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Helminth parasites in small ruminants of sub-urban areas of Rajshahi, Bangladesh

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ABSTRACT

The growth, development and multiplication of small ruminants are greatly hampered by the helminth infections. This study determined the status of helminthic infection in sheep and goat of Rajshahi, Bangladesh. The fecal samples collected from naturally infected animals were examined by direct smear method for detection of eggs followed by modified McMaster technique to determine the intensity of infection. Helminth parasites collected from slaughtered animals were also identified accordingly. Results of coprological examination showed the prevalence of helminthic infection was 86% in sheep and 78% in goat. The females were more prone to helminth infection than male. The eggs of Fasciola, Paramphistomum, Moniezia, Oesophagostomum, Trichuris and strongyle type were detected from both sheep and goat and percentage of infection were ranges from 82 to 11. Quantitative examination of 20 fecal samples (10 goats and 10 sheep) showed highest infection of strongyles group helminth (1883±125.83) followed by Fasciola (1267±28.87), Paramphistomum (734±76.38), Strongyloides (630±38.68), Moniezia (483±86.22) and Trichuris (450±75.50) irrespective of species of animals. Adult Fasciola. Paramphistomum, Moniezia, Haemonchus, Oesophagostomum, Trichuris and metacestode Coenurus cerebralis. Cysticercus taenuicollis and hydatid cyst were identified from different organs of slaughtered sheep and goats. The results suggest that helminth parasites are highly prevalent in sheep and goats of the study area. Effective strategies are warned to control the helminthic infection for improvement of small ruminant's production in Rajshahi, Bangladesh.

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Introduction

Bangladesh is predominantly a country of agriculture in which livestock is an integral part. Share of livestock in agricultural GDP is 13.44%

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Department of Veterinary and Animal Sciences University of Rajshahi, Rajshahi-6205, Bangladesh. E-mail: moizur@ru.ac.bd (Moizur Rahman) *Contributed equally to the work. and contribution of livestock in national GDP is 1.43% (BER, 2020). Small ruminants (sheep and goat) are important part of livestock and directly contribute to the poor man's economy of developing country for poverty reduction through income generation. There are about 263.79 million goats and 35.83 million sheep in Bangladesh (BER, 2020). Production of small ruminants play role in food and nutrition security

and employment generation. Goat especially the black bengal goat rearing is becoming much popular day by day in Bangladesh for their high quality meat, prolificacy and disease resistance capacity. Currently special emphasis is also given to sheep production in different region of Bangladesh (Rahman et al., 2017).

Parasitism is one of the important constraints of livestock production. Parasitic infections cause deleterious and debilitating effects on animals. There may be reduction in weight gain and loss of weight due to utilization of host foods, removal of host blood and tissue fluids, destruction of host's tissues. mechanical interference, abnormal growth of host tissue, secreting and excreting various harmful substance into host, introduce into the host body other pathogenic microorganisms etc. (Rahman et al.,1996). In heavy infections, parasites contribute to reduced milk and meat production (Murthy and Rao, 2014). Helminthic infections are important among the parasitic species as it can easily transmit to one host to another by several mode of transmission. It is a leading cause of hindering small ruminant production worldwide (Dewan et al., 1979). Ecology, geographical location and climatic condition are the key factors regarding prevalence of helminth parasites in an area. Helminths are abundance in Bangladesh as geographical location and climatic condition are mostly favorable for their growth and development (Hossain et al., 2004).

High prevalence of different helminths was reported in sheep and goats in Bangladesh of different region of Bangladesh. It was mentioned that about 85% of sheep and goat infected with helminth parasites (Howlader et al., 2002; Khalid et al., 2004; Mazid et al., 2006; Islam et al., 2008; Hassan et al., 2011; Sangma et al., 2012; Poddar et al., 2017, Islam et al., 2017; Rahman et al 2017). The helminth includes Haemonchus contortus, Trichostrongylus axei, T. colubriformis, Cooperia С. punctata, Oesophagostomum pectinata, radiatum, O. columbianum, Bunostomum bovis, B. phlebotomum and Gaigeria pachyscelis (Mazid et al., 2006; Hassan et al., 2011; Islam et al., 2008). Sangma et al., (2012) reported 81.1% helminth infection in sheep of Tangail district in which Fasciola gigantica (8.4%), Paramphistomum sp. (44.2%). Schistosoma indicum (3.7%),Bunostomum sp. (19.0%), Trichuris sp. (2.1%), Strongyles (62.6%) and Strongyloides sp. (9.5%) were common. Haemonchus sp. (67.45%), Oesophagostomum sp. (43.27%), Gaigeria sp. (41.85%), Trichuris sp. (28.35%), Bunostomum sp. (18.25%) in goat were also reported by Howlader et al., (2002). Hassan et al., (2011) recorded 63.41% goats of Chattagram was infected with helminth parasites where Strongyloides sp. showed highest percentage of infection (51.74%). A study on sheep of Sherpur district revealed helminthic infection in coprological 67.9% examination. The eggs identified were Fasciola gigantica (11.3%), Paramphistomum sp. (13.2%), Schistosoma indicum (3.8%), Moniezia sp. (3.8%), Strongyle-type (24.5%), hook worm (6.6%), Strongyloides sp. (12.3%) and Trichuris sp. (1.9%) (Poddar et al., 2017). A report of Islam et al., (1995) stated the metacestodes Cysticercus tenuicollis (54.54%), Coenurus cerebralis (6.32%) and hydatid cysts (11.13%) in black bengal goats of Bangladesh.

Small ruminants especially goat and sheep are strongly emphasized for their multipurpose production profile. Thus, validation of healthy sheep and goat production is obligatory for livelihood development of poor people as well as to enrich national economy. Detrimental effects of helminthic infection in small ruminants have high economic impact regarding productive and reproductive performance. Several studies have been carried out on helminthic infection in small ruminants of different areas in Bangladesh, but no attempts have been taken to study the prevalence of helminths in small ruminants of Raishahi. The present study revealed the status of helminthic infection in sheep and goat of sub-urban areas of Rajshahi, Bangladesh.

Materials and Methods

Study location and sample collection

To conduct the study, a total of 100 fecal samples were collected from semi-scavenging randomly selected household sheep (n=50) and goat (n=50) of sub-urban areas of Rajshahi City Corporation, Rajshahi. Male female ratio of collected samples was 1:1. We sampled 6 animals (3 sheep and 3 goats) each day early in the morning. Fresh fecal sample (5-10 gm) from each individual was collected during defecation with aseptic condition. Clean vials were used to kept the samples. The unique identification number and basic information (age, sex and body weight) were marked on the vials. A record keeping register was also maintained. Collected fecal samples were transferred immediately to the laboratory of Veterinary and Animal Sciences, University of Rajshahi. The samples were examined immediately after collection or kept in refrigerator at 4°C until the examination.

Fecal sample examination, detection and identification of eggs

Fecal samples were examined directly under microscope following Direct Smear Method described by Urguhart et al., (1996) to screened out the positive samples. Briefly, a very little amount of sample was taken onto the glass slide followed by spreading of sample using normal saline and removal of course particles until making it transparent. Different helminths eggs were detected and identified under compound microscope (10X) based on their characteristic morphological features described by Soulsby (1982) and Rahman et al., (1996). The intensity of helminth infection in 20 randomly selected positive samples (10 sheep and 10 goats) was determined by Modified McMaster Egg Counting technique (Soulsby, 1982; Rahman et al., 1996). In brief, for nematode, 3 gm of each fecal sample was taken in a small beaker followed by adding of floatation fluid (saturated salt solution) upto 45 ml. After mixing and sieving with 150 mm mesh sieve, a small portion of suspension was transfer into the two chambers of McMaster slide using plastic transfer pipette. Then left stand for few minutes to float the eggs and count the eggs under microscope (10X). A multi-plication factor was applied to estimate the number of eggs per gram of feces (EPG) based on the concentration of the suspension "feces/flotation liquid" used to fill the chambers of the McMaster slide. For trematode and cestode eggs, 2 gm of feces was thoroughly mixed with 60 ml of water, sieving with 150 mm mesh sieve and transferred to a 60 ml capacity extra wide mouthed bottle. A special plastic pipette with mixing bowl (1.3 ml capacity of the constriction mark) was immersed in the fecal suspension and taken out after filling of suspension upto the constriction mark. The pipette was then immersed vertically into the mercuric iodide solution (1.630 sp. gr.) upto the constriction mark. The fluid in the pipette was then mixed in the bowl and transferred to the two chambers of the McMaster slide. The counting of eggs floated or adhered to the lower surface of the chambers was started after 1 minute and completed within 5 minutes. A multiplication factor for EPG was 30 (2 gm in 60 mi suspension) for counted number of eggs as 1.0 ml was taken for counting (Rahman et al., 1996).

Collection and identification of helminth parasites from infected animals

gastrointestinal The tracts and other associated organs of highly infected slaughtered animals (5 sheep and 5 goats) were examined. The parasites were collected and preserved by the methods described by Soulsby (1992) and Rahman et al., (1996). Briefly, each part of gastrointestinal tract was opened separately and contents were collected in separate containers. The inner surface of each part was washed gently with physiological saline to detach the parasites from the gut wall. The contents were washed several times using water to make it transparent. The parasites were collected from the transparent contents using needle and petridishes. The liver was cut into small pieces and gently squeeze by finger pressure in normal saline to take away the immature flukes from the parenchyma. Worms found were placed separately in physiological saline. The gross morphological features of collected helminths were studied immediately after collection, washing and simple processing. Briefly, after washing with physiological saline, nematodes were placed in hot glycerin alcohol for straightening which made morphological studies easier. Clearing agent lactophenol was added to the adult nematode to observe the morphology under microscope (4X and 10X). Large and heavy trematodes and the segments of cestodes were pressed between two glass slides and preserved. Before preservation, all the helminths were mounted on a glass slide and examined under microscope with or without lactophenol for preliminary identification. For detail morphological study, the trematodes and cestodes were fixed in alcohol formalin acetic acid solution followed by the removal of fixatives in graded alcohol (50-70%). The iodine solution was added to the specimens and let stand overnight. Removal of iodine was done by washing with 70% alcohol for several times. For staining, working solution of Semichon's carmine was added to the specimens and let stand for 5 hours. De-staining was performed by washing the specimens several times in 70% alcohol with

0.05% HCl. Then the specimens were dehydrated by passing through graded alcohol (30%, 50% and 70%, 80%, 90% and absolute alcohol). The specimens were then placed in cresol or aniline oil until they sunk. Then mounting with Canada balsam was done after momentary washing in xylol. In all cases, adult parasites and metacestodes were identified following the keys given by Khalil et al., (1994) and Rahman et al., (1996).

Results

Helminth parasites were abundant in sheep and goats

The eggs of different helminths from 100 fecal samples (50 sheep and 50 goats) were identified according to the characteristics described by Soulsby (1982) and Rahman et al., (1996). Direct smear method detected eggs of different helminths in 82 fecal samples. Among the 50 goats' feces, 39 were infected (78%). Infection rate was higher in sheep as 43 sheep fecal samples (86%) were positive for eggs of more than one helminth (Fig. 1). The findings suggested that the sheep and goats of the study area were abundantly infected with different helminths.



Fig. 1. Infection status of helminth parasites in sheep and goats

Eggs of five different genera and a group of helminth parasites were detected and identified. Two trematodes (Fasciola and Paramphistomum), one cestode (Moniezia) and three nematodes (Strongyles, Trichuris and Strongyloides) eggs were identified. The rate of Fasciola infection was highest (82% in sheep and 66% in goat) among all identified helminths. Strongyles group showed highest prevalence (68% in sheep and 65% in goat) among nematodes. Moniezia infection was higher in goats (24%) than sheep (18%). Characteristic larva containing eggs of Strongyloides was detected from feces of 26% goat and 11% sheep (Fig. 2).



Fig. 2. Status of specific helminth infection in sheep and goats

Both sexes animals were vulnerable to helminth infection

Determination of sex wise helminthic infection revealed the differences among the infection rate in male and female sheep and goats. Female mostly showed higher infection rate in both sheep and goats (Fig. 3). In female sheep, the infection rate was 88% (Fasciola), 76% (Strongyles), 68% (Paramphistomum), 56% (Trichuris), 32% (Moniezia) and 13% (Strongyloides). The male sheep showed 78% (Fasciola), 68% (Strongyles), 64% (Paramphistomum), 43% (Trichuris), 24% (Moniezia) and 11% (Strongyloides) infection. In female goat, Fasciola infection was 72% followed by Paramphistomum (64%), Strongyles (64%), Moniezia (52%), Strongyloides (52%) and Trichuris (36%). In male goat, Fasciola showed highest (68%) infection whereas Monizia infection was the lowest (36%).



Fig. 3. Eggs of helminths detected under microscope (10X). A) Fasciola B) Paramphistomum C) Moniezia D) Strongyles E) Trichuris F) Strongyloides.

Intensity of infection was varied in different helminthic infection

Twenty (10 sheep and 10 goats) positive fecal samples were selected for EPG count having at least three helminth eggs. Level of infection in sheep was higher in comparison to goat. In sheep, the highest EPG count was found in strongyles group of infection in both sheep and goat (1883±125.83 and 1283±76.38, respectively). Fasciola infection was in second position in EPG count (1467±76.38 in sheep and 734±76.38 in goat). whereas in goat EPG was high in case of both Fasciola and Paramphistomum infection (Table 1). In sheep, the range of EPG were; Fasciola (417-1467), strongyles group (200-1883), Paramphistomum (100-534), Trichuris (183-428), Moniezia (100-483) and Strongyloides (100-150). In goat, EPG range were; strongyles group (183-1283) followed by Fasciola and Paramphistomum (150-734), Strogyloides (333-630), Trichuris (333-450), and Moniezia (250-355). The low to high EPG count of different helminths indicated the level of infection of most of the examined animals is high enough to cause sub-clinical or clinical diseases by most of the helminth identified.



Fig. 4. Sex wise helminth infection. (A) Goat (B) Sheep

Adult and larval helminths were common in slaughtered animals

Ten highly infected animals (5 sheep and 5 goats) determined by coprological examination were slaughtered to observe the presence of adult and larval helminth. Six adult helminth parasites were identified from all carcasses. But minute nematode Strongyloides was not found in any carcasses. The identified helminths were trematode Fasciola and Paramphistomum, cestode Moniezia and nematodes Haemonchus, Oesophagostomum and Trichuris (Fig 5). Adult Fasciola and Paramphistomum were collected from the bile duct and rumen, respectively. Moniezia was found in small intestine. Nematode Haemonchus was collected from abomasum. Oesophagostomum and Trichuris were found in caecum. The adult helminthes were identified grossly according to the characteristics described by Soulsby (1982) and Rahman et al., (1996). Three larval cestodes viz. Cysticercus taenicollis (metacestode of Taenia taenicollis), Coenurus cerebralis (metacestode of Taenia multiceps) and hydatid cyst (metacestode of Echinococcus collected granulosus) were also durina examination of carcasses. C. taenicollis was found in subcutaneous tissues of a goat. The brain of goat contains C. cerebralis. Hydatid cyst was collected from the liver of a slaughtered sheep and lungs of a goat (Fig 5).



Fig. 5. Adult and larval helminths collected from slaughtered animals. Adults: A) Fasciola B) Paramphistomum C) Moniezia D) Haemonchus E) Trichuris F) Oesophagpstomum; Metacestode: G) Cysticercus taenicollis H) Coenurus cerebralis I) Hydatid cyst

Discussion

Ruminants have more prone to helminthic infection in Bangladesh. Small ruminants mainly sheep and goat are easily accessible to helminths as they are roaming everywhere. In this study, fecal samples of 100 small ruminants (sheep and goats) were examined to determine the helminthic infection. Eggs of different helminths were detected and counted to know the infection status. The adult parasites and metacestodes were collected and identified from selected slaughtered animals. The overall infection of helminths found in sheep and goat was 82%. The infection rate in sheep was 86% (Fig.1).

Sample	Fasciola	Paramphistomum	Moniezia	Strongyles	Strongyloides	Trichuris
no.*						
S 09	1267±28.87	ND	ND	1683±115.47	ND	ND
S 12	600±50.00	133±28.87	ND	533±104.08	100±50.00	400±50.00
S 13	ND	ND	150±50.00	516±76.38	ND	183±76.37
S 16	1066±104.08	245±32.79	167±76.38	933±76.38	150±50.00	416±76.38
S 21	700 ±100.00	166±104.08	ND	566±76.38	ND	ND
S 23	1467±76.38	534±56.13	355±32.79	1883±125.83	150±50.00	428±30.14
S 31	ND	100±50.00	100±28.87	ND	ND	ND
S 33	534±56.13	ND	483±86.22	350±50.00	ND	183±76.38
S 39	1250±86.60	534±56.13	167±76.38	1083±76.38	ND	416±76.38
S 46	417±76.38	100±50.00	133±28.87	200±100.00	100±50.00	ND
G 02	ND	250±50.00	ND	166±57.78	366±28.87	ND
G 04	315±76.38	266±76.38	ND	250±50.00	ND	ND
G 06	417±57.74	734±76.38	315±76.38	400±86.60	ND	383±115.47
G 07	734±76.38	ND	ND	633±104.08	630±38.68	ND
G 21	150±50.00	167±38.19	283±76.38	ND	333±15.28	367±87.80
G 19	ND	150±50.00	483±86.22	ND	ND	333±76.38
G 29	266±38.19	ND	250±90.14	616±104.08	616±35.12	ND
G 36	183±57.74	216±41.63	ND	ND	350±20.00	450±75.50
G 38	ND	ND	355±32.79	183±28.86	583±37.86	ND
G 43	734±76.38	534±56.13		1283±76.38	630±38.68	416±76.38

Table 1: Intensity of infection of different helminth parasites in sheep and goats.

*Twenty samples (10 Goats and 10 Sheep) found positive in direct smear method were selected randomly for quantitative examination. S=Sheep, G=Goat, ND = Not detected.

This finding is justified by the report of Sangma et al., (2012) and Mazid et al., (2006), where they stated 81.1% and 94.67% helminth infection in sheep, respectively. In contrast, Khan et al., 2010 and Poddar et al., 2017 reported significantly and infection rate (44.2% lower 67.9%, respectively) in sheep. Seventy-eight percent (78%) helminthic infection of goat in this study is mostly similar to the findings of Islam et al., 2008 (74.55%). Although Hassan et al., (2011) mentioned the infection rate in goat was 63.41%. The variation in infection rate in sheep and goats may be due to the deviations in geoclimatic/environmental and demographic condition which might influence the growth, development, multiplication and fecal-oral transmission of helminths. The considerably higher infection rate in sheep (86%) than goat (78%) was observed under this study. Mostly similar findings were reported in India with the prevalence of 85.16% and 79.24% helminths in sheep and goats, respectively (Singh et al., 2017). In contrast, the prevalence of helminths was higher in goats than that in sheep in West India (98% and 88%) (Chikweto et al., 2018), Pakistan (78.2% and 78%) (Raza et al., 2014), and Bangladesh (77% and 65.9%) (Islam et al., 2017). The higher prevalence of helminthic infections in sheep than goats might be due to close to ground grazing behavior of sheep, thus the risk of ingestion of parasitic infective stage in comparatively higher than that of goats, as they are browsers (Lathamani et al., 2016).

Coprological examination detected eggs of six specific genera and one group of helminths in sheep with significant rate of infections (11-82%) (Fig 2,3). Previous studies in two different regions of Bangladesh reported Fasciola (8.4 and 81.39%), Paramphistomum and (13.2)61.14%). Haemonchus/strongyles (66.2 and 72%). Oesophagostomum (24.5 and 89.89%), Trichuris (1.9 and 2.1%), Strongyloides (9.5 to 12.3%) and Moniezia (3.8 to 28.69%) in sheep (Sangma et al., 2012; Poddar et al., 2017). Fasciola (66%), (49%), Paramphistomum Oesophagostomum (48%), Trichuris (38%), *Haemonchus* (65%), Moniezia (24%) and Strongyloides (26%) were also observed in goat in this study. Whereas Islam et (2008)stated Fasciola (14.28%), al., Oesophagostomum (4.01%), Trichuris (8.03%), Haemonchus (25.89%), Moniezia (16.52%) and Hassan et al., (2011) reported Fasciola (10.95%),

Paramphistomum (39.30%), Haemonchus (41.78%), Strongyloides (51.74%) infection in goat. Variation in climatic condition in diverse geographical location might have effect on infection status of different helminth. Availability of intermediate host, rearing condition, health status, grazing in low land area, hygienic management, lack of public awareness, host immunity are also important factors which might influence helminthic infections in small ruminants.

A number of studies reported that females were usually more susceptible to helminthic infection than male (Mazid et al., 2006, Wani et al., 2011). Current study also showed that in most cases female was relatively more susceptible to helminthic infection than male sheep and goat although the difference was insignificant (Fig. 3). In this study, 76% male sheep was infected with Fasciola whereas in female the infection rate was 86%. Similar infection pattern in male and female were also recorded in goat. Yeasmin et al. (2015) reported male sheep (81.5%) were more infected with helminths than female (72.7%) which is inconsistent with the current findings. Urguhart et al., (1996) mentioned that during parturient and peri-parturient period, females are more susceptible to parasitic infections due to the stress, alteration in the immune status and other physiological condition of the animals. On contrary, the limited grazing behavior of female during parturient period than males might be the cause regarding high infection rate in males than female (Gulland and Fox, 1992). Valcárcel and García et al., (1999) reported sex as the chief factor for influencing parasitic prevalence although Okafor et al., (1988) in Nigeria concluded that prevalence of helminth parasites was not related to sex.

Counting of eggs of 20 (10 sheep and 10 goats) fecal samples by McMaster technique determine the significant level of infection (Table 1). EPG of strongyles group of helminths was found highest (350-1833) and *Strongyloides* was lowest (100-150) in infected sheep samples. Other helminths also showed significant level of infection. In goat, EPG was highest also in case of strongyles group of helminths (166-1283) and lowest in *Moniezia* (250-315) in infected animals. Sangma et al., (2012) reported the highest EPG of *Oesophagostomum* (100-1500) and lowest EPG of

Trichuris (100-1000) in sheep which is not consistent with the present study. Zvinorova et al. (2016) stated the variation in EPG could be affected by factors such as study area, sampling criteria, age, season, rearing and management of animals examined, deworming status of the animals, variations in counting technique etc.

adult Six common helminth parasites (Fasciola, Paramphistomum, Oesophagostomum, Trichuris, Haemonchus., Moniezia) and three larval cestodes (Cysticercus taenuicollis, Coenurus cerebralis, Hydatid cyst) were collected and identified from different location of carcass of sheep and goats (Fig.5). We could not able to collect and identify Strongyloides which was detected in coprological examination. This might be due to limitation of collection and identification techniques or the animals slaughtered were not infected with Strongyloides. Mazid et al., (2006) identified 11 helminth species (Fasciola, Paramphistomum, Cotylophoron, Homologaeter, Gastrothylux, Schistosoma, Trichuris, Haemonchus, Oesophagostomum and two species of Moniezia) from sheep of Tangail district of Bangladesh. Hassan et al., (2011) and Islam et al., (2008) also reported different types of helminths including Gaigeria, Bunostomum and Cooperia those were not found in this study. The variation in presence of adult helminths in sheep and goats might be due to the effect of small sample size, differences in the laboratory technique(s) conducted, rearing and management practices, grazing habits, climatic conditions, unavailability of intermediate host, soil structure and the period, duration, and place of study (Nath et al., 2016; Moussouni et al., 2018). Three metacestodes (Cysticercus taenicollis, Coenurus cerebralis and hydatid cyst) were identified in sheep and goats during this study which is consistent with the report of Islam et al. (1995). Hailu et al., (2019) also recorded Coenurus cerebralis and hydatid cysts from ruminants slaughtered in Ethiopia. An abundant infestation of Cysticercus taenicollis in mesentery of slaughtered sheep and goat of Tunisia was also reported by Khaled et al., (2019). Further large-scale study regarding all aspects of helminthic infections in small ruminants is necessary to elucidate the actual scenario in the study area.

Conclusion

This baseline study demonstrated the prevalence of helminth parasites in sheep and goats of suburban areas of Rajshahi, Bangladesh and revealed the helminth infections are of great concern. Coprological examination detected eggs of five specific genera and a group of helminth parasites. Intensity of infection was significant in most helminth infections to cause subclinical or clinical diseases. Six adult helminths and three metacestodes were detected and identified from slaughtered animals. Thus, it's indeed a great necessity to overwhelmed helminthic infections through regular and strategic use of anthelmintic to minimize economic losses owing to helminthic diseases of sheep and goats.

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Authors' Contribution

Conceptualization, MZH and MR; Methodology, MZH, MMR, MZU, MR; Investigation, MZH, MMR, AK; Write-up: MZH, MMR, MR, SAH: Supervision, MR, LN, MIZM and MIA. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest: The authors declare no conflict of interests.

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