



RESEARCH ARTICLE

Phenotyping and Prevalence of *Haemonchus contortus* (Nematoda: Trichostongylidae) in Ruminants from Endemic Areas of Pakistan: Influence of Host Species and Geographical Area on Phenotypic Traits of Worms

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ARTICLE HISTORY (16-292)

Received: August 15, 2016
Revised: November 09, 2016
Accepted: November 18, 2016
Published online: March 11, 2017

Key words:

Haemonchosis
Multivariate analysis
Pakistan
Phenotyping
Ruminants

ABSTRACT

The objectives of the current study were to find the prevalence and phenotyping of adult *Haemonchus contortus* in sheep, goats and cattle from different geographical areas of Pakistan. We used 300 abomasa collected from slaughtered animals with total worm burden of 1950 and only adult worms were used for morphological measurements. Microscopic method was applied on the standardized measurements. The *H. contortus* size variations were studied by multivariate analyses. The result showed the prevalence of infection was 46.66% (140/300), with sheep (55%), goats (50%) and cattle (35%). The gubernaculum lengths, cuticular ridges and esophagus length of the worms showed significant ($P < 0.05$) difference among three host species. The linguiform morphs were predominant in goats (72%), cattle (70%) and sheep (64%) followed by knobbed and smooth morph. The size variation on phenotypic traits showed principal component I (PCI) 60% and second principal component (PCII) 34% due to geographical areas and the host species. The result reported 14 numbers of isolates shared by sheep, goats and cattle demonstrating the close relationship between domestic animals epidemiology. The study concluded that Punjab province and its adjoining areas are under a high burden of *Haemonchus* infection in livestock animals. Furthermore, it suggests that due to existence of multiple isolates of *Haemonchus* species problem of anthelmintic resistance occurs which demands further investigation.

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To Cite This Article: Bibi R, Afshan K, Khan IA, Iqbal Z, Kayani AR, Mushtaq M, Irfan M, Qayyum M and Hassan MF, 2017. Phenotyping and prevalence of *Haemonchus contortus* (Nematoda: Trichostongylidae) in ruminants from endemic areas of Pakistan: influence of host species and geographical area on phenotypic traits of worms. Pak Vet J, 37(2): 170-174.

INTRODUCTION

In Pakistan, livestock sector contributed almost 58.55% of the agriculture share and about 12% to GDP of Pakistan during 2015-2016. Livestock plays an important role towards rural economy as 35-40 million rural inhabitants are linked to livestock and deriving 30-40% of the income from livestock (Bachaya *et al.*, 2006). Haemonchosis is one of highly pathogenic blood feeding nematode disease of sheep, goats and cattle which causes major damage to the livestock industry and its sustainability. Haemonchosis is mainly caused by three species *Haemonchus contortus*, *H. placei* and *H. similis*.

The livestock sector is facing economic instability due to gastrointestinal worms with high morbidity in terms of reduction in weight, poor growth, infertility, poor wool quality, reduction in meat and milk yield and occasionally causes mortality (Rehman *et al.*, 2016). The adult worm and L4 larval stage of the *Haemonchus* adhere to the abomasa of the host, cause severe anemia which can lead to host mortality. Due to the huge economic threats of haemonchosis on livestock production, accurate identification is critical to their management and control (Ai *et al.*, 2010). The anthelmintics are used against GI nematode infections as prophylactic measures. Due to emergence of resistant strain of *Haemonchus* worm and

over use of anthelmintics for treatment and control purposes has greatly reduced its efficacy across the world in sheep, goats and cattle (Irum *et al.*, 2014; Cotter *et al.*, 2015). The development of resistance in nematodes against anthelmintics and accumulation of extensively used drug residues in food and environment raises public health concerns which divert attention towards proper identification of *Haemonchus* species for prophylactic strategies and in order to determine their role in parasitic gastroenteritis (Amarante, 2011).

The prevalence of *Haemonchus* species in Pakistan is reported by different researchers: 25.6% in Dera Ghazi Khan (Lashari *et al.*, 2015), 21.41% in Multan (Lashari and Tasawar, 2011), 77.7% in Khanewal district (Tasawar *et al.*, 2010), 14.65% in Hyderabad (Akhter *et al.*, 2011), 14.66% in Swat (Nabi *et al.*, 2014) and 58.1% in Rawalpindi (Asif *et al.*, 2008). Therefore, because of high prevalence rates of haemonchosis in the country understanding the epidemiology of infections is essential for the design of efficient programmes of prophylaxis against gastrointestinal nematode infections. Most studies conducted in Pakistan are based on prevalence of infection in different areas but limited attempts were made on morphometry to differentiate *Haemonchus* species. In present study we evaluated the prevalence and morphological characteristics of *Haemonchus contortus* adults infecting the sheep, goats and cattle.

MATERIALS AND METHODS

Experimental site and adult worm collection: Material studied included adult worms from various areas of Pakistan, obtained from several slaughterhouses including mountain, hilly and lowland locations. The study zone comprises the province of Punjab and Khyber Pakhtunkhwa, on the districts of Lahore, Multan, Mandi Bahauddin and Haripur. A total of 300 abomasa containing 1950 adult worms were studied originating from three host species; cattle, sheep and goats. The worms were differentiated as *H. contortus* by using the discriminant function criteria developed by Jacquet *et al.* (1996). Of these worms, a total of 300 undamaged specimens were selected for the morphometric study. The parasites after their recovery from the hosts were washed in normal saline to remove mucus; lactophenol and glycerol were used to observe the clear internal structure of worm on a glass microscope slide (Lichtenfels *et al.*, 1994). The worms analyzed were fixed in hot 70% alcohol, after fixation the nematode parasites were preserved in glycerin alcohol (glycerin: 70% alcohol, 1:3) and were mounted in glycerin.

Measurement techniques: Measurements of specimens were obtained with the aid of a calibrated ocular micrometer on a compound microscope and are recorded in micrometers. Interference contrast light microscopy was used to study the synlophe and other characters at a magnification of 400 X. Adult characteristics studied were length of body, length of cervical papillae, length of right and left spicules, length of esophagus, diameter of esophagus and gubernaculum length. Lichtenfels *et al.* (1994) discriminated *Haemonchus* species by spicule, circular ridges and synlophe analysis.

Data analysis: Geometrical morphometric approaches were used to quantify the morphological discriminations (Rohlf and Marcus, 1993), a technique offering an estimate of size by which different axes of growth are integrated into a single variable, the “centroid size” (Bookstein, 1989). The phenotypic variations among *Haemonchus* adults were calculated with multivariate analyses, by using size-free canonical discriminant analysis on the covariance of log-transformed measurements to assess phenotypic variations between the samples. The principal component analysis is used to summarize most of the variations in a multivariate dataset in a few dimensions (Dujardin and Le Pont, 2004). The resulting “allometry-free”, or size-free, variables were submitted to a canonical variate analysis (CVA), and Mahalanobis distances were derived (Mahalanobis, 1936). Phenotypic analysis of *Haemonchus* adults was conducted by using various modules of the CLIC package (by J.P. Dujardin, <http://momedujardin.wordpress.com>). The dendrogram of Mahalanobis distance was constructed using the UPGMA, a sub program of online software <http://genomes.urv.cat/UPGMA/index.php?entrada> (Garcia-Vallve *et al.*, 2002). The Mann Whitney U test was applied to make a comparison between the morphological characters of *H. contortus* and different host species. The results were statistically significant at $P < 0.05$.

RESULTS

Abomasa examination showed 46.66% prevalence of *Haemonchus* spp, sheep (55%), goats (50%) and cattle (35%) with a total worm burden of 1950 worms (Table 1). Body length of male *H. contortus* in goats were significantly ($P < 0.05$) shorter as compared to that from sheep and cattle (Table 2). The body sizes of female *H. contortus* in cattle were significantly ($P < 0.05$) shorter than sheep and goats. Length of right spicule of *H. contortus* in sheep were significantly ($P < 0.05$) shorter when compared to that from cattle and left spicule length were found statistically non-significant ($P > 0.05$) among sheep, goat and cattle. The cervical papillae lengths of female *H. contortus* in sheep were significantly ($P < 0.05$) larger when compared with goats and cattle. However, there is no significant ($P > 0.05$) difference in the cervical papillae lengths of the male worms in the three host animals. The gubernaculum lengths of the worms in goats were significantly ($P < 0.05$) shorter as compared to that from sheep and cattle. The cuticular ridges and esophagus length of the worms were statistically significant ($P < 0.05$) among three host animals. However, the esophagus diameter of male *H. contortus* in goats were significantly ($P < 0.05$) greater as compared to that from sheep and cattle, and of female *H. contortus* in sheep were significantly ($P < 0.05$) shorter than goats and cattle (Table 2).

The percentage of the different vulvar morphs per host-species is shown in Table 3. The smooth morph was relatively rare in *H. contortus* for three hosts (4, 16 and 5% in goats, sheep and cattle, respectively) whereas linguiform morphs were predominant in goats (72%) followed by in cattle (70%) and sheep (64%). The knobbed vulvar morph was moderate in three host animals (26, 28 and 20% in in goats, sheep and cattle, respectively). The morph distribution was similar in the sheep, goats and cattle.

Table 1: Prevalence and intensity of female and male *H. contortus* in sheep, goats and cattle.

Host species	No. of host examined	No. of infected host %	Density of nematodes	♀ <i>H. contortus</i> (%)	♂ <i>H. contortus</i> (%)
Goat	100	50	1000	800 (60)	200(20)
Sheep	100	55	600	400 (66.66)	200(33.33)
Cattle	100	35	350	250 (71.42)	100(28.57)
Total	300	46.66	1950	1450(74.4)	500(25.6)

Percentages showed in parenthesis

Table 2: Morphometric measurements shown in Mean±SD for male (n=50) and female (n=50) *H. contortus* parasites in ruminants.

Measurements (µm)	Sex	Host		
		Sheep (n=100)	Goats (n=100)	Cattle (n=100)
Body length (BL)	Male	16.58±0.24 ^a	12.42±0.46 ^b	14.7±0.33 ^c
	Female	24.12±0.29 ^a	24.14±0.44 ^a	19.04±0.32 ^b
Cervical papillae (CP)	Male	307.4±2.52 ^a	310±4.87 ^a	314.2±7.62 ^a
	Female	319.8±3.07 ^a	299.4±4.84 ^b	289.6±5.15 ^{bc}
Length of right spicule (LRS)	Male	451.6±3.98 ^a	456.2±2.6 ^{ab}	460.2±2.62 ^{bc}
Length of left spicule (LLS)	Male	431.6±3.98 ^a	435.8±2.66 ^a	439.4±2.6 ^a
Length of gubernaculum (LG)	Male	237.4±2.61 ^a	169.6±2.39 ^b	203.2±4.39 ^c
Cuticular ridges (CR)	Male	22.84±0.18 ^a	20.08±0.18 ^b	26.48±0.22 ^c
	Female	23.48±0.25 ^a	22.36±0.23 ^b	26.5±0.15 ^c
Esophagus length (EsL)	Male	1319.4±12.27 ^a	1546.2±6.33 ^b	1224±15.6 ^c
	Female	1356.2±7.71 ^a	1435±16.49 ^b	1266±15.4 ^c
Esophagus diameter (EsD)	Male	101.8±1.84 ^a	164±2.09 ^b	99±2.04 ^a
	Female	128.8±2.1 ^a	143±3.52 ^b	148.6±4.02 ^b

Smaller superscripts compare means in rows; Means with different letter differ significantly (P<0.05) among each other.

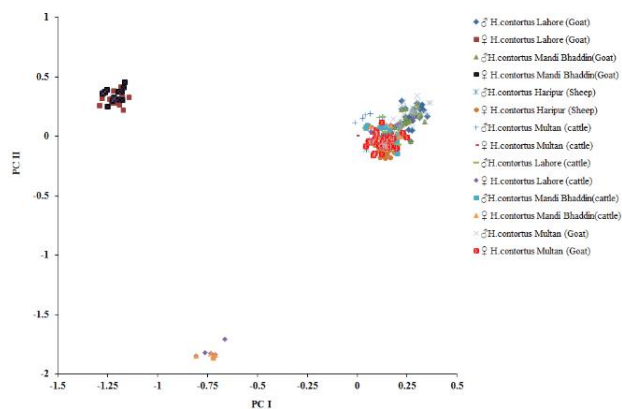
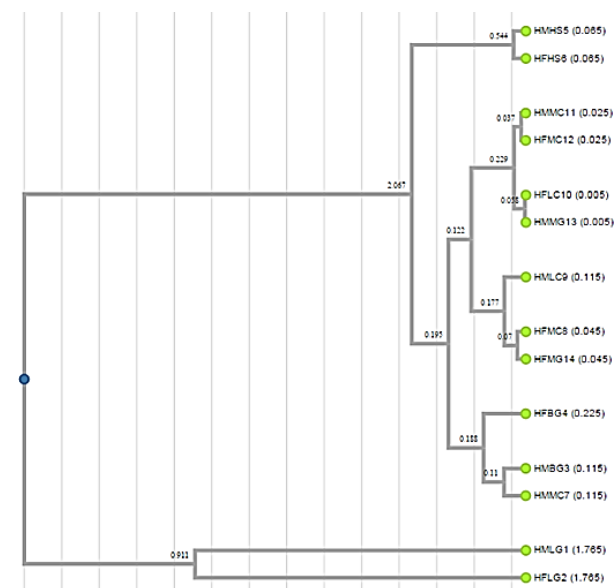
Table 3: Distribution of the *H. contortus* vulvar morphology in goats, sheep and cattle.

Host	Density of worms	Linguiform morph (%)	Knobbed morph (%)	Smooth morph (%)
Goats	200	144 (72)	52 (26)	4 (2)
Sheep	200	128 (64)	56 (28)	16 (8)
Cattle	50	35(70)	10(20)	5(10)

Percentages showed in parenthesis

Prevalence comparison of the *Haemonchus* populations shows a general overlap between them regardless of the different definitive host species, worm type and geographical area. The size and shape of worm bodies were studied by multivariate analyses. A scatter plot of the first two principal components (PC) is shown in Fig. 1. The first common principal component (PCI) of the 14 populations analyzed can be interpreted as a measure of overall size, all significantly correlated with PCI, thus contributing 60% to overall variation. The results show that the female *H. contortus* populations from goats and cattle from Lahore and Mandi Bahauddin have significant size differences as compared to those from sheep and other areas.

Mahalanobis distances between the 14 *H. contortus* adult groups are shown in Table 4. The maximum distances were showed when male and female *H. contortus* worms in goats from Lahore were compared with worms in sheep (from Haripur), cattle (Lahore, Multan, Mandi Bahauddin) and goats (from Multan, Mandi Bahauddin). UPGMA cluster analysis resulted in two major clusters based upon the Mahalanobis distance matrix (Fig. 2). The first group is further divided into 3 subgroups (A, B and C). Sub group A has 2 isolates, whereas subgroup B contains 7 isolates, and subgroup C contains 3 isolates. Group second contain only 2 isolates comprises male and female *H. contortus* in goats from Lahore. The dendrogram illustrates the relatedness of the isolates based upon distance matrix.

**Fig. 1:** Factor map corresponding to worms of *Haemonchus* from naturally infected Sheep, goats and cattle from different areas of Pakistan. Samples are projected onto the first (PC I, 60%) and second (PC 2, 34%) principal components. Each group is represented by its perimeter.**Fig. 2:** Dendrogram based on morphometric data using Dendro UPGMA. The clustering of *H. contortus* isolates represents 14 different isolates based on the data using the Mahalanobis distance metric and (UPGMA) using average linkages. (HMLG1= ♂*Haemonchus* Lahore (Goat); HFLG2= ♀*Haemonchus* Lahore (Goat); HMBG3= ♂*Haemonchus* Mandi Bahauddin (Goat); HFBG4= ♀ *Haemonchus* Mandi Bahauddin (Goat); HMHS5= ♂*Haemonchus* Haripur (Sheep); HFHS6= ♀*Haemonchus* Haripur (Sheep); HMMG7=♂*Haemonchus* Multan (Cattle); HFMG8= ♀*Haemonchus* Multan (Cattle); HMLC9=♂*Haemonchus* Lahore (Cattle); HFLC10= ♀*Haemonchus* Lahore (Cattle); HMMG11 =♂*Haemonchus* Mandi Bahauddin (Cattle); HFMG12= ♀ *Haemonchus* Mandi Bahauddin (Cattle); HMMG13 = ♂*Haemonchus* Multan (Goat); HFMG14= ♀ *Haemonchus* Multan (Goat).

DISCUSSION

Previous studies conducted in Pakistan and different parts of the world have revealed that the prevalence rate of *H. contortus* vary from area to area. In present study, the prevalence of *H. contortus* was 46.66% which might be due to the influence of multifactorial system includes hosts, parasites and environment that affect prevalence rates from area to area. Other possible reason for higher *Haemonchus* prevalence in the study area may be due to extensive management of animals with high stocking density, poor nutritional and veterinary infrastructure

Table 4: Mahalanobis distances between the 14 worms adult groups distinguished in Punjab and KPK province, Pakistan, according to parasite type (σ *H. con* = male *H. contortus*; φ *H. con.* = female *H. contortus*), host species (Sheep, goats and cattle) and geographical altitude (Lahore, 217m; Mandi Bahauddin, 204m; Multan, 124; Haripur, 537m).

	σ <i>H.con.</i> goat 217m	φ <i>H.con.</i> goat 217m	σ <i>H.con.</i> goat 204m	φ <i>H.con.</i> goat 204m	σ <i>H.con.</i> sheep 537m	φ <i>H.con.</i> sheep 537m	σ <i>H.con.</i> cattle 124m	φ <i>H.con.</i> cattle 124m	σ <i>H.con.</i> cattle 217m	φ <i>H.con.</i> cattle 217m	σ <i>H.con.</i> cattle 204m	φ <i>H.con.</i> cattle 204m	σ <i>H.con.</i> goat 124m	φ <i>H.con.</i> goat 124m
σ <i>H.con.</i> goat 217m	0.00													
φ <i>H.con.</i> goat 217m	3.53	0.00												
σ <i>H.con.</i> goat 204m	7.07	3.55	0.00											
φ <i>H.con.</i> goat 204m	6.75	3.22	0.51	0.00										
σ <i>H.con.</i> sheep 537m	6.96	3.51	0.87	1.25	0.00									
φ <i>H.con.</i> sheep 537m	7.02	3.55	0.75	1.16	0.13	0.00								
σ <i>H.con.</i> cattle 124m	6.84	3.31	0.23	0.39	0.87	0.77	0.00							
φ <i>H.con.</i> cattle 124m	7.64	4.12	0.98	0.96	1.82	1.69	1.11	0.00						
σ <i>H.con.</i> cattle 217m	7.5	3.98	0.78	0.78	1.63	1.5	0.92	0.2	0.00					
φ <i>H.con.</i> cattle 217m	7.62	4.08	0.57	0.9	1.25	1.12	0.79	0.66	0.52	0.00				
σ <i>H.con.</i> cattle 204m	7.51	3.98	0.5	0.78	1.26	1.13	0.71	0.59	0.42	0.13	0.00			
φ <i>H.con.</i> cattle 204m	7.5	3.97	0.47	0.78	1.21	1.08	0.69	0.64	0.47	0.12	0.05	0.00		
σ <i>H.con.</i> goat 124m	7.61	4.08	0.56	0.9	1.24	1.1	0.78	0.67	0.53	0.01	0.13	0.12	0.00	
φ <i>H.con.</i> goat 124m	7.61	4.1	1.04	0.97	1.89	1.76	1.16	0.09	0.26	0.75	0.67	0.72	0.76	0.00

(Fentahun and Luke, 2012). The results of present study indicated statistically no significant difference ($P > 0.05$) in the prevalence of haemonchosis between sheep and goat showing that both species are equally prone to the infection. The result was consistent with Fentahun and Luke (2012) who recorded non-significant ($P > 0.05$) prevalence rates between small ruminants.

In present study, three vulvar morphs of *H. contortus* females from sheep, goats and cattle were observed; linguiform morphs were predominant followed knobbed and smooth morphs. The results of some previous studies on vulvar morphology of female *H. contortus* showed that the linguiform vulvar type was the most predominant morph type followed by smooth and knobbed vulvar morphs both in sheep and goats (Demissie *et al.*, 2013). The appearance of some genetic factors is responsible for the different vulvar shape of *Haemonchus* species during worm establishment and development in the host. Eysker and Ploeger (2000) indicated vulvar morphology helps to understand and know more about the biology of *Haemonchus* species and determine the type of population that occurs in sheep and goats of the area. In the previous studies it was found that there were some morphological variations among the *H. contortus* worms collected from different small ruminants (Rahman and Hamid 2007). Troell *et al.* (2005) also reported that *H. contortus* has greater morphological plasticity, and has greater potential to avoid the environmental pressure by showing different epidemiological approaches, and also base on environmental conditions for the pre-parasitic stages.

The current work recorded significant difference in cuticular ridges of male and female worms from sheep, goats and cattle. These findings are consistent with Gharamah *et al.* (2011) study where insignificant difference in cuticular ridges from sheep and goats was reported. The result indicates that cuticular ridges are useful morphometric character in species discrimination. The differences in cuticular ridges are due to either the presence of short extra ridges in a location or cross overs where one ridge reversed positions with an adjacent ridge.

Most of the studies conducted on *Haemonchus* morphometry based on the analysis of male specimens. Three measurements including: total length of the spicule, the distance from the tip to the hook of the right spicule,

and the distance from the tip to the hook of the left spicule are more commonly used in morphometry (Silva *et al.*, 2015). The current findings showed no significant variations in the lengths of both right spicule and left spicule of male worms in goats and sheep, while significant variation was observed in length of right spicule of male in sheep and cattle. The results of current findings are not in agreement with Rahman and Hamid (2007), who reported significantly shorter spicules in goats as compared to sheep. The spicule lengths of *H. contortus* in the present study were smaller than those reported for *H. contortus* in Penang, Malaysia (Rahman and Hamid, 2007). In present study significant variations were observed in body and gubernaculum lengths of male *H. contortus* in three host animals. These finding are inconsistent with study carried out by Rehman and Hamid (2007), revealed non-significant difference in body and gubernaculum lengths of *H. contortus* among goats and sheep.

According to Dujardin *et al.* (2008), quantitative morphological differences inform about both genetic variation and external influences. In the case of endoparasites, it is necessary to distinguish between external environment according to geography and parasitized organ inside the host (Valero *et al.*, 2012). Given that the entire material in this study comes from different geographical area, external environment according to geography and the host species effect on the size and shape of the parasite populations was analyzed. The present study aims to quantitatively characterise the influence of the host species on the morphometric traits of *H. contortus* adults (from sheep, goats and cattle) in natural populations from Pakistan, taking into account standardised measurements. This is the first study on the decisive influence exercised by the host species on metric traits of *H. contortus* adults. Morphological variation has been quantified by geometrical morphometrics (Cooke and Terhune, 2015). The results obtained in the principal component analysis and Mahalanobis distance matrix showed that *Haemonchus* worms in goats from Lahore and in sheep from Haripur present significant variation in size and shape when compared to parasites from different host animals and geographical areas. The animal host species can strongly influence the phenotype of the adult stage, mainly due to the different size of the abomasa

microhabitat and different physiology of host. The ability of organisms to produce different phenotypes under different environmental conditions (phenotypic plasticity) has been an object of evolutionary and ecological studies since the Neo-Darwinian synthesis (Pigliucci, 2005). The current result showed a 14 number of isolates, which indicates close relationship among domestic livestock hosts from different geographical areas. This sympatric distribution of *H. contortus* might be because of absence of mechanical barrier and animals easily move from one area to another and share the grazing pastures. The Indus River and its tributaries are also major agents through which these different areas are interconnected. Through the Indus River the eggs of parasites move from one area to another and show sympatric distribution.

Conclusions: In conclusion, morphometric analysis proved to be very useful for the identification of *H. contortus* in epidemiological studies involving different species of ruminants in shared pastures. The present study confirmed that (i) the Punjab province and its adjoining areas are under a high burden of haemonchosis (ii) a 14 number of isolates shared by sheep, goats, and cattle, demonstrating the close relationship between domestic animals epidemiology. Therefore, research must be carried out to define phenotypic measurements complementing the molecular tools that would enable the analysis of parasite plasticity. Yet, another direction of future research certainly lies at the interface between genetics and environments, in the epigenetic machinery that somehow translates genetic effects and environmental influences (host species, geographical location) into coherent *Haemonchus* phenotypes.

Acknowledgments: The authors would like to thank workers at local abattoirs for assistance with collection of abomasa of slaughtered animals.

Authors contribution: MQ conceived the idea and designed this research experiment. RB, MM, MI, KA and ARK executed the experiment and analyzed the *Haemonchus* samples. MQ, KA, ZI, IAK and MFH involved with data analysis. ARK and IAK provided technical assistance in conducting the study. All authors have participated in the study and concur with the submission and subsequent revisions submitted the corresponding author. The authors declare that they have no competing interests.

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