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Research Article

Factors associated with the hatchability of *Haemonchus* species eggs and molecular identification of *Haemonchus contortus*

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ABSTRACT

Haemonchus species is a common parasitic nematode infecting all ruminants and causing significant economic losses in developing countries. Accurate and reliable identification of various *Haemonchus* species and their molecular epidemiology is essential to formulate effective control strategies against Haemonchosis. The present study was carried out to identify larval stages of *H. contortus* in goat and sheep using morphological features and Polymerase chain reaction. A total of 400 abomasa were collected from different abattoirs for parasitological examination. Only female worms were stored for eggs isolation. The eggs were collected by macerating the whole female worms with adequate phosphate buffer saline (PBS) using a sterile mortar and pestle followed by filtering through a sieve. The collected eggs were counted by modified McMaster technique and incubated in different culture media such as phosphate buffered saline (PBS), normal saline (NS), tap water (TW) and distilled water (DW). The highest hatchability was obtained at PBS (57%) followed by NS (43%) and TW (35%) at fourth day of incubation at around neutral pH (7) and temperature 29°C. Distilled water, Acidic (pH<5) or Alkaline (pH>8) pH and high temperature (>35°C) suppressed the normal hatching rate. Different larval stages were morphologically identified according to their body length, tail and diameter in µm, mouth cavity and esophageal structure. 5-7% blood sera or glucose enhanced the hatchability upto 58.33% and other growth enhancer showed no favourable effects. The amplification of 265 bp from ITS-2 of rDNA of *H. contortus* was successfully conducted to facilitate their molecular identification.

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1. INTRODUCTION

Haemonchus contortus, well known as the barber's pole worm, is very common and one of the most pathogenic nematodes of ruminants (Easwaran *et al.* 2009; Graef *et al.* 2013). Adult worms attach to abomasal mucosa and feed on the blood. Females may lay over 10,000 eggs in a day which pass from the host animal to environment through the feces. After hatching *H. contortus* larvae molt several times. The L_1

stage usually occurs within 4 to 6 days under the optimal conditions of 24ºC–29°C ambient temperature and sheds its cuticle and develops into L_2 (rhabditiform) stage. Again L_2 stages of the larvae sheds its cuticle and physiologically develops into L_3 (filariform), the infective stage (Soulsby 1982). The sheep, goat and other ruminants are infected by ingesting these larvae when grazing. The L_3 larvae shed their cuticles and burrow into the internal layer of the abomasum where they develop into L_4 stage or pre-adult larvae usually within 48 hours. The L_4 larvae then molt and develop into L_5 or adult form. The male and female adults live in the abomasa and mate (Jacquiet *et al.* 1995). The egg is nearly 70–85 µm long by 44 µm wide and the early stages of cleavage contain between 16 and 32 cells. The adult female is 18–30 mm long and is easily recognized by its trademark "barber's-pole" coloration. The red and white appearance is because *H. contortus* is a blood feeder and the white ovaries can be seen coiled around the blood filled intestine. The male adult worm is much smaller about 10–20mm long and displays the distinct feature of a well-developed copulatory bursa containing an asymmetrical dorsal lobe and a Y-shaped dorsal ray (Easwaran *et al.* 2009).

The infection called Haemonchosis causes large economic losses of meat, milk and leather production and showed clinical signs due to blood loss. Sudden death may be observed only in acute infection. Other common clinical signs include pale mucous membrane, anaemia, edema, ill thrift, lethargy and depression. The accumulation of fluid in the submandibular tissue, a phenomenon commonly called "bottle jaw", may be seen. Growth and production are significantly reduced. This infection develops anthelmintic resistance day by day that reduce the socio-economic condition of the farmers around the world (Gasser *et al.* 1993; Gatongi *et al.* 1998; Dallas et al. 2000; Thrusfield 2005; Bott *et al.* 2009; Gharamah *et al.* 2012). The data on epidemiology of various worms are available as an aid to combat infections more effectively in most of the developed countries. In contrast, information on epidemiological data particularly on Haemonchosis is rare in developing countries like Bangladesh which is crucial for studying molecular epidemiology and to develop the control strategies. Moreover few data derived from coprological and morphometric studies were not reliable for further research.

So, Morphometric identification (both gross and microscopic) followed by molecular detection of environmental larval stages of *H. contortus* is important to know the concrete information about the biology of *Haemonchus* parasite, for species specific diagnosis and to develop the effective control strategies against Haemonchosis. Moreover, to study the biology, anthelmintic resistance assay (like egg hatch assay and larval migration inhibition assay) and molecular epidemiology, it is important to have laboratory hatched larvae for better and reliable results. Therefore, in this study, we focused on the effects of some factors associated with hatchability of *H. contortus* eggs. The ambient temperatures (25ºC-35ºC), pH (5-9) of the used media, various culture media such as phosphate buffer saline (PBS), normal saline (NS), tap water (TW) and some growth enhancer (blood, sera, glucose, table sugar) were considered in this study. So, the present research study was conducted to observe the morphometric changes in the free living stage of larvae of *H. contortus* hatched at various growth media and studied the effects of some factors associated with hatchability and provided with some accurate information for future research work.

2. MATERIALS AND METHODS

Study area and population

The present study was carried out on goat and sheep reared in various geographic regions (coastal, plain, hilly) of Bangladesh but slaughtered in local abattoirs of Chattogram Metropolitan area (The Chattogram city in Bangladesh; geographical coordinates: 22°21´94˝ North, 91°48´12˝ East). The abomasal samples were collected from the abattoirs situated at Jautala, Firingi bazar, Pahartali, Colonel hat and Halishahar wet market. A total of 400 abomasa were collected from the period January to December, 2015.

Study design and sample collection

Simple random sampling method was followed during sample collection from different abattoirs in Chattogram city. The both ends of each abomasum was ligated and put it in to a plastic zipper bag and then transported to Parasitology laboratory of department of Pathology and Parasitology of Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh.

Parasite collection and egg recovery

The adult worms were collected from the abomasa according to the protocol described by Hansen and Perry (1994) and Iqbal *et al.* (1993) with some modifications. The collected parasites were washed by normal saline (NS) and placed in sterile petri dishes. The worms (male and females) were identified by

observing their gross and microscopic features described by Soulsby (1982). Eggs were recovered from female *Haemonchus spp*. For this purpose, the female worms were washed , macerated using a mortar pastel. The recovered eggs from uterus were further filtered using through a sieve of 150 µm pore size then mixed with PBS and strained through a sieve and transferred into another container. Then, it was filtered through a tea strainer and was transferred into another container according to the procedure described by Belle *et al.* (1957); Rahman *et al.* (1975); Hubert and Kerboeuf (1992). Quantification of eggs were performed using the modified McMaster technique described previously by Thienpont *et al.* (1979) and the procedure described by Iqbal *et al.* (1993).

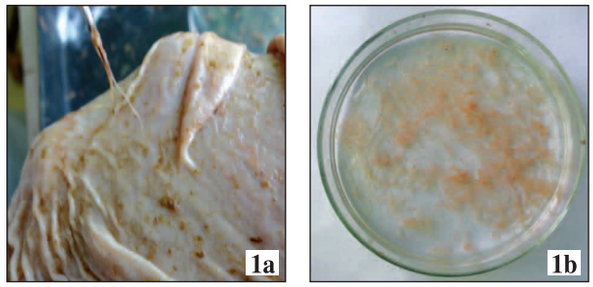


Figure 1. Collection of *Haemonchus* species from abomasum **(1a)** and worms were cultured at PBS in a sterile petri dish **(1b)**

Effect of liquid media on hatchability of eggs (experiment no. 1)

The suspension of the eggs was cultured in phosphate buffer saline (PBS), normal saline (NS), tap water (TW) and distilled water (DW) to observe their effects of them on hatchability. Total volume of egg culture was 10 mL that contains 2000 eggs. 300 µl of the eggs culture (that contain 60 eggs) from total volume of culture was taken on a glass slide and observed under microscope in every 6 hours interval. The hatchability was recorded in each observation.

Egg culture in various growth enhancers (experiment no. 2)

The effect of various growth enhancers was also reported during research study. The blood, sera, glucose and table sugar used as growth enhancer. These growth enhancers were added only with PBS media at 5%, 7% and 10% level. Because the maximum hatchability was recorded in PBS media from the previous experiment. Again, the eggs culture was examined under light microscope to observe the effect of growth enhancer on hatchability.

Effect of temperature and pH on hatchability (experiment no. 3)

We also observed the effect of different temperature (25°C-35°C) and pH (5-9) on the growth rate of *Haemonchus* species in PBS medium by the procedure as described by Iqbal *et. al.* (1993). The hatchability was recorded at the spreadsheets in each observation.

During the experiment no.1, 2 and 3, the L_1, L_2 and L_3 larval stages were observed under microscope and subsequently identified by their morphometric structures. Larval developmental changes were observed at 6^{th}, 12^{th} , 18^{th} , 24^{th}, 36^{th}, 48^{th} hour and 3^{rd}, 4^{th}, 5^{th}, 6^{th}, and 7^{th} days during incubation period by the procedure as described by Iqbal *et. al.* (1993). with some modifications. Average body length, total diameter and tail length of each larva (L_1-L_3) was measured using a calibrated microscope.

DNA extraction and conventional polymerase chain reaction (PCR)

Total genomic DNA was extracted from eggs, larvae and worms separately using G-spin™ Total DNA Extraction Kit (REF-17045, iNtRON Biotechnology, Korea) according to the manufacturer's instruction. Extracted DNA samples were stored at -20°C until use. A single step PCR was conducted to amplify the 265 bp of ITS-2 gene of nuclear rDNA of *H. contortus* using the forward primer, HAE-F (5´-CAA ATG GCA TTT GTCTTT TAG-3´) and universal reverse primer, NC2-R (5´-TTA GTT TCT TTT CCT CCG CT-3´) according to Dallas *et al.* (2000) and Bott *et al.* (2009). Each PCR was performed in a 20µl reaction, containing master mix = 10µl (iNtRON Biotechnology), Template = 2µl (above 30 ng/ µl), primer (F+R = 1 µl + 1 µl) = 2 µl (10 pmol/ µl), distilled water = 6 µl. The PCR was carried out in Thermal Cycler (2720 Thermal Cycler; Applied Biosystem). The cycling programme included initial denaturation at 94°C for 5 minutes and followed by 35 cycle of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extention at 72°C for 30 seconds and final extention at 72°C for 7 minutes. PCR amplicons were stored at 4°C temperature (Thrusfield 2005; Bott *et al.* 2009). The amplified PCR products were visualized in 1.5% agarose gel electrophoresis and compared with gene marker in UV light chamber.

Statistical analysis

All data were collected and recorded in MS excel sheet and analyzed using SPSS version 19. Chi-square test (χ^2) was done to evaluate the significant effects of associated factors with hatchability. The confidence level was 95% and $P<0.05$ was set for significance (Schwartz D. 1993).

3. RESULTS

Morphological identification of *Haemonchus* species

The collected *Haemonchus* species were examined by naked eye and then under microscope. In naked eye, "barber's pole appearance" of female worm was observed. The body was filliform (slender) shaped, tapering towards the anterior end in male and towards both ends in female. Male worm measured 10.65 mm in average length and 0.22 mm in width. Female worm was 21.33 mm in average length and 0.48 mm in width. Microscopically, the tail ends of male carried out a bursa. The bursa consists of three lobes (two lateral lobes and a poorly developed dorsal lobe). Vulvar flap was situated in the posterior third of the body in female worm. (Figure 2)

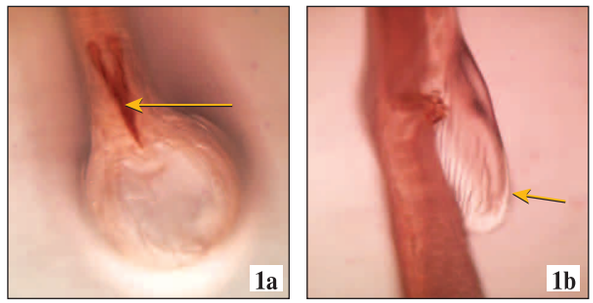


Figure 2. Identification of male and female *Haemonchus* species. Figure shows that the tail ends of male worm with a copulatory bursa **(2a)** and vulvar flap **(2b)** in female worm located at posterior third of the body.

Effects of culture media on hatchability

The effects of various media (such as PBS, NS, TW and DW) on the hatchability of eggs were presented in Figure 3. It was observed that the maximum hatchability (57%) was recorded in PBS followed by 43% in NS and 35% in TW at day 4 of incubation at 29ºC temperature. On the other hand, the hatchability (28%) in DW was comparatively lower than other media from the beginning of incubation (Figure 3).

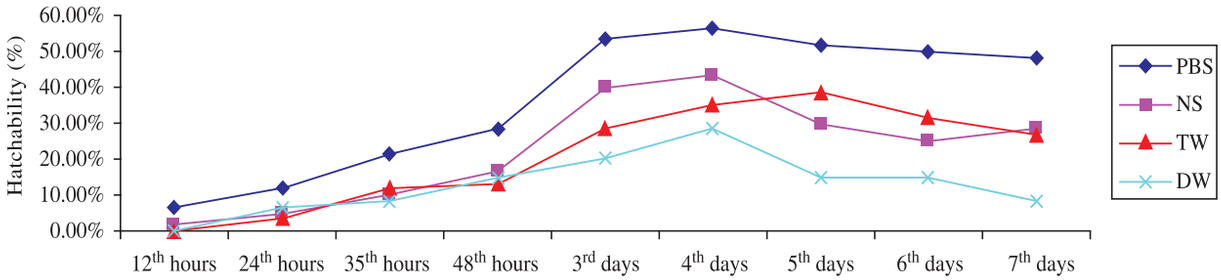


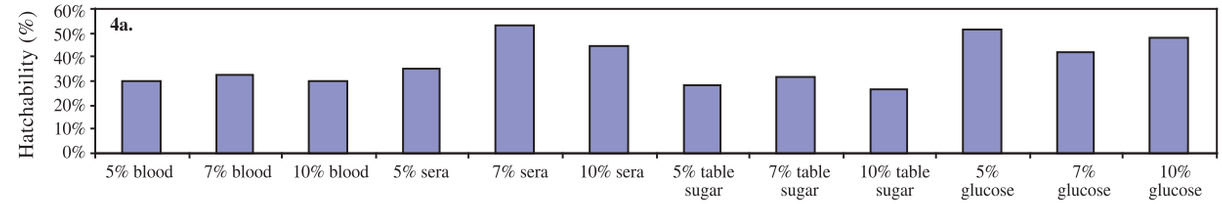
Figure 3. Hatchability of *H. contortus* eggs in various media (PBS, NS, TW and DW) at different time interval. Maximum hatchability was recorded at PBS at day 4 of incubation at 29°C temperature and the lowest hatching rate was observed at DW in same condition

Effects of growth enhancer on hatchability

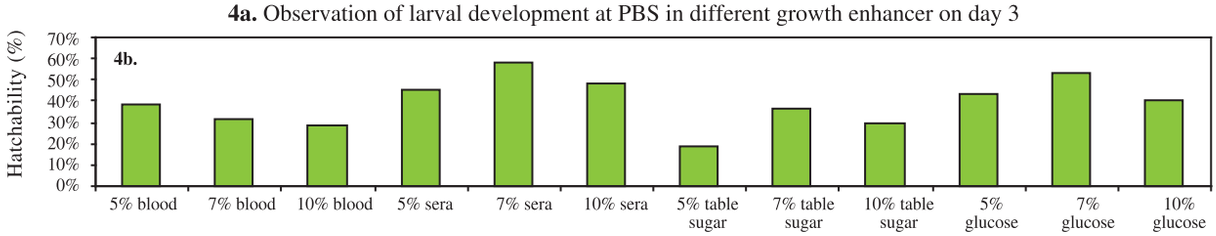
The growth enhancer had the variable effects on the hatchability. 5%, 7% and 10% of blood, sera, glucose and table sugar were used in PBS media. 7% sera and 7% glucose showed highest results, upto 58.33% and 53%, respectively at 29°C temperature at day 4 of incubation whereas 7% blood and 7% sugar enhanced the growth rate upto 32% and 37%, respectively. Other growth enhancer showed no extra effects on the hatchability (Figure 4).

Effects of pH of the culture media and ambient temperature

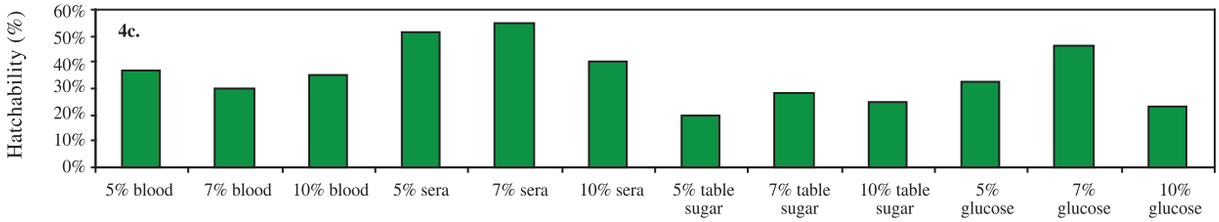
PH of the culture media and environmental temperature influenced the hatching rate of the eggs during observation as well. Neutral pH (7) and 29°C ambient temperature showed nearly 58.33% hatchability in PBS media and then 55% at 27°C and 48% at 25°C temperature at day 4 of incubation (Figure 5). The hatching rate in pH 6 (43%) and pH 8 (42%) was almost similar. The hatchability was relatively low at pH 5 and pH 9.



4a. Observation of larval development at PBS in different growth enhancer on day 3



4b. Observation of larval development at PBS in different growth enhancer on day 4



4c. Observation of larval development at PBS in different growth enhancer on day 5

Figure 4. Effect of different growth enhancer (**4a**, **4b** and **4c**) on hatchability cultured in PBS media at 29°C temperature

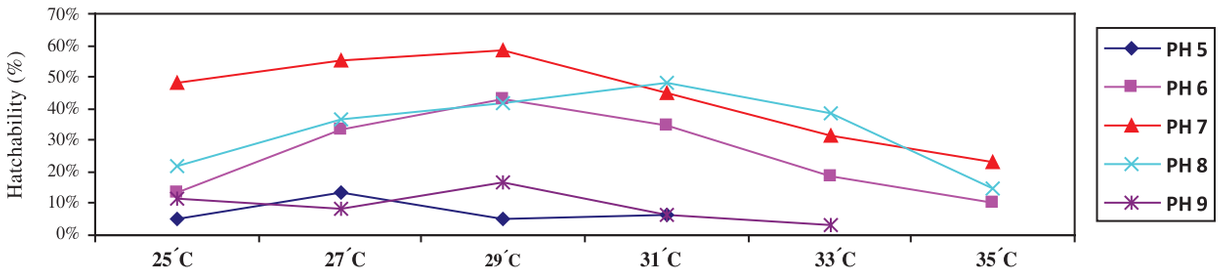
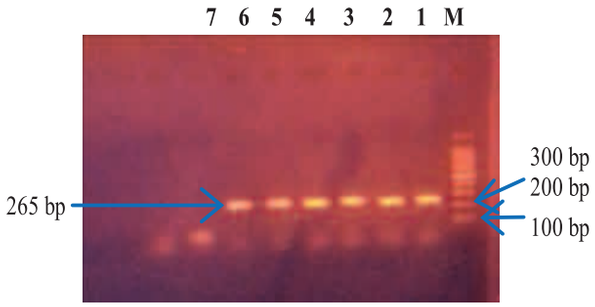


Figure 5. Effect of PH (5-9) and ambient temperature (25°C-35°C) on hatchability at PBS media.



Molecular identification of larvae using conventional PCR

1.5% of agarose gel displayed amplicon length produced by conventional PCR. A fragment of 265 bp of ITS-2 gene of nuclear ribosomal DNA was amplified (Figure 6).

Figure 6. 1.5% of agarose gel showing amplicons produced by conventional PCR using primers, HAE-NC2. A fragment of 265 bp of ITS-2 of rDNA was amplified. Here, Lane 1 = Positive control, Lane 2= L1 larva, Lane 3= L2 larva, Lane 4=L3 larva, Lane 5=adult worm, Lane 6= eggs, Lane 7 = Negative control, M = DNA Marker. The PCR amplicons of genomic materials extracted from L1, L2, L3 stage of larvae, adult male worm and eggs were loaded into the lane from 2 to 6, lane 1 and lane 7 was loaded by positive known samples and negative (without DNA materials) samples, respectively. The individual amplicons lengths in the lane from 2 to 6 were expected compared with known amplicons length of 265 bp of ITS-2 in lane 1. The lane 7 showed nil bands due to lack of desired genome

Table 1. Comparison among L_1, L_2 and L_3 stage of larvae based on their morphometric features under light microscope (10 x 10)

Parameters	L_1 stage	L_2 stage	L_3 stage
Stage	First free living stage	Second free living stage	Third free living stage
Size	Smaller	Moderate	Larger
Activities	Less active	Active	More active
Outer cuticles	Less visible	Visible	More visible
Esophagus shape	Slander	Rhabditiform	Filiariform
Mouth cavity	Less visible	Visible	More visible
Visceral part	No/less visible	Less visible	More visible
Average body length (µm)	340.00	522.30	672.30
Average tail length (µm)	38.30	60.00	55.00
Average diameter (µm)	18.30	28.30	28.33
Tail end	Somewhat blunted/pointed	Less pointed	More pointed

4. DISCUSSION

In this study, there was notable effect of some selected factors on the hatching rate of larvae of *H. contortus*. Detection at the species level along with various larval stages was confirmed. "Barber's pole appearance" of female worm was observed. The body was filliform (slender) shaped. Male worm measured 10.65 mm in average body length and female worm was 21.33 mm. The tail ends of male carried out a bursa and "vulvar flap" was situated in the posterior third of the body in female. These findings were similar with Soulsby (1982). He found that male worm were 10-20 mm in long and female were 18-30 mm in long. Barberpole appearance and vulvar flap in female were also more visible in his observation. While comparing hatchability in different media and supplements, the maximum hatchability was recorded at PBS (57%) followed by NS (43%) and TW (35%) at 4 days of incubation in 29°C. The hatching rate was very negligible in DW (28%) which might be due to lack of nutrient component or mineral of the culture media. Ashad *et al.* (2011) found that the maximum hatchability in PBS was 40% at day 5 of incubation in 26°C and 25% hatchability was recorded in TW at same condition. The hatching rate of the larvae was increased when additional growth supplements were mixed with culture media. 7% sera with the PBS media performed the best hatching rate (58.33%) followed by 7% glucose (53.33%) at day 4 of incubation in 29°C. On the other hand, Ashad *et al.* (2011) found 42% hatching rate when he added 15% blood sera with PBS at same condition. Our hatching rate was relatively higher than the findings of Ashad *et al.* (2011). It might be due to ensure a balanced environment and fresh nutrients of the culture media. Paul (1965) used liver extracts (0.5%) and vitamins with culture media and also observed an elongated survival period (9 days) and high hatchability of the larvae *H. contortus.*

Temperature and pH also played a vital role in their hatchability. It was observed that the hatchability increased gradually when temperature of the incubator was increased from 25°C to 29°C at pH 7. The highest hatchability was reported in 29°C (58%) followed by 27°C (55%) at day 4 of incubation. On the other hand, Ashad *et al.* (2011) and Moinuddin *et al.* (2011) reported that maximum hatching rate of *H. contortus* and *Oesophagostomum columbianum* was 39% and 40%, respectively at pH 6.5 in 26°C at day 6 and no development of eggs were seen at 4°C and low pH (<3) during the 15 days of incubation. It might be due to cold shock that squeezes the germinal mass of the gravid eggs. Soulsby (1982) recorded that lower temperature retarded the development of the eggs and very few eggs hatched below 9°C. Laha *et al.* (2000) observed 37.55% hatching by 48 hours at 25°C. We found that the hatching rate was gradually decreased from 31°C and sharply decreased at 35°C. Hernandez *et al.* (1992) and Tembely (1998) found that the eggs failed to develop and hatchability was lowest at 37°C. The mentioned conditions might be arrest the embryonic developmental process and also killed the larvae after hatching. Surprisingly, we observed the

highest hatching rate at neutral pH (7) compared to other authors. It might be occur due to available of fresh nutrients in the media, good surrounding ambient condition and survival power of the gravid eggs. Sommerville and Murphy (1983) observed that eggs failed to hatch at low (<3) or high (>10) pH due to disfunction of enzymatic reaction. Stringfellow (1986) and Misra and Ruprah (1973) observed the maximum hatching (54%) at pH 6.5-8.5 in research work.

The L_1, L_2 and L_3 larval stages were detected separately on the basis of their morphometric features under light microscope followed by molecular detection. The L_1 stages of larva were smaller, less active and outer cuticle was not visible or less visible. The L_2 was more active, outer cuticle was visible and esophageal shape was rhabditiform. The L_3 was more active, outer cuticle was more visible compared to other larval stages. The esophageal shape of L_3 stages of larvae were filiariform. The tail sheath of L_3 was pointed. The average body length (µm) of L_1, L_2 and L_3 stage of larvae was 340, 522 and 672, respectively. We also measured the average tail length of the among stage of larvae and it was 38, 60 and 55 in µm in case of L_1, L_2 and L_3 stage, respectively. Dikman and Andrews (1933) provided key information about the infective nematode larvae (L_3) of sheep and goat. Whole body length and tail length of the L_3 larvae was 650-751 µm and 54-68 µm, respectively in his observation. We observed the L_1 stage after 12 hours of incubation. Verlia (1916) observed the L_1 larvae from the gravid eggs within 15 hours. The L_2 stage of larvae was detected from 2 to 3 days of incubation in same condition. The L_3 stage was also found after 3 days of incubation. Mizelle and Berberian (1953) reported that the L_2 and L_3 stages of the larvae of *H. contortus* observed at day 3 or 4 of incubation in 35°C at the laboratory research. A product of 265 bp from ITS-2 of rDNA was amplified using HAE-F and NC2-R primer to identify the *H. contortus*. Tiong *et al.* (2014) and Nathan *et al.* (2009) used both primers for molecular diagnosis of the *H. contortus*.

Finally, we detected the free living stages of larvae of *H. contortus* at molecular level that was the first molecular detection in Bangladesh. Our findings on the hatchability were also more favourable and higher than other authors regarding some selected factors. This study will very helpful for larvae culture to trial the anthelmintic resistances. However, modern and sophisticated instruments were not available during study. Continuous supervision was hardly possible. Further research study in this area may necessary to improve and extend the technology for egg culture within short period from which we can obtain our desired volume of larvae for anthelmintic resistant trial as well as early detection of drug resistant gene.

5. CONCLUSIONS

The amplification of 265 bp from ITS-2 gene of the nuclear rDNA of *H. contortus* was successfully conducted to facilitate molecular detection of each larval stage. The highest hatchability (58.33%) observed in PBS media along with 7% sera or 7% glucose at 29°C ambient temperature in neutral pH (7) at research laboratory. It was the first molecular detection of *H. contortus* in Bangladesh. These findings have vital application for studying the molecular epidemiology and control strategies of Haemonchosis.

6. COMPETING INTEREST

The authors declare that they have no any competing interest.

7. ACKNOWLEDGEMENT

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