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Expression of The Transient Receptor Potential Channel 4 (TRPC4) Gene in Goats Naturally Exposed to *Haemonchus contortus* Infection

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Abstract

Expulsion of gastrointestinal nematodes (GIN) requires gut contractions and glycoprotein hyper-secretion for detachment from the gut wall. The Transient receptor potential cation channels (TRPC) facilitates contraction of smooth muscle. A mutation in the TRPC4 of mice significantly reduces contraction and motility of the intestine. Thus far, the correlation between TRPC4 and GIN infection has not been evaluated in goats or any other species. This study evaluated gene expression of TRPC4 in *Haemonchus contortus* exposed resistant goats. Goats that were naturally susceptible and resistant to *Haemonchus contortus* were sacrificed and intestinal tissues collected. From conserved regions of human, mouse, rat, and bovine TRPC4 gene alignments, oligonucleotide primers were generated using CLC Main Workbench bioinformatics software. The RT-PCR and quantitative real time pcr were performed using total RNA extracted from intestinal tissues. The expected 388bp cDNA product was amplified and sequenced. The goat TRPC4 showed 88 and 87% homology to rat and mouse and 98%, 92%, 91% and 90% homology to the bovine, horse, pig and human TRPC4 genes, respectively. The TRPC4 expression increased ($P<0.05$) in naturally susceptible goats. There were breed and gender effects ($P<0.05$) on TRPC4 expression. A strong ($P<0.05$) correlation was evident when the variables TRPC4 gene expression, clinical anemia, and parasite load were compared in goats. These data indicate that TRPC4 may aid in elucidation of the mechanism of action of the TRPC genes involved in gastrointestinal contraction and motility and their link to GIN infection.

Keywords: *Haemonchus contortus*, gastrointestinal motility, TRPC4, goat

1. Introduction

1.1 Significance of the Problem

Persistence in the development of control measures to target gastrointestinal nematode infection (GIN) in small ruminants remains a high priority for the small ruminant industry. Infection by *Haemonchus contortus*, a blood sucking gastrointestinal nematode (GIN), is the leading cause of economic losses in small ruminant production. *Haemonchus contortus* causes a disease known as haemonchosis in all domesticated ruminants (Asanji, 1988; Hogg et al., 2010; Schillhorn van Veen, 1978; van Dijk & Morgan, 2006). Young animals are most susceptible, become heavily infected, and manifest anemia, hypoproteinemia and edema (Kaplan et al., 2004; Le Jambre, 1995). Treatment of GIN infection is controlled primarily by the use of anthelmintics which has lead to the development of drug resistance (Chandrawathani, Yusoff, Wan, Ham, & Waller, 2004; Corley & Jarmon, 2012; Terrill, Miller, Burke, Mosjidis, & Kaplan, 2011). Focus has been geared towards identification of biomarkers that influence gut expulsion of GIN (Artis, 2006; Bancroft, Artis, Donaldson, Sypek, & Grencis, 2000; Corley & Jarmon, 2012; Khan et al., 2003; Perrigoue, Zaph, Guild, Du, & Artis, 2009; Vallance et al., 1999). Very few have dealt with studies on biomarkers of gut expulsion of *Haemonchus contortus* in small ruminants (Corley & Jarmon, 2012). Transient receptor potential (TRP) cation channels are involved in several cellular functions (Freichel et al., 2005; Gonzalez-Cobos & Trebak, 2010). They are of interest because they are involved in contraction of smooth muscle (Gonzalez-Cobos & Trebak, 2010; Ambudkar, 2009) and have been linked to many diseases (Nilius, Voets, & Peters, 2005) including that of the digestive system (Holzer, 2011). As a result the TRPC family has been used for drug targets in neurogastroenterology (Boesmans, Owsianik, Tack, Voets, & Vanden Berghe, 2011; Holzer, 2011). The TRPC channels function as calcium cation channels that cause membrane depolarization and entry of calcium into the cell, thereby resulting in smooth muscle contraction (Patel et al., 2010). Specifically, it has been shown that TRPC4 and TRPC6 deletions in mice, impairs intestinal

motility and contractility (Tsvilovskyy et al., 2009). Goats are plagued with the gastrointestinal nematode *Haemonchus contortus*, that costs the small ruminant industry millions of dollars per annum in livestock loss and drug treatment (Corwin, 1997).

1.2 Justification

The ability of the intestine to expel worms is dependent on many factors, one of which is intestinal contractility (Artis, 2006; Hasnain et al., 2011). The TRPC4 in particular has been linked to gastrointestinal contraction and motility (Kim, So, & Kim, 2006; Unno et al., 2006). Although the role of TRPC4 has been identified as crucial in intestinal contractility in mice, the TRPC4 gene has not been isolated in goats, nor evaluated in *Haemonchus contortus* infection. To date, the relationship between gene expression of TRPC4 and the response to GIN infection has not been assessed in goats or any other species. Moreover, the relationship between TRPC4 control over gastrointestinal motility and contractility and *Haemonchus contortus* infection in goats has not been investigated. Therefore the objectives of this study were to identify and characterize the TRPC4 gene of the goat and examine gene expression of TRPC4 in selected pasture exposed goats.

2. Method

2.1 Experimental Animals and Haemonchus Contortus Detection

Animals used in this study were Spanish and Myotonic goats. These animals were housed at VSU Randolph farm in accordance with institutional animal care and use guidelines. More than 100 Goats were screened for parasite load and clinical anemia status via fecal egg counts (FEC), packed cell volume (PCV) and FAMACHA eye color charts (FAM), and divided into susceptible and resistant groups accordingly. Our previously published work describe these procedures in detail (Corley & Jarmon, 2012). The FEC, FAM and PCV data collected were analyzed using SAS version 9.1.3, (Cary, North Carolina). We determined that goats with > 2000 eggs per gram of feces (EPG) with PCV $\leq$18 were naturally susceptible and those goats with > 2000 FEC with PCV $\geq$18 were resistant. Animals were sacrificed and intestinal tissue samples collected and stored at -80^{o}C for nucleic analysis. *Haemonchus contortus* spp. was verified via nucleotide sequencing as previously published (M. Corley & A. Jarmon, 2012). Nucleotide sequences were analyzed using sequence analysis software (NCBI-BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990), CLC Main Workbench).

2.2 Goat Intestinal Tissue Collection and Preparation

Animals were sacrificed as previously published (Corley & Jarmon, 2012) in accordance with national humane euthanasia guidelines. Intestinal including jejunal tissues were collected in sterile PBS and RNAlater (Invitrogen, NY) and placed at -80^{o}C for further molecular analysis. Tissues were homogenized in sterile PBS, centrifuged at 10,000 g and supernatants collected for gene expression analysis.

2.3 Total RNA Extraction from Goat Intestinal Tissue

Total RNA was isolated from goat tissue samples previously stored at -80^{o}C using a modified RNA isolation procedure (Gauthier, Madison, & Michel, 1997). Total RNA extraction procedures were performed according to previously published methods (Corley & Jarmon, 2012). Concentration and purity of total RNA were measured using a Nanodrop ND-1000 spectrophotometer (Thermoscientific). The RNA was stored at - 80^{o}C for later use in RT-PCR and qRT-PCR.

2.4 Reverse Transcriptase PCR (RT-PCR) of Goat TRPC4

Oligonucleotide primers were designed from mRNA of the bovine human, horse, pig, mouse and rat TRPC4 nucleotide sequences using the bioinformatics software, CLC Main Workbench (http://www.clcbio.com). Primers and target regions used for isolation of the goat TRPC4 gene are given in Table 1. The RT-PCR was conducted using the recommended protocol of the Verso 1-step RT-PCR kit (Thermo Scientific). Modified thermocyling conditions for 40 cycles were as follows: 50^{o}C 15 minutes, 95^{o}C, 2 minutes (initial denaturation), 95^{o}C, 30 secs, 55^{o}C, 1 minute, 72^{o}C, 1 minute repeated 39 times and a final extension at 72^{o}C for 5 minutes. The target TRPC4 cDNA was visualized by 1.5% agarose gel electrophoresis and a UGenius UV gel documentation system (SynGene, Fredericksburg, MD) equipped with a high resolution CCD camera.

2.5 Nucleotide Sequencing of Goat TRPC4 cDNA

For TRPC4 nucleotide sequencing, the cDNA (388bp) products were cut out and purified from agarose gels (Qiagen and Bio-Rad). The purity and concentration of gel purified cDNA was measured and prepared for nucleotide sequencing per commercial instructions. Samples were sent for sequencing at GeneWiz (South Plainfield, New Jersey). Raw nucleotide sequences were analyzed using sequence analysis software

(NCBI-BLAST, CLC Main Workbench). Subsequently, qRT-PCR analysis was conducted to determine gene expression of goat TRPC4 cDNA.

2.6 Measurement of Goat TRPC4 Gene Expression

Gene expression of TRPC4 in goat intestinal tissues was measured using qRT-PCR. The analytical parameters were as previously published (M. Corley & A. Jarmon, 2012). In brief, the analysis was conducted using the iScript One Step RT-PCR kit with SYBR Green (BioRad). For each reaction 100ng of total RNA was used. The Actin gene was used as an internal standard for relative quantitative analysis of TRPC4 gene expression.

2.7 Statistical Analysis

Relative fold quantitation data were analyzed using the General Linear Model procedure of SAS. Trial differences (n=3) were accounted for by using a Randomized Complete Block Design. Means were considered significant at $P<0.05$. Pearson Correlation Coefficient analysis was performed to compare variables measured (FEC, FAMACHA, PCV, TRPC4).

3. Results

Haemonchus contortus sp. verification was performed by direct gene detection (M. Corley & A. Jarmon, 2012). Goats in susceptible and resistant groups were significantly ($P<0.05$) different, indicating that the criteria used to determine susceptibility and resistance to *Haemonchus contortus* was valid. After RNA isolation from the goat tissue, RT-PCR was performed. Agarose gel electrophoresis was performed to visualize the expected 388bp goat TRPC4 cDNA (Figure 1). The goat TRPC4 cDNA was sequenced and aligned to the human, horse, bovine, pig, mouse and rat TRPC4 genes to identify sequence similarities (Figure 2). The goat TRPC4 amino acid sequence was aligned with the human, horse, bovine, Pig, mouse and rat TRPC4 AA sequences (Figure 3). The Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990, 1997) of the goat TRPC4 showed 98%, 92%, 91% and 90% nucleotide sequence homology to the bovine, horse, pig and human TRPC4 genes respectively and 88 and 87% homology to rat and mouse TRPC4 respectively (Figure 2). In the partial amino acid sequence alignment of goat TRPC4, showed that on the translational level, bovine and goat TRPC4 differed only by 1% among the bovine, human horse mouse rat and the pig differed by 2% (Figure 3). Gene expression of TRPC4 was lower ($P<0.05$) in *Haemonchus contortus* resistant goats (Figure 4). Myotonic goats expressed more ($P<0.05$) TRPC4 than Spanish goats (Figure 5). The TRPC4 was also upregulated ($P<0.05$) in male goats compared to female goats (Figure 6). There was no significant difference in TRPC4 gene expression relative to age, but older goats (5-7 years old) tended to express more TRPC4 than younger goats (1-5 years old) (Figure 7). Overall, there was a strong positive ($P<0.05$) correlation between TRPC4 gene expression and FAMACHA eye color chart scores and FEC, and a strong negative correlation ($P<0.05$) with PCV in goats.

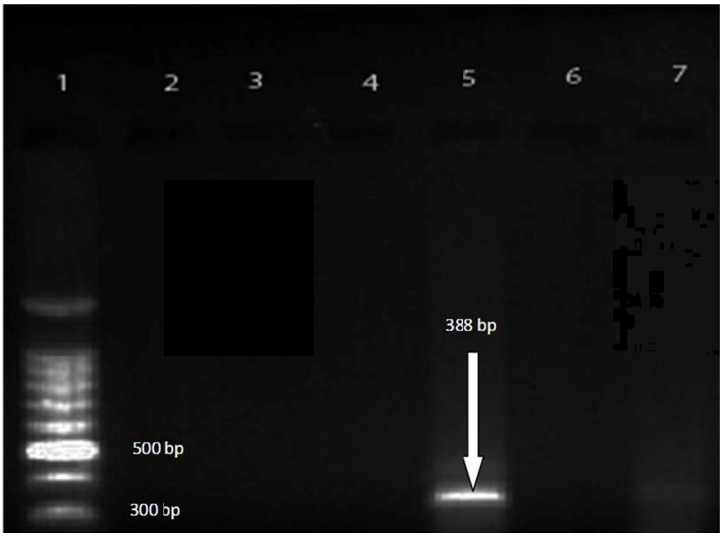

Figure 1. Agarose Gel Electrophoresis of Goat TRPC4 cDNA after Reverse Transcriptase PCR

Gel electrophoresis of Goat TRPC4 cDNA. The gel is 1.5% agarose with 0.05mg/ml ethidium bromide. Lane 1=Molecular Weight Marker, Lane 5 = Goat TRPC4 cDNA (388 bp).

Table1. Primers and Target Regions used for isolation of the goat TRPC4 gene from goat tissue

Accession No.	Primer name	Conserved Primer sequence	Target Region	Fragment Length (bp)
NM174478	Forward	CCCCACGAGGTCCGCTGTAA	795-814	388
NM016179	Reverse	GCCATTAAGTACCGTCAGAAA	1163-1182 (Rev-comp)	
NM001145868				
XM001495912				
NM016984				
NM080396				

Table showing a summary of the Accession numbers of the TRPC4 genes used for nucleotide alignments and design of oligonucleotide primers from conserved regions among the species (human, mouse, rat, pig, horse, cattle). The CLC Main Workbench Bioinformatics software was used to generate primers.

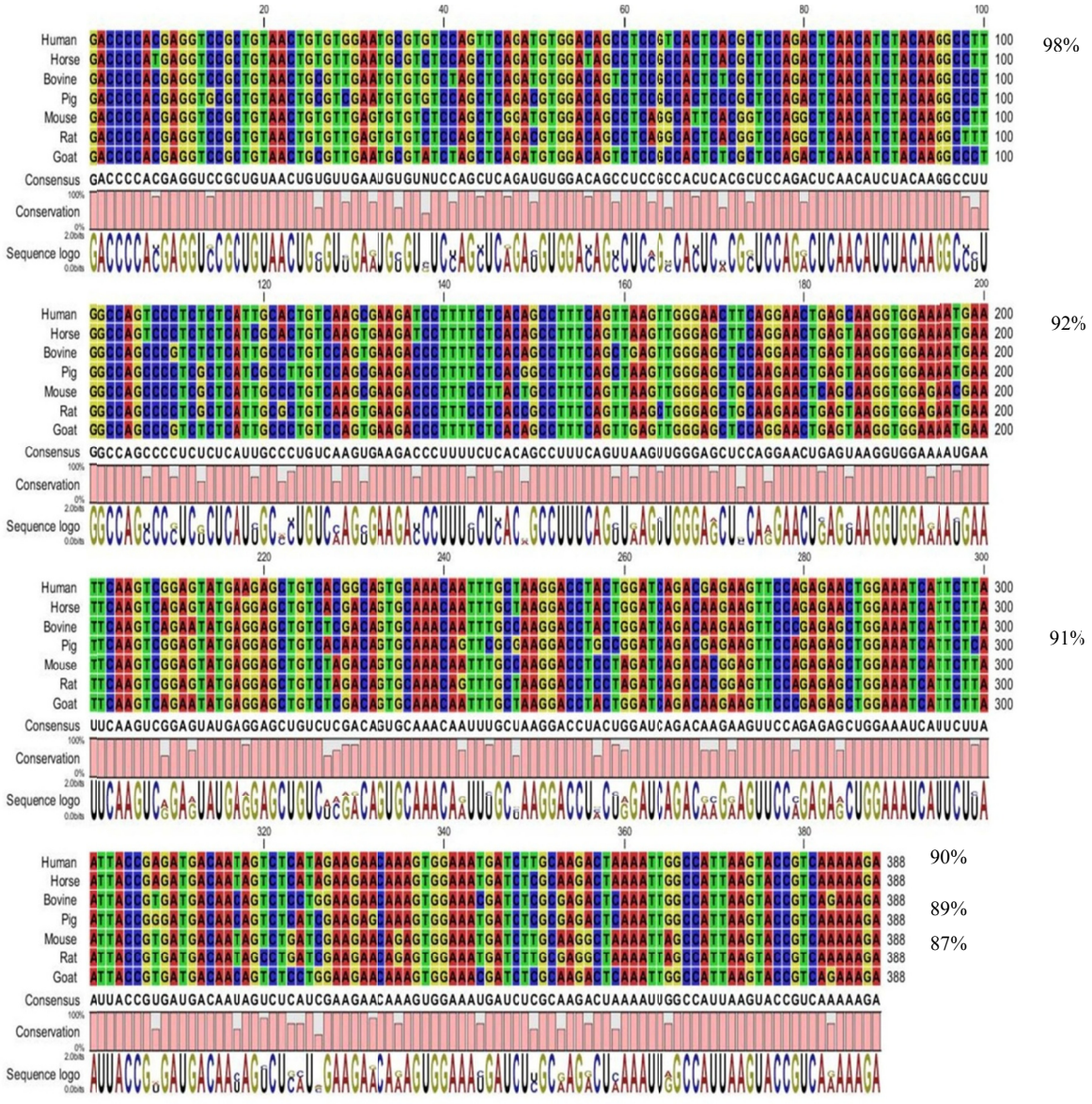

Figure 2. Nucleotide sequence alignment showing homology of goat, human, horse, bovine, pig, mouse and rat TRPC4 genes

Nucleotide sequence alignment of partial goat TRPC4 gene is shown. Alignment is shown to validate the conserved regions used to design the cross species oligonucleotide primers. Generated using CLC Main

Workbench Bioinformatics software Percentage represents the degree of homology among partial nucleotide sequences of the TRPC4 genes.

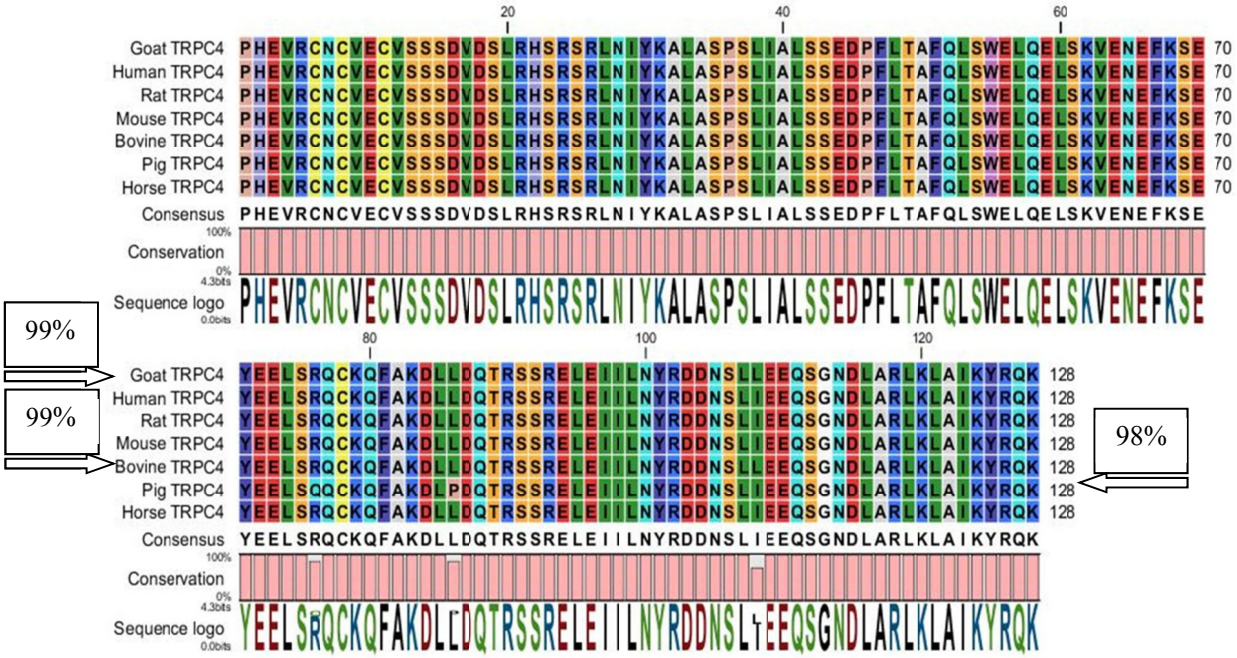

Figure 3. Amino acid alignment of translated goat, human, horse, bovine, pig, mouse and rat TRPC4

Amino acid translated sequence alignment of partial goat TRPC4 gene is shown. Alignment is shown to validate the conserved regions used to design the cross species oligonucleotide primers. Degeneracy lends to a greater amino acid sequence homology of the partial TRPC4 genes. Generated using CLC Main Workbench Bioinformatics softwarePercentage represents the degree of homology among partial amino acid sequences of the TRPC4 genes.

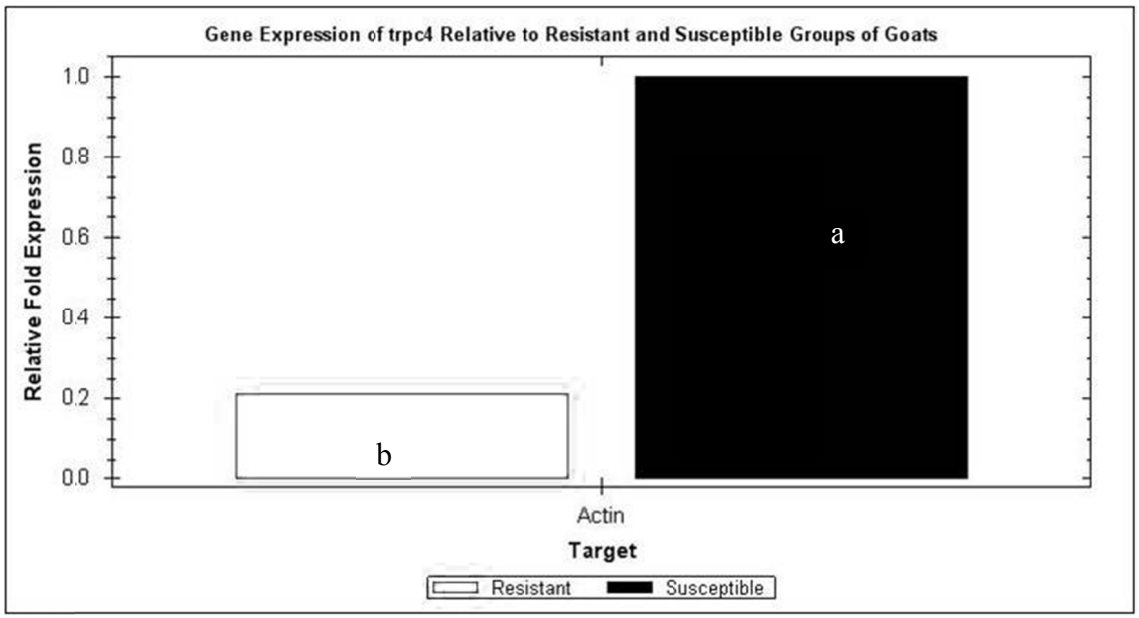

Figure 4. Gene expression of TRPC4 in the intestine in resistant and susceptible groups of goats

Relative fold expression of TRPC4 gene in resistant and susceptible goats as measured by qRT-PCR.

ab, Means with different letters differ ($P<0.05$).

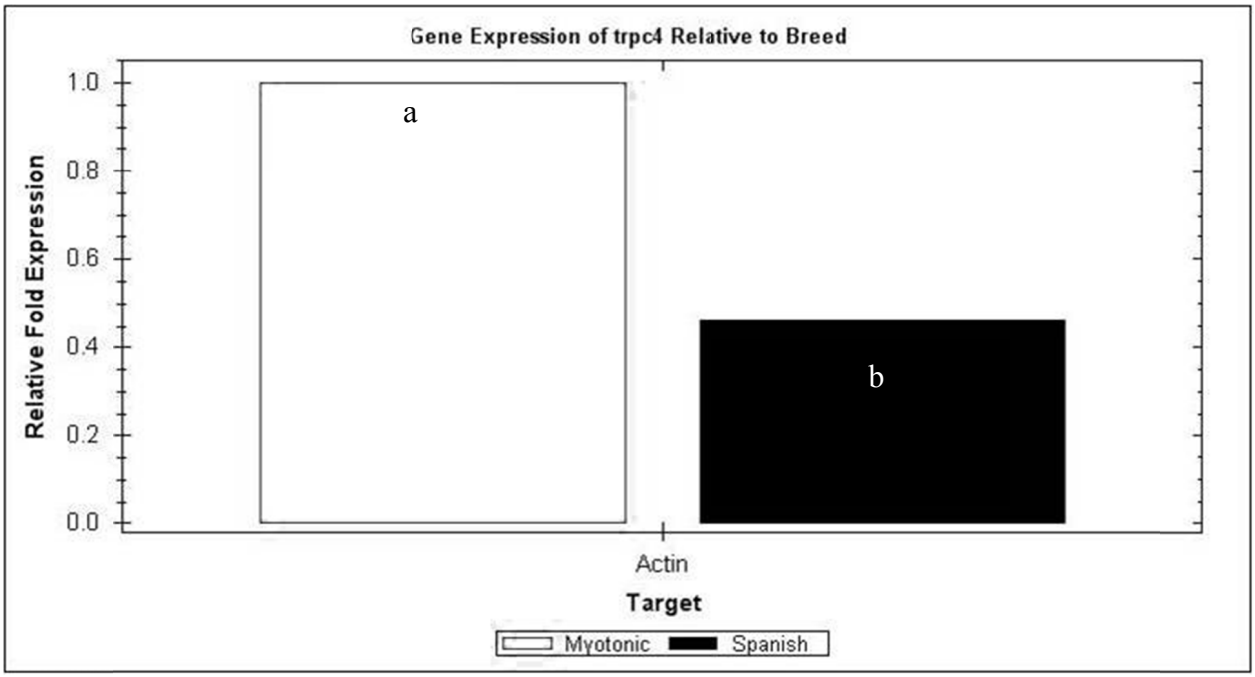

Figure 5. Gene expression of TRPC4 in the intestine of goats naturally exposed to *Haemonchus contortus* as influenced by breed

Relative fold expression of TRPC4 gene in Spanish and Myotonic goats as measured by qRT-PCR.

ab, Means with different letters differ (P<0.05).

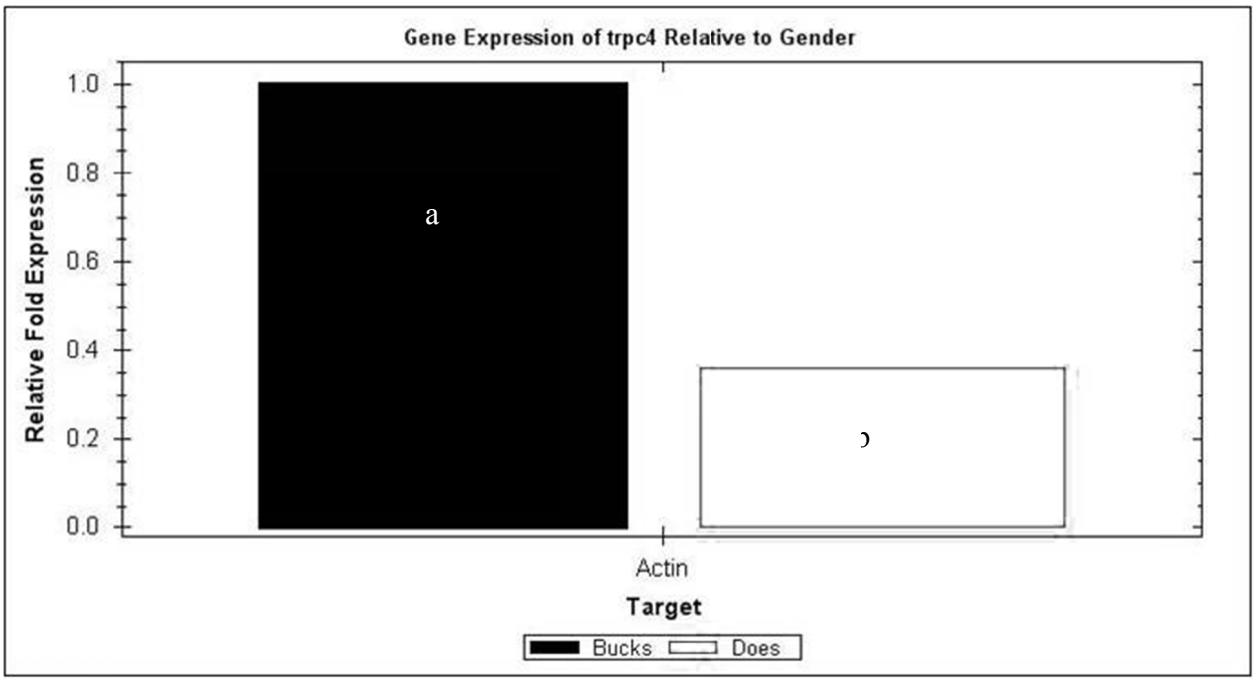

Figure 6. Gene expression of TRPC4 in the intestine of goats naturally exposed to *Haemonchus contortus* as influenced by gender

Relative fold expression of TRPC4 gene in male and female goats as measured by qRT-PCR.

ab, Means with different letters differ (P<0.05).

Figure 7. Gene expression of TRPC4 in the intestine of goats naturally exposed to *Haemonchus contortus* as influenced by age

Relative fold expression of TRPC4 gene in goats of different ages (older = 3-5 yrs old, younger = 1-3 yr old) as measured by qRT-PCR.

ab, Means with different letters differ ($P<0.05$).

4. Discussion

The ability of the intestine to expel worms is dependent on many factors, one of which is intestinal contractility. Although the role of TRPC4 (Tsvilovskyy et al., 2009) and TRPC6 (Tsvilovskyy et al., 2009 have been identified as crucial in intestinal contractility in mice, no sequence data for the TRPC4 nor the TRPC6 gene had been reported in goats, nor evaluated in *Haemonchus contortus* infection. We were able to isolate a partial sequence of the goat (GenBAnk Accession No. JX962344) TRPC4 and studies are under way with TRPC6. Based on previous studies TRPC4 gene knockout mice exhibited impaired gastrointestinal motility and contractility (Tsvilovskyy et al., 2009). This led to testing the hypothesis that goats showing resistance to *Haemonchus contortus* would upregulate gene expression of TRPC4. The rationale was that since TRPC4 is involved in intestinal contractility and motility then goats should have more motility and contractility during infection by *Haemonchus contortus* and therefore be prone to more gut expulsion of the nematodes. However, these data showed that TRPC4 was down regulated in the resistant group of goats. This response is complex but may be explained by other studies that have demonstrated that TRPC4 protein is down regulated in smooth muscle as a protective mechanism in response to muscle stretch (Lindsey, Tribe, & Songu-Mize, 2008). This down regulation of TRPC4 was demonstrated in aortic and mesenteric smooth muscle cells isolated from male Sprague-Dawley rats. The TRPC4 expression was decreased after 5 hour stretch and remained suppressed through a 24 h stretch and, recovered after removal of the stretch stimulus within 2 hours (Lindsey et al., 2008). In this study the down regulation of TRPC4 gene expression in the resistant groups of goats may have been a result of this protective mechanism existing in the intestine after *Haemonchus contortus* infection. This hypothesis is yet to be tested, as it has not been demonstrated in the intestine, particularly in goats with *Haemonchus contortus* infection. The mode of action of these genes in GIN infection is a more complex paradigm. Myotonic goats expressed more TRPC4 than Spanish goats. This supports the inference that TRPC4 is upregulated in goats more susceptible to *Haemonchus contortus* infection as this was the case in the susceptible group of goats. On the other hand, our previous studies have shown that interleukin 13, another biomarker of GIN resistance (Artis, 2006; Grencis & Bancroft, 2004), involved in gut expulsion of nematodes (Artis, 2006), was down regulated in myotonic goats (M. M. Corley & A. A. Jarmon, 2012). This makes a strong case that

TRPC4 is upregulated in more *Haemonchus contortus* susceptible goats. The TRPC4 was also upregulated in male goats compared to female goats. This response is logical, as it has been previously demonstrated that male goats are more susceptible to *Haemonchus contortus* infection than female goats (M. Corley & A. Jarmon, 2012). There was no significant difference in TRPC4 gene expression relative to age, but older goats (5-7 years old) tended to express more TRPC4 than younger goats (1-5 years old). This response could be explained by the fact that older goats have a weaker immune system than younger but a closer look would have to be made by widening the age gap in different groups of goats to see any significant effect in age groups for TRPC4 gene expression. Overall, there was a strong positive correlation between TRPC4 gene expression and FAMACHA eye color chart scores and FEC, and a strong negative correlation with PCV in goats. An increase in TRPC4 gene expression correlated with a high parasite load and anemia. On the other hand TRPC4 was down regulated in goats that were not experiencing anemia. This indicated that TRPC4 is upregulated in goats more susceptible to *Haemonchus contortus* infection. These data indicate thatTRPC4 gene expression correlates with susceptibility in goats pasture exposed to *Haemonchus contortus.* This would indicate that TRPC4 may be a potential biomarker for susceptibility rather than resistance to *Haemonchus contortus* infection in goats.

5. Conclusion

The results of this study indicated that the cross species oligonucleotide primers designed from the conserved regions of the TRPC4 genes can successfully be used to isolate a partial sequence of the goat TRPC4, from intestinal tissue samples, and used as a biomarker to evaluate gene expression in response to *Haemonchus contortus* infection in goats. Based on our findings, the TRPC4 gene could potentially be studied as a genetic marker for susceptibility to GIN infection, specifically *Haemonchus contortus* and could potentially be targeted for drug development in the treatment of GIN infection.

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