

In vitro* study of environmental and nutritional factors on the hatching and development of eggs of *Haemonchus contortus

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Abstract

To determine the *in vitro* effects of environmental and nutritional factors on the development and hatching of eggs of *Haemonchus contortus*, eggs were cultivated at different temperatures, humidity and pH, in phosphate buffered saline (PBS), tap water, or normal saline, with and without faecal material and soil. No development of eggs occurred at 4°C up to 15 days. Hatching was maximum (40.7%) at 30°C on Day 5. Maximum hatching (39.5%) was observed on Day 6 at pH 6 and hatching was minimum (3.3%) on Day 4 at pH 3. Hatching did not occur at pH 2. At a relative humidity of 80 - 90%, maximum hatching (42.2%) was recorded on Day 5. Light had no effect on development and hatching. Maximum proportion of eggs hatched in PBS containing faecal material (40.1%). It is suggested that PBS containing faecal material may be used for the incubation of eggs of *Haemonchus contortus*. Best results may be obtained at 30°C, pH 6 and relative humidity 80 - 90%. (*Bangl. vet.* 2013. Vol. 30, No. 1, 1 - 9)

Introduction

The hot humid climate of Bangladesh greatly favours the development and survival of parasitic nematodes. *Haemonchus contortus* is the most important nematode parasite in small ruminants, causing severe anaemia and high mortality. The percentage of infestation in different species of *Haemonchus* ranged from 50 - 85% (Qadir, 1967). The principal feature of haemonchosis is anaemia, where the adult and the fourth larval stage suck hosts' blood. The average blood loss has been calculated as 0.05 mL/ parasite/ day (Clark *et al.*, 1962). Haemonchosis predominantly occurs during the rainy season all over the world (Travassos *et al.*, 1974; Gupta *et al.*, 1987). Qadir (1981) reported that the peak of infestation extended from June to September in Bangladesh. The pre-parasitic development of *Haemonchus contortus* in pasture is typical of trichostrongyles and depends upon the environmental temperature and moisture. Infective L₃ larvae develop within 4 - 6 days in a suitable environment. The optimum temperature for development is 27°C with little or no development below 9°C (Soulsby, 1982). Developmental time varies between regions depending on prevailing weather. The eggs laid by the female *Haemonchus contortus* are passed in the faeces. First-stage larvae (L₁) hatch and develop to second-stage (L₂) and third-stage larvae (L₃). The L₃ are free-living and able to survive for two weeks depending on

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environment and do not feed and moult until they are ingested by a suitable definitive host.

Laboratory culture of L₃ is considered useful for parasitological, immunological and chemotherapeutic studies. Although the factors influencing the hatching of eggs and survival of larvae have been thoroughly studied in natural conditions, precise reports are not available in laboratory conditions. The present study was designed to find-

- The effects of temperature, pH, humidity and light on the hatching of eggs and the development of larvae of *Haemonchus contortus*.
- Suitable media for the incubation of eggs
- Effects of nutrients (faecal material and soil) on the development and hatching of eggs

Materials and Methods

The study was carried out from November 2011 to April 2012. Abomasa of goats were collected from Kamal Ranjit Market of Bangladesh Agricultural University (BAU), Mymensingh.

Female parasites were crushed using pestle and mortar containing phosphate buffered saline (PBS). Eggs were counted by modified McMaster technique (Thienpont *et al.*, 1979) using McMaster counting chamber.

Total number of eggs/mL = (Total eggs × 10)/0.3.

Study of the effects of temperature

Eggs were incubated at 35°C, 30°C, 20°C, 10°C and 4°C. To achieve 4°C, the culture media were kept in a refrigerator. A counted number of eggs were suspended in a petridish containing PBS. The petridishes were kept in an aluminium tray covered with a thin layer of moist cotton to prevent the evaporation of culture media. They were incubated up to 7 days and examined regularly at 24 hour intervals using a dissecting microscope. A drop of culture media was examined to see the development of eggs and the larval stages (L₁, L₂ and L₃). After 15 days observation, the culture media were kept at room temperature to record the further development as described by Soulsby (1982).

Study of the effects of pH

To study the effects of pH, the pH of the culture media were adjusted to 2, 3, 4, 5, 6, 7, 8 and 9 by adding hydrochloric acid (HCl) into PBS drop by drop with the help of micropipette while stirring the media. The pH was detected by pH meter. Observations were made every 24 hours up to seven days using a dissecting microscope.

Study of the effects of humidity

To determine the effects of humidity a counted number of eggs was mixed with PBS and incubated at 70 - 80% and 80 - 90% relative humidity at constant 30°C. Hygrometer was used to measure the relative humidity in the incubator. Humidity was controlled by adding water in the petridishes. Developmental stages were observed by dissecting microscope at 24 hour intervals up to six days.

Study of the effects of light

The culture media with a counted number of eggs were covered with black cloth to exclude light but facilitate air exchange, at room temperature. Control media (PBS) was kept at room temperature in light. Observations were made in similar manner up to seven days.

Study of the effects of different media

To find suitable media for the cultivation of eggs of *Haemonchus contortus*, a counted number of eggs was cultured in PBS, normal saline and tap water and incubated and observed as previously described.

For the preparation of nutrient media, faeces or soil were added to the PBS. A counted number of eggs was suspended in each culture medium and incubated at room temperature up to six days. The percentage of hatching eggs was calculated every 24 hours.

Statistical analysis

Student t-test was used to determine the significance of differences between variables (Steel and Torrie, 1980).

Results and Discussion

Effects of temperature

There was no development of eggs at 4°C up to 15 days. Mizelle and Berberian (1953) recorded that 4°C was lethal to the ova of *Haemonchus contortus*. Tripathi (1980) observed no development of eggs at 4°C for 30 days. Miro *et al.* (1991) failed to collect larvae from eggs incubated at 4°C and these did not develop when returned to warmer temperatures. The exact mechanism of inactivation of eggs at 4°C could not be explained. However, it was observed that germinal mass was squeezed. Possibly low temperature caused injury to the germinal mass.

At 10°C eggs started to hatch on Day 4 and at 20°C on Day 3, and at 30 and 35°C eggs from Day 2. Hatching continued up to Day 5. The proportion of eggs hatching was maximum (40.7%) at 30°C at Day 5. This finding conformed to the findings of Soulsby (1982) who recorded that low temperature retarded the development of eggs and below 10°C little or no development took place. Islam and Ahmad (1987) recorded the hatching of eggs on Day 11 at 10°C. At 20°C, hatching of the eggs started at Day 3 and

16.5% developed to infective stage by Day 7, similar to the findings of Misra and Ruprah (1973b) who recorded 10-15% at 17 - 19°C in outdoor grass plot in Hissar, India.

Moulting started from Day 4 except at 30°C and 35°C where moulting started from Day 3. Maximum number of L₂ (29.1%) were observed on Day 5 at 30°C and the highest number of L₃ (26.8%) on Day 7 at 30°C. The number of larvae decreased gradually from Day 6. First moulting was slightly delayed at 10°C, at Day 5 (Table 1). Conway (1964) and Narain and Chowdhury (1979) recorded Day 4 for the hatching of eggs at 15 - 17°C, and these attained L₃ stage. At 30°C, 15.3% eggs hatched at Day 2 and maximum 40.7% of eggs hatched at Day 5 and larvae attained infective stage at Day 4. Rahman *et al.* (1996) found that eggs hatched within 1 - 2 days at 26°C and reached infective stage within 3 - 4 days. In contrast Laha *et al.* (2000) recorded 17.2%, 37.6% and 24.8% of larvae at 24, 48 and 72 hours, respectively, at 25 - 30°C. Embryonation ceased after 72 hours of incubation. This might be due to the media and procedure. Shahiduzzaman *et al.* (1999) found that hatching of eggs of *Haemonchus contortus* required 4 days at 20 - 30°C. L₁ to L₂ development required 4 to 12 days. Moulting of L₁ to L₂ required a mean of 13.5 days. The percentage of infective larvae was 26.2% at Day 8, whereas Shahiduzzaman *et al.* (1999) recovered 24.7% of infective larvae at 20 - 30°C. Islam and Ahmad (1987) recorded the hatching of larvae by second day in laboratory at 20 - 30°C. Eggs reached L₃ stage by four days. Tripathi (1977) found that 20 - 30°C was the most suitable for hatching and development of eggs of *Haemonchus contortus*. Similarly Misra and Ruprah (1973a) found that 22 - 35°C was suitable for development and survival of pre-parasitic stages of *Haemonchus contortus*.

At 35°C, the hatching of eggs and the percentage of infective larvae were markedly depressed. Narain and Chowdhury (1979) similarly recorded 4 - 8 days at 37°C for hatching and found that few larvae attained infective stage. Tripathi (1977) observed some eggs hatched at 40°C within 24 hours but few became infective larvae. Islam *et al.* (2005) found that most of the eggs did not hatch and L₃ did not form at 40°C. Hernandez *et al.* (1992) found the larval development was low and sporadic over hot and dry summer period. It is suggested that the high temperature prevents embryonic development and can kill the larvae once they are embryonated.

Effects of pH

Hatching did not occur at pH 2. Hatching started from Day 3 except at pH 3 and 4, when hatching started from Day 4: at pH 6 hatching started on Day 3. The proportion hatching was minimum (3.3%) at pH 3 on Day 4 and maximum (39.5%) at pH 6 on day six (Table 2). The failure of hatching at pH 2 might be due to coagulation of the germinal mass. Sommerville and Murphy (1983) similarly observed the failure of hatching of eggs at pH 2, due to inactive enzymes in unsheathing fluid. A negligible number of eggs (3.3%) hatched at pH 3. Best results were 39.5% at pH 6, at Day 6. Stringfellow (1986) similarly obtained the best hatching at pH 6.4. Sommerville and Murphy (1983) recorded exsheathing of larvae at pH 6. Misra and Ruprah (1973a)

found the maximum hatching of eggs and the optimal development of *Haemonchus contortus* occurred at pH 6.5 to 8.5 in laboratory conditions, similar to the present findings. On the other hand, hatching was markedly reduced at pH 9, which might be due to the denaturation of the egg mass in alkaline pH.

Table 1. Effects of temperature on the development and hatching of eggs of *H. contortus*

Temperature (°C)	Hatching eggs (%)					Development of larval stages (%)										
	D1	D2	D3	D4	D5	D3		D4		D5		D6		D7		
						L2	L3	L2	L3	L2	L3	L2	L3	L2	L3	
4																
10				6.0	8.0a					2.5		5.2	3.4	1.9		4.1a
20			19.1	24.4	31.2b			11.3		18.9	4.7	9.6	12.2	2.0		16.5b
30		15.3	29.2	33.3	40.7b	17.6		22.0	9.0	29.1	14.0	13.3	19.1	6.1		26.8ab
35		7.0	12.8	22.8	20.5ab	14.9		16.5	5.6	23.5	12.3	13.3	2.7	9.8		10.1b

Values in the same row having different superscript differ significantly ($P < 0.05$)

Table 2. Effects of pH on the hatching of eggs of *Haemonchus contortus*

Observations	Proportion of eggs hatching								
pH	2	3	4	5	6	7	8	9	
Day 1	-	-	-	-	-	-	-	-	
Day 2	-	-	-	-	5	-	-		
Day 3	-	-	-	22.0a	23.0a	20.0a	18.6b		4.1ab
Day 4	-	3.3a	7.2b	23.5a	29.4a	23.1a	20.9b		7.3ab
Day 5	-	5.8a	12.3b	25.3a	32.3a	28.8a	23.0b		10.2ab
Day 6	-	8.1a	13.5b	25.1a	39.5a	34.5a	-26.2b		
Day 7	-	8.9a	17.0b	-	-	-	-		

Values in the same row having different superscript differ significantly ($P < 0.05$)

Effects of humidity

Hatching of eggs started at Day 3 at relative humidity 70 - 80% and 80 - 90% at 30°C. Hatching ceased after Day 6 in relative humidity of 70 - 80% and was one day earlier in case of relative humidity of 80 - 90%. About 24% eggs hatched at Day 4 at humidity of 70 - 80% compared to 35% in humidity of 80 - 90%. Maximum (42.2%) hatching was recorded at relative humidity 80 - 90% at Day 5 (Table 3). These results conform to the findings of Rahman *et al.* (1996) who found 80% relative humidity was favourable for hatching eggs of *Haemonchus contortus*: infective stage was reached within 3-4 days. Levine and Todd (1975) found more than 50% of the eggs developed into infective stage in 90% humidity in laboratory. This differed from Shahiduzzaman *et al.* (1999) who recorded 24.7% infective larvae at 86% relative humidity: hatching

required 2.5 to 7.5 days. Krecek *et al.* (1992) showed that as relative humidity increased, the number of larvae increased, in pasture. Fernandez *et al.* (1994) showed a significant difference in the time of development from eggs to infective larvae with respect to the seasons of the year: the highest recovery of first and second stage larvae obtained at the end of rainy season when the humidity was high.

Table 3. Effects of humidity on the hatching of eggs of *Haemonchus contortus*

Observation	Proportion of eggs hatching	
	70- ≤ 80	≥ 80-90
Relative humidity (%)		
Day 1	-	-
Day 2	-	-
Day 3	20.1a	31.2b
Day 4	23.7a	35.0b
Day 5	29.1a	42.2b
Day 6	34.5	-

Values in the same row having different superscript differ significantly ($P < 0.05$)

Effects of light

There was no variation in dark and light condition for the development and hatching of eggs of *Haemonchus contortus*. Hatching started at Day 3 in both dark and light conditions and ceased after Day 5 (Table 4). Similar finding was observed by Islam and Ahmad (1987).

Table 4. Effects of dark and light on the hatching of eggs of *Haemonchus contortus*

Observation	Proportion of eggs hatching	
	Dark	Light
Condition		
Day 1	-	-
Day 2	-	-
Day 3	25.5	26.3
Day 4	28.8	30.2
Day 5	33.3	34.0
Day 6	-	-

The differences are not statistically significant ($P > 0.05$)

Effects of media

Hatching of eggs began at Day 4 in normal saline whereas at Day 3 in PBS and tap water. Hatching occurred at Day 2 in PBS mixed with faecal material or soil. The proportion hatching was higher (31.8%) in PBS with faecal material followed by PBS with soil followed by PBS, tap water and then normal saline. Hatching continued up

to 5 days. The highest proportion hatching was 40.1% in PBS containing faecal material and the lowest was 12.2% in normal saline, at Day 5. The number of larvae decreased gradually from Day 6 (Table 5).

Of the three culture media used, the highest proportion of eggs hatching was in PBS (33.5%) followed by tap water (24.7%) and normal saline (12.2%) at Day 5. Of the media containing nutrients, the best results were in PBS containing faecal material, in which 20.2% hatched at Day 2 and 40.1% by Day 5. Percentage of eggs hatching, survival and longevity of the larvae were higher in the media containing faecal material or soil. These conform to the findings of Paul (1965). The present study suggests that PBS containing faecal material and PBS containing soil may be suitable for the incubation of eggs of *Haemonchus contortus*. Temperature and humidity were important factors for *in vitro* cultivation of eggs of *Haemonchus contortus*.

Table 5. Effects of media on the hatching of eggs of *Haemonchus contortus*

Media	Proportion of eggs hatching					
	D1	D2	D3	D4	D5	D6
Day of observation						
Normal saline (NS)	-	-	-	9.3	12.2a	10.3
Phosphate Buffered Saline (PBS)	-	-	28.0	30.7	33.5b	26.8
Tap water	-	-	17.8	21.7	24.7ab	19.5
PBS + feces	-	20.2	31.8	38.0	40.1a	37.5
PBS + soil	-	16.8	28.3	32.4	38.7a	34.3

Values in the same row having different superscript differ significantly ($P < 0.05$)

Conclusions

Temperature, humidity, pH and light were important factors for *in vitro* cultivation of eggs of *Haemonchus contortus*. These factors influenced the development and hatching of the eggs. Media containing nutrients (PBS + faeces) improved the development and hatching of eggs.

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