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Molecular Method to Diagnosis of some Strongylide Nematode of goats in Nyala Area South Darfur State- Sudan

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Abstract: *A total of 100 positive faecal samples were examined using floatation technique to detect strongylide eggs, were confirmed by PCR technique, faecal samples were storage by frozen in -20°C and DNA extraction by using phenol/chloroform/isoamylalcohol protocol. PCR leading to amplify the region of the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA (r DNA) of strongylide species, amplification by three different primers in separate reactions, in each reaction used species specific primer forward and a universal reverse primer to srongylide species NC2. Haemonchus contortus, Trichostrongylus spp and Oesophagostomum columbianum prevalence rates were recorded as 47(47%), 4(4%) and (0%) respectively, Most commonly, small ruminants are affected by multiple strongylide nematodes, mixed infection between Haemonchus contortus and Trichostrongylus spp was recorded 3(3%)*
Keyword*: Strongylide, PCR, ITS-2, NC2, goats, Sudan.*

I. Introduction

Parasitic worms of livestock cause diseases of major socioeconomic impact worldwide (Roeber et.al 2013), goats play an important role in the economy of rural communities where they are commonly raised by poor families and farmers for livelihood (Bushara, et.al, 2011). Gastrointestinal Nematodes (GINs) of goats remain one of the main constraints to goats production; they cause reduction of animal productivity by reduction of body weight and milk yield (Soulsby.1986) and Amarante and Amarante (2016), especially Haemoncus contortus, Trichostrongylus spp and Oesophagostomum columbianam are most common, pathogenic and economic impacts of small ruminants (Bowman, 2009). (GINs) have a worldwide distribution and more commonly affect goats in the tropical and sub- tropical regions of the world (Soulsby, 1986; Kusiluka and kambarage, 1996). Usually diagnosis of (GINs) depends on microscopic technique to detect eggs or larvae on the animal faeces (Zajac and Conby2006). Problem facing faecal egg count as diagnostic method for the fact that many parasites lay quite similar eggs which are very difficult to differentiate from one another, but differentiation and identification of the third stage larvae can be carried out by faecal culture (Hansen and Perry, 1994). Molecular techniques such as PCR and DNA sequencing have enabled the accurate identification of parasites (Gasser, et.al, 1993). These advanced techniques are highly sensitive, providing highly accurate identification of strongylide up to species level(Tan,et.al,2014) by using genomic DNA isolation from strongylide eggs from fresh or frozen faecal samples(Bott,et.al,2009.; Tan,et.al,2014). This study was conducted to identification and differentiation of some strongylide nematode species using PCR technique using DNA extracted from faecal samples.

II. Materials and methods

A total of 100 faecal samples positive for strongylide by flotation technique was stored by freezing at -20°C for extracting DNA. For DNA extraction, 1 g stool, frozen at -20°C, diluted in 10 ml of lysis buffer (Tris-HCL, 0.5 M; EDTA, 20 mM; NaCl, 10 mM; SDS, %0.1; pH 9.0) (TEN-9) in 50 ml tube. After vortexing for 5 minutes, samples are then homogenized by shaking for10 minutes. Samples were then diluted again (1:2) with 10 ml lysis buffer and homogenized for 5 minutes. Particulate materials were removed by centrifugation at 4500 ×g for 10 minutes. After transferring the supernatant to a new tube, approximately 10 ml of supernatant, DNA was precipitated by adding 5 ml ammonium acetate 7.5M (half of the sample volume) and 25 ml of ice-cold ethanol 96-100% (twice the sample volume). Then incubation at -20°C for 20-30 minutes will render a better precipitation. DNA was collected following centrifugation at 4500 ×g for 15 minutes at room temperature. In this step, precipitated DNA is coloured and contains the bile salts. The DNA pellet was re-suspended in 600 µl of TE (pH 8) and incubated at 65°C for 15 minutes. Then, DNA was extracted organically and also purified using conventional single step phenol/chloroform/isoamylalcohol protocol. Phenols will dissolve the colorful materials. After isopropanol precipitation, the colourful DNA pellet was collected and dissolved in 300 µl of

Tris-EDTA buffer following an overnight incubation at 37°C. This method was described by Machiels et al. (2000) and Abbaszadegan et al. (2007). PCR causing amplification of the region of the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA (rDNA) of strongylide species. Amplification by three different primers in separate reactions, in each reaction species specific primer forward and universal reverse primer to srongylide species NC2 (Bott et.al, 2009) are used.

PCR was carried out in the Bio-rad MyCyler^{TM} Thermal Cycler by using initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 s (denaturation), 55-C for 30 s (annealing) and 72 -C for 30 s (extension), followed by a final extension at 72 °C for 7 minutes. (Bott, et.al., 2009 and Tan.et.al., 2014).

To verify PCR amplification 5µl of PCR product was loaded in gel containing 1.5% agarose dissolved in 1x TBE (65mM Tris-HCL,27Mmboric acid,1m M EDTA,PH 9). Staining with 5µl of Ethidium bromide solution at voltage 100 volts for 30 minutes in 1x TBE buffer, 3µl of 100 Pb DNA ladder was run alone with amplicons.
Species specific primer forward and a universal reverse primer to strongylide species table.(1)

Species	Primer	Forward sequence	Reverse sequence	Amplicon length (pb)
Haemonchus contortus	HAE	CAAATGGCATTTGTCTTTTAG	TTAGTTTCTTTTCCTCCGCT	265
Trichostrongylus spp	TRI	TCGAATGGTCATTGTCAA		267-268
Oesophagostomum columbianum	OEC	TGTCGAACGATGCTTGCT		251

Statistical analysis:
Data analyzed using Microsoft Excel 2007.

III. Results

PCR reaction was optimized for the amplification of DNA of H. contortus Trichostrongylus sp and Oesophagostomum columbianam using a specific oligonucleotide primer located in the ITS-2 of rDNA. Out of 100 samples examined, Haemonchus contortus, Trichostrongylus spp and Oesophagostomum columbianum prevalence rates were recorded as 47(47%), 4(4%) and (0%) respectively, Most commonly, small ruminants are affected by multiple strongylide nematodes, mixed infection between Haemonchus contortus and Trichostrongylus spp was recorded 3(3%) as shown as in table (2), figure (1) and (2).

Table (2) Prevalence of Haemonchus contortus, Trichostrongylus sp and Oesophagostomum columbianam of goats by PCR technique in the study area

Species	No. Samples examined	No.positive
Haemonchus contortus	100	47(47%)
Trichostrongylus sp	100	4(4%)
Oesophagostomum columbianam.	100	(0%)
Mixed infection H.contortus and Trichostrongylus spp	100	3(3%)

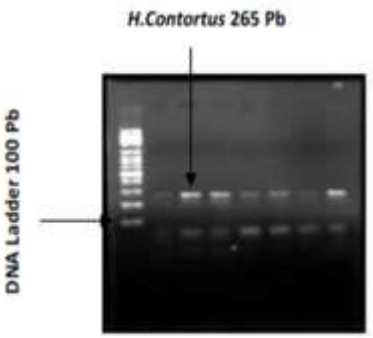

Figure (1): Agarose gel electrophoresis (1.5%) amplification of *H. contortus* DNA

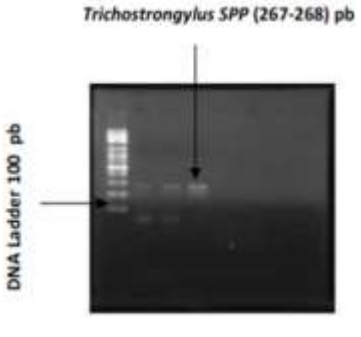

Figure (2) gel electrophoresis (1.5%) amplification of *Trichostrongylus sp* DNA band size (267-268 Pb)

IV. Discussion

Specific diagnosis of nematode infection has major implication for monitoring of drug resistance in nematode population and can underpin studies of the biology, ecology and epidemiology of strongylid (Roeber et.al 2011). This investigation revealed that the study area conspicuously lack molecular data focusing on strongylide nematode parasites of small ruminants.

In the present study the positive faecal samples which were diagnosed for strongylide nematodes by using McMaster Technique were further tested by PCR technique. Thus (47%) of 100 samples were recorded positive for Haemonchus contortus. This result was different from that of (Anwar et.al 2014) who recorded (66%) of 44 a positive diagnosis of samples, and is smallest than that reported by Tan et.al (2014) and Roeber et.al (2011). Furthermore the present results trichostrongylus spp are also smallest. Those shown by Tan et.al (2014) for the same parasite which recorded as (79.8 %) of 99 samples. However no amplification with Oesophagostomum colmbianum was recorded in the present study. Occassionally, small ruminants are affected by multiple strongylide nematodes (Zajac 2006), hence mixed infection by Haemonchus contortus and Trichostrongylus spp (3%) was recorded in this study. This result was in favorable with that of Tan et.al (2014) who reported (1.9) in one study but was strikingly different for the (45.2%) shown by them partially for another mixed infection.

Contrary to our general results Sweeny et.al (2011), observed very similar results for McMaster and PCR techniques for genomic DNA extracting directly from faeces, it is seen that such variations may results from differences in techniques and the biological materials. These are known to cause problems when PCR is used as diagnostic methods for the probable existence of impurities that inhibit PCR amplification as was suggested by (Abbaszadegan et.al, 2007).

V. Conclusion

In the present study demonstrated that flotation technique revealed higher strongylide infection result, furthermore PCR was identification genera of strongylide infection, using phenol/chloroform/isoamylalcohol protocol in faecal DNA extraction leading to PCR inhibitor. In addition, the study concluded that the Haemonchus contortus is prevalent in goats in the study area.

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