG and the heterozygous allele GA were found segregating in both populations. The genotypic frequency of GG was 0.943 in the N group and 0.941 in the P group. The genotype GA was segregated with a genotypic frequency of 0.114 and 0.118 in the N and P groups respectively. Genotypic and allelic frequencies are not statistically different in both groups (p=0.735).

Primer set CD3e_2 was used to amplify exon 9 of the CD3e gene. This exon is part of the un-translated region **(UTR)** of the CD3e gene. Two published SNPs are found in this exon, an

insertion/deletion in the base 101 and a transition in the base 104. Of these two, only Rs136656580, an insertion/deletion in the base 101 was found to be segregating in both populations. No significant difference (p=0.554) was observed in the distribution of genotypes between both groups (Table 2.8). Heifers in both populations segregate the homozygous genotype for the insertion of AGG with a frequency of 0.745 and 0.771, followed by the heterozygous genotype with frequencies of 0.235 and 0.213 and last the homozygous for the deletion with frequencies of 0.0207 and 0.016 in the N and P groups respectively.

Additionally, a new SNP in the base 112 was found in exon 9 of the CD3e gene (Figure 2.8). This polymorphism consists in a transition of cytosine to thymine. Only one homozygous allele CC and the heterozygous alleles were found segregating in both populations. In both populations, the highest segregating allele was the homozygous CC allele with a 90.34% in the N group and 78.69% in the P group. Both groups significantly differ between each other (p=0.024). The evaluation of the H-W equilibrium of both populations for these unreported SNPs is found in Table 2.10. Both N and P populations do not follow the H-W equilibrium for the reported genotypes of CD3e-112. On the contrary, observed genotypes for CD3e-258 in the N group are not in H-W equilibrium (p=0.0004), while heifers in the P group are in equilibrium (p=0.840).

Frequencies and single nucleotide polymorphisms (SNP) in the ITGA4 gene

Four different regions of the ITGA4 gene were analyzed using PCR. The information of each primer used can be found in Table 2.11. Primer set ITGA4_1 was used to amplify exon 1, intron 1, exon 2 and part of intron 2. DNA sequence analysis of this fragment revealed the segregation of 4 different SNPs, all of them described for different cattle breeds, including Holstein-Friesian (Barris et al., 2012;

Kawahara-Miki et al., 2011; Stothard et al., 2011). Information of each SNP found in the ITGA4_1 amplified fragment can be found in table 2.12. Three of these polymorphisms are located in the intron 1 of this gene. Rs42239893 and Rs133348759 are transitions of adenine to guanine in the base position 301 and 529 of the fragment and Rs42239894 is a transversion of cytosine to adenine in the base position 360. No statistical difference was detected in the genotypic and allelic frequencies between both analyzed populations for SNPs in intron 1 (Table 2.13). The distribution of genotypes and alleles of Rs42239893 and Rs42239894 follow the H-W equilibrium in both populations, while in Rs133348759, only the N group is in equilibrium (Table 2.15). This is because in all SNPs, the heterozygous allele is the one being segregated with the highest frequency. Another SNP was found in this amplified fragment. Rs133643873 is a transversion of thymine to guanine found in the base position 780 of the ITGA4_1 amplified fragment. No significant difference was detected in the allelic and genotypic distribution of this SNP (p=0.307). This SNP is also found in H-W equilibrium. Haplotypes of the ITGA4_1 fragment do not differ statistically between each other (p=0.192).

Two other fragments of the ITGA4 gene were also analyzed with PCR, but due to an absence of SNPs in these regions, no statistical analyses were made. Primer set ITGA4_2 was used to amplify and sequence exon 3, intron 3, and exon 4 of this gene. ITGA4_3 primer set was used to amplify and sequence intron 7, exon 8, intron 8 and exon 9 of the ITGA4 gene.

ITGA4_4 primer set was used to amplify and sequence the entire exon 28 of this gene. DNA sequence analysis of this exon revealed the presence of 4 SNPs. Rs42240844 and Rs42240845. Both these SNPs are transitions found in base positions 415 and 456 respectively (Table 2.12). For both SNPs, no significant differences were found between both populations (Table 2.13) and they are found in H-W equilibrium (Table 2.15). Two new SNPs were also found in the evaluated populations. ITGA4-192 (Figure 2.13) is an insertion/deletion of two thymines in the base position 192. The heterozygous

genotype of this polymorphism was only found in one individual the Positive population and had a genotypic frequency of 0.008 (Table 2.14). The other new polymorphism observed in evaluated heifers is ITGA4-298. This SNP consists of a transition of guanine to adenine (Figure 2.14) in the position 298 of exon 28. No statistical difference was found in the genotypic and allelic frequencies between both populations (p=0.495) and both populations are in H-W equilibrium (Table 2.15). There is also no statistical difference in haplotype distribution between both populations as well (p=0.303).

Frequencies and single nucleotide polymorphisms (SNP) in the IgHE gene

The heavy constant region of IgE consists of four exons and their respective introns. Three of these exons were amplified and sequenced in this investigation. The information of the primers used for this analysis can be found in Table 2.16. Sequencing results of this gene indicated the presence of 9 different polymorphisms (Table 2.17). The analysis of the distribution of genotypes and alleles for each SNP can be found in Tables 2.18 and 2.19 respectively. H-W equilibrium test results are presented in table 2.20 and the distribution of haplotypes is presented in Table 2.21.

Two published SNPs are located in the intron 2 of the IgHE gene. Rs208453779 and Rs2011220021 are both transversions of cytosine to adenine found in the base positions 107 and 135 respectively. The segregation of Rs208453779 is dominated in both populations by the AA allele with frequencies of 0.857 and 0.868 for the N and P groups respectively. In the case of Rs2011220021, the majority of the heifers evaluated in both populations were segregating the CC allele with frequencies of 0.866 and 0.974 for the N and P groups respectively. No statistical difference was detected in the genotype and allele distribution of both SNPs in the two evaluated populations. H-W equilibrium test indicates that the distribution of these SNPs was not in equilibrium in both populations as well. This is

because over 85% of the animals in both populations are segregating only one homozygous allele and the other homozygous and the heterozygous allele are barely being segregated in the populations.

M. Pagán et al., 2013 reported the presence of a new polymorphism found in the second intron of this gene in Senepol*Charolais crossbreed heifers in Puerto Rico. IgHE-40IR is a transition of adenine to guanine in the base position 30 of the IgHE gene amplified fragment. In the evaluated populations in this study, the distribution of this SNP was dominated by the presence of both homozygous alleles. No statistical difference was detected between both populations (p=0.487) and both populations are not in H-W equilibrium.

Four new SNPs were also found by Pagán et al., 2013 in the exon 2 of the IgHE gene of the evaluated Senepol*Charolais heifers. In the evaluated dairy heifer populations on this study, only 3 of these SNPs were being segregated. IgHE-280IR is a transversion of cytosine to adenine in the base position 291 of the amplified fragment. This SNP results in the change of amino acid sequence of glutamine to lysine. The second SNP found, IgHE-310IR, is a transition of guanine to adenine that results in a codon change from arginine to glutamine. IgHE-320IR is a silent mutation product of the transition of cytosine to thymine. Both genotypes codify for the codon proline. The fourth SNP found in exon 2 by Pagán et al., 2013, IgHE-380IR, was not found to be segregating in the evaluated population of dairy heifers. This polymorphism is transversion of cytosine to guanine that results in the replacement of glutamine to glutamic acid in the codon sequence. The only genotype observed for this SNP was CC.

The last two exons of each immunoglobulin heavy constant region contain sequences encoding the secreted and the transmembrane regions of an antibody (Murphy, 2012). IgHE-840IR is a transition of adenine to guanine found in exon 3 of the IgHE gene. This polymorphism was also found in the Senepol*Charolais cattle evaluated by Pagán et al., 2013. In this study, it was found that both evaluated population segregate mainly the GG genotype with a frequency of 0.908 and 0.921 in the N and P

groups respectively. Both alleles AA and GG codify for the same codon, threonine, meaning that there is no change in the amino acid reading sequence. No significant difference was detected in the distribution of genotypes and alleles between these two populations (p=0.907). Both populations also are found in H-W equilibrium.

Discussion

Chemokine (C-C motif) ligand 5 (CCL5)

A total of 4 different polymorphisms have previously been described in the exon 4 of the CCL5 gene. This exon contains the stop codon TAG, which indicates that all polymorphisms found in this exon are in the UTR of this gene. Polymorphisms Rs136242974, Rs109200822, Rs109769870 and Rs110457788 have been reported in different cattle breeds (Barris et al., 2012; Kawahara-Miki et al., 2011; Stothard et al., 2011), including Holstein-Friesian. Information on each of these previously described SNP can be found in Table 2.2, including the location and type of SNP. For the evaluated SNPs found in the exon 3 of the CCL5, it was observed that a significant difference in the genotypic frequencies between both populations was present for the previously published SNPs (Rs136242974, Rs109200822, Rs109769870, and Rs110457788). The segregation of these SNPs is characterized by a higher segregation of the heterozygote allele.

CCL5 has been mostly studied in mouse and human models due to its important role in the mediation of inflammatory responses that include the degranulation of effector cells and the activation of T-cells. CCL5 functions in the chemotaxis of T-cells, dendritic cells, eosinophils, NK cells, basophils, mast cells and the activation of T-cells and leukocytes (Appay et al., 2001; Levy, 2009). The role of CCL5

in the immune response against GI nematodes in cattle is not completely understood, but it has been reported that during infections with GI nematodes, there is an increase in the expression of this pro-inflammatory chemokine in the small intestine mucosa of susceptible calves (Araujo et al., 2009).

CD3-epsilon (CD3e)

The CD3e gene codifies for the ε chain of the CD3-TCR complex. The ε chains are involved in the signaling and activation of the T-cell once an antigen is presented by an effector cell in the TCR (Hagens et al., 1996). Signaling produces a serious of phosphorylation and oxidation reactions that result in the activation of the T-cell. The activation of T-cells is essential for the immune response of the host against GI nematodes, which will result in changes in the mucosa secretion and an increase in contractions of the gut with the purpose of the expulsion of adult worms that are established (Onah and Nawa, 2000). Although the clear function of CD3e in the immune response in cattle is not known, it has been documented that deficient expression in the membrane of T-cells or structural changes in CD3e gene are linked to severe immunodeficiency characterized by an absence of T-cell proliferation and activation (Basile et al., 2004; Le Deist, et al., 1991; Roberts et al., 2007; Soudais, et al., 1993; Thoenes et al., 1992). It is known that during a natural infection with trychostrongyle parasites, calves have shown increased levels in the regulation of CD3e in the small intestine mucosa of susceptible animals (Araujo et al., 2009).

In this study, only one SNP in the CD3e gene, CD3e-112, which had not been previously described, was the only polymorphism which showed a statistical difference between both evaluated populations. Both populations show a higher genotypic difference for the homozygote allele CC, but the N population has a higher segregation of this allele. The segregation of the heterozygote allele was found to be segregating with a higher frequency in the population with higher FEC (group P) than heifers

with low FEC (group N), meaning that the presence of the heterozygote allele can be associated with animals with high FEC.

Integrin alpha 4 (ITGA4)

Seven different SNPs were found in the amplified fragments of the ITGA4 gene, all of which showed no statistical difference between evaluated populations. Thus, the evaluated polymorphisms do not serve as candidate SNPs for the differentiation and selection between resistant and susceptible populations. ITGA4 is involved in inflammatory responses and is partially responsible for eosinophil recruitment (Rothenberg et al., 2001) and a strong up-regulation of several integrins, including ITGA4, has been observed at 42 days post infection with *Cooperia oncophora* (Li and Gasbarre, 2009). Additionally, Arujo et al., 2009 reported an increase in the regulation of this gene in the mesenteric lymph nodes of resistant calves. The presence of ITGA4 is also necessary for the diapedesis of leukocytes through the endothelium (Murphy, 2012).

Immunoglobulin E Heavy Chain Constant Region (IgHE)

Immunoglobulin E is an important mediator of the immune response against GI nematodes. Like all antibodies, IgE is composed of a heavy and light chain and each chain has a constant and variable region. The variable regions of both chains play an important role in the recognition and binding of an antigen to the antibody molecule. The heavy constant region of an antibody determines its effector function: how it will interact with various immune cells to dispose of an antigen (Clarke et al., 2001).

The immune response against GI nematodes is characterized by the production of IgE-mediated immediate hypersensitivity in the gut (Claerebout and Vercruysse, 2000). High levels of IgE expression are observed in the small intestine mucosa of parasite susceptible calves (Araujo et al., 2009). It has been found in sheep infected with *Haemonchus contortus* that IgE levels in blood serum increase within 2 to 3 weeks after infection (Kooyman et al., 1997). Similarly, cattle infected with *Cooperia*, show an increase of parasite-specific IgE titres in blood serum (Kanobana et al., 2003). Receptors for IgE can be found in mast cells, basophils, eosinophils and macrophages. Interaction between IgE and its receptor in the effective cell results in the degranulation of these cells during nematode infections (Meeusen et al., 2005).

Conclusions

The analysis of allelic frequencies between a population of dairy cattle that has low EPG and another population of dairy heifers with high EPG was made through the use of PCR to evaluate SNPs in the CCL5, ITGA4, CD3e, and IgE genes. All the evaluated genes are involved in different mechanisms of protective immunity against GI nematodes; from cell attracting molecules such as chemokines to antibodies and components of T-cell receptors. These genes might serve as candidate genes for the genetic evaluation of resistant or susceptible cattle and assist in the selection of a population of cattle that have a higher degree of resistance against infection. No evaluation of polymorphisms found in each of the evaluated genes in association to GI nematode susceptibility or resistance has had not been previously made in cattle. Of all evaluated genes, the segregation of 4 SNPs in the CCL5 and one SNP in CD3e were found to be statistically different between populations, making these genes possible candidate genes for the selection of a resistant cattle population against GI nematodes.

Table 2. 1: Description of primer sequences used in the evaluation of each single nucleotide polymorphisms (SNP) in the CCL5 gene.

Gene	Forward (5' – 3')	Reverse (3' – 5')	Fragment size (bp)	Reference
CCL5	AGAAGTGGGTGCGAGAGTACATCAAC	TTCTTATTTGCCAGTGCAGCCCTC	409	NM_175827.2

Table 2. 2: List of SNPs found in the amplified fragment of the CCL5 gene.

Gene	SNP I.D.	N	Location	SNP	Base position
CCL5	Rs136242974	155	Exon 3	G/A	48
CCL5	Rs109200822	155	Exon 3	C/T	55
CCL5	Rs109769870	155	Exon 3	G/A	145
CCL5	CCL5-187	155	Exon 3	G/A	187
CCL5	Rs110457788	155	Exon 3	A/G	219

Table 2. 3: Genotypic frequencies of SNP found in the CCL5 gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P	p-value of X^2
		Genotypic frequency	Genotypic frequency	
Rs136242974	AA	0.064	0.192	0.010
	AG	0.596	0.452	
	GG	0.340	0.356	
Rs109200822	CC	0.092	0.206	0.014
	CT	0.634	0.452	
	TT	0.275	0.343	
Rs109769870	AA	0.057	0.192	0.007
	GA	0.603	0.480	
	GG	0.340	0.329	
CCL5-187	AA	0.071	0.096	0.241
	GA	0.532	0.411	
	GG	0.397	0.493	
Rs110457788	AA	0.078	0.219	0.010
	AG	0.638	0.493	
	GG	0.284	0.288	

Table 2. 4: Allelic frequencies of SNP found in the CCL5 gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P
		Allelic frequency	Allelic frequency
Rs136242974	A	0.362	0.418
	G	0.638	0.582
Rs109200822	C	0.409	0.436
	T	0.592	0.569
Rs109769870	A	0.358	0.432
	G	0.642	0.569
CCL5-187	A	0.337	0.301
	G	0.663	0.699
Rs110457788	A	0.397	0.466
	G	0.603	0.534

Table 2. 5: Hardy-Weinberg equilibrium test. Calculated X^2 value of SNP found in the CCL5 gene for animals in the "Negative" group (N) and the "Positive" group (P) along with p-values for this test (df=2). P-values<0.05 are statistically significant, meaning that the population does not follow the H-W equilibrium.

SNP	Genotype	Group N			Group P		
		n	X^2	p-value	n	X^2	p-value
Rs136242974	AA	9	11.87	0.003	14	0.37	0.831
	AG	84			33		
	GG	48			26		
Rs109200822	CC	13	14.26	0.001	15	0.45	0.799
	CT	90			33		
	TT	38			25		
Rs109769870	AA	8	13.65	0.001	14	0.04	0.842
	GA	85			35		
	GG	48			24		
CCL5-187	AA	10	5.12	0.024	7	0.04	0.980
	GA	75			30		
	GG	56			36		
Rs110457788	AA	11	15.63	0.0004	16	0.01	0.995
	AG	90			36		
	GG	40			21		

Table 2. 6: Description of primer sequences used in the evaluation of each single nucleotide polymorphisms (SNP) in the CD3e gene.

Primer name	Forward (5' – 3')	Reverse (3' – 5')	Fragment size (bp)	Reference
CD3e_1	TCTCAAACGTAGAACCCTTCAGGC	TTCTCGGAGGCACAAAGATCTCCT	577	U25687
CD3e_2	TGTATGCTGGCCTGAATCAGAGAG	ATCTAGGAGTCTGCAGTGGACACA	565	U25687
CD3e_3	TTGTTCCTTCCTCATCTCCGGTGT	ATAGTCCCACCTGTCCCTTTCT	700	U25687

Table 2. 7: List of SNPs found in different amplified fragments of the CD3e gene.

Gene	SNP I.D.	N	Location	SNP	Base position
CD3e_2	Rs136656580	206	Exon 9	Ins/Del	101
CD3e_2	Rs209286808	206	Exon 9	A/G	104
CD3e_2	CD3e-112	206	Exon 9	C/T	112
CD3e_3	Rs13576648	216	Exon 7	T/G	215
CD3e_3	Rs132883476	216	Intron 7	T/G	227
CD3e_3	CD3e-258	216	Intron 7	G/A	258

Table 2. 8: Genotypic frequencies of SNP found in the CD3e gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P	p-value of X^2
		Genotypic frequency	Genotypic frequency	
Rs136656580	Del/Del	0.021	0.016	0.554
	Ins/Del	0.235	0.213	
	Ins/Ins	0.7448	0.771	
Rs209286808	AA	1.000	1.000	-
	AG	0.000	0.000	
	GG	0.000	0.000	
CD3e-112	CC	0.903	0.787	0.024
	CT	0.097	0.213	
	TT	0.000	0.000	
Rs13576648	GG	0.000	0.000	-
	GT	0.000	0.000	
	TT	1.000	1.000	
Rs132883476	GG	0.000	0.000	-
	GT	0.000	0.000	
	TT	1.000	1.000	
CD3e-258	AA	0.000	0.000	0.735
	AG	0.114	0.118	
	GG	0.886	0.882	

Table 2. 9: Allelic frequencies of SNP found in the CD3e gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P
		Allelic frequency	Allelic frequency
Rs136656580	Del	0.138	0.123
	Ins	0.862	0.877
Rs209286808	A	1.000	1.000
	G	0.000	0.000
CD3e-112	C	0.952	0.893
	T	0.048	0.107
Rs13576648	G	0.000	0.000
	T	1.000	1.000
Rs132883476	G	0.000	0.000
	T	1.000	1.000
CD3e-258	A	0.057	0.059
	G	0.943	0.941

Table 2. 10: Hardy-Weinberg equilibrium test for segregating SNPs. Calculated X^2 value of SNP found in the CD3e gene for animals in the "Negative" group (N) and the "Positive" group (P) along with p-values for this test (df=2). P-values<0.05 are statistically significant, meaning that the population does not follow the H-W equilibrium.

SNP	Genotype	Group N			Group P		
		n	X^2	p-value	n	X^2	p-value
Rs136656580	Del/Del	3	0.03	0.985	1	0.24	0.887
	Ins/Del	34			13		
	Ins/Ins	108			47		
CD3e-112	CC	131	0.37	0.831	48	0.87	0.647
	CT	14			13		
	TT	0			0		
CD3e-258	AA	0	15.63	0.0004	0	0.35	0.840
	GA	17			58		
	GG	132			9		

Table 2. 11: Description of primer sequences used in the evaluation of each single nucleotide polymorphisms (SNP) in the ITGA4 gene.

Primer name	Forward (5' – 3')	Reverse (3' – 5')	Fragment size (bp)	Reference
ITGA4_1	ACACGCTGAGCCTCTTCTCTT	TTTCCAGGACTTGTCTACCGTGGG	849	NP_777173.1
ITGA4_2	ATGGTATTCCACAGAAGCTGGAGG	AGATCCTCCCACTGCTAACCAAGT	538	NP_777173.1
ITGA4_3	TGAGAGCTTGGTTCCCTAAACCT	TCTCAGAACTTTGGAGTAAGTAGACC	531	NP_777173.1
ITGA4_4	ACAAGGCTGTATCTCCCAAACT	TGAGTCATTGCCTACTGGGTGGAA	670	NP_777173.1

Table 2. 12: List of SNPs found in the amplified fragment of the ITGA4 gene.

Primer name	SNP I.D.	N	Location	SNP	Base position
ITGA4_1	Rs42239893	200	Intron 1	A/G	310
ITGA4_1	Rs42239894	200	Intron 1	C/A	360
ITGA4_1	Rs133348759	200	Intron 1	G/A	529
ITGA4_1	Rs133643873	200	Intron 2	T/G	780
ITGA4_4	ITGA4-192	195	Exon 28	Ins/Del	192
ITGA4_4	ITGA4-298	195	Exon 28	G/A	298
ITGA4_4	Rs42240844	195	Exon 28	T/C	415
ITGA4_4	Rs42240845	195	Exon 28	G/A	456

Table 2. 13: Genotypic frequencies of SNP found in the CD3e gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P	p-value of X^2
		Genotypic frequency	Genotypic frequency	
Rs42239893	AA	0.090	0.076	0.321
	GA	0.388	0.500	
	GG	0.522	0.424	
Rs42239894	AA	0.463	0.424	0.866
	AC	0.403	0.439	
	CC	0.134	0.136	
Rs133348759	AA	0.008	0.000	0.567
	AG	0.075	0.044	
	GG	0.918	0.957	
Rs133643873	CC	0.508	0.439	0.307
	CT	0.410	0.515	
	TT	0.082	0.046	
ITGA4-192	Ins/Ins	0.000	0.000	0.152
	Ins/Del	0.000	0.016	
	Del/Del	1.000	0.984	
ITGA4-298	AA	0.008	0.000	0.495
	GA	0.069	0.109	
	GG	0.924	0.891	
Rs42240844	CC	0.183	0.109	0.121
	CT	0.504	0.438	
	TT	0.313	0.453	
Rs42240845	AA	0.099	0.063	0.160
	AG	0.405	0.297	
	GG	0.496	0.641	

Table 2. 14: Allelic frequencies of SNP found in the ITGA4 gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P
		Allelic frequency	Allelic frequency
Rs42239893	A	0.284	0.326
	G	0.716	0.674
Rs42239894	A	0.664	0.644
	C	0.336	0.356
Rs133348759	A	0.045	0.022
	G	0.955	0.978
Rs133643873	C	0.713	0.697
	T	0.287	0.303
ITGA4-192	Ins	0.000	0.008
	Del	1.000	0.992
ITGA4-298	A	0.042	0.055
	G	0.958	0.945
Rs42240844	C	0.435	0.328
	T	0.565	0.672
Rs42240845	A	0.302	0.211
	G	0.699	0.789

Table 2. 15: Hardy-Weinberg equilibrium test for segregating SNPs. Calculated X^2 value of SNP found in the ITGA4 gene for animals in the "Negative" group (N) and the "Positive" group (P) along with p-values for this test (df=2). P-values<0.05 are statistically significant, meaning that the population does not follow the H-W equilibrium.

SNP	Genotype	Group N			Group P		
		n	X^2	p-value	n	X^2	p-value
Rs42239893	AA	12	0.27	0.874	5	1.26	0.533
	GA	52			33		
	GG	70			28		
Rs42239894	AA	62	1.25	0.535	28	0.12	0.942
	AC	54			29		
	CC	18			9		
Rs133348759	AA	1	2.18	0.336	63	55.02	0.0001
	AG	10			3		
	GG	123			0		
Rs133643873	CC	68	0.00	1.000	29	3.18	0.204
	CT	55			34		
	TT	11			3		
ITGA4-298	AA	1	2.79	0.248	0	0.21	0.900
	GA	9			7		
	GG	121			57		
Rs42240844	CC	24	0.08	0.961	7	0.02	0.990
	CT	66			28		
	TT	41			29		
Rs42240845	AA	13	0.20	0.905	4	0.75	0.687
	AG	53			19		
	GG	65			41		

Table 2. 16: Description of primer sequences used in the evaluation of each single nucleotide polymorphisms (SNP) in the IgHE gene.

Gene	Forward (5' – 3')	Reverse (3' – 5')	Fragment size (bp)	Reference
IgHE_2	ACACCCTGAGACCAACTCCAACTT	TTTCTGGATCATGCAGGTGAGGGT	1,064	U63640.2
IgHE_4	TCCTGAGAGAAGCCTGACTAA	GGAGGCTGGGCGTTATTT	392	U63640.2

Table 2. 17: List of SNPs found in the amplified fragment of the IgHE gene.

Gene	SNP I.D.	N	Location	SNP	Base position
IgHE_2	IgHE-40IR	154	Intron 1	A/G	39
IgHE_2	Rs208453779	157	Intron 1	C/A	107
IgHE_2	Rs2011220021	157	Intron 1	C/A	135
IgHE_2	Rs209632518	157	Exon 2	A/C	188
IgHE_2	IgHE-280IR	157	Exon 2	C/A	291
IgHE_2	IgHE-310IR	157	Exon 2	G/A	313
IgHE_2	IgHE-320IR	157	Exon 2	T/C	324
IgHE_2	IgHE-380IR	157	Exon 2	C/G	371
IgHE_2	IgHE-840IR	157	Exon 3	A/G	832
IgHE_4	Rs134183307	240	Exon 4	C/T	263

Table 2. 18: Genotypic frequencies of SNP found in the IgHE gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P	p-value of X^2
		Genotypic frequency	Genotypic frequency	
IgHE-40IR	AA	0.556	0.541	0.487
	AG	0.034	0.000	
	GG	0.410	0.460	
Rs208453779	AA	0.857	0.868	0.222
	CA	0.092	0.026	
	CC	0.050	0.105	
Rs2011220021	AA	0.034	0.026	0.119
	AC	0.101	0.000	
	CC	0.866	0.974	
Rs209632518	AA	0.076	0.026	0.227
	AC	0.042	0.000	
	CC	0.882	0.974	
IgHE-280IR	AA	0.916	0.947	0.519
	CA	0.050	0.053	
	CC	0.034	0.000	
IgHE-310IR	AA	0.000	0.000	0.724
	GA	0.017	0.000	
	GG	0.983	1.000	
IgHE-320IR	CC	0.008	0.026	0.366
	CT	0.034	0.000	
	TT	0.958	0.974	
IgHE-380IR	CC	1.000	1.000	-
	CG	0.000	0.000	
	GG	0.000	0.000	
IgHE-840IR	AA	0.042	0.026	0.907
	AG	0.050	0.053	
	GG	0.908	0.921	

Table 2. 19: Allelic frequencies of SNP found in the IgHE gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P
		Allelic frequency	Allelic frequency
IgHE-40IR	A	0.573	0.541
	G	0.427	0.460
Rs208453779	A	0.903	0.8816
	C	0.097	0.118
Rs2011220021	A	0.084	0.026
	C	0.916	0.974
Rs209632518	A	0.097	0.026
	C	0.903	0.974
IgHE-280IR	A	0.941	0.974
	C	0.059	0.026
IgHE-310IR	G	0.008	0.000
	A	0.997	1.000
IgHE-320IR	C	0.025	0.026
	T	0.975	0.974
IgHE-380IR	C	1.000	1.000
	G	0.000	0.000
IgHE-840IR	A	0.067	0.053
	G	0.933	0.947

Table 2. 20: Hardy-Weinberg equilibrium test for segregating SNPs. Calculated X^2 value of SNP found in the IgHE gene for animals in the "Negative" group (N) and the "Positive" group (P) along with p-values for this test (df=2). P-values<0.05 are statistically significant, meaning that the population does not follow the H-W equilibrium.

SNP	Genotype	Group N			Group P		
		n	X^2	p-value	n	X^2	p-value
IgHE-40IR	AA	65	101.23	<0.0001	20	37.00	<0.0001
	AG	4			0		
	GG	48			17		
Rs208453779	AA	102	26.35	<0.0001	33	29.02	<0.0001
	CA	11			1		
	CC	6			4		
Rs2011220021	AA	4	14.16	0.001	1	38.00	<0.0001
	AC	12			0		
	CC	103			37		
Rs209632518	AA	9	68.62	<0.0001	1	38.00	<0.0001
	AC	5			0		
	CC	105			37		
IgHE-280IR	AA	109	35.3	<0.0001	36	0.03	0.985
	CA	6			2		
	CC	4			0		
IgHE-310IR	AA	0	0.01	0.995	0	-	-
	GA	2			0		
	GG	117			38		
IgHE-320IR	CC	1	11.89	0.003	1	38.00	<0.0001
	CT	4			0		
	TT	114			37		
IgHE-380IR	CC	119	-	-	38	-	-
	CG	0			0		
	GG	0			0		
IgHE-840IR	AA	5	42.55	<0.0001	1	8.47	0.016
	AG	6			2		
	GG	108			35		

Table 2. 21: Haplotype distribution between N and P populations in the CCL5 gene. P-value was obtained from a Chi-square test (contingency table).

	Haplotype						p-value
	ACAGA	GTGAG	GTGGG	GTGRG	RYRGR	RYRRR	
N group	7	9	15	9	23	56	0.116
P group	11	7	8	6	9	21	
Total	18	16	23	15	32	77	

Table 2. 22: Haplotype distribution between N and P populations in the CD3e gene. P-value was obtained from a Chi-square test (contingency table).

	Haplotypes			P-value
	ECG	ICG	IYG	
N group	30	98	10	0.204
P group	11	36	11	
Total	41	134	21	

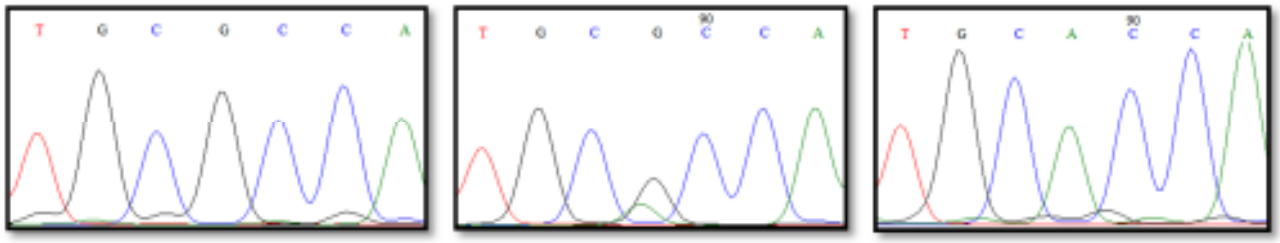
Table 2. 23: Haplotype distribution between N and P populations in the ITGA4 gene. P-value was obtained from a Chi-square test (contingency table).

	Haplotypes				P-value
	GACGCDGTG	GACGCDGYR	RMCGYDGTG	RMCGYDGYR	
N group	10	15	10	7	0.255
P group	10	4	5	6	
Total	20	19	15	13	

Table 2. 24: Haplotype distribution between N and P populations in the IgHE gene. P-value was obtained from a Chi-square test (contingency table).

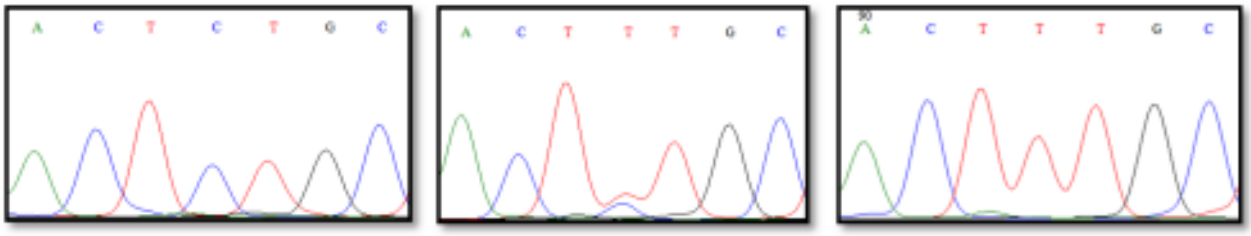
	Haplotypes		P-value
	AAGCAGTCG	GAGCAGTCG	
N group	48	47	0.275
P group	10	16	
Total	58	63	

CCL5



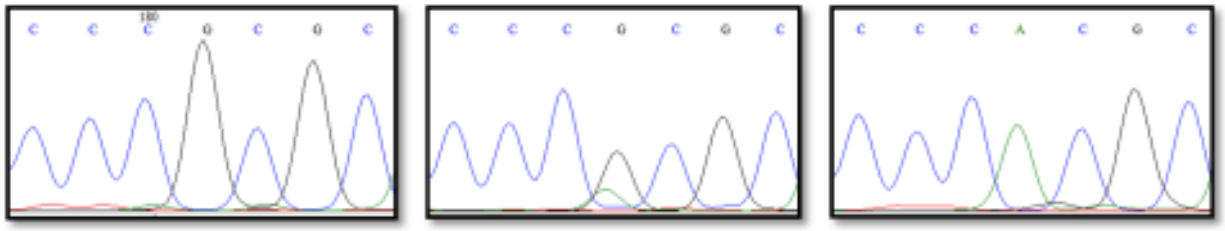
Genotype GG Genotype GA Genotype AA

Figure 2. 3: Fragment of the genomic sequence of the CCL5 gene containing SNP Rs136242974. A transition of guanine to adenine is found in the base 48 in the exon 3 of the CCL5 gene (NCBI accession number NM_175827.2).



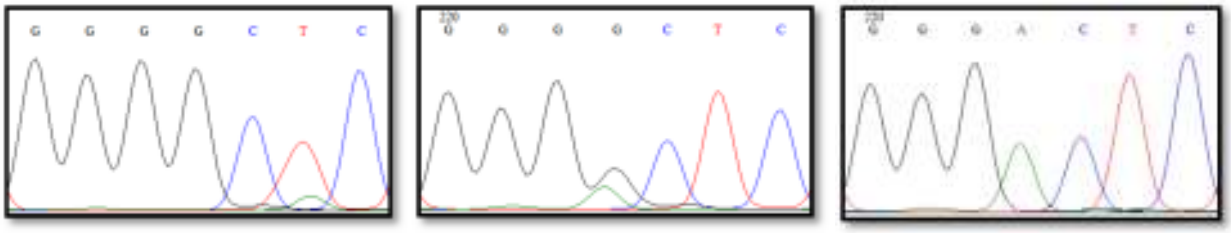
Genotype CC Genotype CT Genotype TT

Figure 2. 4: Fragment of the genomic sequence of the CCL5 gene containing SNP Rs109200822. A transition of cytosine to thymine is found in the base 55 in the exon 3 of the CCL5 gene (NCBI accession number NM_175827.2).



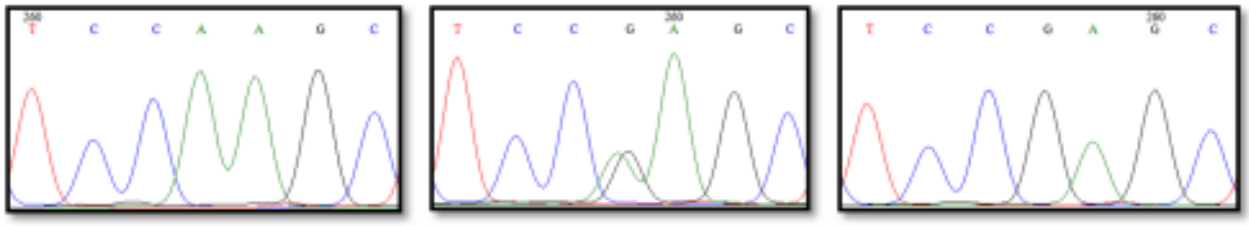
Genotype GG Genotype GA Genotype AA

Figure 2. 5: Fragment of the genomic sequence of the CCL5 gene containing SNP Rs109769870. A transition of guanine to adenine is found in the base 145 in the exon 3 of the CCL5 gene (NCBI accession number NM_175827.2).



Genotype GG Genotype GA Genotype AA

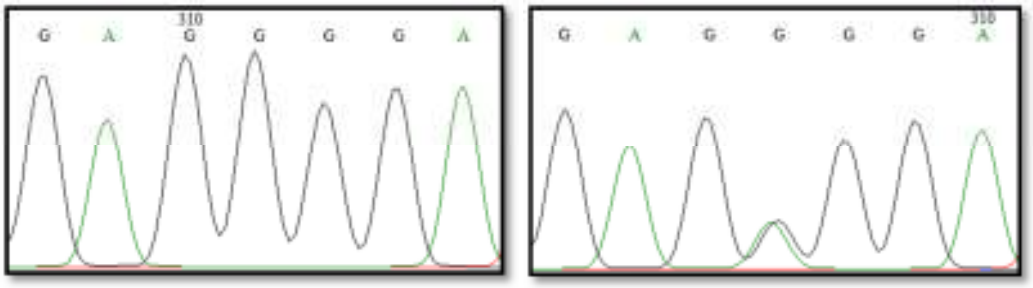
Figure 2. 6: Fragment of the genomic sequence of the CCL5 gene containing SNP CCL5-187. A transition of guanine to adenine is found in the base 187 in the exon 3 of the CCL5 gene (NCBI accession number NM_175827.2).



Genotype AA Genotype GA Genotype GG

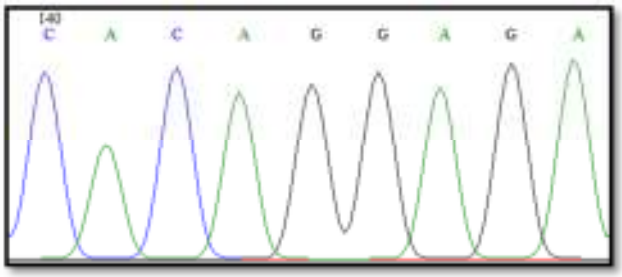
Figure 2. 7: Fragment of the genomic sequence of the CCL5 gene containing SNP Rs110457788. A transition of guanine to adenine is found in the base 48 in the exon 3 of the CCL5 gene (NCBI accession number NM_175827.2).

CD3e

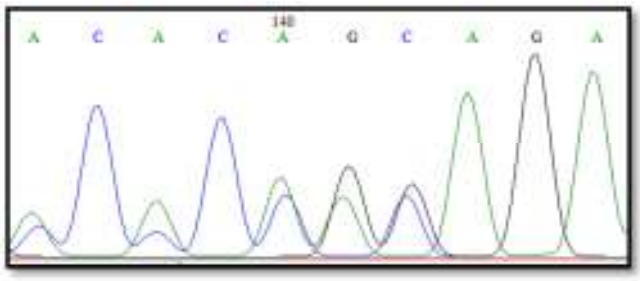


Genotype GG Genotype GA

Figure 2. 8: Fragment of the genomic sequence of the CD3e gene containing SNP CD3e-258. A transition of guanine to adenine is found in the base 258 in the intron 7 of the CD3e gene (NCBI accession number U25687).

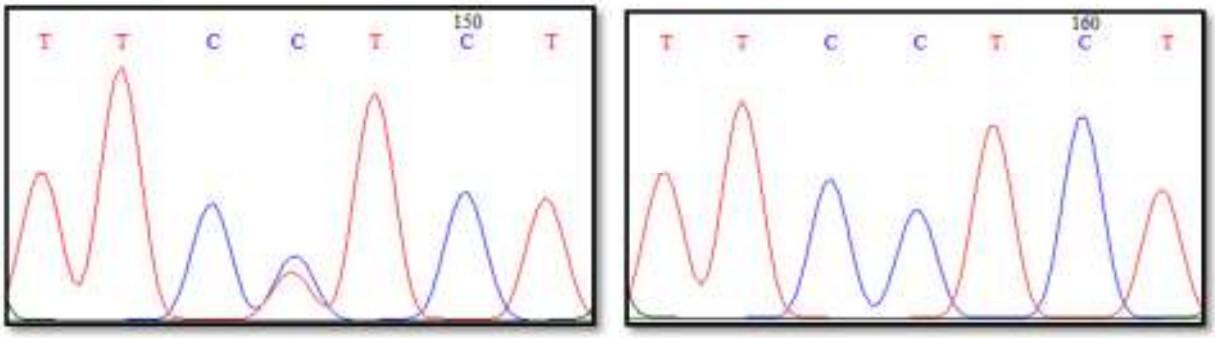


Homozygous for the insertion of AGG



Heterozygous +AGG/-AGG

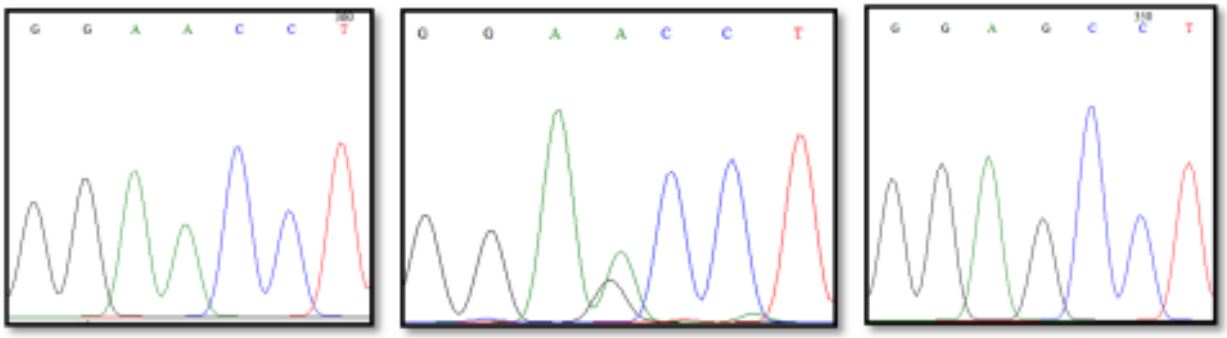
Figure 2. 9: Fragment of the genomic sequence of the CD3e gene containing SNP Rs136656580. An insertion/deletion of two consecutive adenines followed by guanine is found in the base 101 in the exon 9 of the CD3e gene (NCBI accession number U25687).



Genotype CT Genotype CC

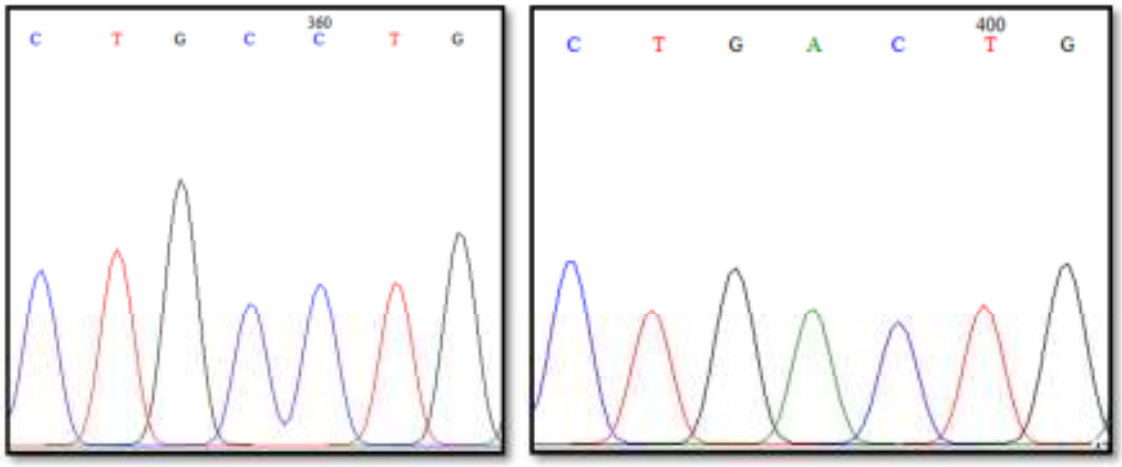
Figure 2. 10: Fragment of the genomic sequence of the CD3e gene containing SNP CD3e-112. A transition of cytosine to thymine is found in the base 112 in the exon 9 of the CD3e gene (NCBI accession number U25687).

ITGA4



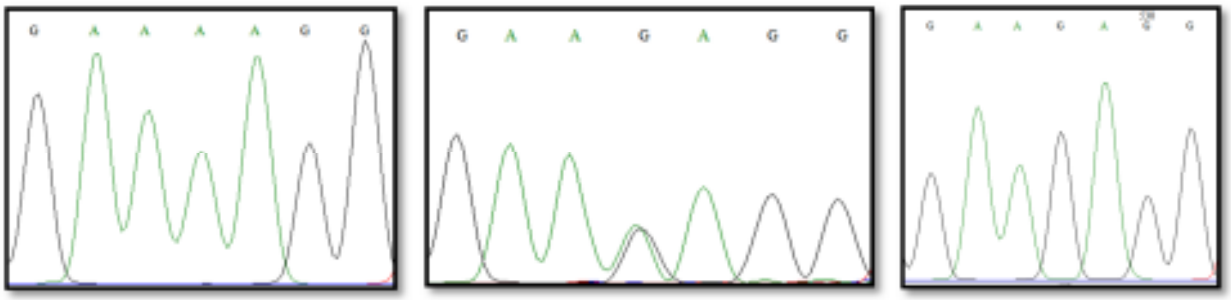
Genotype AA Genotype AG Genotype GG

Figure 2. 11: Fragment of the genomic sequence of the ITGA4 gene containing SNP rs42239893. A transition of adenine to guanine is found in the base 310 in the intron 1 of the ITGA4 gene fragment amplified (NCBI accession number NP_777173.1).



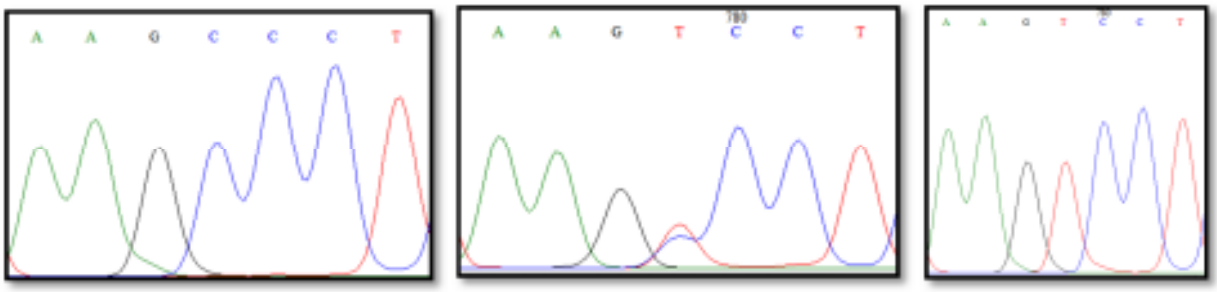
Genotype CC Genotype AA

Figure 2. 12: Fragment of the genomic sequence of the ITGA4 gene containing SNP rs42239894. A transversion of adenine to cytosine is found in the base 360 in the intron 1 of the ITGA4 gene fragment amplified (NCBI accession number NP_777173.1).



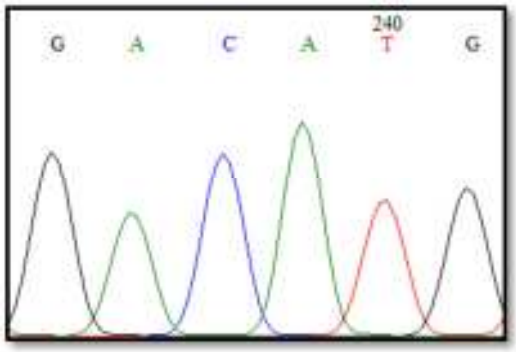
Genotype AA Genotype AG Genotype GG

Figure 2. 13: Fragment of the genomic sequence of the ITGA4 gene containing SNP rs133348759. A transition of adenine to cytosine is found in the base 529 in the intron 1 of the ITGA4 gene fragment amplified (NCBI accession number NP_777173.1).

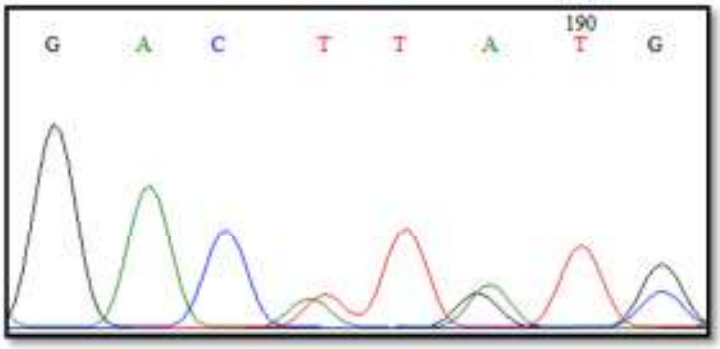


Genotype CC Genotype CT Genotype TT

Figure 2. 14: Fragment of the genomic sequence of the ITGA4 gene containing SNP rs133643873. A transition of cytosine to thymine is found in the base 780 in the intron 1 of the ITGA4 gene fragment amplified (NCBI accession number NP_777173.1).

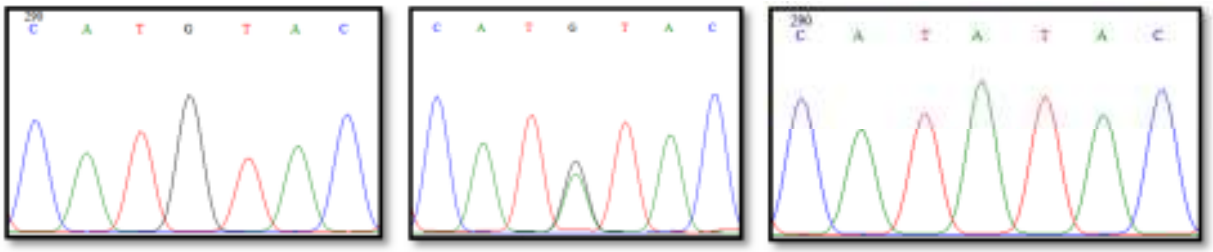


Homozygous for the deletion of TT



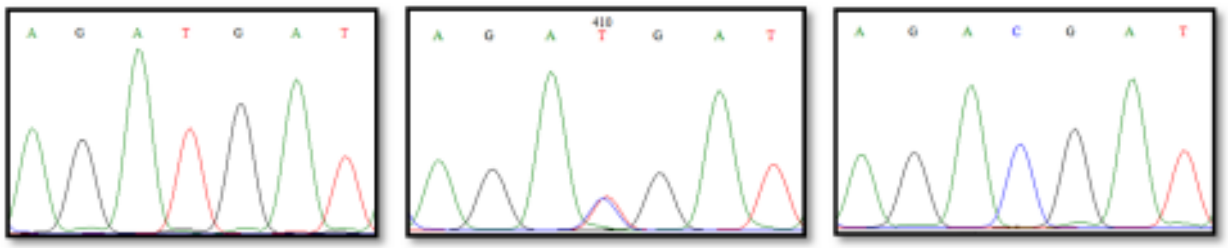
Heterozygous +TT/-TT

Figure 2. 15: Fragment of the genomic sequence of the ITGA4 gene containing SNP ITGA4-192. An insertion/deletion of two thymines is found in the base 192 in the exon 28 of the ITGA4 gene (NCBI accession number NP_777173.1).



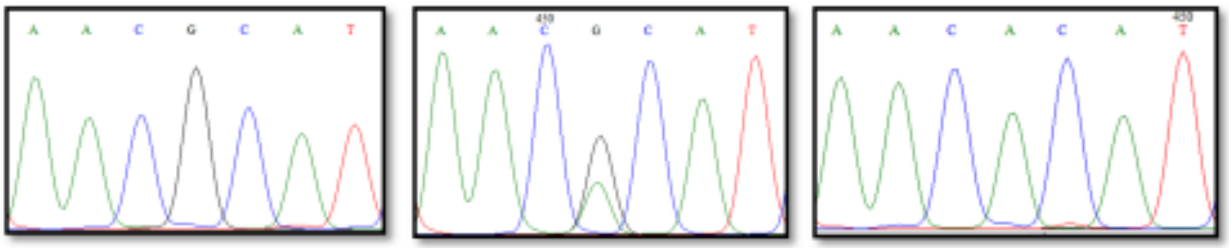
Genotype GG Genotype GA Genotype AA

Figure 2. 16: Fragment of the genomic sequence of the ITGA4 gene containing SNP ITGA4-298. A transition of guanine to thymine is found in the base 298 in the exon 28 of the ITGA4 gene fragment amplified (NCBI accession number NP_777173.1).



Genotype TT Genotype CT Genotype CC

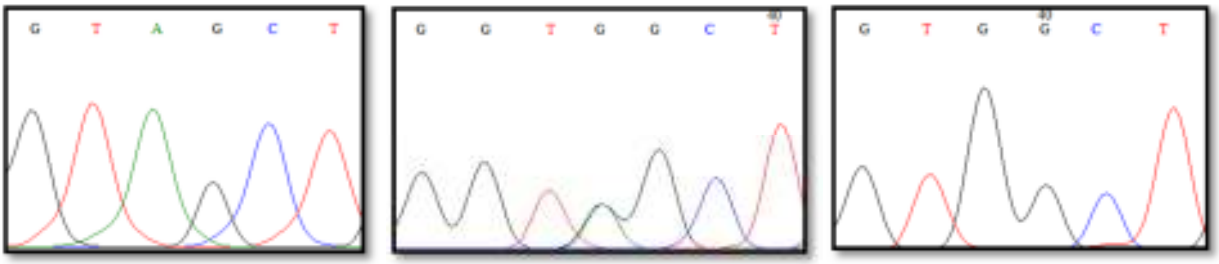
Figure 2. 17: Fragment of the genomic sequence of the ITGA4 gene containing SNP Rs42240844. A transition of cytosine to thymine is found in the base 415 in the exon 28 of the ITGA4 gene fragment amplified (NCBI accession number NP_777173.1).



Genotype GG Genotype GA Genotype AA

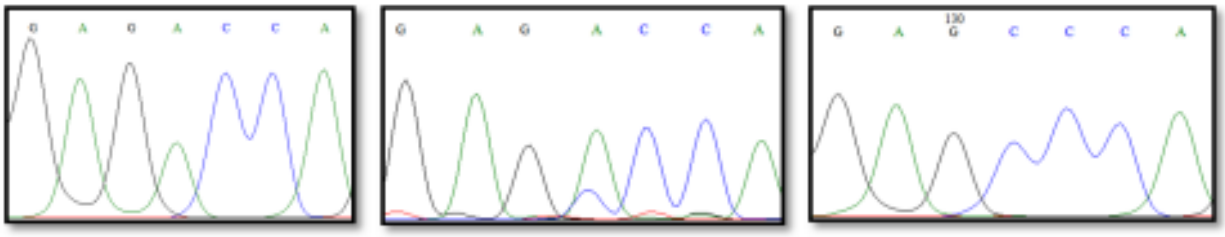
Figure 2. 18: Fragment of the genomic sequence of the ITGA4 gene containing SNP Rs42240845. A transition of adenine to guanine is found in the base 456 in the exon 28 of the ITGA4 gene fragment amplified (NCBI accession number NP_777173.1).

IgHE



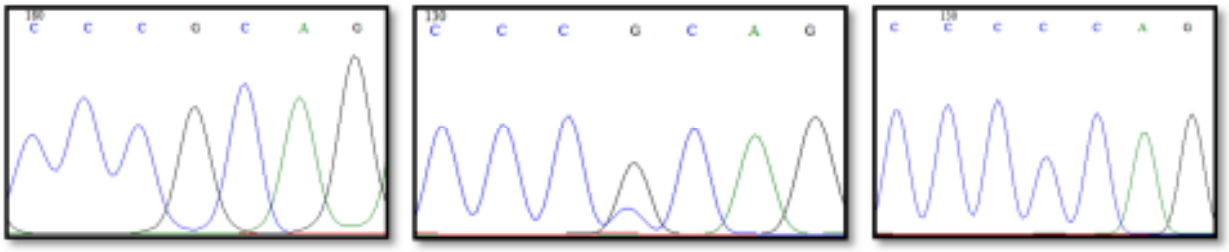
Genotype AA Genotype AG Genotype GG

Figure 2. 19: Fragment of the genomic sequence of the IgHE gene containing SNP IgHE-40IR. A transition of adenine to guanine is found in the base 39 in the intron 1 of the IgHE gene fragment amplified (NCBI accession number U63640.2).



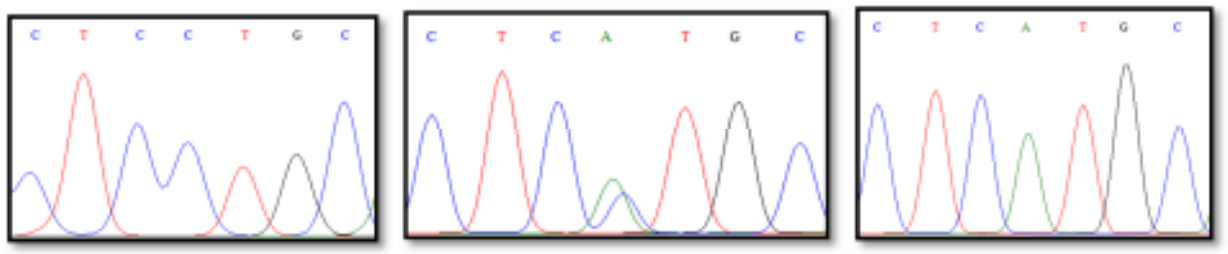
Genotype AA Genotype AC Genotype CC

Figure 2. 20: Fragment of the genomic sequence of the IgHE gene containing SNP Rs208453779. A transversion of adenine to cytosine is found in the base 107 in the intron 1 of the IgHE gene fragment amplified (NCBI accession number U63640.2).



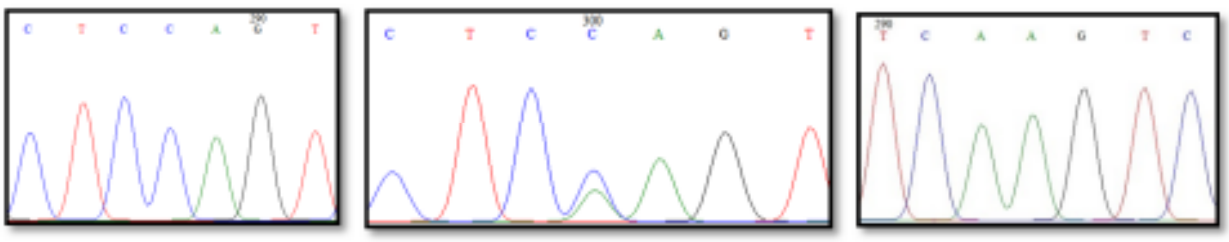
Genotype AA Genotype AC Genotype CC

Figure 2. 21: Fragment of the genomic sequence of the IgHE gene containing SNP Rs2011220021. A tranversion of adenine to cytosine is found in the base 135 in the intron 1 of the IgHE gene fragment amplified (NCBI accession number U63640.2).



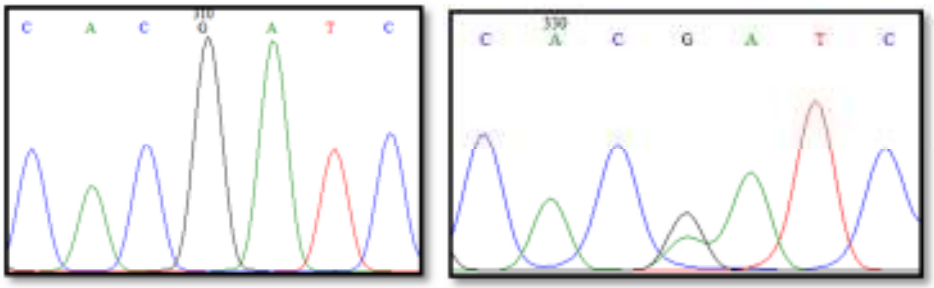
Genotype CC Genotype CA Genotype AA

Figure 2. 22: Fragment of the genomic sequence of the IgHE gene containing SNP Rs209632518. A transversion of cytosine to adenine is found in the base 188 in the exon 2 of the IgHE gene fragment amplified (NCBI accession number U63640.2).



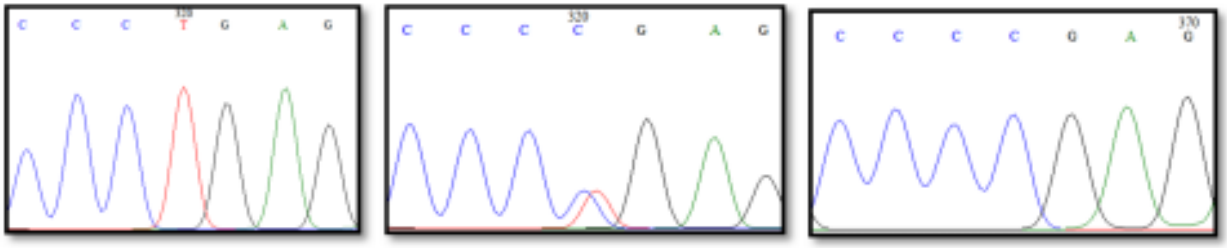
Genotype CC Genotype AC Genotype AA

Figure 2. 23: Fragment of the genomic sequence of the IgHE gene containing SNP IgHE-280IR. A tranversion of adenine to cytosine is found in the base 291 in the exon 2 of the IgHE gene fragment amplified (NCBI accession number U63640.2).



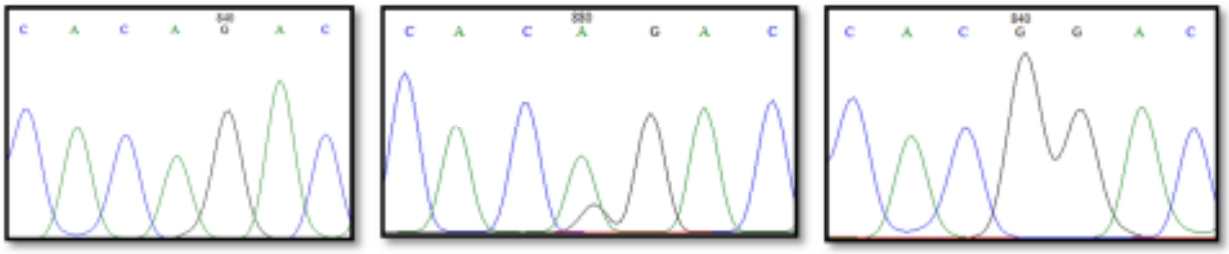
Genotype GG Genotype AG

Figure 2. 24: Fragment of the genomic sequence of the IgHE gene containing SNP IgHE-310IR. A transition of adenine to guanine is found in the base 313 in the exon 2 of the IgHE gene fragment amplified (NCBI accession number U63640.2).



Genotype TT Genotype TC Genotype CC

Figure 2. 25: Fragment of the genomic sequence of the IgHE gene containing SNP IgHE-320IR. A transition of thymine to cytosine is found in the base 324 in the exon 2 of the IgHE gene fragment amplified (NCBI accession number U63640.2).



Genotype AA Genotype AG Genotype GG

Figure 2. 26: Fragment of the genomic sequence of the IgHE gene containing SNP IgHE-840IR. A transition of adenine to guanine is found in the base 832 in the exon 3 of the IgHE gene fragment amplified (NCBI accession number U63640.2).

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Appendices

Appendix A: Use of Larval migration inhibition assay for the evaluation of anthelmintic resistance in dairy heifers from Puerto Rico

Abstract

Ivermectin **(IVM)** resistance of *Cooperia spp.* in dairy heifers from Puerto Rico was evaluated using the larval migration inhibition assay **(LMIA)**. Third stage larvae **(L_3)** were recovered from fecal cultures prior to the administration of treatment with IVM in 8 of the evaluated farms where anthelmintic resistance had previously been evaluated with the use of a fecal egg count reduction test **(FECRT)** with 3 different anthelmintics: albendazole **(ALB)**, doramectin **(DOR)**, and IVM. The effective concentration where 50% of larval migration is inhibited **(EC_{50})** was obtained from susceptible and resistant *Cooperia spp.* isolates. The susceptible isolate had an EC_{50} of 0.7224 µM and the resistant isolate had an EC_{50} of 7.0778 µM. The evaluation of field isolates using LMIA was not able to discriminate between susceptible and resistant larvae from field isolates of *Cooperia spp*.

Resumen

La resistencia antihelmíntica de ivermectina **(IVM)** en *Cooperia spp.* de novillas lecheras de Puerto Rico fue evaluada mediante el ensayo de inhibición de la migración de las larvas **(LMIA)**. La tercera etapa larval **(L_3)** fue recuperada a partir de cultivos fecales pre tratamiento de 8 fincas donde se evaluó previamente la resistencia antihelmíntica con el uso de un "fecal egg count reduction test" **(FECRT)**. La concentración efectiva donde se inhibe el 50% de la migración de las larvas **(EC_{50})** se obtuvo a partir cepas susceptibles y resistentes de *Cooperia spp.* Las larvas susceptibles se caracterizaron por un

EC_{50} de 0.7224 µM, mientras que las larvas resistentes tuvieron un EC_{50} de 7.0778 µM. La evaluación larvas resistentes y susceptibles de muestras del campo utilizando el LMIA no fue capaz de discriminar entre las larvas susceptibles y resistentes.

Introduction

It is important to be able to detect anthelmintic resistance at low levels and as early as possible in order to delay the spread of anthelmintic resistance in parasite species that have an impact in the host's development and health (Demeler et al., 2010a). As an alternative for the early detection of anthelmintic resistance, several assays for the detection of resistance *in vitro* have been developed. *In vitro* tests involve the incubation of one of the parasite's free living stages in a range of concentrations of a drug and the measurement of a variable such as the vitality, motility or migration to generate a dose response value (Demeler et al., 2010a). Some *in vitro* methods for the detection of anthelmintic resistance include the egg hatch test, micromotility test, larval development test and larval migration inhibition assay. These tests are available for the detection of anthelmintic resistance for macrocyclic lactones, benzimidazoles and imidazothiazoles/tetrahydropyrimidines (Coles et al., 2006).

The Larval Migration Inhibition Assay **(LMIA)** is an *in vitro* assay that involves the incubation of the L_3 larval stage in different concentrations of an anthelmintic followed by migration of the larvae through a fine mesh sieve. This assay enables the separation drug resistant larvae that are able to migrate through the sieve from drug susceptible larvae that remain trapped within the sieve, thus not being able to migrate. A dose response can then be calculated in order to determine the relative susceptibility of the parasites to the drug and whether the parasite population is resistant (Taylor et al., 2002). Since the infective third stage larvae do not feed, the effects of the drug in the ability of the larvae to migrate through the sieve are thought to be related to paralysis of the body musculature

(Demeler et al., 2010b), thus, this assay is available for the detection of anthelmintic resistance in macrocyclic lactones only.

Materials and Methods

Ivermectin resistant and susceptible Cooperia spp. isolates

Resistant **(R)** *Cooperia spp.* L_3 isolates were provided by Dr. Gil Myers' Lab (Myers Parasitology Services) in Magnolia, KY. Larvae were recovered from calves treated with IVM that had a negative drug efficacy. Susceptible (S) *Cooperia spp.* isolates were obtained from recovered larvae from farm 7 (characterized as susceptible by an IVM FECR of 96.67% in a FECRT).

Field isolates of L_3 recovered from fecal samples from dairy farms in Puerto Rico

From September 2012 to December 2012, fecal samples were collected from 10 of the evaluated farms in the FECRT and sent to the University of Georgia in Athens, GA. Pooled feces from selected farms were mixed with vermiculite (Sargent-Welch, Buffalo, NY) and incubated for 10 days at room temperature (25° C). The cultures were examined and stirred every day. To prevent larvae desiccation, distilled water was added as needed. After 10 days of incubation, the L_3 were recovered using the Baermann technique (Dinaburg, 1942) in which the pooled fecal matter is suspended over a sieve inside a funnel with lukewarm water for 24 hours. Recovered larvae were stored at 10° C in deionized water and used within 3 months after collection. Using a 10% lugol iodine solution, larvae

species were stained and identified using morphological characteristics of the anterior, tail sheath extension and overall body length (Wyk, et al., 2004).

Larval Migration Inhibition Assay procedure

A 10 mM stock solution of ivermectin (IVM) was prepared using 100% dimethyl sulfoxide (DMSO) (Fisher Chemical, Rochester, New York) and stored in aliquots at -30° C for a maximum of 3 months. Working solutions were prepared using four-fold dilutions of IVM in 100% DMSO for a final concentration in the plate of 20 µM, 5.0 µM, 1.25 µM, 0.3125 µM, 0.0781 µM, 0.0195 µM and 0.0049 µM. Negative controls consisted in live larvae that were incubated in a no drug solution consisting of 1% DMSO in Phosphate Buffered Saline (PBS) 0.01 M (Sigma BioReagent).

LMIA procedure was performed using standard 24 well culture plates (Costar, NY). The plate consisted of triplicate concentrations of ivermectin (IVM, Sigma 18898, St. Louis, MO) and no drug control using the following plate design:

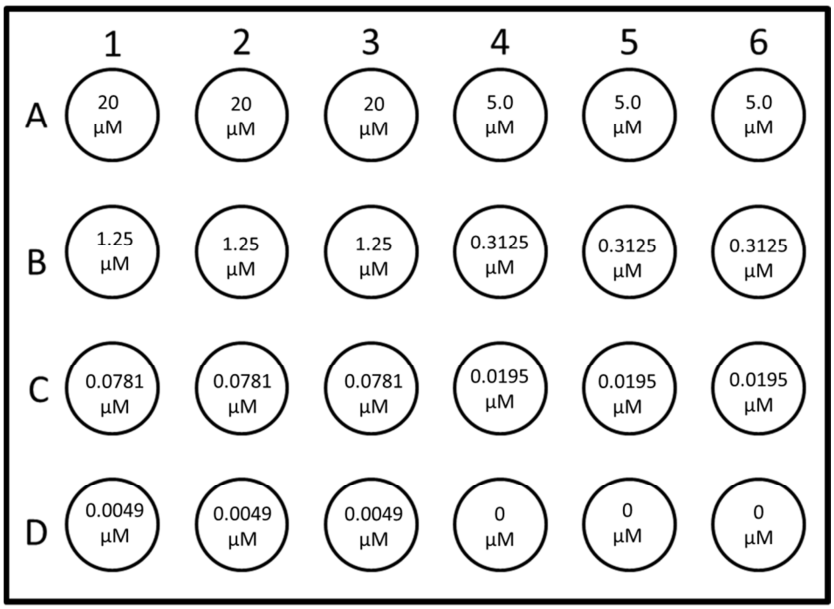

Figure A-1. 1: Experimental design of the 24well plate format used for LMIA

Third stage larvae were counted and aliquots of 80 L_3/100 µL of dH_2O were prepared and pipetted into each well following the experimental design. Additionally, 100 µL of an incubation solution at 2x the final plate concentration of 1% DMSO in PBS were prepared for each drug concentration and pipetted into each of the 24 wells of the incubation plate. Both solutions were vortexed vigorously frequently to ensure homogeneity in each well.

After 24 hours of incubation, the L_3 were transferred to a migration plate containing 4 migration racks. Each rack consisted of 6 sieves made from clear Plexiglas XT tubes (Brinke KG, id = 8-9 mm/od = 11.5 mm) cut to the length of 2 cm and glued to a Plexiglas stick. At the end of each sieve, a 28 µm precision woven nylon polyamid mesh was glued. Each well in the migration plate was loaded with 1.8 ml of a migration solution at 1x the final plate concentration of 1% DMSO in PBS. The contents of the incubation plate (larvae suspension and incubation solution) were transferred onto the top of the sieves following the plate design. Plates were examined under a stereoscope to confirm that no larvae remained in the plates. L_3 were left to migrate for 24 hours in the dark at room temperature (25° C).

Figure A-1. 2: Migration plate used for LMIA. The plate consists of a 24 well tissue culture plate with 4 migration racks, each of these composed of 6 circular plexiglass tubes with a 28 µm mesh glued to the bottom.

Another 24 well plate was prepared to receive the non-migrated larvae. The migration racks were gently removed from the migration plate and inverted over the non-migration plate. The non-migrated larvae were carefully rinsed to the non-migrated larvae plate using a washing bottle with dH_2O.

Statistical analysis of LMIA

The number of migrated larvae and non-migrated larvae in the plates were counted for each drug concentration and the negative controls. The percentage of migration and migration inhibition were calculated. Dose response data to determine the effective concentration where 50% of larval migration is inhibited **(EC_{50})** were analyzed using the "One population" logistic regression model of the Fit Logit Program (Dobson et al., 1987; Waller et al., 1985).

Results

Using L_3 recovered from the fecal cultures, LMIA with ivermectin **(IVM)** were performed for 8 of the 10 samples in duplicates. Two of the 10 fecal samples did not yield enough larvae to perform an LMIA. The effective anthelmintic concentration required to paralyze 50% of the larvae **(EC_{50})** for the 8 farms evaluated obtained by a non-linear regression are presented in Table A-1.1 For the R and S *Cooperia spp.* isolates, EC_{50} were 7.0778 µM and 0.7224 µM respectively. Dose response curves were generated using a logistic regression model. A shift in curves towards the right of the IVM susceptible isolate indicates the presence of drug resistance. Curves that are displaced towards the left of the susceptible isolate curve are described as susceptible. Isolates obtained from farms 2, 16 and 18 had EC_{50} that were below the EC_{50} of the susceptible isolate.

Discussion

The larval migration inhibition assay has been contemplated as an alternative for the early detection of anthelmintic resistance. This assay has been recently standardized for the use in cattle (Demeler et al., 2010a; Demeler et al., 2010b), but published data available regarding the use of this assay is scarce due to problems with the reproducibility of this method. Most evaluations of anthelmintic resistance using LMIA are done with monocultures of *Cooperia oncophora* isolates. Demeler et al., 2010a reported an EC_{50} of 123 nM for susceptible isolates and 621 nM for resistant isolates for this specie. Similarly, evaluations of *C. oncophora* resistance to IVM using LMIA in Germany have shown that susceptible larvae have an EC_{50} of 0.107 µM (Demeler et al., 2010b). Similar studies in

Belgium, where LMIA was used to evaluate the IVM resistance of *C. oncohora* showed that susceptible isolates of this specie had an EC_{50} of 0.120 µM (El-Abdellati et al., 2010).

Recently, LMIA was used to evaluate IVM resistance of *Cooperia spp.* in cattle in Brazil (Almeida et al., 2013). In this study, statistical difference was detected between susceptible and resistant isolates and their susceptible isolate was characterized by an EC_{50} of 1.16 ηmol. *Cooperia spp.* is considered the dose-limiting specie, meaning that compared to other GI nematode species, a higher dose of a drug is necessary to eradicate this parasite (Vercruysse, 2002). It has also been documented that L_3 are thought to be less susceptible to the effects of macrocyclic lactones (Demeler et al., 2010b).

In this study, the LMIA was unable to discriminate between susceptible and resistant larvae obtained from evaluated dairy farms in Puerto Rico. The EC_{50} obtained from the susceptible isolate in this study was and 0.7224 µM. The resistant isolate had an EC_{50} of 7.0778 µM. This indicates that larvae from the resistant isolate are 10X more resistant than the larvae form the susceptible isolate. A discrepancy in the obtained results from LMIAs is that the EC_{50} of farm 2 (0.7263 µM) that had a FECR of -136.19 is close to that of the susceptible isolate. In this case, the assay was unable to differentiate between the susceptible and resistant larvae. Another similar case in which the assay is unable to discriminate between resistant and susceptible larvae was found in the LMIAs of farms 16 and 18. In this case, both EC_{50} were lower than the EC_{50} of the susceptible isolate and their FECRT results indicate that resistance to IVM is present in both farms.

Conclusion

The LMIA has been recently standardized and adapted for the use in cattle mostly with L_3 isolates of *Cooperia oncophora*. LMIA results made with *Cooperia spp*. in Brazil indicate that the assay can be used with this specie. Although LMIAs were performed using L_3 recovered from fecal cultures from heifers from dairy farms in Puerto Rico, the assay could not differentiate between susceptible and resistant larvae. Results obtained from FECRT are not in agreement with the EC_{5o} obtained for each farm isolate in the LMIA.

Table A-1. 1: Effective anthelmintic concentration required to paralyze 50% of the larvae (EC_{50}) for each farm isolate evaluated with LMIA. Susceptible (S) and resistant (R) *Cooperia spp.* larvae are also presented in this table.

Farm	EC_{50} (µM)	FECR (%)
R	7.0778	negative
S	0.7224	96.67
0	0.4637	---
2	0.7263	-136.19
5	1.9080	65.94
11	8.3016	-414.13
13	2.9415	-37.63
16	0.3650	24.45
18	0.3959	85.50

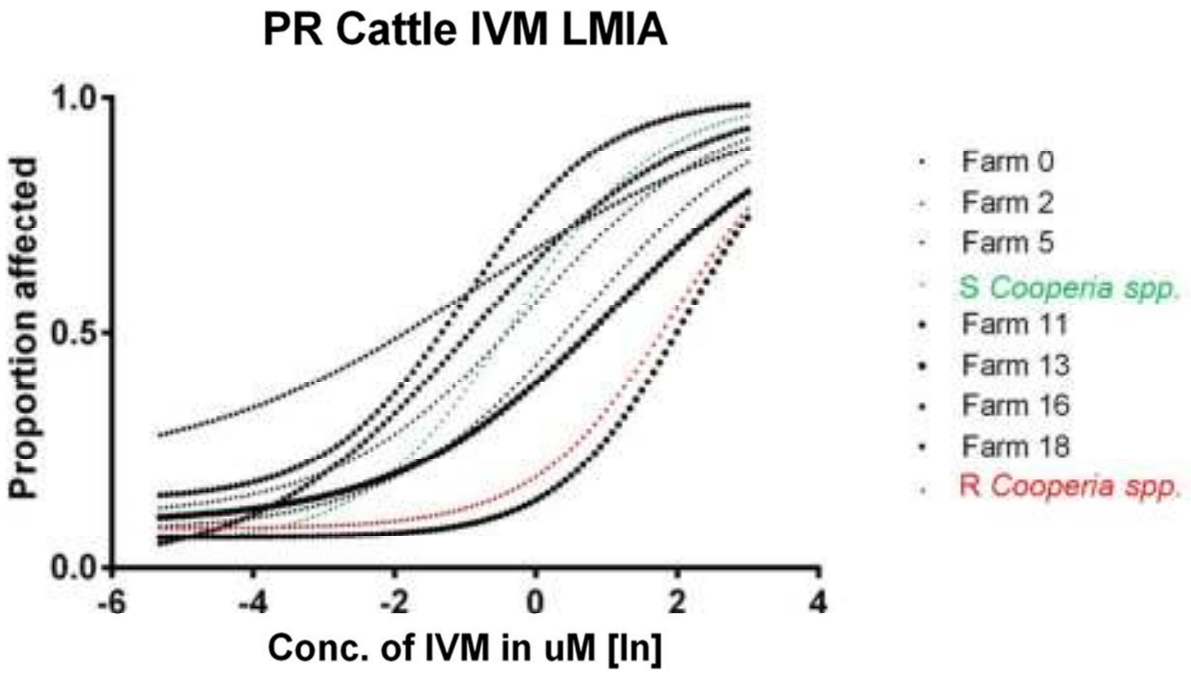

Figure A-1. 3: Dose-response curves of susceptible *Cooperia spp.* and farm isolates of *Cooperia spp.* from farms from Puerto Rico for IVM (µM) obtained in the LMIA. Susceptible (S) *Cooperia spp*. EC_{50} and resistant (R) *Cooperia spp.* EC_{50} isolates are also presented.

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Appendix B: Evaluation of molecular markers presumably associated with nematode parasite resistance in Senepol*Charolais crossbreed heifers

Abstract

Gastrointestinal parasitism is a serious affecting problem of the cattle industry. The use of molecular markers associated with resistance against gastrointestinal parasites has been contemplated as an alternative for the control of gastrointestinal parasites. Four candidate genes associated with the immune response against gastrointestinal parasites in cattle were evaluated: **CD3e** (CD3-epsilon), **CCL5** (chemokine (C-C motif) ligand 5), and **ITGA4** (integrin alpha 4). Senepol*Charolais crossbreed heifers were grouped into 2 populations based on fecal egg count **(FEC)** and allelic and genotypic frequencies were evaluated using a Chi square **(X^2)** test. A total of 15 single nucleotide polymorphisms **(SNPs)** were found to be segregating collectively in all evaluated genes. Of all evaluated SNPs, only 2 polymorphisms found in the CCL5 gene were found to be adequate candidates for the selection of resistant heifers in the evaluated beef heifers.

Resumen

El uso de marcadores moleculares asociados con la resistencia contra los parásitos gastrointestinales ha sido contemplado como una alternativa para el control de estos parásitos en el ganado vacuno. Se evaluaron cuatro genes candidatos asociados con la respuesta inmune contra los parásitos gastrointestinales en el ganado: **CD3e** (CD3-épsilon), **CCL5** (quimiocina (motivo CC) ligando 5), y **ITGA4** (integrina alfa 4). Novillas cruce Senepol*Charolais se agruparon en 2 poblaciones basadas en el

recuento fecal de huevos **(FEC)** y las frecuencias alélicas y genotípicas se evaluaron usando un prueba Chi cuadrado **(X^2)**. Un total de 15 polimorfismos de nucleótido único **(SNP)** se encontraron segregando colectivamente en todos los genes evaluados. De todos los SNPs evaluados, sólo se encontraron 2 polimorfismos en el gen CCL5 que mostraron ser candidatos adecuados para la selección de las novillas resistentes en la población cruce Senepol*Charolais evaluada.

Introduction

The presence of gastrointestinal parasitism in ruminants usually presents itself as subclinical infections that can result in significant economic losses. In the United States, gastrointestinal nematode parasitism costs the cattle industry more than $2 billion per year (Li and Gasbarre, 2009). Clinical signs of parasitism include stunted growth, tissue edema and severe diarrhea (Gasbarre et al., 2001). In turn, these clinical signs translate into economic losses in cattle productivity such as reduced weight gain, weight loss, stunted growth rates, and delays in puberty, breeding, and age at calving (Stromberg et al., 2012) and decreased milk production in cows (Perri et al., 2011). The beef industry in Puerto Rico accounts for 3.43% of the agricultural production, representing a gross income of $25,604,000 in 2010-2011 (División de Estadísticas Agrícolas, 2012). Calves and young heifers are the most susceptible to parasite infections due to a low resistance to gastrointestinal parasitic worm burdens. Usually, infections with low parasitic burdens are asymptomatic, but high parasitic burdens can produce clinical signs (Claerebout and Vercruysse, 2000).

The development of anthelmintic resistance, the public concern of chemical residues in food and the environment, and the high cost of drug treatment have made the use of alternative parasite control strategies imperative. The selection of cattle populations that show resistance to GI parasites based on

genetic variations within the host's genome is one of these alternative methods for the control of gastrointestinal **(GI)** parasite infections. Understanding the immune responses that contribute to the development of protective immunity, immunosuppression and resistance is a key component on the development of alternative parasite control strategies (Li and Gasbarre, 2010).

However, knowledge of the protective immune responses against GI nematodes is limited by the fact that parasites have evolved to evade the host's protective immune responses. The immune response against an infection will depend on the parasite's specie as well as age and health status of the host. Infections with *Oesophagostomum radiatum* produce protective immune response early in the host's life, while other parasites such as *Ostertagia ostertagi, Cooperia spp.* and *Haemonchus contortus* require longer periods of exposure to generate an immune response (Gasbarre et al., 2001).

Materials and methods

Animal Selection

Senepol*Charolais crossbred heifers (n=41) weighing 250.85±9.24 kg were selected from the Beef Cattle Research and Teaching Farm of the University of Puerto Rico located in Aguadilla. Upon weaning, heifers were placed on a rotational pasture system in a single group. Fecal samples were taken every two weeks for a period of 16 weeks. Heifers were placed in two groups based on fecal egg count (FEC) prior to the administration of anthelmintic treatment. The "Negative" group (n=14) was composed of heifers whose FEC was <33 eggs per grams of feces **(EPG)**. The "Positive" group (n=27) was composed of heifers whose FEC was >100 EPG.

Blood collection and DNA isolation

Blood samples were collected from heifers by jugular venipuncture. 15 mL of blood were collected for DNA extraction using the protocol established by Sambrook et al., 1989. Using this method, red blood cells are lysed and eliminated through centrifugation. DNA was extracted from the buffy coat of blood which is composed of leukocytes and platelets which are then treated with different buffers to lyse the cell's membrane and extract the DNA within. Quantification of DNA samples was performed by spectrophotometry at 260 nm. Aliquots of 120 ng of DNA were used as a template for polymerase chain reaction.

Single nucleotide polymorphisms (SNP) genotyping

Polymerase chain reaction **(PCR)** was performed using an Eppendorf thermocycler (Model No. 950040015, Hauppauge, NY). Single nucleotide polymorphism **(SNP)** detection was performed using primers at a final concentration of 0.3 µM for the genes:

CD3e (CD3-epsilon),

CCL5 (chemokine (C-C motif) ligand 5), and

ITGA4 (integrin alpha 4).

The amplified PCR product was sequenced at MacroGen Inc. Laboratory at Seoul, Republic of Korea.

Statistical Analysis

Differences in genotypic and allelic frequencies between the two evaluated populations were determined and analyzed with contingency tables using InfoStat statistical analysis software (Di Rienzo et al., 2012). Additionally, genotype and allele frequencies were analyzed following the Hardy-Weinberg (H-W) equilibrium (Rodríguez, 2009). Haplotypes for all genes was also analyzed using contingency tables with InfoStat.

Results

Frequencies and single nucleotide polymorphisms (SNP) in the CCL5 gene

The genotypic frequencies and allelic frequencies of SNPs found in the CCL5 gene can be found in Table B-1.1, Table B-1.2, and Table B-1.3 respectively. Five different SNPs were found in the exon 3 of this gene. For each of these SNPs, Chi-square test **(X^2)** was performed to evaluate the distribution of each genotype in the two evaluated populations. Rs109200822, a transition of cytosine to thymine and 3 transitions of adenine to guanine (Rs136242974, Rs109769870 and Rs110457788) were found segregating in both evaluated populations (Table B-1.2). A statistical difference was detected in the genotypic segregation between both populations for the SNP Rs109200822 (p=0.025). Results of the H-W equilibrium test show that SNPs segregating in the population P follow the H-W equilibrium (Table B-1.4). In the population N, all SNPs are in equilibrium, except Rs109200822 which is in disequilibrium due to a higher segregation of the heterozygote allele in this population.

SNP CCL5-187 is a transition of guanine to adenine in the base 187 of the amplified fragment of the exon 3 of the CCL5 gene. Contrary to dairy heifers previously evaluated, where all genotypes were

being segregated, the genotypic distribution of this SNP in beef heifers evaluated was dominated by the segregation of the heterozygote allele and the GG homozygote. A significant difference was also detected in the distribution of this polymorphism (p=0.001). Frequency of the allele A was 0.107 and 0.389 for groups N and P respectively. For the guanine allele, the allelic frequencies were 0.893 and 0.611 for the N and P groups respectively (Table B-1.3). H-W equilibrium test for both populations suggests that group N is in H-W equilibrium (p=0.031), while group P is not (p=0.985).

Frequencies and single nucleotide polymorphisms (SNP) in the CD3e gene

The summary of all SNPs found in all amplified fragments of the CD3e gene can be found in Table B-1.5. Genotypic and allelic frequencies for all SNPs detected in this gene can be found in Table B-1.6 and Table B-1.7 respectively. The results of H-W equilibrium test are in Table B-1.8 and the analysis of haplotype distribution can be found in Table B-1.14. No significant difference was found in the distribution of haplotypes (p=0.583).

Primer set CD3e_3 was used to amplify part of Intron 6, exon 7, intron 7, and exon 8 of this gene. Two published SNPs are found in this fragment, both of them transversions of thymine to guanine. Rs13576648 is located in exon 7. This transversion causes a silent mutation since both genotypes codify for the amino acid glycine. Rs132883476 is another transversion of thymine to guanine that is found in intron 7. All evaluated heifers in both groups (n=35) were only segregating the TT genotype of these two SNPs. A new polymorphism was found in intron 7 of the CD3e gene in dairy heifers (Figure 2.6). This SNP, CD3e-258, consists of a transition of guanine to adenine. In the evaluated beef heifer populations, only the GG allele was found segregating.

Primer set CD3e_2 was used to amplify exon 9 of the CD3e gene. This exon is part of the UTR of this gene. Two published SNPs are found in this exon, an insertion/deletion in the base 101 and a transition in the base 104. Of these two, only Rs136656580, an insertion/deletion in the base 101 was found to be segregating in both populations. No significant difference (p=0.554) was observed in the distribution of genotypes between both groups (Table B-1.6). Heifers in both populations segregate the homozygous genotype for the insertion of AGG with a frequency of 0.643 and 0.688, followed by the heterozygous genotype with frequencies of 0.357 and 0.250 and last the homozygous for the deletion with frequencies of 0.000 and 0.063 in the N and P groups respectively.

Additionally, a new SNP in the base 112 was found in exon 9 of the CD3e gene. This polymorphism consists in a transition of cytosine to thymine. Only two individuals segregating the homozygous allele CC and the heterozygous alleles were found in both populations. For both groups, the highest segregating allele is homozygous CC allele with a 90.63% in the N group and 96.43% in the P group. No significant difference was found between both populations (p=0.351). The evaluation of the H-W equilibrium of both populations for these unreported SNPs if found in Table B-1.8. Both N and P populations do not follow the H-W equilibrium for the reported genotypes of CD3e-112 and Rs136656580.

Frequencies and single nucleotide polymorphisms (SNP) in the ITGA4 gene

Primer set ITGA4_1 was used to amplify exon 1, intron 1, exon 2 and part of intron 2. DNA sequence analysis of this fragment revealed the segregation of 4 different SNPs, all of them described for different cattle breeds (Barris et al., 2012; Kawahara-Miki et al., 2011; Stothard et al., 2011). Three of these polymorphisms are located in the intron 1 of this gene. Rs42239893 and Rs133348759 are

transitions of adenine to guanine in the base position 301 and 529 of the fragment and Rs42239894 is a transversion of cytosine to adenine in the base position 360. No statistical difference was detected in the genotypic and allelic frequencies between both analyzed populations for SNPs in intron 1 (Table B-1.11). The distribution of genotypes and alleles of these SNPs is in H-W equilibrium (Table B-1.12). Another SNP was found in this amplified fragment. Rs133643873 is a transversion of thymine to guanine. No significant difference was detected in the allelic and genotypic distribution of this SNP (p=0.432). This SNP is also found in H-W equilibrium.

DNA sequence analysis of exon 28 of the ITGA4 gene revealed the presence of 4 SNPs. Rs42240844 and Rs42240845 are both transitions found in base positions 415 and 456 respectively. For both SNPs, no significant differences were found between both populations (Table B-1.10) and these SNPs follow the H-W equilibrium (Table B-1.12). A new polymorphism, ITGA4-298, that was detected in the dairy heifer population was also observed in evaluated beef heifers. This SNP consists of a transition of guanine to adenine in the position 298 of exon 28. No statistical difference was found in the genotypic and allelic frequencies between both populations (p=0.551) and both populations are in H-W equilibrium (Table B-1.12). Another new SNP that had been detected in the dairy heifer population, ITGA4-192, was not found to be segregating in the evaluated Senepol*Charolais populations. The homozygote allele for the deletion was the only genotype found in this population.

Discussion

Chemokine (C-C motif) ligand 5 (CCL5)

A total of 4 different polymorphisms have previously been described in the exon 4 of the CCL5 gene. This exon contains the stop codon TAG, which indicates that all polymorphisms found in this exon

are in the untranslated region **(UTR)** of this gene. Polymorphisms Rs136242974, Rs109200822, Rs109769870 and Rs110457788 have been reported for different cattle breeds (Barris et al., 2012; Kawahara-Miki et al., 2011; Stothard et al., 2011). For the evaluated SNPs found in the exon 3 of the CCL5, it was observed that a significant difference between both populations was present in SNP Rs109200822. The segregation of these SNPs is characterized by a higher segregation of the heterozygote allele.

CCL5 has been mostly studied in mouse and human models due to its important role in the mediation of inflammatory responses that include the degranulation of effector cells and the activation of T-cells. CCL5 functions in the chemotaxis of T-cells, dendritic cells, eosinophils, NK cells, basophils, mast cells and the activation of T-cells and leukocytes (Appay, et al., 2001; Levy, 2009). The role of CCL5 in the immune response against GI nematodes in cattle is not completely understood, but it has been reported that during infections with GI nematodes, there is an increase in the expression of this pro-inflammatory chemokine in the small intestine mucosa of susceptible calves (Araujo et al., 2009).

CD3-epsilon (CD3e)

The CD3e gene codifies for the ε chain of the CD3 T-cell receptor (TCR) complex. The ε chains are involved in the signaling and activation of the T-cell once an antigen has been presented by an effector cell (Hagens et al., 1996). Signaling produces a serious of phosphorylation and oxidation reactions that result in the activation of the T-cell. The activation of T-cells is essential for the immune response of the host against GI nematodes, which will result in changes in mucosa secretion and an increase in contractions of the gut with the purpose of the expulsion of adult worms that are established (Onah and Nawa, 2000). Although the clear function of CD3e in the immune response in cattle is not known, it has

been reported that deficient expression in the membrane of T-cells or structural changes in CD3e gene are linked to severe immunodeficiency characterized by an absence of T-cell proliferation and activation (Basile et al., 2004; Le Deist, et al., 1991; Roberts et al., 2007; Soudais, et al., 1993; Thoenes et al., 1992). It is known that during a natural infection with trichostrongyle parasites, calves have shown increased levels in the regulation of CD3e in the small intestine mucosa of susceptible animals (Araujo et al., 2009).

None of the SNPs found in the evaluated Senepol*Charolais crossbreed population were found to be statistically different between populations based on FEC and no statistical difference was found in the segregation of haplotypes present in this gene (Table B-1.14). In the previously evaluated dairy population in this study, the SNP CD3e-112 was the only polymorphism which showed a statistical difference between both evaluated populations based on FEC. The segregation of the heterozygote allele was found to be segregating with a higher frequency in the population with higher FEC (group P) than heifers with low FEC (group N), meaning that the presence of the heterozygote allele can be associated with animals with high FEC. For this to be validated with a similar population of beef heifers, a much numerous population is needed.

Integrin alpha 4 (ITGA4)

Seven different SNPs were found in the amplified fragments of the ITGA4 gene, all of which showed no statistical difference between evaluated populations. Thus, the evaluated polymorphisms do not serve as candidate SNPs for the differentiation and selection between resistant and susceptible populations in either dairy or beef cattle. ITGA4 is involved in inflammatory responses and is partially responsible for eosinophil recruitment (Rothenberg et al., 2001) and a strong up-regulation of several integrins, including ITGA4, has been observed at 42 days post infection with *Cooperia oncophora* (Li and

Gasbarre, 2009). Additionally, Arujo et al., 2009 reported an increase in the regulation of this gene has also been observed in the mesenteric lymph nodes of resistant calves. The presence of ITGA4 is also necessary for the diapedesis of leukocytes through the endothelium (Murphy, 2012).

Conclusions

Two populations of beef heifers, one that had low EPG and another population with high EPG, were used for the evaluation SNPs in the CCL5, CD3e, and ITGA4 genes. All the evaluated genes are involved in different mechanisms of protective immunity against GI nematodes; from cell recruitment, molecules such as chemokines to antibodies, and components of T-cell receptors. These genes might serve as candidate genes for the genetic evaluation of resistant or susceptible cattle and assist in the selection of a population of cattle that have a higher degree of resistance against infection. No evaluation of polymorphisms found in each of the evaluated genes in association to GI nematode susceptibility or resistance has been previously made in cattle. The evaluated SNPs found segregating in the CD3e and ITGA4 genes showed to be inadequate candidates for the selection of resistant heifers based on FEC. In the CCL5, 2 SNPs showed to be statistically different between heifers with high and low EPG. This confirms that of all evaluated genes, CCL5 is the best candidate gene for the differentiation of resistant and susceptible heifers.

Table B-1. 1: List of SNPs found in the amplified fragment of the CCL5 gene.

Gene	SNP I.D.	N	Location	SNP	Base position
CCL5	Rs136242974	41	Exon 3	G/A	48
CCL5	Rs109200822	41	Exon 3	C/T	55
CCL5	Rs109769870	41	Exon 3	G/A	145
CCL5	CCL5-187	41	Exon 3	G/A	187
CCL5	Rs110457788	41	Exon 3	A/G	219

Table B-1. 2: Genotypic frequencies of SNP found in the CCL5 gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P	p-value of X^2
		Genotypic frequency	Genotypic frequency	
Rs136242974	AA	0.214	0.074	0.198
	AG	0.643	0.556	
	GG	0.143	0.370	
Rs109200822	CC	0.429	0.074	0.025
	CT	0.071	0.815	
	TT	0.500	0.111	
Rs109769870	AA	0.214	0.074	0.379
	GA	0.571	0.593	
	GG	0.214	0.3333	
CCL5-187	AA	0.000	0.000	0.001
	GA	0.214	0.778	
	GG	0.786	0.2222	
Rs110457788	AA	0.286	0.074	0.174
	AG	0.643	0.778	
	GG	0.071	0.148	

Table B-1. 3: Allelic frequencies of SNP found in the CCL5 gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P
		Allelic frequency	Allelic frequency
Rs136242974	A	0.536	0.352
	G	0.464	0.648
Rs109200822	C	0.464	0.482
	T	0.536	0.519
Rs109769870	A	0.500	0.370
	G	0.500	0.630
CCL5-187	A	0.107	0.389
	G	0.893	0.611
Rs110457788	A	0.607	0.463
	G	0.393	0.537

Table B-1. 4: Hardy-Weinberg equilibrium test. Calculated X^2 value of SNP found in the CCL5 gene for animals in the "Negative" group (N) and the "Positive" group (P) along with p-values for this test (df=2). P-values<0.05 are statistically significant, meaning that the population does not follow the H-W equilibrium.

SNP	Genotype	Group N			Group P		
		n	X^2	p-value	n	X^2	p-value
Rs136242974	AA	3	0.03	0.985	2	2.57	0.277
	AG	9			15		
	GG	2			10		
Rs109200822	CC	6	6.96	0.031	2	0.03	0.985
	CT	1			22		
	TT	7			3		
Rs109769870	AA	3	0.21	0.900	2	3.62	0.164
	GA	8			16		
	GG	3			9		
CCL5-187	AA	0	0.42	0.517	0	0.20	0.655
	GA	3			21		
	GG	11			6		
Rs110457788	AA	4	4.61	0.010	2	0.32	0.852
	AG	9			21		
	GG	1			4		

Table B-1. 5: List of SNPs found 3 different amplified fragments of the CD3e gene.

Gene	SNP I.D.	N	Location	SNP	Base position
CD3e_2	Rs136656580	35	Exon 9	Ins/Del	101
CD3e_2	Rs209286808	35	Exon 9	A/G	104
CD3e_2	CD3e-112	35	Exon 9	C/T	112
CD3e_3	Rs13576648	35	Exon 7	T/G	215
CD3e_3	Rs132883476	35	Intron 7	T/G	227
CD3e_3	CD3e-258	35	Intron 7	G/A	258

Table B-1. 6: Genotypic frequencies of SNP found in the CD3e gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P	p-value of X^2
		Genotypic frequency	Genotypic frequency	
Rs136656580	Del/Del	0.000	0.063	0.554
	Ins/Del	0.357	0.250	
	Ins/Ins	0.643	0.688	
Rs209286808	AA	1.000	1.000	-
	AG	0.000	0.000	
	GG	0.000	0.000	
CD3e-112	CC	0.813	0.929	0.351
	CT	0.188	0.071	
	TT	0.000	0.000	
Rs13576648	GG	0.000	0.000	-
	GT	0.000	0.000	
	TT	1.000	1.000	
Rs132883476	GG	0.000	0.000	-
	GT	0.000	0.000	
	TT	1.000	1.000	
CD3e-258	AA	0.000	0.000	-
	AG	0.000	0.000	
	GG	1.000	1.000	

Table B-1. 7: Allelic frequencies of SNP found in the CD3e gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P
		Allelic frequency	Allelic frequency
Rs136656580	Del	0.179	0.188
	Ins	0.821	0.813
Rs209286808	A	1.000	1.000
	G	0.000	0.000
CD3e-112	C	0.906	0.964
	T	0.094	0.036
Rs13576648	G	0.000	0.000
	T	1.000	1.000
Rs132883476	G	0.000	0.000
	T	1.000	1.000
CD3e-258	A	0.000	0.000
	G	1.000	1.000

Table B-1. 8: Hardy-Weinberg equilibrium test for segregating SNPs. Calculated X^2 value of SNP found in the CD3e gene for animals in the "Negative" group (N) and the "Positive" group (P) along with p-values for this test (df=2). P-values<0.05 are statistically significant, meaning that the population does not follow the H-W equilibrium.

SNP	Genotype	Group N			Group P		
		n	X^2	p-value	n	X^2	p-value
Rs136656580	Del/Del	0	0.66	0.720	1	0.52	0.721
	Ins/Del	5			4		
	Ins/Ins	9			11		
CD3e-112	CC	13	0.02	0.888	13	0.17	0.680
	CT	3			1		
	TT	0			0		
CD3e-258	AA	0	-	-	0	-	-
	GA	0			0		
	GG	14			21		

Table B-1. 9: List of SNPs found in the amplified fragment of the ITGA4 gene.

Primer name	SNP I.D.	N	Location	SNP	Base position
ITGA4_1	Rs42239893	33	Intron 1	A/G	310
ITGA4_1	Rs42239894	33	Intron 1	C/A	360
ITGA4_1	Rs133348759	33	Intron 1	G/A	529
ITGA4_1	Rs133643873	31	Intron 2	T/G	780
ITGA4_4	ITGA4-192	39	Exon 28	Ins/Del	192
ITGA4_4	ITGA4-298	39	Exon 28	G/A	298
ITGA4_4	Rs42240844	39	Exon 28	T/C	415
ITGA4_4	Rs42240845	39	Exon 28	G/A	456

Table B-1. 10: Genotypic frequencies of SNP found in the ITGA4 gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P	p-value of X^2
		Genotypic frequency	Genotypic frequency	
Rs42239893	AA	0.3571	0.3684	0.644
	GA	0.5000	0.3684	
	GG	0.1429	0.2632	
Rs42239894	AA	0.0714	0.1579	0.723
	AC	0.3571	0.3684	
	CC	0.5714	0.4737	
Rs133348759	AA	0.0000	0.0000	0.384
	AG	1.0000	1.0000	
	GG	0.0000	0.0000	
Rs133643873	CC	0.1429	0.2941	0.432
	CT	0.5000	0.2941	
	TT	0.3571	0.4118	
ITGA4-192	Ins/Ins	0.0000	0.0000	-
	Ins/Del	0.0000	0.0571	
	Del/Del	1.0000	0.9429	
ITGA4-298	AA	0.0000	0.0400	0.551
	GA	0.1429	0.2400	
	GG	0.8571	0.7200	
Rs42240844	CC	0.2143	0.5600	0.102
	CT	0.5000	0.2400	
	TT	0.2857	0.2000	
Rs42240845	AA	0.0714	0.0000	0.375
	AG	0.4286	0.4000	
	GG	0.5000	0.6000	

Table B-1. 11: Allelic frequencies of SNP found in the ITGA4 gene for animals in the "Negative" group (N) and the "Positive" group (P).

SNP	Genotype	Group N	Group P
		Allelic frequency	Allelic Frequency
Rs42239893	A	0.607	0.553
	G	0.393	0.447
Rs42239894	A	0.250	0.342
	C	0.750	0.658
Rs133348759	A	0.000	0.000
	G	1.000	1.000
Rs133643873	C	0.393	0.441
	T	0.607	0.559
ITGA4-192	Ins	0.000	0.000
	Del	1.000	1.000
ITGA4-298	A	0.071	0.160
	G	0.929	0.840
Rs42240844	C	0.464	0.680
	T	0.536	0.320
Rs42240845	A	0.286	0.200
	G	0.714	0.800

Table B-1. 12: Hardy-Weinberg equilibrium test for segregating SNPs. Calculated X^2 value of SNP found in the ITGA4 gene for animals in the "Negative" group (N) and the "Positive" group (P) along with p-values for this test (df=2). P-values<0.05 are statistically significant, meaning that the population does not follow the H-W equilibrium.

SNP	Genotype	Group N			Group P		
		n	X^2	p-value	n	X^2	p-value
Rs42239893	AA	5	0.46	0.795	7	1.23	0.541
	GA	7			7		
	GG	2			5		
Rs42239894	AA	1	0.03	0.985	3	0.63	0.730
	AC	5			7		
	CC	8			9		
Rs133348759	AA	0	-	-	0	-	-
	AG	0			0		
	GG	14			19		
Rs133643873	CC	2	0.46	0.795	5	2.77	0.250
	CT	7			5		
	TT	5			7		
ITGA4-298	AA	0	0.08	0.961	1	0.29	0.865
	GA	2			6		
	GG	12			18		
Rs42240844	CC	3	2.00	0.368	14	5.03	0.081
	CT	7			6		
	TT	4			5		
Rs42240845	AA	1	0.03	0.985	0	1.56	0.458
	AG	6			10		
	GG	7			15		

Table B-1. 13: Haplotype distribution between N and P populations of Senepol*Charolais crossbreed heifers in the CCL5 gene. P-value was obtained from a Chi-square test (contingency table).

	Haplotypes			P-value
	GYGRR	RYRGR	RYRRR	
N group	2	3	1	0.097
P group	4	3	12	
Total	6	6	13	

Table B-1. 14: Haplotype distribution between N and P populations of Senepol*Charolais crossbreed heifers in the CD3e gene. P-value was obtained from a Chi-square test (contingency table).

	Haplotypes			P-value
	ECG	ICG	IYG	
N group	4	8	3	0.583
P group	5	8	1	
Total	9	16	4	

Table B-1. 15: Haplotype distribution between N and P populations of Senepol*Charolais crossbreed heifers in the ITGA4 gene. P-value was obtained from a Chi-square test (contingency table).

	Haplotypes		P-value
	ACCAMGYCA	GAGCAGTCG	
N group	2	0	0.053
P group	1	4	
Total	3	4	

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