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A PCR/Sequencing Technique to Identify Species and Genotypes of *Haemonchus* from Mixed Trichostrongyle Egg DNA Recovered from Livestock Fecal Samples

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A PCR/SEQUENCING TECHNIQUE TO IDENTIFY SPECIES AND GENOTYPES OF *HAEMONCHUS* FROM MIXED TRICHOSTRONGYLE EGG DNA RECOVERED FROM LIVESTOCK FECAL SAMPLES

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biology with specialization in Microbiology degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This thesis is dedicated to Hildreth's Parasitology lab where I started my life as a researcher in the field of molecular diagnostics and spent two productive years to generate data for my thesis and to my dear parents for everything.

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ABSTRACT

A PCR/SEQUENCING TECHNIQUE TO IDENTIFY SPECIES AND GENOTYPES OF *HAEMONCHUS* FROM MIXED TRICHOSTRONGYLE EGG DNA RECOVERED FROM LIVESTOCK FECAL SAMPLES

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Six primer pairs, each with a pair of genus-specific and universal second internal transcribed spacer (ITS2) primers were evaluated to amplify and sequence the ITS2 region of nuclear ribosomal DNA to identify the species and genotypes of *Haemonchus* from livestock fecal samples containing mixtures of trichostrongyle nematode eggs. Based upon the use of universal ITS2 primers on *Haemonchus* adults, the ITS2 region has been shown to contain three fixed single-nucleotide differences between *H. placei* and *H. contortus*. It is clinically useful to identify the species and genotypes of *Haemonchus* infecting livestock, but it has not been possible in fecal samples containing trichostrongyle eggs from multiple species. Three primers came from publications showing genus-level specificity for *Haemonchus*. Two primers were published universal ITS2 primers. A genus specific and a universal primer was developed based upon analyses of sequences available on the National Center for Biotechnology Information website. PCR products from these primers amplifying individual worm DNA were sent to GenScript USA Inc. for Sanger sequencing, and then compared with egg DNA recovered from mixed infections. Comparisons included sequence quality values (QV) coupled with analyses of their ability to differentiate *H. placei* from *H. contortus*. A set composed of a forward universal primer in the 5.8S subunit gene coupled with a genus-specific reverse primer (at the ITS2 3' end, extending into the

28S gene) provided excellent QV values over most of the ITS2 region except for the 3' end. Two other primer sets provide good QV values in the 3' half of ITS2. These whole-worm DNA results remained consistent, and so did egg DNA from mixed infections for most samples except one fecal egg sample from one ewe. Two egg DNA samples from a cattle herd and two egg DNA and one adult worm DNA from a sheep flock were identified as *H. placei* and *H. contortus*, respectively. Three distinct genotypes were identified based on nucleotide diversity. Two of them are already reported and one is unique but needs further study to confirm the finding.

Chapter 1

Literature Review

1.1 Introduction

1.1.1 *Haemonchus*

Evolutionary biology studies suggests that members of the genus *Haemonchus* originated in sub-Saharan Africa (Gilleard & Redman, 2016). It is one of the 22 genera included in the family, trichostrongylidae. . There are 12 valid species of *Haemonchus* according to Eric et al., (2004). These species were identified based on phylogenetic analysis of 25 morphological characteristics of the adult stage for this parasite. Out of these twelve species of *Haemonchus*, 3 species (i.e. *H. placei*, *H. contortus,* and *H. similis*) are prevalent in North America and are more globally distributed as well, with the movement of their hosts especially the livestock host (Eric et al., 2004; Gilleard, 2013). It is believed that there were no species of *Haemonchus* endemic to North America before the Europeans first traveled to this area (Eric et al., 2004).

1.1.2 Clinical and Economic Impact of Haemonchosis

It is clinically and economically useful to diagnose the species and genotypes of *Haemonchus* infecting livestock (Gibbons, 1979). The development of drug resistance and evidences of cross-infection, coinfection, and hybridization between the two species of the genus, *H. contortus* and *H. placei* over the period has complicated the diagnosis and control of this parasite. Parasitic gastroenteritis caused by *Haemonchus* is a major cause of economic losses in livestock industries across the globe. According to Lane et

al., (2015), as mentioned in Emery et al., (2016), the Australian sheep industry losses AUD 436 million (total production loses and nematode control costs) annually. A major proportion of this amount is spent on control and treatment of *Haemonchus.* In addition to the number of worms present and rate of infection, the severity of infection depends on the immunological and nutritional health of the infected animals. Adult worms invade the submucosa of abomasum and pierce the capillaries by means of their single buccal tooth. This invasion leads to blood loss. In addition, there is more blood loss due to reduced clotting of blood. Each worm releases anti-hemostatic secretion when it pierces and wounds sub-mucosal capillaries. Each worm is estimated to result a loss of 0.05ml of blood per day either by ingestion or leakage from invaded tissue (Urquhart, 1996). Blood loss causes anemia and bottle jaw condition due to the dramatic reduction of packed cell volume, and loss of serum proteins and hypoproteinemia respectively (Hildreth & Harmon, 2013). Eventually, the herd health is affected resulting in production losses due to stunted growth, impaired weight gain, wool loss/ poor quality wool, and mortality of the severely infected cattle (*Bos taurus*), sheep (*Ovis aries*), and goats (*Capra hircus*) (Amarante, 2011).

1.1.3 Importance of Identification of Species

Haemonchus contortus and *H. placei* was considered synonymous until Blouin et al. (1997) identified them as two distinct species based on mtDNA evidence. Later Hoberg et al., (2004) confirmed this finding, recognizing *H. placei* as a valid species. Considerable morphological, molecular, and genetic differences have been described to validate them as separate species (Chaudhry et al., 2015; Amarante, 2011). *Haemonchus contortus* typically infects sheep and goats (Lichtenfels et al., 1994) but can also infect a

wide variety of ruminant host (including cattle, deer (*Cervidae*)) and camelids (Hoberg & Zarlenga, 2016; Hogg et al., 2010). *H. placei* is more host specific than *H. contortus*, mostly infecting cattle and rarely sheep (Hoberg & Zarlenga, 2016; Lichtenfels et al., 1994). Pure *H. placei* and pure *H. contortus* infections have been reported in small ruminants and cattle respectively under both experimental and field conditions, suggesting occurrence of cross infection and wide range of host species for *Haemonchus spp.* (Chaudhry et al., 2015; Akkari et al., 2013; Hogg et al., 2010; Achi et al., 2003; Jacquiet et al., 1998). Since *H. contortus* is more adapted to sheep than cattle and *H. placei* more adapted to cattle than sheep, it is believed that with time, animals will apparently eliminate the species of parasites that are not well adapted to them (Amarante et al., 1997). *Haemonchus placei*, however, was seen to survive very well in small ruminant hosts contrary to *H. contortus* surviving well only in its usual dominant host (Jacquiet et al., 1998).

Many populations of *H. contortus* have become resistant to most of the commercially available and major classes of anthelmintic (Emery et al., 2016; Kotze & Prichard, 2016; Gasbarre, 2014), seriously impacting the global economy. A study conducted in Brazil to compare the sensitivity of different diagnostic techniques of anthelminthic resistance of gastrointestinal nematodes in cattle, *H. placei* is found resistant to levamisole and ivermectin (Neves et al., 2014). However extensive resistance has not been reported yet. Though not any gene flow was reported earlier, it's evident now (Chaudhry et al., 2015). The developing resistance on *H. placei* can be either due to the flow of gene carrying resistance traits or just due to the selection of resistant genes through uncontrolled uses of anthelminthic to control *Haemonchus* infections. These

evidences of coinfection, cross infection and anthelmintic resistance create a threat for gene transfer between the two species; the transfer of gene carrying wider host adaptability trait for *H. contortus* to *H. placei* or the transfer of anthelminthic resistant alleles from *H. contortus* to *H. placei*. Thus, development of reliable identification system is necessary to design better treatment and control strategies for *Haemonchus* infections to keep the livestock industry sustained.

1.2 Identification Approach

Diagnosis of trichostrongyle infections can be made by finding their eggs in fecal samples. The genera and species of these trichostrongyles have morphologically similar eggs which makes it impossible to identify them even at the genus level.

1.2.1 Morphological and Cytological

Populations of *H. contortus* and *H. placei* adults can be differentiated from each other based on the distribution of longitudinal ridges on the synlophe/surface of cuticle and length of spicule. *Haemonchus placei* males have longer spicules than *H. contortus* (Lichtenfels et al., 1994). The two species can be even differentiated based on morphology of the infective larva (L3). According to Van Wyk et al., (2004), L3s of *H. placei* are longer with longer sheath tail than those of *H. contortus*. In addition to this, there are cytological karyotypic differences in that *H. placei* has a larger X- chromosome than its autosomes, in contrast to similar size of all the chromosomes in *H. contortus* (Amarante et al., 1997; Le Jambre & Royal, 1980). This was described based on visualization of DAPI (4′,6-diamidino-2-phenylindole) stained metaphase spreads. Because we are needing to diagnose the species of *Haemonchus* from fecal samples with

mixed eggs of trichostrongyles, it is not possible for us to use morphological or cytological techniques.

1.2.2 Molecular

Many studies have defined fixed molecular differences in the ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) sequences between *H. contortus* and *H. placei*. Stevenson et al., (1995) detected three nucleotide differences between *H. contortus* and *H. placei* in the 24th, 205th and 219th base positions on comparing their ITS2 sequences. For this study, samples of *H. contortus* were collected from Australia, China, the UK, and Switzerland and those of *H. placei* were collected from three regions in Australia. Later, (Brasil et al., 2012) identified species of *Haemonchus* based on these three nucleotide differences in 156 specimens of individual adult worms and L3s obtained from some cattle, buffalo (*Bison bison*), sheep and goat herds in Brazil. Also, *H. placei* can be separated from *H. contortus* based on differences in mtDNA NADH dehydrogenase subunit 4 (ND4) gene sequences between them. (Blouin et al., 1997) collected individual species sample from around the United States to conduct this study. PCR amplification of ETS also carries potential to differentiate *H. contortus* and *H. placei* based on differences in the size of the PCR products (Zarlenga et al., 1994). Recently, with the objective of differentiating *H. contortus, H. placei*, and also, their hybrids, (Amarante et al., 2017), described a conventional PCR based approach to amplify External Transcribed Spacer (ETS) using species-specific primer pairs.

Though, PCR primers have been already published to diagnostically differentiate *Haemonchus* eggs from the other trichostrongyle nematodes of sheep at the genus level, it has not been possible for species level identification in fecal samples containing

trichostrongyle eggs from multiple species. In the ITS2 region, there are only 3 fixed single-nucleotide differences between *H. placei* and *H. contortus*. Sequencing this region from *Haemonchus*-specific PCR products will enable us to identify both species and genotypes.

1.3 rDNA and ITS2

Nuclear ribosomal DNA has been a focus for defining species specific markers for the specific identification of strongylid nematodes of livestock. It is due to lower sequence variation in its genes and spacer regions among individuals within a population and between populations (Gasser et al., 2008) compared to genes in mitochondrial DNA (Hu & Gasser, 2006). This comparatively higher mutation rates in mitochondrial genes make it suitable to study genetic structures of nematode populations below the level of species (Gasser et al., 2008). The evolution of rDNA is concerted, and results in homogeneity of the rDNA sequences. The sequence similarity is greater among species than between species. This means the rDNA carries useful genetic markers for identification of species of parasites and can be explored well, as reviewed by Gasser, (1999).

Ribosomal DNA in all eukaryotes contains 3 rRNA genes (the 5.8S gene, 18S gene, and 28S gene) along with 4 non-coding spacer regions; an external transcribed spacer (ETS), a non-transcribed spacer (NTS), first internal transcribed spacer (ITS-1) and second internal transcribed spacer (ITS-2) region. (Hwang & Kim, 1999; Polanco et al., 2000). Usually, ETS and NTS are collectively referred as Intergenic spacers (IGS). Structurally, rDNA occurs as tandem repeats with each repeat unit arranged as shown in figure 1.

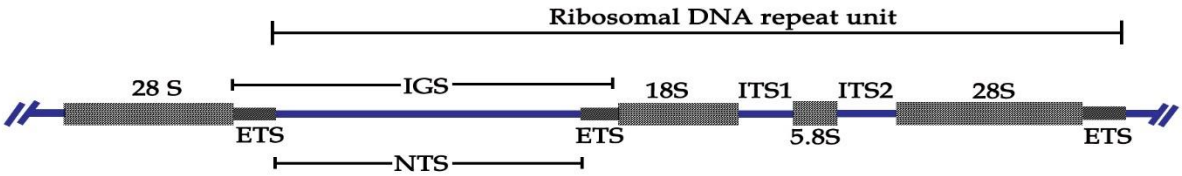

Figure 1. Schematic diagram of rDNA tandem repeat unit (Modified diagram by (Hwang & Kim, 1999)).

The IGS separates the large-subunit rRNA (28S gene) and small subunit rRNA (18S gene). The 5.8S gene lies between the18S and 28S genes and is separated from each by ITS1 and ITS2 respectively. The presence of multiple repeats of rDNA in the genome makes PCR amplification from single juvenile and adult nematodes possible (Powers et al., 1997) which was later pointed out by (Hung et al., 1999) as well. The rDNA array of multiple species of trichostrongylids like *Haemonchus contortus* (6.9-7.2kb), *H. placei* (7.2kb), *H. similis* (7.4 kb) (Zarlenga et al., 1994), *Ostertagia ostertagi* (7.5kb) (Dame et al., 1991) has been well explored. According to (Zarlenga et al., 1994), a minimum of two classes of sizes (1.32 and 1.63kb) of different copies of the ETS within the genome is responsible for the variation of the length in the rDNA array of *H. contortus*.

The study of phylogenetic relationship calls for appropriate selection of molecular markers or gene regions. The choice of molecular marker depends on the hierarchy of the categorical levels in the phylogenetic analysis (Waikagul & Thaenkham, 2014). The spacer regions and the gene regions have variable rate of evolution, and ITS region is the fastest evolving region (Hillis & Dixon 1991). The three ribosomal genes are considered very conserved regions as they are the coding regions of the ribosomal DNA. Among them, 18S gene is most highly conserved making its application to the identification of higher categorical levels like kingdoms, phyla, classes, or orders; whereas, the 28S gene

has comparatively more variation in the rate of evolution suggesting its application on identification of middle categorical levels such as orders or families. The 5.8S gene is considered too short to carry enough phylogenetic information, hence is not advisable for phylogenetic studies (Hwang & Kim, 1999). Chilton et al., (1997) found that the 5.8S gene has the same sequences for the gastrointestinal nematodes *Trichostrongyle axei*, *T. colubriformis*, *T. probolorus*, *T. retortaeformis*, *T. rugatus*, and *T. vitrinus*. Though *Haemonchus contortus* and *H. placei* have different sequences of ITS1 (Zarlenga et al., 1998), ITS2 (Stevenson et al., 1995) and IGS region, the sequences of their 18S gene are same (Zarlenga et al., 1994). Thus, lesser variability of sequences of the rRNA genes among species makes its diagnostic value/ species identification potential low (Gasser et al., 2008).

As ribosomal DNA spacer regions do not code for any protein, they are highly variable compared to coding regions of DNA and evolve at a comparatively faster rate. This property of spacer regions makes them suitable for phylogenetic study of identification of lower categorical levels like genera, species and populations (Hwang & Kim, 1999). The most divergent region of all, ITS is considered suitable for phylogenetic studies of closely related taxa, identification of species or strain variation, and/or hybrids, or as molecular marker in the study of population genetics (Kochzius et al., 2008; Hillis & Dixon, 1991). Fast- evolving marker is required for study in a species level (Schultz et al., 2005). As our study aims for identification of species and probable genotyping of genus *Haemonchus*, we considered rDNA spacer region for our study.

The ITS region is preferred to the IGS region due to larger size of the latter (ca. 4-5 kb versus ca. 1 kb) as explained in (Hwang & Kim, 1999). Also, IGS consists of

internal repetitive structures also known as sub-repeats differing in sequences and number of copies in contrast to ITS without any internal repetitive structures. This makes the ITS region more suitable to direct sequence analysis (Ryu et al., 1999). Moreover, the presence of ITS regions between conserved region in rDNA (18S, 5.8S and 28S gene) makes design of universal primers for PCR amplification of ITS region possible and has already been applied to the study of closely related taxa, like Protozoa, Coelenterata, Platyhelminthes, Nemathelminthes, Mollusca and Arthropoda (Shen et al., 2012; Chow et al., 2008). According to Powers et al., (1997), not any single species of nematode has failed to amplify with Universal PCR primer sets.

There has been consistent demonstration of ITS1 and ITS2 as reliable genetic markers for the identification of species for a variety of strongylid nematodes of livestock by large number of studies. Some of the examples of these nematodes include *Haemonchus, Teladorsagia* and *Ostertagia* (abomasum), *Trichostrongylus* (abomasum/small intestine), *Cooperia, Nematodirus, Bunostomum* (small intestine), *Oesophagostomum* and *Chabertia* (large intestine), *Dictyaocaulus, Protostrongylus* and *Metastrongylus* (lungs), as cited by (Gasser et al., 2008). Gasser et al., (2008) concluded that the magnitude of variation of both the ITS1 and ITS2 sequences within a species is considerably less (generally, <1.5%) than between the species based on the findings of several studies done on variability of ITS sequences within and among species of different genera of strongylids of livestock. This suggests that ITS region can be considered for specific identification of trichostrongyle nematodes of livestock. Nucleotide sequence differences within the ITS1 and ITS2 have already been used for accurately delineating and differentiating closely related species in the genus

Trichostrongylus (Family: *Trichostrongylidae*). Also, the length of the sequences of ITS1 and ITS2 have been determined by comparing the ITS sequences of different strongylid nematodes of livestock. ITS 2 (215-484 bp) is smaller in size than the ITS1 (364-522 bp) (Chilton, 2004). Along with smaller size, there is a wealth of resources (a large number of sequences in the NCBI database and level of confidence to rely on them) with ITS2 compared to ITS1 to refer to, which made us choose ITS2 for our study. More importantly, several studies have verified that ITS2 has invariably fixed differences between *H. placei* and *H. contortus*, three nucleotide bases substitution in 24th, 205th and 219th base positions (exchange between purines A/G) within a short fragment of 231 bases (4) which adds value to the choice of ITS2 for our study. The sequences of ITS2 are widely applied for phylogenetic reconstruction on genus and species level, as described in (Schultz et al., 2005).

1.4 Project Objective

The main objective of the research is to identify 2 sets of primers capable of generating PCR products that collectively span the entire ITS2 region of *Haemonchus*. Each set should contain a genus-specific primer towards one end of the ITS2 region and a universal primer located in the ribosomal gene extending past the opposite end of the ITS2 region. Collectively, PCR products from the 2 sets must span the entire ITS2 region.

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Chapter 2

Design of a PCR/ Sequencing Technique to Identify Species of *Haemonchus*

2.1 Introduction

The genus, *Haemonchus,* is a blood feeding trichostrongyle nematode of ruminants. Two of its 12 species (*H. contortus* and *H. placei*) are a principal abomasal pathogen in domestic ruminants. Though this parasite originated in Sub-Saharan Africa (Gilleard & Redman, 2016), these two species have expanded their distributions globally due to the movements of their hosts (Gilleard, 2013; Hoberg, 2004). *Haemonchus contortus* most often parasitizes sheep and goats, where it can cause a lethal anemia if not treated, whereas, *H. placei* is more common in cattle, and generally causes much lower mortality.

It is possible to identify the anemia associated with haemonchosis by matching the color of the inner mucous membrane of the eye to a colored guided FAMACHA chart, however, it is difficult to specifically diagnose it in live animals because the diagnostic egg stage of *Haemonchus* in fecal samples is visually identical to the other trichostrongyle nematodes (Preston et al., 2014). PCR primers have been published to diagnostically differentiate fecal samples containing *Haemonchus* eggs from those produced by the other trichostrongyle genera in sheep, goats and cattle (Bisset et al., 2013; Harmon et al., 2007; von Samson-Himmelstjerna et al., 2002). Most of the PCR methods for identifying the genera and species of nematodes are based upon unique differences in sequences within the spacer regions of nuclear ribosomal DNA.

These spacer regions between the genes coding for the ribosomal proteins are particularly popular because they are highly repetitive in the genome (making them easy to detecting with PCR), and there is less sequence variation within its genes and spacer regions among individuals within a population and between populations (Gasser et al., 2008) compared to genes in mitochondrial DNA (Hu & Gasser, 2006). In the rDNA the spacer regions between the ribosomal DNA genes have served as useful molecular markers for species level identification of trichostrongyle nematodes such as *Haemonchus* (Amarante et al., 2017; Brasil et al., 2012; Stevenson et al., 1995; Zarlenga et al., 1994). There is a wealth of sequence information available for second internal transcribed spacer (ITS 2) region on the National Center for Biotechnology Information (NCBI) website to identify sequence variations among the different *Haemonchus* genotypes. Also, there are only 3 fixed single-nucleotide differences between *H. placei* and *H. contortus* in the ITS2 region and has been successfully used to differentiate these species (Brasil et al., 2012; Stevenson et al., 1995). Sequencing this region from *Haemonchus*-specific PCR products will enable us to identify both species and genotypes.

Schnieder et al., (1999) designed genus specific primers for 5 genera of gastrointestinal nematodes of ruminants including, *Haemonchus* using their ITS2 sequences to differentiate eggs or larvae of these nematodes from each other. The identification was based on the differences in the sequences of ITS2 for the selected genera of nematodes. Zarlenga et al., (2001) developed a multiplex PCR assay based on External Transcribed Spacer (ETS) and First Internal Transcribed Spacer (ITS1) and Second Internal Transcribed Spacer (ITS2) to identify and semi-quantify eggs of

common GI nematodes infecting cattle using worm DNA. Subsequently, von Samson-Himmelstjerna, (2002) developed a real time PCR assay to identify and quantify DNA from larval stage of *Haemonchus* contortus along with other common trichostrongyles using genus specific primer-probe combination. Harmon et al., (2007) then tested the linear relationship between trichostrongyle egg numbers and CT values to determine the potential usefulness of QPCR techniques for measuring the parasite egg output of specific trichostrongyles in ruminants. Later, Bott et al., (2009) evaluated species specific real time PCR for the identification of *Haemonchus* from egg DNA along with 11 keys other trichostrongyle species in sheep using a species or genus specific forward oligonucleotide primer located in the ITS2 coupled with a conserved reverse primer in the 28S subunit of rDNA. Subsequently, Bisset et al., (2013) designed generic- and species-specific primers for use in a multiple assay to identify key trichostrongyle larvae found in the sheep from New Zealand. They used an approach like that of Bott et al., (2009) in that one of the primer pairs was located in the ITS2 spacer while the other pair was located in one of the conserved rDNA gene subunits. If the specific primer is the reverse pair, then the universal primer is located in the small subunit; if the specific primer is the forward pair, then universal primer is in the large subunit of rDNA. All the primers specific for *Haemonchus* are based on the ITS2 region designed from one or two ITS2 sequences data from adult worm or juvenile derived DNA isolated from a specific geographical region (Bisset et al., 2013; Bott et al., 2009; Zarlenga et al., 2001; Schnieder et al., 1999). It is not possible to obtain a complete sequence of ITS2 with genus specific forward and reverse primers published until now as they lie within the ITS2. Also, the first 25 nucleotide bases or so in the sequences obtained after sequencing are unreliable

(Swanson, 2014). So, incorporation of universal primer in each pair of primer and the use of two primer pairs to amplify the genomic egg DNA from feces containing multiple genera of gastrointestinal nematodes should enable researchers to produce a complete sequence of ITS2 facilitating the identification of species and genotypes of *Haemonchus*.

It is clinically useful to diagnose the species and genotypes of *Haemonchus* infecting livestock. The evidences of coinfection, cross infection (Achi et al., 2003; Akkari et al., 2013; Chaudhry et al., 2015; Hogg et al., 2010; Jacquiet et al., 1998) and anthelmintic resistance (Emery et al., 2016; Gasbarre, 2014; Kotze & Prichard, 2016; Neves et al., 2014) alarms a threat for chances of gene transfer between the two species; transfer of gene carrying wider host adaptability trait for *H. contortus* to *H. placei* or the transfer of anthelminthic resistant allele from *H. contortus* to *H. placei*. Thus, development of reliable identification system is necessary to design better treatment and control strategies for *Haemonchus* parasite infection and eventually, keep the livestock industry sustainable. The main objective of the research in this thesis is to identify two sets of primers that are genus-specific for *Haemonchus* and are capable of generating PCR products that collectively span the entire ITS2 region such that the region can be reliably sequenced. For this, each set will contain a genus-specific primer towards one end of the ITS2 region and a universal primer located in the ribosomal gene extending past the opposite end of the ITS2 region. Collectively, PCR products from the two sets must span the entire ITS2 region.

2.2 Materials and Methods

Primers were evaluated using DNA extracted from *Haemonchus contortus* adults and from trichostrongyle eggs harvested from sheep infected with *Haemonchus contortus, Teladorsagia circumcincta* and *Trichostrongylus* spp. mixed infections.

2.2.1 Sources of Samples and Extraction of Genomic DNA

2.2.1.1 Adult worm

Adult worms were collected from a ewe whose death was attributed to haemonchosis based upon necropsy findings. The flock containing this ewe was described by Grosz et al., (2013). The worms were preserved in 70% ethanol until used. Genomic DNA was extracted from a single worm using the Qiagen DNeasy Plant Mini DNA extraction kit (Valencia, CA, USA) as per manufacturer's protocol and stored at -20°C until further use.

2.2.1.2 Eggs

Trichostrongyle eggs (including *Haemonchus* spp., *Teladorsagia* spp. and *Trichostrongylus* spp.) were harvested from feces collected from the same commercial flock of sheep as adult worms were isolated and a different commercial cattle herd from South Dakota. Appropriate 3 g fecal samples were taken and the Wisconsin sucrose flotation method (Cox & Todd, 1962) as modified by Harmon et al., (2007) was used to harvest the eggs. This technique uses sucrose (specific gravity 1.26) and centrifugation to separate the eggs from the fecal pellet. Following egg extraction, the eggs were counted with an Olympus AX70 microscope (at 100X total magnification). The eggs were then

washed off from the coverslip with distilled water and stored at -20˚C until further analysis. Genomic DNA was extracted from the stored egg samples using the Qiagen DNeasy Plant Mini DNA extraction kit (Valencia, CA, USA) as per manufacturer's protocol and stored at -20°C until further use.

2.2.2 Confirmation of Identity for Genomic DNA

Upon extraction of genomic DNA, PCR analyses were performed to confirm the genera of trichostrongyles present in each sample. Table 1 lists the PCR primers used for each genus, *Haemonchus.* These primers target the ribosomal DNA second internal transcribed spacer (ITS2) region.

2.2.2.1 PCR Amplification

PCR amplification was performed using a 25 µL reaction mixture containing 12.5 µL GoTaq® Hot Start Green Master Mix, 2X (Cat. #M5123 from Promega, Inc. consisting of 50 U/mL Taq DNA Polymerase, 400 µL dATP, dGTP, dCTP, dTTP, and 4 mM $MgCl_2$, yellow and blue dye), 7 µL nuclease-free H_2O, 3 µL of respective primer (forward and reverse concentration was 0.2 µM), and 2.5 µL genomic DNA. Thermocycling parameters for the amplification were 1 cycle of initialization at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min with a single cycle of final elongation at 72°C for 7 min.

All PCR products were resolved in agarose gels (2%) stained with ethidium bromide in Tris EDTA (TE) buffer for 90 min at 125 V to verify that they represented single bands of accurate molecular weight. A ladder was used to confirm the size/

molecular weight of the PCR amplified products separated by agarose gel electrophoresis (exACTGene, 50bp Mini DNA Ladder from Fisher Scientific International Inc.). Bands were visualized, and the image was captured by Odyssey® Fc gel imaging system using image studio software.

2.2.3 Selection of *Haemonchus* Primers for ITS2 Sequencing

Two universal nematode primers (NC1 and NC2) suggested by Gasser et al., (1993) were selected for this study among the four universal primers reviewed, since couple of studies have already successfully used it for the molecular characterization of *Haemonchus* (Bandid et al., 2014; Yin et al., 2013). The forward universal primer (NC1) lies in the 5.8S subunit gene and the reverse universal primer (NC2) in the 28S subunit gene (Table 2). The 5.8S gene lies between 18S and 28S genes and is separated from each by ITS1 and ITS2 respectively. Three previously published, genus-specific primers were selected from studies published by Bisset et al., (2013), Harmon et al., (2007) and von Samson-Himmelstjerna et al., (2002) (Table 3). These five primers were arranged in five different primer sets, each primer set either contained a forward universal primer in the 5.8S subunit gene coupled with a genus-specific reverse primer (toward the ITS2 3' end) or a genus-specific forward primer (toward the ITS2 5' end) coupled with a reverse universal primer in the 28S gene. One additional specific primer was custom developed based upon one of the other primer, Haco Fd1 suggested by Bisset et al., (2013) but moved 5 base positions behind the starting of the sequence of primer, Haco Fd1 to include a more conserve region; this was determined from analyses of sequences available on the NCBI website. The locations of the ITS2 primers were determined

looking at the *Haemonchus* sequences spanning 5.8S- ITS2- 28S region in the rDNA retrieved from NCBI in MEGA software.

2.2.4 Validation of primers

2.2.4.1 PCR Amplification using Adult Worm DNA

Each primer sets were tested for their ability to amplify PCR products using *Haemonchus* DNA harvested from adult worms or isolated juveniles. Since the adult worms used in this study were collected from an ewe whose death was attributed to haemonchosis based upon necropsy findings, they were speculated to be *H. contortus*. The genomic DNA obtained from these worms were PCR amplified using a Universal primer pair (NC1-NC2) to validate this speculation. This speculation turned out true when the ITS2 sequences of these worms were aligned with the standard ITS2 sequences for *H. contortus*. One complete ITS2 sequence of H. contortus was selected randomly from GenBank to use it as a reference sequence to map the sequences obtained from worm genomic DNA amplified using universal primers NC1-NC2.

PCR amplification conditions consisted of 1 cycle of initialization at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min with a single cycle of final elongation at 72°C for 7 min. PCR products were separated in agarose gels (2%) stained with ethidium bromide in TE buffer for 90 min at 125 V to verify that the product consisted of a single band with the proper molecular weight. Bands of appropriate molecular weight were then excised, and PCR products were extracted and purified using Gel/PCR DNA Fragment Extraction Kit (MIDSCI^{TM} Cat. No. IB47020) as per manufacturers protocol. Purified PCR products

were sequenced on both strands using Sanger sequencing with the appropriate forward and reverse primers in a separate reaction by GenScript (Piscataway, NJ).

2.2.4.2 Analyses of *Haemonchus* ITS2 Sequences in NCBI

All the DNA sequences of *H. contortus* and *H. placei* spanning the ITS2 interspacial region were retrieved from the National Center for Biotechnology Information (NCBI) data base in FASTA format. There were 297 accession entries in NCBI database till April 23, 2018 that contained the complete ITS2 region. Multiple sequence alignment was performed on those sequences using the MUSCLE tool from MEGA 7.0 (Kumar et al., 2016) to verify whether all sequences in the database were consistent for the three single nucleotide polymorphisms (SNPs) between the two species at 24^{th}, 205^{th} and 219^{th} base positions. These 297 entries were used to examine the level of variance (% variability of the nucleotide bases in particular region) within the ITS2 region of both *H. contortus* and *H. placei* (Table 4)% variability was calculated dividing total number of variable nucleotide positions in a range of base positions by multiple of range of particular range of base positions and total number of sequences examined. Based on level of variance determined, an ITS2 bar was prepared with color gradient indicating 100% conserved region to highly variable.

2.2.4.3 Comparison of Product Sequences with Established Species and Genotypes

Resultant sequences from primer applications of adult and juvenile DNA were compared to each other using Mega7.0 software (Kumar et al., 2016), and with the genotype sequences from the NCBI data base for *H. contortus* and *H. placei*. DNA Baser sequence assembly software (DNA Baser Assembler v4, 2013) was used to analyze

chromatogram of the sequences obtained and to trim off sequences that had less than 60% good bases in a window of 20 bases. A base must have a quality value above 20 to be considered a good/trusted base. Base caller embedded in the program was also enabled to double check a chromatogram obtained from the Genscript by recomputing the confidence score for bases (Quality Values/QVs) and recalling the undetermined bases in a chromatogram. This also allows to correct ambiguities while building consensus sequence from forward and reverse sequences. (DNA Baser Assembler v4,2013)

After trimming, average quality values for a pair of sequences obtained from each of the primer sets were determined using DNA Baser Assembler v4 (2013). Also, the length of consensus sequences obtained from each pair of sequences was noted. Based on these parameters, primers were selected.

2.2.4.5 Primer Specificity

The sequences of Harmon Reverse primer, Haco Fd1, and Custom Fd primers were BLASTed (BLASTn) against the NCBI database to test their specificity. Alignment score for the sequences of the primers with the search hits were looked upon. Primers specific to only genus *Haemonchus* is highly desirable to inhibit the amplification of other genera of trichostrongyles that could be present in the egg DNA isolated from feces containing eggs of multiple genera of them.

2.2.5 Verification of PCR Amplification and Sequences from DNA Isolated from Mixed Egg Samples

PCR product production and purification, and product sequencing was as described about. Only three primer pairs were tested on egg DNA for their specificity to

amplify *Haemonchus* DNA from a mixed DNA obtained from fecal samples with eggs from multiple genera of trichostrongyles. Also, the calculation of average quality values and total mismatches from a pair of sequence obtained for each of the primer sets was as described about for adult worm samples.

Table 1. Genus-level Primers

Primers used to identify genus of trichostrongyle eggs, larvae and adult worm.

Genus	Primer Identity	Forward Primer 5′ to 3′	Reverse Primer 5′ to 3′	Base Pairs
Haemonchus	*Haemonchus contortus*/**	CATATACATGCAACGT GATGTTATGAA**	GCTCAGGTTGCATTAT ACAAATGATAAA*	157
Cooperia	*Cooperia oncophora***	GTGTGGCTAACGTTTT AACACTGT	TCTTGAACTATAACGG GATTTGTCAAA	77
Ostertagia/ Teladorsagia	*Ostertagia leptospicularis**	ATGAAACTACTACAGT GTGGCTAACA	TTCTTGAACTGAAATG GGAATTATCA	92
Trichostrongylus	*Trichostrongylus colubriformis**	CTTACGTCTGGTTCAG GGTTGTT	ACTGAAATGGGAATCA TCACAATATTT	105

*According to von Samson-Himmelstjerna et al. (2002)

**According to Harmon et al. (2007). Harmon et al. (2007) made slight modification in the forward primers for *H. contortus* and *C. oncophora* described by von Samson-Himmelstjerna et al (2002) (Grosz et al. 2013)

Table 2. List of Published Universal Primers for *Haemonchus*

Primer Name	Sequence	Location	Length	Reference
ITS2GF (generic)	CACGAATTGCAGACGCTTAG	5.8S	20	Bissett et al. (2014)
ITS2GR (generic)	GCTAAATGATATGCTTAAGTTCAGC	28S	25 28	
NC1	ACGTCTGGTTCAGGGTTGTT	5.8S	20	Gasser et al. (1993)
NC2	TTAGTTTCTTTTCCTCCGCT	28S	20	

Table 3. List of Published Genus-specific primers for *Haemonchus*

Primer Name	Sequence 5'-3'	Location	Length	Reference
Genus specific F	GAGGGCTAATTTCAACATTG	17	20	Schnieder et al. (1999)
Genus specific R	CAAATGATAAAAGAACATCGTC	205	22	
Hc 2 multi 272F	GCGAATATTGAGATTGACTTAGATAGAGAC	166	30	von Samson-Himmelstjerna et al., (2002)
Hc 2 multi 349R	GCTCAGGTTGCATTATACAAATGATAAA	216	28	
Forward	CATATACATGCAACGTGATGTTATGAA	85	27	Harmon et al. (2007)
HacoFd1	CAACGTGATGTTATGAAATTGTAACA	96	26	Bisset et al. (2014)
HacoFd3	CATGTATGGCGACGATGTTCTT	195	22	
HacoRv2	TCATATCATTCAGGGATGTTACAATTT	110	27	
HacoRv3	CTTGAACTGAAATGGGAATTGTCT	59	24	
HAE	CAAATGGCATTTGTCTTTTAG	41	21	Bott et al. (2009)

Table 4. Determination of variable and conserved regions in the *Haemonchus* ITS2 between the three species-specific SNPs

Nucleotide Base Positions	BPs in Range	No. of Variations	Range × 288 Sequence Entries in GenBank	Variability
1-17	17	54	4896	1.10%
18-23	6	892	1728	51.62%
24th SNP Position				
25-58	34	20	9792	0.20%
59-65	7	628	2016	31.15%
66-122	67	55	19296	0.28%
123	1	TVTC	288	>50%
124-195	30	26	8640	0.30%
196	1	TVTC	288	>50%
197-204	8	23	2304	1.0%
205th SNP Position				
206-218	13	5	3744	0.13%
219th SNP Position				
220-231	12	0	3456	0.00%

Abbreviation: BP - base pairs, SNP - Single Nucleotide Polymorphism, TVTC – Too Variable To Count

Table 5. Universal and species-specific primers for *Haemonchus*

Universal Primers 5′ to 3′	Species-specific Primers 5′ to 3′
NC1 ACGTCTGGTTCAGGGTTGTT	Harmon Fd* CATATACATGCAACGTGATGTTATGAA
NC2 TTAGTTTCTTTTCCTCCGCT	Harmon Rev.** GCTCAGGTTGCATTATACAAATGATAA A
ITS2 GR1**** CTTTTCCTCCGCTAAATGATATG C	HacoFd1*** CTTTTCCTCCGCTAAATGATATGC
	Custom Fd**** TCAACATTGTTTGTCAAATGGC

* According to Harmon et al. (2007)

** According to von Samson-Himmelstjerna et al. (2002)

*** According to Bisset et al. (2013)

**** Custom developed

Slight modification was made in the forward primer for *H. contortus*, Haco Fd1 and a generic/conserved reverse primer, ITS2GR described by Bisset et al. (2013). Primer Custom Fd was moved 5 base positions behind the starting of the sequence of primer, Haco

Fd1 and primer ITS2 GR1 was moved 11 base positions behind the starting of the sequence of primer, ITS2GR1.

2.3 Results

2.3.1 Resources Available for 3 rDNA Spacers in NCBI

Among the three rDNA interspacial regions, GenBank contains the largest number of *Haemonchus* complete sequences for ITS2 compared to ITS1 and ETS (Fig. 2). As of June 1, 2018, there were 247, 40 and 9 complete ITS2 sequences of *H. contortus*, *H. placei* and *H. contortus* × *H. placei* hybrid respectively. In contrast, ITS1 has only 37 and 1 complete ITS1 sequences of *H. contortus* and *H. placei*. There are no complete ITS1 sequences for hybrids of *H. contortus* and *H. placei* deposited in the GenBank database. Also, only one complete ETS sequence exist for *H. contortus* thus far. Of all the spacer regions, ITS2 is the only spacer region, which has larger number of complete sequence entries in NCBI and the sequences reported include sequences for both *H. contortus* and *H. placei*, and their hybrids. However, total of 66, 324 and 2 sequences for ITS1, ITS2 and ETS sequences respectively covering *H. contortus*, *H. placei* and their hybrids were found in GenBank database. This provides confidence to have all the complete ITS2 *Haemonchus* sequences in GenBank database as standard sequences to compare our result sequences of ITS2 and validate our technique

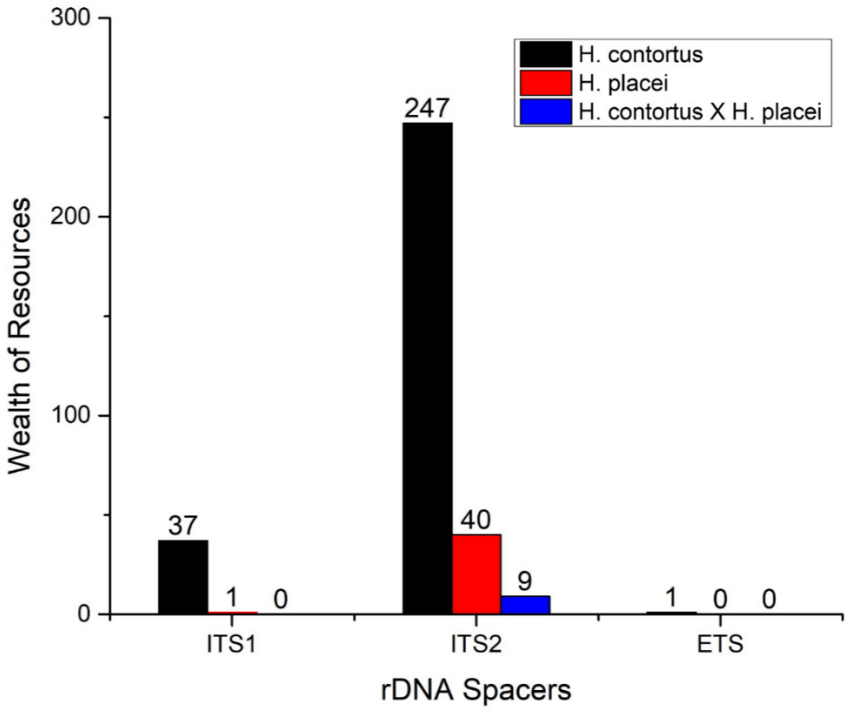

Fig.2 Demonstration of comparative wealth of resources in terms of complete sequences for three spacer regions in rDNA.

2.3.2 Analyses of *Haemonchus* ITS2 Sequences in NCBI

Apparently, there were not any variations within the typical 231BP ITS2 region for all the ITS2 sequences for *Haemonchus* listed in the GenBank database. The start and end of ITS2 was determined based on that defined by Bissett et al., (2013) in a *H. contortus* sequence outlining the 5.8S-ITS2-28S region of rDNA. The beginning of the ITS2 spacer for *Haemonchus* (AACCAT/AACAAT) is unique to other trichostrongyles like *Teladorsagia* and/ *Ostertagia* (AATGAA), *Cooperia* (AACGAT) and *Trichostrongylus* (AATTTC). So is the end of ITS2 for *Haemonchus*, ends on TAAT

which is slightly different from other trichostrongyles (Bisset et al., 2013). Among the 247 *H. contortus* ITS2 sequences listed in GenBank, 97.98% contained adenine in the 24th position, 99.6% contained guanine in the 205th position, and 99.6% contained adenine in the 219th position (Fig. 3). 4 sequence entries had a gap in the 24th position. All these four sequences came from a same study and all of their first 30 nucleotide base pairs were deviated from expected alignment for the species. Interestingly, one sequence matching the identity for *H. placei* was reported as *H. contortus* under an accession number KX829170.1 in the GenBank database. Similarly, among the 40 *H. placei* ITS2 sequences listed, 97.5% contained guanine in the 24th position, 100% contained adenine in the 205th position, and 99.6% contained guanine in the 219th position. 1 sequence reported as *H. placei* was heterozygous (A/G) for expected identity (G) at the 24th and 219th position. The consistency of the base pair substitutions for these two species at the three ITS2 positions (24, 205, 219) should provide a reliable basis for identification of these two species of *Haemonchus*. For the 9 hybrids listed in GenBank, all of them are heterozygous for all three nucleotide base substitutions confirmed by previous studies as having fixed difference between two species of *Haemonchus*, *H. contortus* and *H. placei* at positions; 24(A/G), 205 (G/A), 219(A/G).

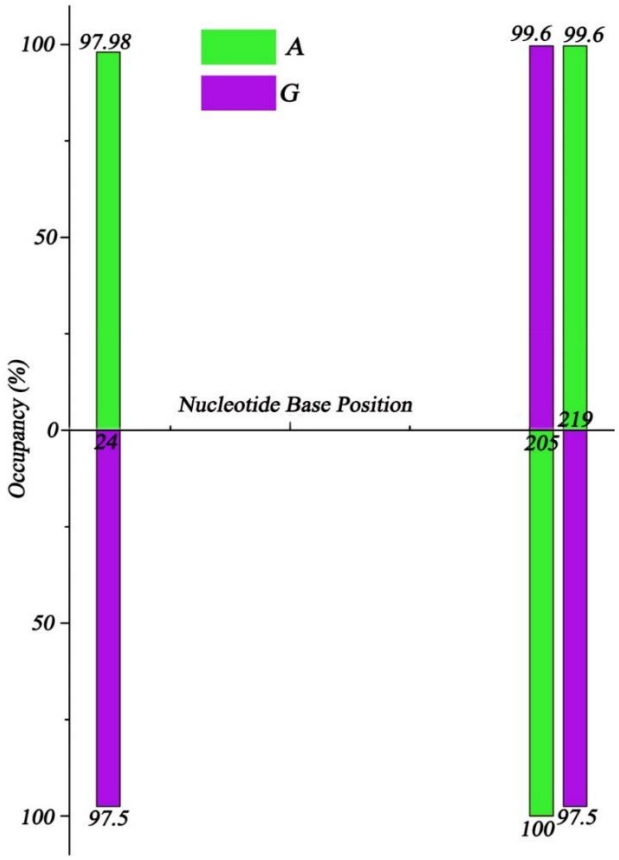

Fig. 3 Column chart showing consistence of SNPs at 25th, 204th and 219th base positions in all the sequences observed.

Using 288 complete ITS2 sequences of *H. contortus* and *H. placei* from GenBank database it was possible to create 11 categorized regions and base positions based on the level of variance of the nucleotides within the ITS2, conserved and variable region within it can be defined on a scale from 100% conserved to highly variable (Table 4). The level of variations in the ITS2 sequences among the various *Haemonchus* genotypes is represented by five different colors (Fig. 4). Region in the ITS2 with percentage variability greater than 30 is marked red and equal to or approximately 1, 0.28, and 0.15 is marked orange, yellow and lime green respectively. Region with no variability and conserved gene regions are marked lawn green and forest green.

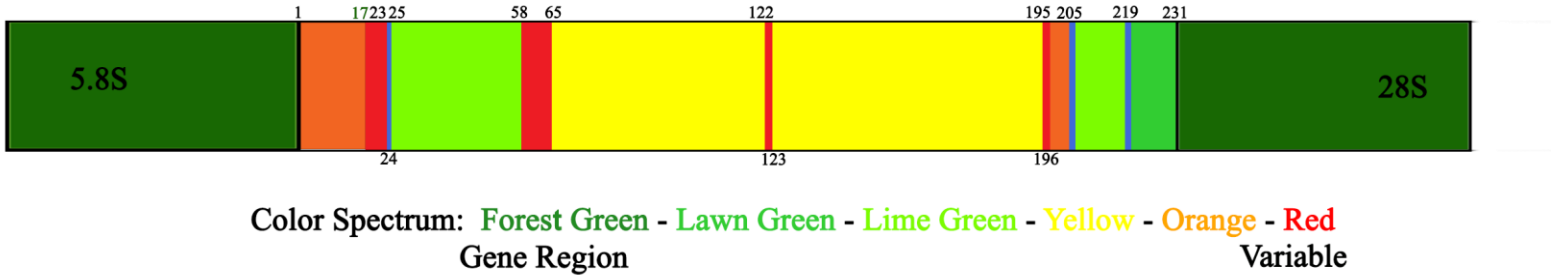

Fig. 4 ITS2 bar indicating variable- conserved regions in the ITS2 with color code

2.3.3 Confirmation of Identity of Genomic DNA

Anwar 3 was identified as positive for *Cooperia, H. contortus, T. axei, and T. colubriformis*. Anwar 6 and 8 were positive for only *H. contortus* (Fig. 5).

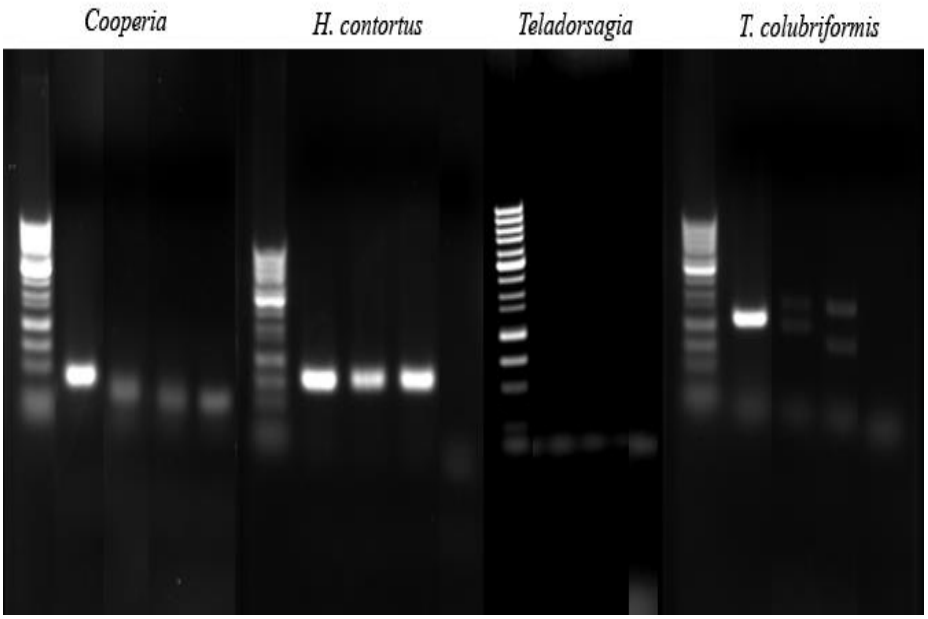

Fig. 5 Gel image showing confirmation of identity of genome of egg sample, Anwar 3,6 and 8 with Genus and Species-specific primers of common gastrointestinal nematodes in sheep for instance, *Cooperia, H. contortus, Teladorsagia, T. axei, T. colubriformis* and *T. colubriformis*.

2.3.4 Building a Standard ITS2 Sequence of *Haemonchus*

The three consensus sequences obtained from worm genomic DNA amplified using universal primers NC1-NC2 represented a complete sequence of ITS2 except one. It missed the first 34 nucleotide bases in the 5´end of the ITS2. Other than that, the remnant sequence had good quality values making it trustworthy. There are zero mismatches between the three sequences. Two of the three sequences had two same

mismatches with the reference sequence at base position 21(C/G) and 22 (C/T). As the third consensus sequence lacked the first 34 sequences in the 5´end of the ITS2, its rational why the third consensus sequence did not show any mismatches to the reference sequence. As these three sequences were identical, they were merged to create a single standard sequence against which all the product sequences can be mapped to build consensus sequences from respective pair of product sequences. The standard sequence is identified as complete ITS2 sequence of *H. contortus* on comparing it with established species.

2.3.5 Selection of Primers for ITS2 Sequencing

Initial five primer-pair combinations produced PCR products that were then sequenced resulting in quality values that ranged from 0 to 47. Zero is defined as extreme poor quality of the sequences obtained. Three primer pairs (Haco Fd1-ITS2 GR1, NC1-Harmon Rev and Harmon Fd-NC2) provided good quality values and no mismatches with the reference sequence in the first trial (Fig. 6).

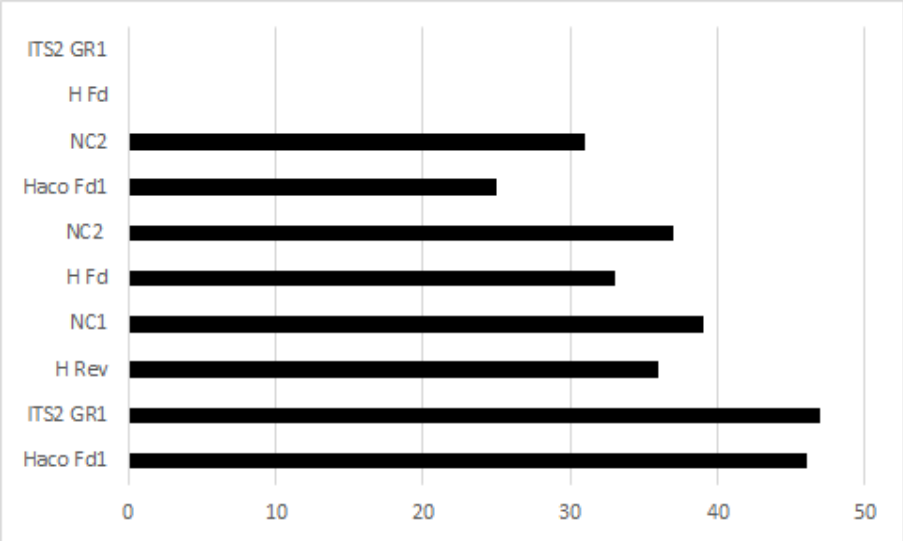

Fig. 6 Quality value assessment of 5 primer pairs on amplifying ITS2 of Worm genomic DNA (First Trial)

Three primer pairs with best results in the first trial were re-evaluated along with another set of custom developed forward primer with ITS2 GR1. Universal forward primer NC1 lies in the 3´end of 5.8S gene region and universal reverse primer ITS2 GR1 is in the 5´end of 28S gene region of rDNA (Fig. 7). Three primer sets; NC1-Harmon Rev, Custom Fd-ITS2 GR1, and Haco Fd1-ITS2 GR1 gave good confidence scores for nucleotide bases in the sequence obtained. Also, on statistical evaluation of the confidence scores given by the triplicates sequenced in two directions, the data for each of them are not significantly dispersed from their mean except for the sequences in a 5´-3´direction obtained using Harmon Fd-NC2 primer pair (Fig. 8). Though Haco Fd1-ITS2 GR1 has higher mismatches with the reference sequence than Harmon Fd-NC2 (Table 6), the former produced sequences with higher quality values than the latter (Fig. 8). This gives confidence to trust the nucleotide bases in their product sequences than that of latter primer pair. Also, the primer pair Harmon Fd-NC2 failed to consistently produce primer

products every time (Fig. 10). Quality values obtained from its product sequences are highly deviated from their mean value. This also makes Haco Fd1-ITS2 GR1 worth to trust than this primer pair. Primer set NC1-Harmon Rev gave the longest and Harmon Fd-NC2 the shortest consensus sequence among all the four primer sets. Primer NC1-Harmon Rev and Custom Fd-ITS2 GR1 gave consistent length of consensus sequence for triplicates performed (Fig. 9).

Thus, a primer-pair consisting of the forward universal primer (NC1) in the 5.8S subunit gene and a genus-specific reverse primer (Harmon Rev.) at the ITS2 3' end provided good quality values (QVs) over most of the ITS2 region except for the 3' end and this was consistent every time for the second trial to validate the efficiency and consistency of each primer sets. Two other primer sets (Haco Fd1- ITS2 GR1 and Custom Fd- ITS2 GR1) provide good QVs in the 3' half of ITS2.

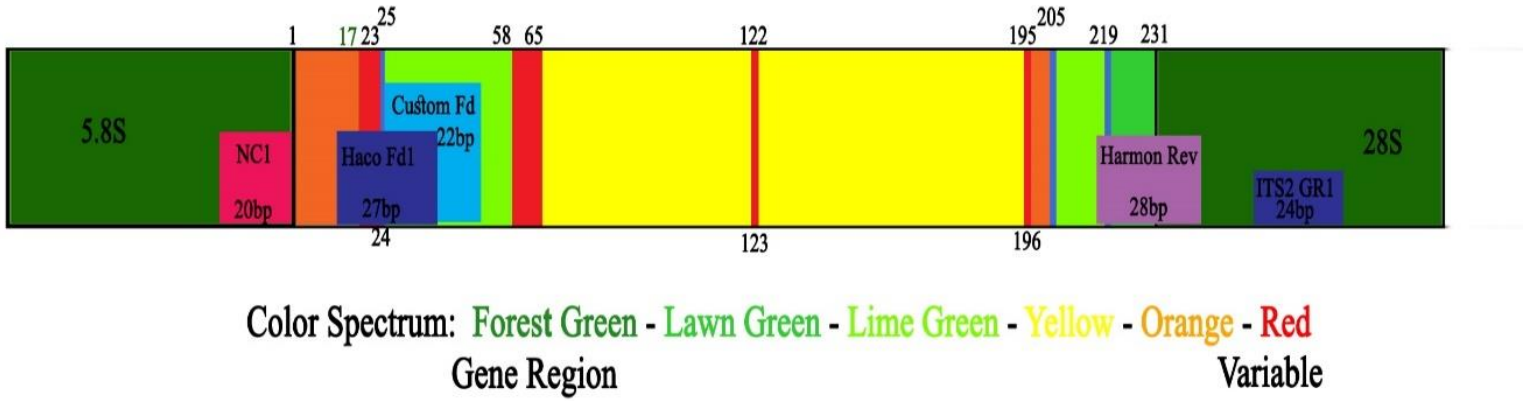

Fig. 7 ITS2 bar showing location of the selected primers

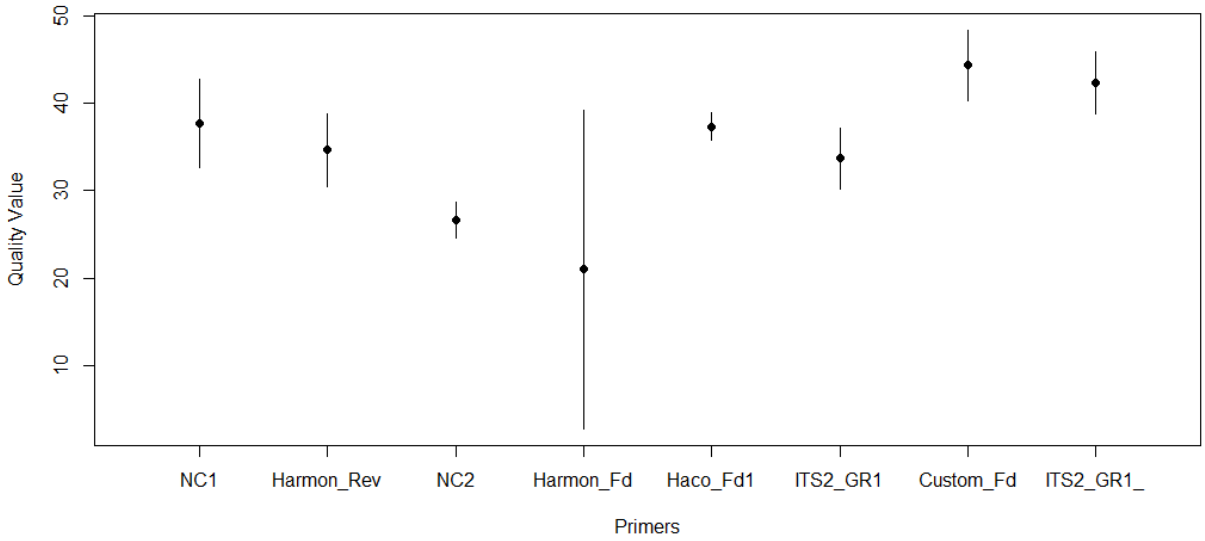

Fig.8 Mean and SD plot for quality values of worm sequences obtained with four primer-pairs showing within sample consistency.

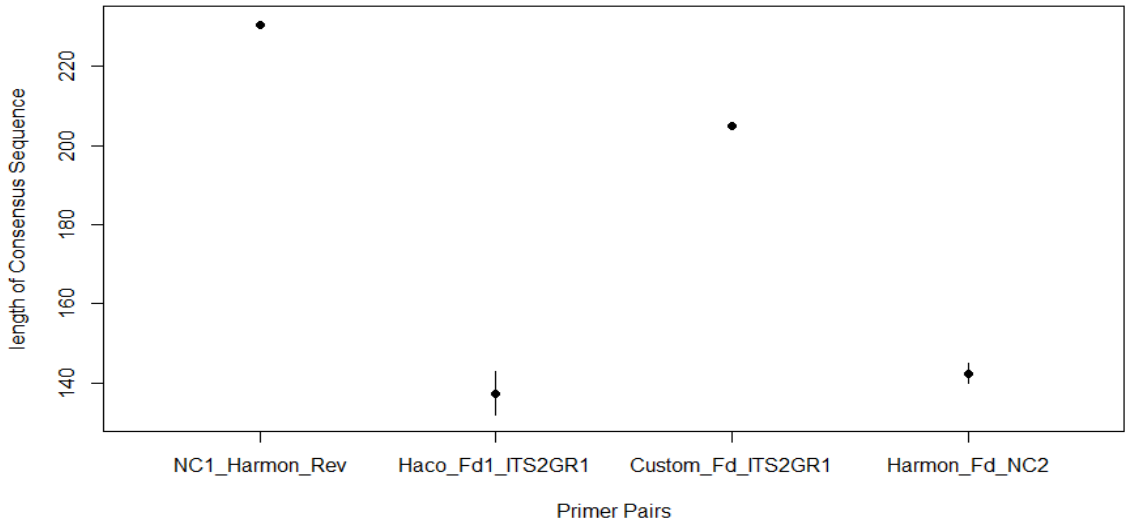

Fig.9 Mean and SD plot for length of consensus sequences of worm obtained from a set of primer with four primer-pairs showing within sample consistency.

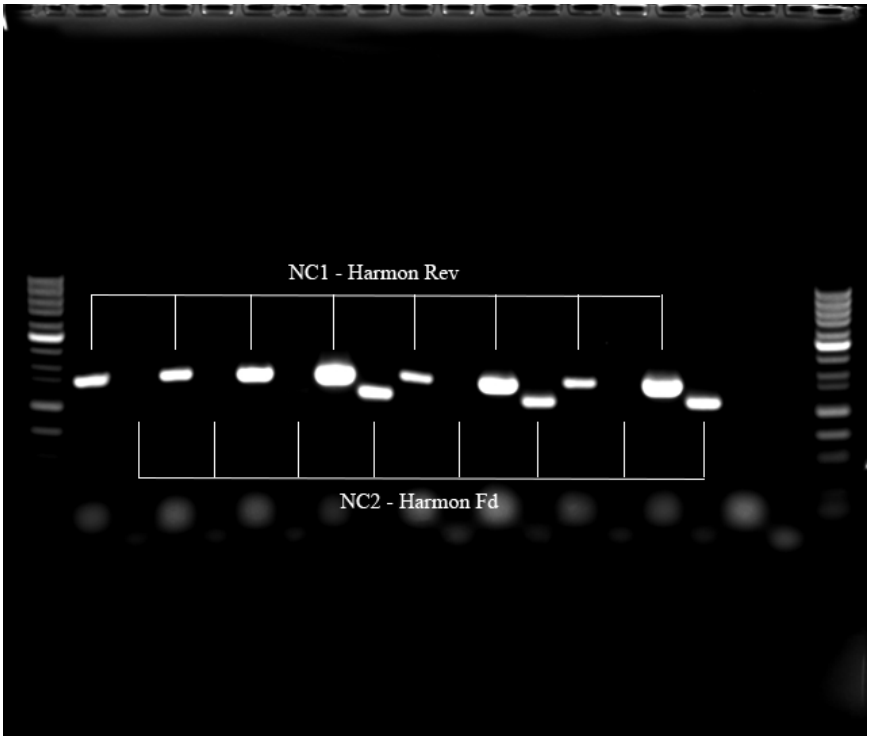

Fig. 10 Agarose gel image showing inconsistency of Harmon Fd-Nc2 primer pair to primer products for same sample being amplified by another primer pair, for instance, NC1-Harmon Rev.

Table 6 Mismatches among the triplicates from each primer pair and with the reference sequence

NC1 and Harmon Rev		
Mismatches Between the Triplicates	Mismatches with the Ref.	Location in the ITS2
2 (0.9%)	0	
	1 (0.4%)	18-23 bp
	1 (0.4%)	66-122 bp
Harmon Fd and NC2		
Mismatches between the triplicates	Mismatches with the Ref.	Location in ITS2
3 (1.3%)	0	
	2 (0.9%)	124-195, 196 bp
	1 (0.4%)	66-122 bp
Haco Fd1 and ITS2GR1		
Mismatches between the triplicates	Mismatches with the Ref.	Location in ITS2
4 (1.7%)	4 (1.7%)	66-122 bp
	5 (2.2%)	
	0	
Custom Fd and ITS2 GR1		
Mismatches between the triplicates	Mismatches with the Ref.	
0	0	
	0	
	0	

2.3.6 Specificity of Primers

When BLASTed on NCBI, the sequences of the three primers (Harmon Rev, Haco Fd1, and Custom Fd) gave highest alignment score for genus *Haemonchus* and *Uncinaria*. *Uncinaria* is supposed to be a small-intestinal nematode in dog. *Haemonchus* gave larger number of hits.

Table 7 Alignment score for search hits of the sequences of 3 genus-specific primers

Primer	Total Hits	Description	
		Taxonomy	Alignment Score
Harmon Rev	222	*H. contortus*	56
	24	*H. longistipes*	56
	1	*H. bedfordi*	56
	4	*H. contortus*×*H. placei*	52
	1	*Uncinaria* Sample 3	52
	1	*Uncinaria* Sample 4	52
Custom Fd	319	*H. contortus*	44.1
	47	*H. placei*	44.1
	1	*H. bedfordi*	44.1
	9	*H. contortus*×*H. placei*	44.1
	1	*H. placei placei*	44.1
	1	*H. placei africanus*	44.1
	1	*H. placei argentinensis*	44.1
	1	*Uncinaria* Sample 3	44.1

Primer	Total Hits	Description	
		Taxonomy	Alignment Score
	1	*Uncinaria* Sample 4	44.1
Haco Fd1	336	*H. contortus*	52
	46	*H. placei*	52
	1	*H. bedfordi*	52
	9	*H. contortus*×*H. placei*	52
	1	*H. placei placei*	52
	1	*H. placei africanus*	52
	1	*H. placei argentinensis*	52
	1	*Uncinaria* Sample 3	52
	1	*Uncinaria* Sample 4	52

2.3.7 Verification of PCR Amplification of DNA Isolated from Mixed Egg Samples by Evaluating Product Sequences

The three primer pairs (NC1 with Harmon Rev., Custom Fd with ITS2 GR1 and Haco Fd1 with ITS2 GR1) were then evaluated using DNA isolated from a mixed population of trichostrongyle eggs, and collectively they provide the data needed to construct the entire ITS2 region for the *Haemonchus* DNA in the eggs (Fig. 11-17). On determing consistency of quality values of sequences in both between triplicates of each sample and between all the samples tested, quality values of product sequences of ITS2 varied considerably, and were not statistically different among the six primer pairs. GR1

paired with Haco Fd1 seems to be more deviated from their mean value. Two very poor (QV=0) sequences from this primer-pair were responsible for this high variation.

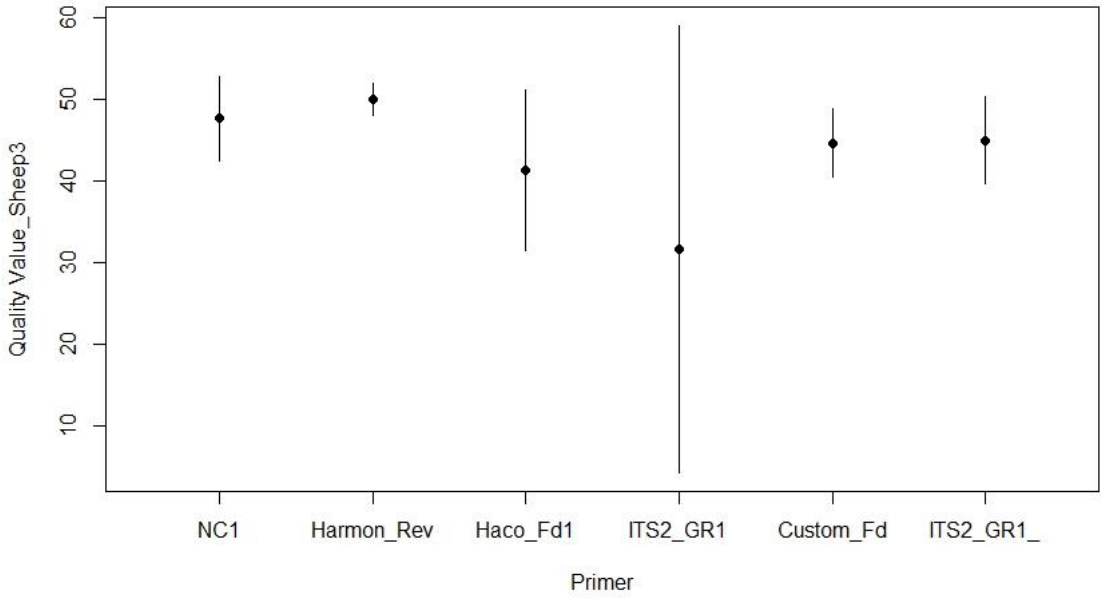

Fig. 11 Mean and SD plot for quality values of Sheep 3 sequences obtained with three primer-pairs showing within sample consistency. Quality values of two sequences (each running in forward and reverse direction) obtained from each primer pair for triplicates of sample, Sheep 3 were plotted to obtain the graph.

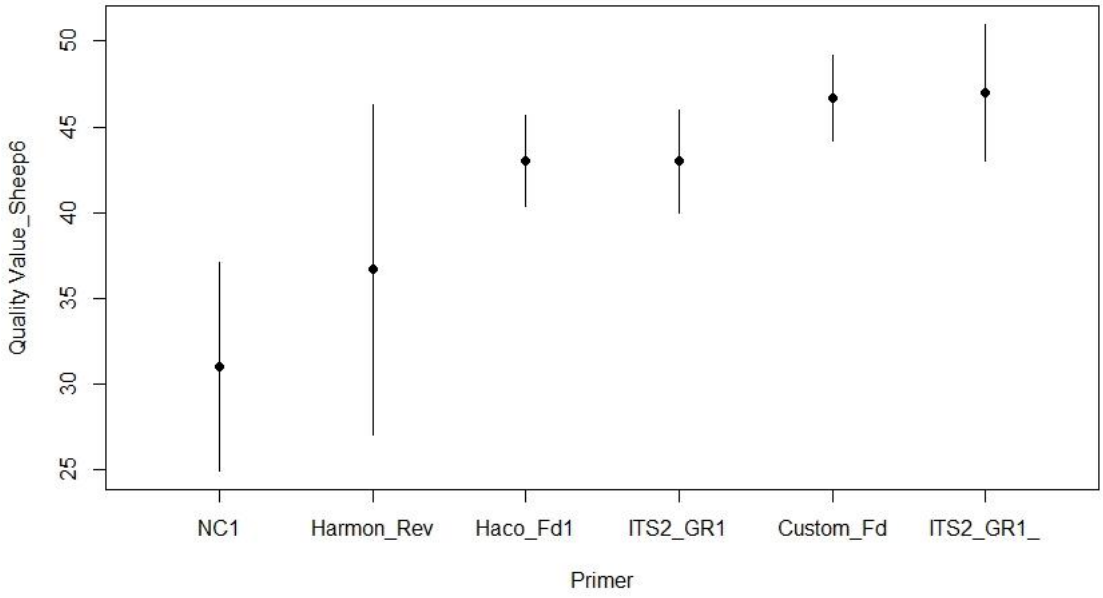

Fig. 12 Mean and SD plot for quality values of Sheep 6 sequences obtained with three primer-pairs showing within sample consistency. Quality values of two sequences (each running in forward and reverse direction) obtained from each primer pair for triplicates of sample, Sheep 6 were plotted to obtain the graph.

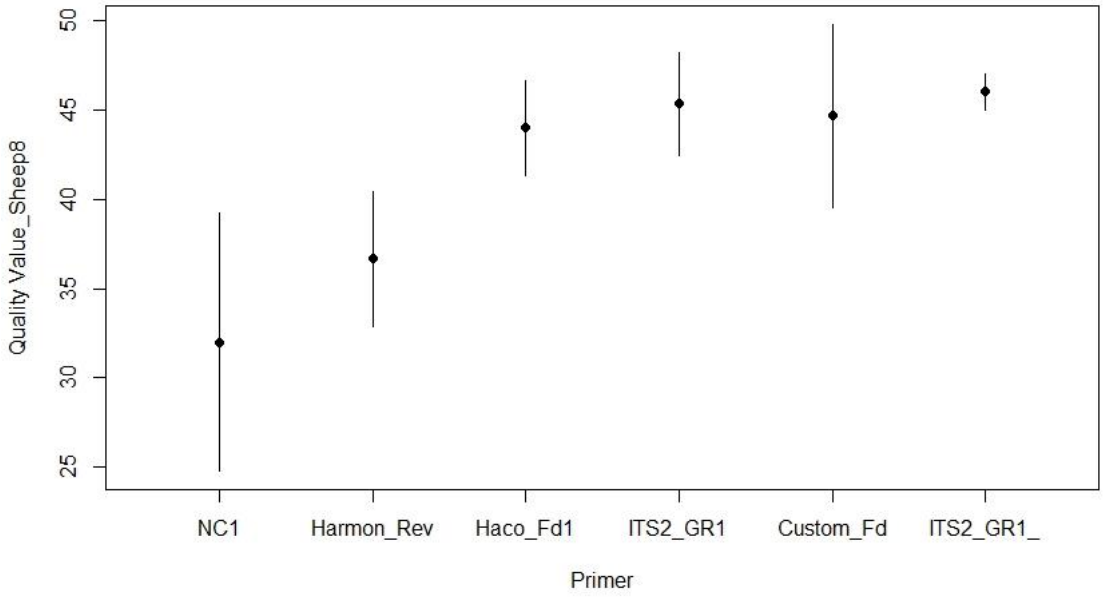

Fig. 13 Mean and SD plot for quality values of Sheep 8 sequences obtained with three primer-pairs showing within sample consistency. Quality values of two sequences (each running in forward and reverse direction) obtained from each primer pair for triplicates of sample, Sheep 8 were plotted to obtain the graph.

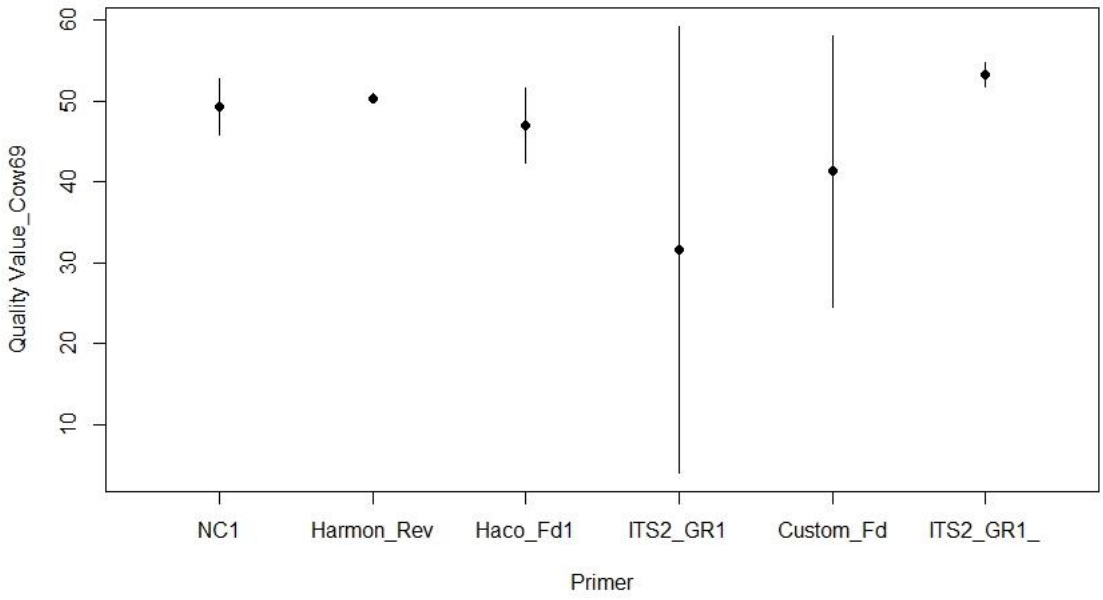

Fig. 14 Mean and SD plot for quality values of Cow 69 sequences obtained with three primer-pairs showing within sample consistency. Quality values of two sequences (each running in forward and reverse direction) obtained from each primer pair for triplicates of sample, Cow 69 were plotted to obtain the graph.

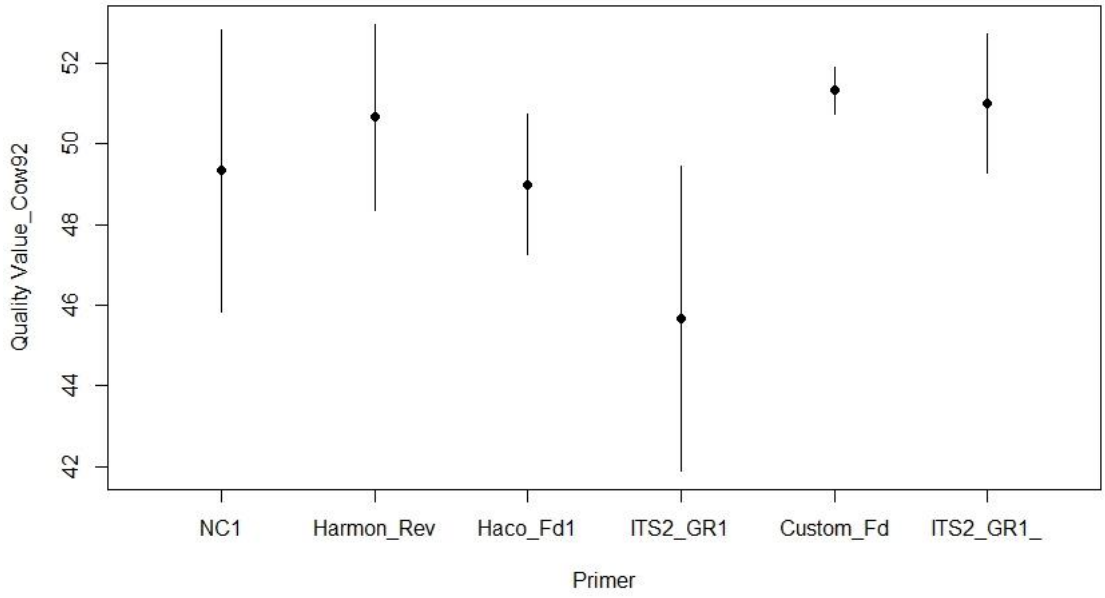

Fig. 15 Mean and SD plot for quality values of Cow 92 sequences obtained with three primer-pairs showing within sample consistency. Quality values of two sequences (each running in forward and reverse direction) obtained from each primer pair for triplicates of sample, Cow 92 were plotted to obtain the graph.

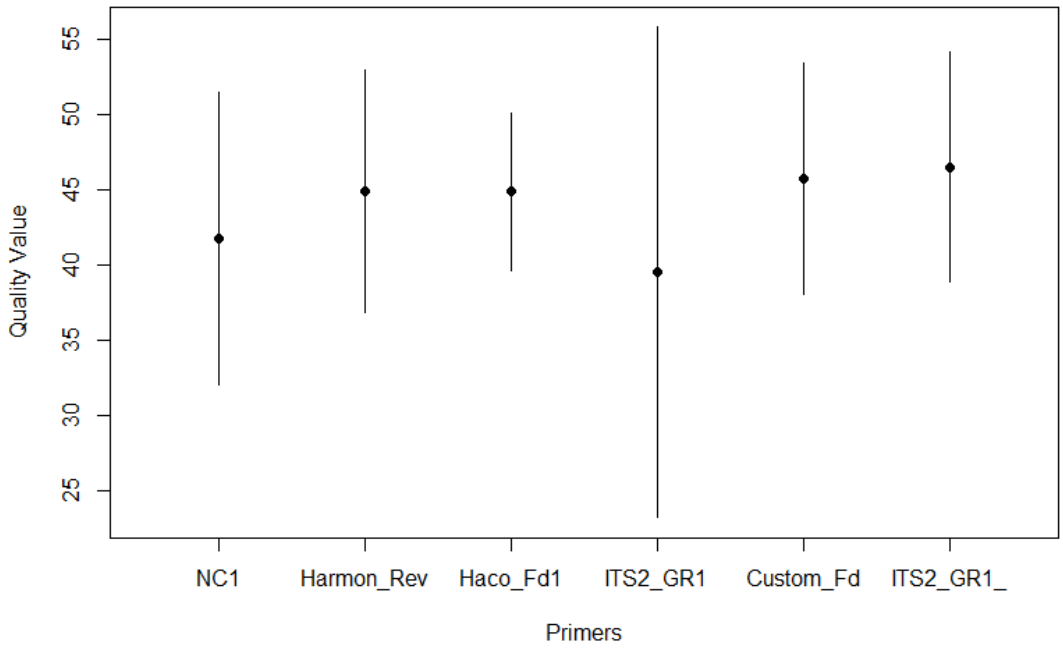

Fig. 16 Mean and SD plot for quality values of total sequences for all of the samples tested obtained with three primer-pairs showing between sample consistency. Quality values of two sequences (each running in forward and reverse direction) obtained from each primer pair for triplicates of all the samples were plotted to obtain the graph.

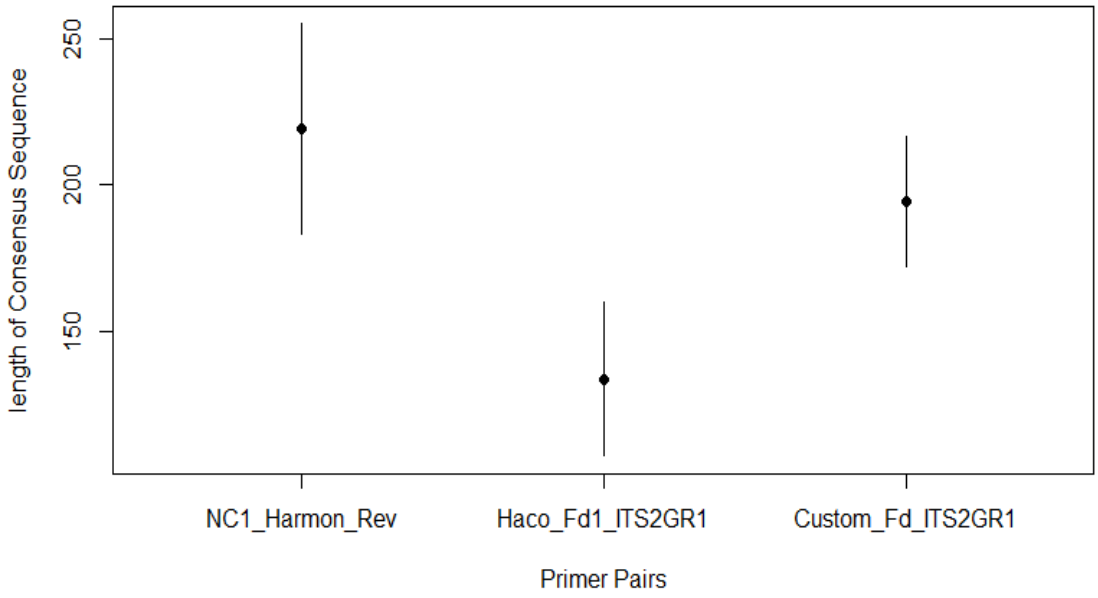

Fig. 17 Mean and SD plot for length of consensus sequences produced by three primer pairs showing the ability of the primer pairs to produce a consistent length of consensus sequence every time they were used. Lengths of all the consensus sequences of all sample obtained by using individual primer pairs were plotted to obtain this graph.

2.3.8 Identification of Species of *Haemonchus*

For species identification, it is only the data from the three species-specific SNPs that need to be considered. Using the consensus sequence data from the PCR amplified products resulting from the selected two primer pairs (NC1-Harmon Rev with Custom Fd-ITS2 GR1 and/ Haco Fd1- ITS2 GR1) on egg DNA isolated two cattle samples could be used to identify them as being infected with *H. placei* based on data from all three SNPs. This identification is consistent among the triplicates of both samples, and both

samples from a cattle herd represent the same genotype based upon the complete alignmnet of their sequences. The three samples from sheep flock, two samples are identified as *H. contortus* and they share a same genotype with the standard reference sequence from worm. One sample Sheep 3 shows heterozygous A/G and G/A genotype at the SNP, rDNA ITS2 base postion, 24 and 205 respectively. This might be due to the presence of *H. contortus/H. placei* hybrids. This sample relates to the genotype identified for two samples from cattle herds. One replicate of sample Cow 69 contradicts with its identification as *H. placei* in the third SNP because of missing data towards the 3´end of ITS2 (Fig. 19).

```
                                1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
#NC1-NC2_{Standard_Ref}         AACCATATAC TACAATGTGG GTAATTTCAA CATTGTTTGT CAAATGGCAT TTGTCTTTTA
#COW_69_III_P1P3_{Cow_69}       .......... .......... C..G...... .......... .......... ..........
#COW_69_I_P1P3_{Cow_69}         .......... .......... C..G...... .......... .......... ..........
#COW_69_II_P1P3_{Cow_69}        .......... .......... C..G...... .......... .......... ..........
#COW_69_III_P1P2_{Cow_69}       .......... .......... C..G...... .......... .......... ..........
#COW_69_I_P1P2_{Cow_69}         .......... .......... C..G...... .......... .......... ..........
#COW_69_II_P1P2_{Cow_69}        .......... .......... C..G...... .......... .......... ..........
#COW_92_III_P1P3_{Cow_92}       .......... .......... C..G...... .......... .......... ..........
#COW_92_II_P1P3_{Cow_92}        .......... .......... C..G...... .......... .......... ..........
#COW_92_I_P1P3_{Cow_92}         .......... .......... C..G...... .......... .......... ..........
#COW_92_III_P1P2_{Cow_92}       .......... .......... C..G...... .......... .......... ..........
#COW_92_II_P1P2_{Cow_92}        .......... .......... C..G...... .......... .......... ..........
#COW_92_I_P1P2_{Cow_92}         .......... .......... C..G...... .......... .......... ..........
#SHEEP_8_II_P1P3_{Sheep_8}      .......... .......... .......... .......... .......... ..........
#SHEEP_8_III_P1P3_{Sheep_8}     ---------- ---------- ---------- ---------- ---------- ----------
#SHEEP_8_I_P1P3_{Sheep_8}       .......... .......... .......... .......... .......... ..........
#SHEEP_8_III_P1P2_{Sheep_8}     ---------- ---------- ---------- ---------- ---------- ----------
#SHEEP_8_II_P1P2_{Sheep_8}      .......... .......... .......... .......... .......... ..........
#SHEEP_8_I_P1P2_{Sheep_8}       .......... .......... .......... .......... .......... ..........
#SHEEP_6_III_P1P2_{Sheep_6}     ---------- ---------- ------.... .......... .......... ..........
#SHEEP_6_I_P1P2_{Sheep_6}       .......... .......... .......... .......... .......... ..........
#SHEEP_6_II_P1P2_{Sheep_6}      .......... .......... .......... .......... .......... ..........
#SHEEP_6_I_P1P3_{Sheep_6}       .......... .......... .......... .......... .......... ..........
#SHEEP_6_II_P1P3_{Sheep_6}      .......... .......... .......... .......... .......... ..........
#SHEEP_6_III_P1P3_{Sheep_6}     ---------- ---------- -----..... .......... .......... ..........
#SHEEP_3_II_P1P2_{Sheep_3}      .......... .......... C..G...... .......... .......... ..........
#SHEEP_3_I_P1P2_{Sheep_3}       .......... .......... C..G...... .......... .......... ..........
#SHEEP_3_III_P1P3_{Sheep_3}     .......... .......... C..G...... .......... .......... ..........
#SHEEP_3_II_P1P3_{Sheep_3}      .......... .......... C..G...... .......... .......... ..........
#SHEEP_3_III_P1P2_{Sheep_3}     .......... .......... C..G...... .......... .......... ..........
#SHEEP_3_I_P1P3_{Sheep_3}       .......... .......... C..G...... .......... .......... ..........
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                                1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
#NC1-NC2_{Standard_Ref}         GACAATTCCC ATTTCAGTTC AAGAACATAT ACATGCAACG TGATGTTATG AAATTGTAAC
#COW_69_III_P1P3_{Cow_69}       ....T..... .......... .......... .......... .......... ..........
#COW_69_I_P1P3_{Cow_69}         ....T..... .......... .......... .......... .......... ..........
#COW_69_II_P1P3_{Cow_69}        ....T..... .......... .......... .......... .......... ..........
#COW_69_III_P1P2_{Cow_69}       ....T..... .......... .......... .......... .......... ..........
#COW_69_I_P1P2_{Cow_69}         ....T..... .......... .......... .......... .......... ..........
#COW_69_II_P1P2_{Cow_69}        ....T..... .......... .......... .......... .......... ..........
#COW_92_III_P1P3_{Cow_92}       ....T..... .......... .......... .......... .......... ..........
#COW_92_II_P1P3_{Cow_92}        ....T..... .......... .......... .......... .......... ..........
#COW_92_I_P1P3_{Cow_92}         ....T..... .......... .......... .......... .......... ..........
#COW_92_III_P1P2_{Cow_92}       ....T..... .......... .......... .......... .......... ..........
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#COW_92_I_P1P2_{Cow_92}         ....T..... .......... .......... .......... .......... ..........
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#SHEEP_8_III_P1P3_{Sheep_8}     ---------- ---------- ---------- ----T..... .......... ..........
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#SHEEP_8_II_P1P2_{Sheep_8}      .......... .......... .......... .......... .......... ..........
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#SHEEP_3_I_P1P2_{Sheep_3}       .......... .......... .......... .......... .......... ..........
#SHEEP_3_III_P1P3_{Sheep_3}     ....T..... .......... .......... .......... .......... ..........
#SHEEP_3_II_P1P3_{Sheep_3}      ....T..... .......... .......... .......... .......... ..........
#SHEEP_3_III_P1P2_{Sheep_3}     ....T..... .......... .......... .......... .......... ..........
#SHEEP_3_I_P1P3_{Sheep_3}       .......... .......... .......... .......... .......... ..........
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                                1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
#NC1-NC2_{Standard_Ref}         ATTCCTGAAT GATATGAACA TGTTGCCACT ATTTGAGTGT ACTCAGCGAA TATTGAGATT
#COW_69_III_P1P3_{Cow_69}       ..C....... .......... .......... .......... .......... ..........
#COW_69_I_P1P3_{Cow_69}         ..C....... .......... .......... .......... .......... ..........
#COW_69_II_P1P3_{Cow_69}        ..C....... .......... .......... .......... .......... ..........
#COW_69_III_P1P2_{Cow_69}       ..C....... .......... .......... .......... .......... ..........
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#COW_92_II_P1P3_{Cow_92}        ..C....... .......... .......... .......... .......... ..........
#COW_92_I_P1P3_{Cow_92}         ..C....... .......... .......... .......... .......... ..........
#COW_92_III_P1P2_{Cow_92}       ..C....... .......... .......... .......... .......... ..........
#COW_92_II_P1P2_{Cow_92}        ..C....... .......... .......... .......... .......... ..........
#COW_92_I_P1P2_{Cow_92}         ..C....... .......... .......... .......... .......... ..........
#SHEEP_8_II_P1P3_{Sheep_8}      .......... .......... .......... .......... .......... ..........
#SHEEP_8_III_P1P3_{Sheep_8}     .......... .......... .......... .......... .......... ..........
#SHEEP_8_I_P1P3_{Sheep_8}       .......... .......... .......... .......... .......... ..........
#SHEEP_8_III_P1P2_{Sheep_8}     .......... .......... .......... .......... .......... ..........
#SHEEP_8_II_P1P2_{Sheep_8}      .......... .......... .......... .......... .......... ..........
#SHEEP_8_I_P1P2_{Sheep_8}       .......... .......... .......... .......... .......... ..........
#SHEEP_6_III_P1P2_{Sheep_6}     .......... .......... .......... .......... .......... ..........
#SHEEP_6_I_P1P2_{Sheep_6}       .......... .......... .......... .......... .......... ..........
#SHEEP_6_II_P1P2_{Sheep_6}      .......... .......... .......... .......... .......... ..........
#SHEEP_6_I_P1P3_{Sheep_6}       .......... .......... .......... .......... .......... ..........
#SHEEP_6_II_P1P3_{Sheep_6}      .......... .......... .......... .......... .......... ..........
#SHEEP_6_III_P1P3_{Sheep_6}     .......... .......... .......... .......... .......... ..........
#SHEEP_3_II_P1P2_{Sheep_3}      ..C....... .......... .......... .......... .......... ..........
#SHEEP_3_I_P1P2_{Sheep_3}       ..C....... .......... .......... .......... .......... ..........
#SHEEP_3_III_P1P3_{Sheep_3}     ..C....... .......... .......... .......... .......... ..........
#SHEEP_3_II_P1P3_{Sheep_3}      ..C....... .......... .......... .......... .......... ..........
#SHEEP_3_III_P1P2_{Sheep_3}     ..C....... .......... .......... .......... .......... ..........
#SHEEP_3_I_P1P3_{Sheep_3}       ..C....... .......... .......... .......... .......... ..........
```

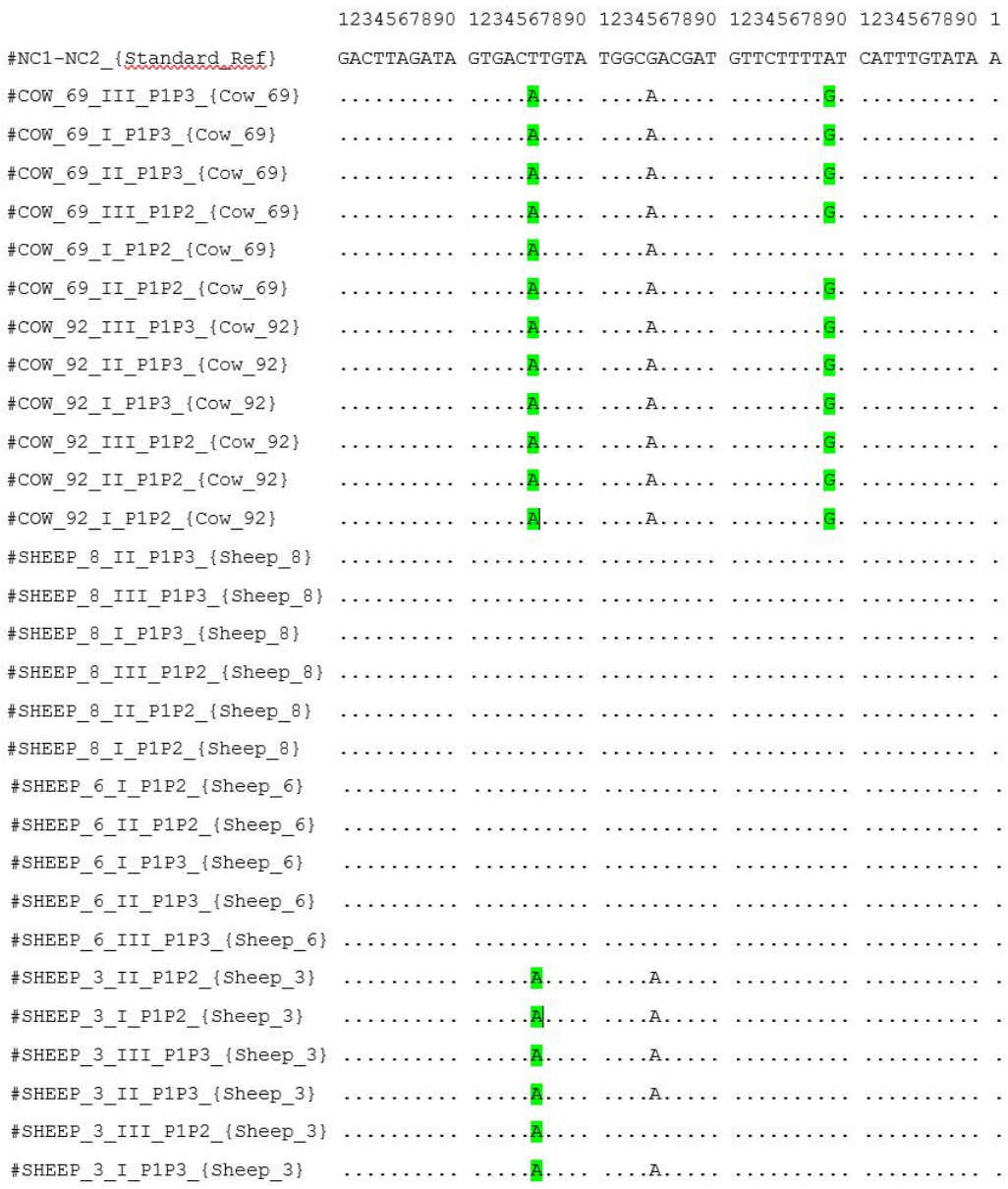

Fig. 18 Complete consensus sequences of ITS2 obtained from amplification of egg DNA samples with the selected two primer pairs (NC1-Harmon Rev with Custom Fd-ITS2 GR1 and/ Haco Fd1- ITS2 GR1) using 2 *H. placei* samples (Lieferman 69 and 92) and 3 *H. contortus* samples (Anwar 3, 6 and 8). The three SNPs at 24th ,205th and 219th base positions, markers to differnetiate *H. contortus* from *H. placei* are highlighted.

2.4 Discussion

Several PCR based studies have described approaches to diagnose the species of *Haemonchus* utilizing differences in the ITS2 region that exists among the different genera and species of nematodes (Chaudhary et al., 2015; Gasser, 1999; Miriam et al., 1999; Stevenson et al., 1995). None of these studies used DNA from mixed genera and species of trichostrongyle eggs that are typically obtained in ruminant fecal samples as they attempted to differentiate *H. contortus* and *H. placei* based on the three SNPs in ITS2 that exists between these two species. Until our study, no one has used multiple sets of genus-specific primer coupled with universal primers to generate PCR products that could be sequenced to identify the species of *Haemonchus*. At least two studies have developed diagnostic approaches to identify the genera of trichostrongyle eggs in fecal samples using a primer pair consisting of one universal primer located in the rDNA subunit and a genus-specific primer in the ITS2 region (Bisset et al., 2014; Bott et al., 2009). Yet, no one has attempted to sequence these products to further determine the species and genotype of these eggs.

Our study describes a technique that employs two *Haemonchus*-specific primers, each located on opposite ends of the ITS2 region of rDNA, that are paired to two universal nematode primers in the rDNA gene located on the opposite ends of ITS2 from its paired genus-specific primer. These two primer pairs generate two PCR products that collectively span the entire ITS2 region so that when these products are sequenced it is possible to determine the entire *Haemonchus* ITS2 sequence of eggs isolated from a given fecal samples. With this information, it is possible to determine the species and genotype of *Haemonchus* infecting that animal.

The universal primers, NC1 and NC2 used as standard way to genotype *Haemonchus* present in the selected herd in South Dakota was as suggested by Gasser et al., (1993) and it has been efficiently used to study the genetic variability of *Haemonchus* (Bandid et al., 2014; Yin et al., 2013). Another universal primer tested in this study, ITS2 GR1 was a slight modification of generic reverse primer (ITS2GR) as designed by Bisset et al., (2013). It was moved 11 base positions behind the starting of the sequence of primer, ITS2GR.

The *Haemonchus*-specific forward primers tested in this study were Haco Fd1 and Custom Fd. Haco Fd1 is as designed by Bisset et al., (2013) and Custom Fd was slightly modified. Primer Custom Fd was moved 5 base positions behind the starting of the sequence of primer, Haco Fd1 to include a more conserved region; this was determined from analyses of sequences available on the NCBI website. The *Haemonchus*-specific reverse primer tested in this study was as described by von Samson-Himmelstjerna et al., (2002). Though there were several other published *Haemonchus*-specific primers available we did not try each of them due to time constraint. Also, we did not want to select any species-specific primers lying on the variable regions identified upon analyzing 288 complete ITS2 sequences of *H. contortus* and *H. placei* from the GenBank database.

Examination of level of variance in the ITS2 sequences among different individuals is vital to make the primer choices to amplify the ITS2 interspacial region. The primers need to be highly specific and sensitive for genus *Haemonchus* to be able to selectively amplify the ITS2 of only *Haemonchus* species. As ITS2 lies between two conserved areas (5.8S and 28S gene) in the rDNA, they are supposed to be somewhat

conserved but obviously they are not 100% conserved and there is some level of variance among different individuals. (Gasser et al., 2008) concluded that the magnitude of variation of both the ITS1 and ITS2 sequences within a species is considerably less (generally, <1.5%) than between the species based on the findings of several studies done on variability of ITS sequences within and among species of different genera of trichostrongyles of livestock. Since the Harmon Rev. primer contains the 3rd SNPs for differentiating *H. contortus* from *H. placei*, the specificity of this primer for *H. contortus* is supposed to be logically true. The sequences for the Haco Fd1 primer is in a region of the ITS2 that is variable for some genotypes located globally. The Custom Fd primer binds to sequences in a more stable portion of the ITS2 and will likely provide more reliable results throughout the globe. Also, the Custom Fd primer with ITS2 GR1 gives comparatively a longer length of consensus sequence than that of Haco Fd1- ITS2 GR1, larger region of sequences is overlapped, providing higher confidence for the consensus sequence being developed. Since, we are interested only in the last 30 nucleotide bases of the 3' end which includes 2 SNPs for differentiating the two species of *Haemonchus*, we chose to use both the primers to sequence the 3´end of the ITS2. The Custom Fd- ITS2 GR1 primer pair was selected despite the sequences obtained from NC2- Harmon Fd primer pair had less mismatches than those from it, because of the inconsistent quality of sequences obtained from the former pair.

Not any non-specific PCR amplification was evident with these three primer sets. Also, the sequences of these primers correctly annealed to the target sequence of ITS2 for genus *Haemonchus* and there was no any evidence of PCR inhibition with these primers. This holds true to the observation made by Zhu & Clark, (1996) as discussed by

(Zarlenga et al., 2001) that the presence of competing primers (in this case, universal and genus-specific primers), the specificity of primer annealing/ amplification is improved.

Our findings suggest that it is possible to generate the complete sequence of ITS2 for *Haemonchus* with decent quality values even when *Haemonchus* DNA is contaminated by DNA from other trichostrongyle species if the proper two primer pairs are used for amplifying the DNA. Therefore, it should be possible to use this PCR-sequencing approach to identify the species and genotype of *Haemonchus* eggs isolated from all livestock fecal samples.

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Chapter 3

Application of the Primer Sets to Identify the Genotypes of *Haemonchus*

3.1 Introduction

The two species of *Haemonchus, H. contortus* and *H. placei* are the two globally distributed and most economically important species of *Haemonchus* (Hoberg et al., 2004). They feed on blood capillaries in the abomasum, making the animals anemic and emaciated. Haemonchosis is recognized as one of the most serious diseases in small ruminants leading to mass mortality (Hildreth & Harmon, 2013; Amarante, 2011). Most of the farm management practices focus majorly on the treatment and prophylaxis measures to control haemonchosis.

Identification of the genotypes and estimations of genetic diversity are very important to understand the disease dynamics of any infectious agent; transmission potential, host adaptation, pathogenicity, environmental survival, resistance to host immune system and drugs which holds true for *Haemonchus* as well (Yin et al., 2013; Jacquiet et al., 1995). Better understanding of genetic diversity of *Haemonchus* spp will help improve the efficacy of control strategies for infected ruminants (Bandid et al., 2014; Yin et al., 2013; Gasser et al., 2008).

Ribosomal DNA spacer regions do not code for any protein, so their sequences are highly variable compared to coding regions of DNA, and evolve at a comparatively faster rate. This property of spacer regions makes them suitable for phylogenetic studies at lower categorical levels such as genera, species and populations (Hwang & Kim,

1999). This also makes them diagnostically important. The second internal transcribed spacer region carries enough information to differentiate two species of *Haemonchus, H. contortus* and *H. placei* (Chaudhry et al., 2015; Stevenson et al., 1995). Thus, it can be used to identify the genotypes of the nematodes as well, based on nucleotide variations obtained in the resultant sequences. Several studies has been conducted to examine genetic variation of the species of *Haemonchus* in different geographical region throughout the world using different markers in mitochondrial DNA (Yin et al., 2016; Yin et al., 2013) and ribosomal DNA (Bandid et al., 2014; Yin et al., 2013). Though the ITS2 region of rDNA has already proved its usefulness for identifying the genotypes of *Haemonchus* species, it has not been yet possible with the egg DNA isolated from fecal samples containing trichostrongyle eggs from multiple species.

We aim to study the genetic variability of the species of *Haemonchus* that we identified with our diagnostic approach as described in the first chapter. This method will use two sets of primers to amplify the ITS2 region of the ribosomal DNA of *Haemonchus* by means of conventional PCR. The primer sets are validated as capable of generating PCR products that collectively span the entire ITS2 region. Each set includes a genus-specific primer towards one end of the ITS2 region and a universal primer located in the ribosomal gene extending past the opposite end of the ITS2 region. Collectively, PCR products from the 2 sets will span the entire ITS2 region. The sequences obtained from within population and between population will be looked upon for examining the degree of sequence variations within and between populations, reporting a new or already identified genotype. These sequences will be used in a phylogenic analysis.

3.2 Materials and Methods

3.2.1 Sources of Samples

3.2.1.1 Adult worm

Adult worms were collected from an ewe that died of severe anemia from a South Dakotan sheep flock that was known to be infected with H. *contortus* and exhibiting symptoms of haemonchosis. The worms were preserved in 70% ethanol until future use.

3.2.1.2 Eggs

Trichostrongyle eggs (including *Haemonchus* spp., *Teladorsagia* spp. and *Trichostrongylus* spp.) were harvested from feces collected from the same commercial flock of sheep from which the adult worms were taken. Two fecal samples were also collected from a commercial cattle herd known to be infected with *Haemonchus spp*. Both sheep flock and cattle herd are from South Dakota. Appropriate 3 g fecal samples were taken and the Wisconsin sucrose flotation method (Cox & Todd, 1962) as modified by Harmon et al., (2007) was used to harvest the eggs. This technique uses sucrose (specific gravity 1.26) and centrifugation to separate the eggs from the fecal pellet. Following egg extraction, the eggs were counted under an Olympus BX53 microscope (at 100X total magnification). The eggs were then washed from the coverslip with distilled water and stored at -20˚C until further analysis.

3.2.2 Extraction of Genomic DNA

3.2.2.1 Adult worm

A single adult worm was selected and suspended in distilled water in a microcentrifuge tube. Genomic DNA was extracted from it using the Qiagen DNeasy

Plant Mini DNA extraction kit (Valencia, CA, USA) as per manufacturer's protocol and stored at -20°C until further use.

3.2.2.2 Eggs

Genomic DNA was extracted from the stored five egg samples using the Qiagen DNeasy Plant Mini DNA extraction kit (Valencia, CA, USA) as per manufacturer's protocol and stored at -20°C until further use.

3.2.3 Generation of PCR Products

3.2.3.1 Experimental Design

Worm genomic DNA was amplified in triplicates at first using universal primers, NC1 and NC2. NC1 and NC2 is a forward and reverse universal primer and NC2 is a reverse universal primer, respectively. Then after five egg DNA samples were amplified using three different primer pairs as described in chapter two. A total of three replicates were tested for each sample. Among those five egg samples, three samples came from three different sheep from same flock and two egg samples from two cows from a herd. Each experiment included *H. contortus* DNA either from the adult worm as a positive control to ensure that the primer sets were specifically amplifying *Haemonchus*.

3.2.3.2 Primers Used

Three sets of primers, NC1-Harmon Reverse, Custom Fd-ITS2 GR1 and Haco Fd1-ITS2 GR1 are used to amplify the ITS2 region of rDNA for *Haemonchus* species (Table 7). Each set has a unique genus-specific primer paired with a universal primer running in the forward and reverse directions.

3.2.3.3 PCR Amplification

PCR amplifications of the ITS2 using individual primer sets were performed using a 25 µL reaction mixture containing 12.5 µL GoTaq® Hot Start Green Master Mix, 2X (Cat. #M5123 from Promega, Inc. consisting of 50 U/mL Taq DNA Polymerase, 400 µL dATP, dGTP, dCTP, dTTP, and 4 mM $MgCl_2$, yellow and blue dye, 7 µL nuclease-free H_2O, 3 µL of respective primer (forward and reverse concentration was 0.2 µM), and 2.5 µL genomic DNA. Thermocycling parameters for the amplification were 1 cycle of initialization at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min with a single cycle of final elongation at 72°C for 7 min.

Table 7. Universal and spesies-specific primers used for identifying *Haemonchus*

Universal Primers 5' to 3′	Genus-specific Primers 5′ to 3′
NC1* ACGTCTGGTTCAGGGTTGTT	**Harmon Fd**** CATATACATGCAACGTGATGTTATGAA
NC2* TTAGTTTCTTTTCCTCCGCT	**Harmon Rev.***** GCTCAGGTTGCATTATACAAATGATAAA
ITS2 GR1**** CTTTTCCTCCGCTAAATGATATGC	**HacoFd1******** CTTTTCCTCCGCTAAATGATATGC
	Custom Fd******* TCAACATTGTTTGTCAAATGGC

*According to Gasser et al. (1993)

** According to Harmon et al. (2007)

*** According to von Samson-Himmelstjerna et al. (2002)

**** According to Bisset et al. (2013)

***** Custom developed

Harmon et al., (2002) made slight modification in the forward primers for *H. contortus* and *C. oncophora* described by von Samson-Himmelstjerna et al (2002) (Grosz et al., 2013). Slight modification was made in the forward primer for *H. contortus*, Haco Fd1 and a generic/conserved reverse primer, ITS2GR described by Bisset et al. (2013). Primer Custom Fd was moved 5 base positions behind the starting of the sequence of primer, Haco Fd1 and primer ITS2 GR1 was moved 11 base positions behind the starting of the sequence of primer, ITS2GR.

All PCR products were resolved in agarose gels (2%) stained with ethidium bromide in TE buffer for 90 min at 125 V to verify that they represented single bands of accurate molecular weight. A ladder (50bp Mini DNA Ladder, Cat. #BP2570100 from Fisher Scientific International, Inc.) was used to confirm the size/ molecular weight of the PCR amplified products separated by agarose gel electrophoresis. Bands were visualized, and each image was captured by Odyssey® Fc gel imaging system using image studio software. Bands of appropriate molecular weight were then excised, and PCR products were extracted and purified using Gel/PCR DNA Fragment Extraction Kit (MIDSCI™ Cat. No. IB47020) as per manufacturers protocol. Purified PCR products were sequenced on both strands using Sanger sequencing with the appropriate forward and reverse primers in a separate reaction by GenScript (Piscataway, NJ).

3.2.4 Building Consensus Sequences from the Product Sequences

The product sequence obtained from universal forward and reverse primer (NC1-NC2) for worm sample was nBLASTed in NCBI to look for existing matches in the GenBank database. This sequence was then used as a standard reference sequence to trim and edit all the sequences that would be obtained following the technique described in Chapter 2. ITS2 sequences were determined using each primer-pairs; NC1-Harmon Rev, Custom Fd-ITS2 GR1, and Haco Fd1-ITS2 GR1. Each experiment was run in triplicates. Hence, adult worm DNA generated 3 sequences from each primer pair. Similarly, ITS2 sequences for each egg samples; cow 69, cow 92, sheep 3, sheep 6 and sheep 8 using each of the three primer pairs were generated as described.

A consensus sequence was built from two sets of primer pairs to obtain a whole length of ITS2 using the DNA Baser sequence assembly software (DNA Baser Assembler v4, 2013) which was also used to analyze chromatogram of the sequences obtained and to trim off sequences that had less than 60% good bases in a window of 20 bases. A base must have a quality value above 20 to be considered a good/trusted base. Base caller embedded in the program was also enabled to double check chromatograms obtained from the Genscript by recomputing the confidence score for bases (Quality Values/QVs) and recalling the undetermined bases in a chromatogram. This also allows to correct ambiguities while building consensus sequence from forward and reverse sequences. A standard reference sequence (described earlier) was used as reference in the program to build all the consensus sequences. For this, sequences from NC1-Harmon Rev and Custom Fd-ITS2 GR1, and NC1-Harmon Rev and Haco Fd1-ITS2 GR1 were merged together for all the samples tested.

Hence, each egg sample and a worm DNA sample had a total of 6 consensus sequences from both sets of primer pairs (3 replicates with each primer pair), and these were evaluated as a single group to determine the ability of this approach to sequence whole length ITS2 sequences (231bp) from mixed egg and a worm samples in a consistent fashion. The mean distance of all consensus sequences within a sample group were calculated in MEGA 6.0 to dtermine whether all the consensus sequences within that group completely align with each other.

3.2.5 Comparing Consensus Sequences with Established Genotypes

All the sequences obtained from worm DNA as well as 5 egg DNA samples were BLASTed in NCBI to compare them with other sequences lodged in the GenBank database. A Maximum likelihood method was used to create a phylogenetic tree based on the Tamura -Nei model in MEGA 7.0. Reliability of the tree was estimated by setting the number of Bootstrap replicates to 1000 (Kumar et al., 2016).

3.3 Results

3.3.1 Identity of Standard Sequence on Blasting

On blasting the product sequence obtained from the universal primer amplification of the sheep adult *Haemonchus* genomic DNA (standard reference sequence) in NCBI, the reference sequence is 100 % identical to 3 sequences listed under accession numbers; JX869066.1, KU558755.1, KY3057801.1, which represent *H. contortus* ITS2 sequences. These sequences were reported from Czech Republic, China and Bangladesh, respectively. This South Dakota sequence does not completely align with any of the many sequences previously reported from the USA. Twenty-eight ITS2

sequences for *H. contortus* and fifteen *H. placei* ITS2 sequences has been reported from the USA in the GenBank database so far. When all the search hits for this reference sequence were built in a tree in NCBI, it showed to be particularly different from a sequence reported for *H. contortus* isolate listed under accession number MF398448.

3.3.2 Evaluation of Nucleotide Variation of the Product Sequences within a Sample Group

ITS2 sequences were determined using each set of primer- pairs from the adult *H. contortus* DNA and DNA from each of the five mixed egg samples. These were evaluated in triplicate, therefore, generating six sequences for each sample. On performing manual comparison of these sequences with each other to determine the number of mismatches within each sample, the adult worm and two of the samples (one from the cow and one from the sheep) showed no mismatches (i.e. 100% identity) among all six sequences (Table 2). All the sequences within these three samples completely align with each other. For the remaining three samples, sequences for one of the three replicates did not match the other two. Among the 36 sequences of 231 base-pairs sequences (8,316 total pair-pairs), only 6 mismatches occurred at four different base positions. The mismatch for the Cow 69 sample consisted of one nucleotide at BP219 that did not match with its other 5 replicates. This resulted in a 99.93% identity for this sample (Table 8). All the other sequences for Cow 69 was homozygous to sequences generated for Cow 92. This mismatched sequence was assumed to be incorrect, and therefore, not considered when comparing the sequences with GenBank database. There was a single mismatch among the six sequences obtained for sheep 8, making the sequences for that sample, 99.93% identical. For Sheep 3, there were 4 mismatches in

two base positions, making the sequences for that sample 99.71% identical. Among the six consensus sequences obtained for sample, Sheep 3, three of those obtained from both primer combinations contradicts with rest of the three replicates in base position 65 (A/T) (Table 8). Since the base estimated for this position (T), had a quality value zero, estimation of this base substitution could not be trusted. Also, the estimation of this base position for one of the replicate was based on only the sequences obtained from one set of primer (NC1-Harmon Rev), as ITS2 GR1 generated an extremely poor-quality sequence. For Sheep 8, only one sequence out of six sequences obtained, had one nucleotide mismatch (G/T) and this sequence was not considered when comparing the sequences with GenBank database because this was the first base (BP 95) in the consensus sequence generated by aligning sequences obtained from two sets of primers, NC1-Harmon Rev and Haco Fd1-ITS2 GR1. Practically, the first 25 nucleotide bases or so in the sequences obtained after sequencing are unreliable (Swanson, 2014).

On comparing all 6 sample groups tested, the nucleotide diversity between the sequences obtained for each group was ranging from 0.000 to 0.030 (Table 9). Sequences for Sheep 6 completely align with the sequences for the sheep adult *Haemonchus* worm and the standard reference sequence generated from two universal (NC1 and NC2) primers. Replicates of two samples, Sheep 8 and Cow 92 showed higher variation in nucleotide composition from each other compared to all replicates of remaining samples (Table 8).

Table 8 Percent identify among the six paired triplicates for each sample

Sample Name	Identity (%)	Mismatches among triplicates (BP & Numbers)	Mismatch with Ref. (BP & Numbers)
Worm	100.00	0	0
Cow 69	99.93	1 (219)	7 (21, 24, 65, 123, 196, 205, 219)
Cow 92	100.00	0	7 (21, 24, 65, 123, 196, 205, 219)
Sheep 3	99.71	3 (65), 1 (205)	5 (21, 24, 123, 196, 205)
Sheep 6	100.00	0	0
Sheep 8	99.93	1 (95)	0

Table 9 Mean distance between 6 sample groups; Worm, Cow 69, Cow 92, Sheep 3, Sheep, 6 and Sheep8

	1	2	3	4	5	6	7
1. Standard Ref		0.000	0.013	0.013	0.001	0.000	0.011
2. Worm	0.000		0.013	0.013	0.001	0.000	0.011
3. Cow 69	0.028	0.028		0.001	0.013	0.013	0.006
4. Cow 92	0.029	0.029	0.001		0.013	0.013	0.007
5. Sheep 8	0.001	0.001	0.029	0.030		0.001	0.011
6. Sheep 6	0.000	0.000	0.028	0.029	0.001		0.011
7. Sheep 3	0.021	0.021	0.007	0.009	0.022	0.021	

3.3.3 Comparison of Genotype with the NCBI Database

Since all the ITS2 sequences obtained from adult worm, sheep 6, and cow 92 were identical among all the resultant six sequences from these samples (Fig. 19), they were each BLASTed in NCBI to compare them with other sequences lodged in GenBank database. Six NCBI sequences described as *H. placei* showed 100% identity to our Cow 92 ITS2 sequence; KU891908.1, KU891907.1, KU891906.1, KC503916.1, AM410068.1, AM410068.1, AJ577466.1, KP688064.1, and JN128896.1. The first three are sequences from Nigerian isolates, Uzbekistan, Burkina Faso (*H. placei africanus*), France, South Africa. the same was true for Cow 69 since the sequences for Cow 69 were identical to Cow 92 (Fig. 19). Only one sequence out of six sequences obtained, had one nucleotide mismatch (BP 219) and this sequence was not considered when comparing the sequences with GenBank database because of missing data towards the 3´end of ITS2.

Though the sequence obtained for Sheep 3 was 99% identical to sequence (X78812.1) identified by Stevenson et al., (1995) as *H. contortus*, not any of the sequences in the GenBank were 100% identical to our sequence. However, three *H. contortus* sequences completely align with our sequence for Sheep 6 (KY305780.1, KU558755.1, and JX869066.1). They represent sequences from China, Bangladesh and

Czech Republic, respectively. The same was true for Sheep 8 since the sequences for Sheep 8 were identical to Sheep 6 (Fig. 19). Only one sequence out of six sequences obtained, had one nucleotide mismatch (G/T) and this sequence was not considered when comparing the sequences with GenBank database for the reasons as described above.

Based on nucleotide diversity of ITS2 sequences obtained from 5 egg DNA samples,\ and a worm DNA (Fig. 19), 3 distinct genotypes have been identified. Two of them are already reported and one is described for the first time by us.

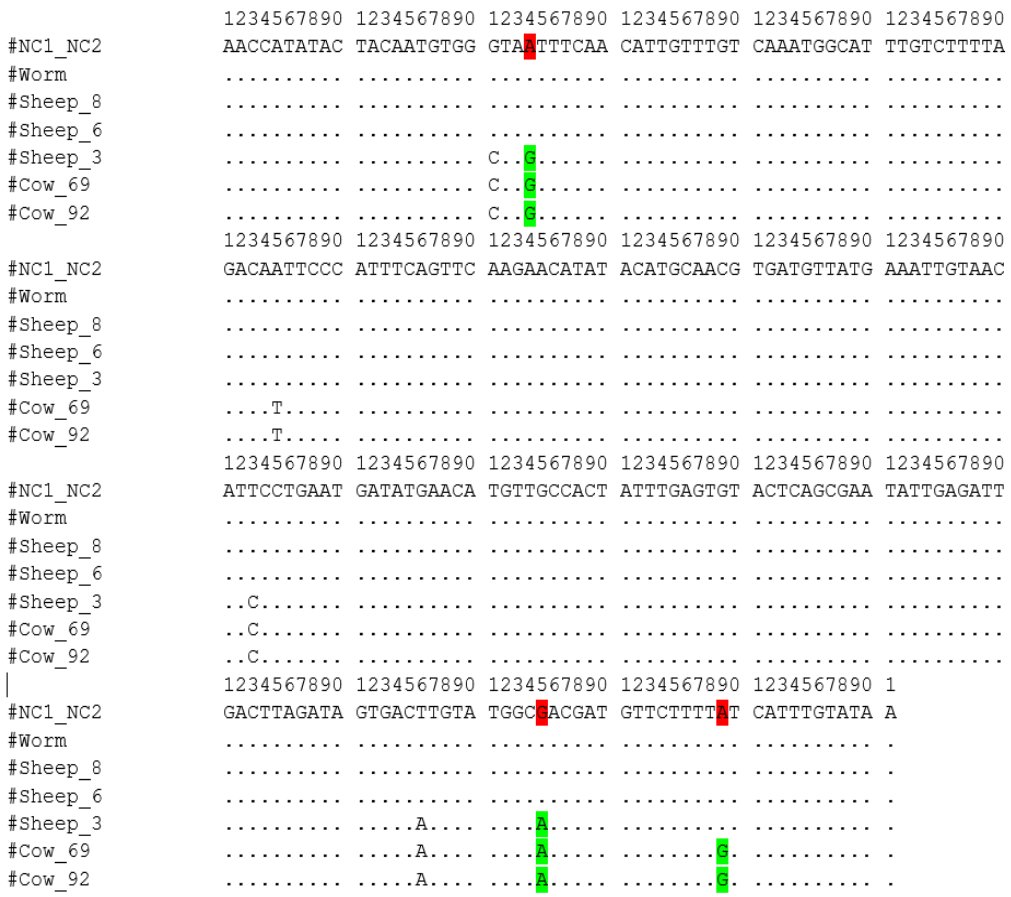

Fig. 19 Nucleotide diversity of all the samples revealed by an ITS2 sequence from each sample

3.3.4 Construction of a Phylogeny Tree

A maximum likelihood tree was constructed with our sequences and standard *H. contortus* accession number X78803) and *H. placei* (accession number X78812) ITS2 sequence identified by Stevenson et al., (1995) to understand the phylogenic relationship of our sequences in relation to two species of *Haemonchus*. Altogether they are divided into two major clads one representing *H. contortus* and another, *H. placei*. Though the Sheep 3 sample branches out from the *H. placei* clad it sits close to *H. contortus* compared to other sheep samples (Fig. 20). Since, it represents a hybrid in terms of the three SNPs that differentiates *H. contortus* from *H. placei* , its location in the tree seems legitimate.

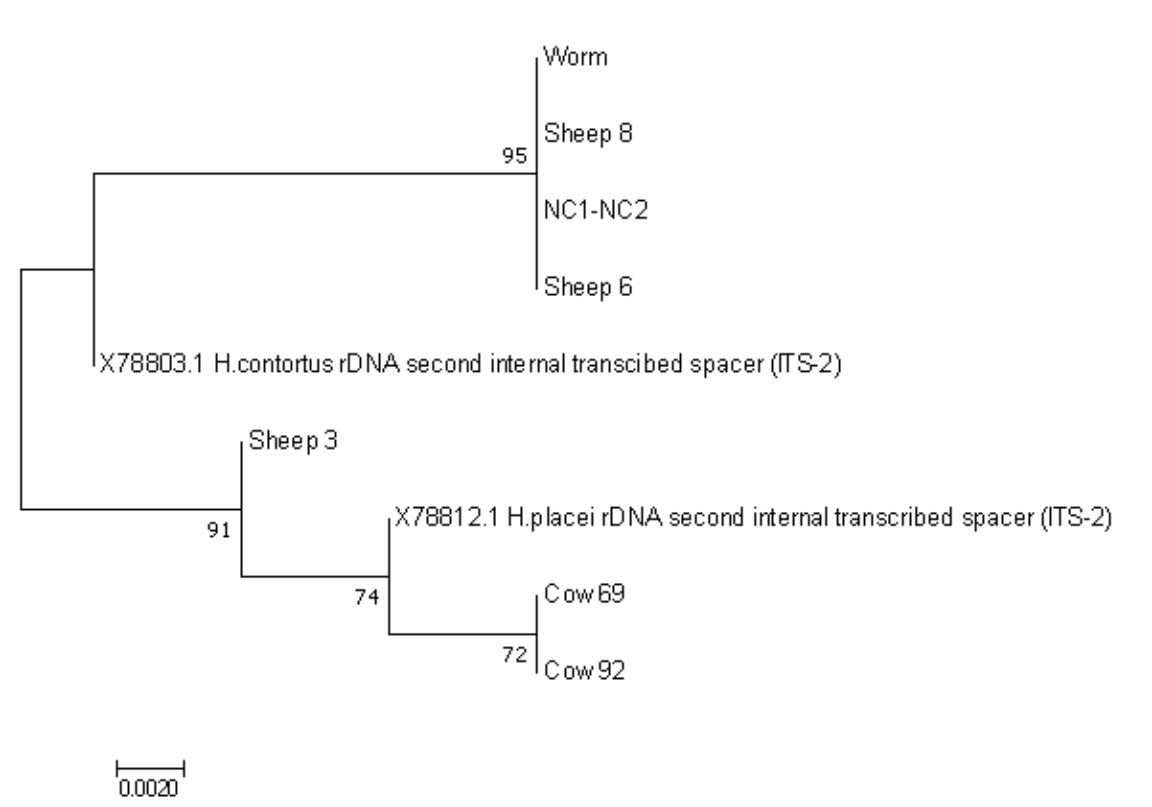

Fig. 20 Maximum likelihood tree showing phylogenic relationship of our sequences in relation to two species of *Haemonchus*.

Seven substitutions at base positions 21, 24, 65, 123, 196, 205, and 219 were identified which included 3 transversions (1: G<->C, 2: A<->T) and 4 transitions (3: A<->G, 1: T<->C).

3.4 Discussion

Genotyping is a method of identifying genetic variation in the region of target sequences within a population of organisms. Many studies have focused on genotyping *Haemonchus* populations obtained for the same target sequence in adult worm specimens collected from different hosts to evaluate the level of genetic diversity among specimens from the same type of host and from different hosts. For instance, 21 genotypes have been identified based on the analysis of ITS2 sequences obtained from 158 *H. contortus* worm samples collected from small ruminants located in 11 provinces in Thailand. This study used the universal forward and reverse primers, NC1 and NC2 to produce the amplification products. Similar study in China characterized 18 genotypes of *H. contortus* with 2.6% sequence variation among all the 152 ITS2 sequences from 152 adult specimens of *H. contortus* collected from seven geographical locations in tropical to subtropical climate zones in the country. Bandid et al., (2014), listed one isotype/genotype/clone based upon the diagnostic stage/ egg stage of *H. contortus* in NCBI under accession number KJ188206. However, the source of this information seems unknown.

We have established that it is possible to correctly speciate and genotype *Haemonchus* eggs isolated from fecal samples containing mixed populations of

trichostrongyle nematodes and used this technique to generate one set of genotypes from a sheep and a cattle sample. The analysis of 7 ITS2 sequences obtained from worm and egg samples in our study revealed a mean nucleotide variation of 0.016%. Further analysis revealed nucleotide diversity of 1.5% between samples identified as *H. placei* and *H. contortus*. Not any intraspecific variation was seen. This is contradicting to the magnitude of variation detected in sequences of *H. contortus* populations reported by different countries till date. Previous studies have reported 2. 6% nucleotide diversity in *H. contortus* populations in countries like in China, Germany, Sweden/Kenya 5.4% and 5.2% was detected in Iran and in Australia, France, Germany, New Zealand, Switzerland, The Netherlands and the UK, respectively (Bandid et al., 2014; Meshgi et al., 2015; Yin et al., 2013) It could be because we had very few samples and all the samples identified as one species comes from a single herd. Yin et al., (2013) studied 152 worm specimens of *H. contortus* from 7 geographical location in China. Similarly, Bandid et al., (2014) used 158 adult worms from 11 provinces in Thailand. Samples identified as *H. contortus* comes from a sheep herd and those identified as *H. placei* from a cattle herd which rationalizes the host specific infection nature of *Haemonchus* species. Future studies are needed to look for the existing interspecies and intraspecies variation for *H. contortus* and *H. placei*. More herds from different geographical regions in the USA need to be studied to ensure the wider applicability of this technique to identify genotypes irrespective of geographical and climatic variations. Also, this technique should be applied to identify species and genotypes of *Haemonchus* infecting several other hosts like bison, giraffe, etc. Garretson et al., (2009) identified anthelminthic resistant *H. contortus* from giraffe in Florida. Since the phylogenetic analysis revealed three genotypes based on nucleotide

diversity of the sequences, the technique can be applied to decent number of samples from different herds to investigate genotypes present in each herd.

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Chapter 4

Conclusion

Morphological methods are still considered gold standard technique for the identification of parasite species. Since, eggs are typically the diagnostic stage of most gastrointestinal nematode's infection, the use of morphological characteristics of eggs to identify them would be of great value clinically. Reliable morphological differences have been reported to exist among adults and larvae of various parasites, but similar morphological identification techniques are generally not possible to distinguish trichostrongyles beyond the level of family. For all the genera except *Nematodirus*, it is not possible to visually differentiate the eggs even to the genus level.

Development of Molecular DNA techniques, particularly the genus/species-specific PCR techniques, have proven useful and reliable for the identification/differentiation of different genera and species of trichostrongyles from egg samples. The spacer regions between the ribosomal DNA genes have served as useful molecular markers for genus (and occasionally species) level identification of trichostrongyle nematodes such as *Haemonchus* (Amarante et al., 2017; Brasil et al., 2012; Stevenson et al., 1995; Zarlenga et al., 1994). Out of the many species of *Haemonchus*, *H. contortus* and *H. placei* are the two species which are globally distributed and have serious impacts on the economy of livestock industries. They feed

on blood capillaries and their infection is recognized as one of the most serious diseases in small ruminants leading to mass mortality (Hildreth & Harmon, 2013; Amarante, 2011). A better understanding of genetic diversity and identification of *Haemonchus* spp is required to understand its infection dynamics and works towards improving the efficacy of control strategies for their infected hosts (Bandid et al., 2014; Gasser et al., 2008; Yin et al., 2013). There is a wealth of sequence information available for the second internal transcribed spacer (ITS 2) region in the NCBI website to identify sequence variations among the different *Haemonchus* genotypes. Also, there are only 3 fixed single-nucleotide differences between *H. placei* and *H. contortus* in the ITS2 region and this has been successfully used to differentiate these species (Brasil et al., 2012; Stevenson et al., 1995). Sequencing this region from *Haemonchus*-specific PCR products was anticipated to help us identify both its identity relative to species and genotypes.

Hence, we targeted this region to design a PCR technique to identify the species and genotypes of *Haemonchus* from livestock fecal samples containing mixtures of trichostrongyle nematode eggs. Two *Haemonchus*-specific primers, each located on opposite ends of the ITS2 region of rDNA were paired to two universal nematode primers in the rDNA gene located on the opposite ends of ITS2 from its paired genus-specific primer. Six primer pairs, each with a pair of genus-specific and universal ITS2 primers were first evaluated to identify two primer pairs that will generate two PCR products that collectively span the entire ITS2 region; so that when these products are sequenced it is possible to determine the entire *Haemonchus* ITS2 sequence of eggs isolated from a given fecal sample. Out of these six, three primer pairs were selected for further study based on their consistent ability to generate PCR products for sequencing, good quality

values within the resultant sequences and fewer mismatches among triplicate sequences both from worm DNA and DNA isolated from a mixed population of trichostrongyle eggs.

From three primer pairs, one primer set was eliminated for final consideration because it was in a region of ITS2 that varied in some regions of the world. Therefore, two primer pairs (NC1-Harmon Rev with Custom Fd-ITS2 GR1 and/ Haco Fd1- ITS2 GR1) were selected to build a consensus sequences that could be used to test the proposed proof of concept for this approach to diagnosing *Haemonchus* species. One of the pairs includes a published primer for the genus *Haemonchus*, but the other was similar to another of the publish primers but moved several base-pairs to a more conserved region of the ITS2. Consensus sequences generated from both sets of primer pairs for a worm DNA and 5 egg DNA samples were able to identify the species of *Haemonchus* based on the three invariable fixed SNPs between *H. contortus* and *H. placei.* Two cattle egg DNA samples were identified as *H. placei* and the adult worm DNA, two of the sheep samples were identified as containing *H. contortus*, but results from a third sample suggests that it might be infected with a hybrid of the two species. The identification was consistent among the triplicates of all the samples, except a few. These exceptions could not be trusted because of their quality value and location in the sequence generated. According to Swanson (2014), the first 25 nucleotide bases or so in the sequences obtained after sequencing are unreliable.

Hence, we were able to show that this PCR technique involving two *Haemonchus*-specific primers is reliable for generating ITS2 sequences to identify the two species of *Haemonchus, H. placei* and *H. contortus,* and their hybrids. Further

studies must be carried out to confirm its reliability to identify species of *Haemonchus* in a wide variety of samples. Also, there are many more primers designed to amplify the ITS2 region of *Haemonchus* by previous studies. All of them could not be tested due to time constraint. Further studies can be done to see the usefulness of those primers in generating *Haemonchus* ITS2 sequences. On constructing a phylogeny tree using product sequences from each sample (total six sequences), three genotypes were revealed based on nucleotide diversity of the sequences. This suggests that the technique can be applied to large number of samples from different herds to investigate genotypes present in each herd.

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