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FACTORS AFFECTING THE DEVELOPMENT AND HATCHING OF EGGS AND THE SURVIVAL OF INFECTIVE LARVAE OF *HAEMONCHUS CONTORTUS* IN LABORATORY CONDITION

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ABSTRACT

Haemonchus contortus Rudolphi, 1803, a blood-sucking worm living in abomasum, poses possibly the greatest threat to the livestock production. Here, we studied the effects of various environmental and nutritional factors on the development and hatching of eggs, development and survival of larvae of *H. contortus*. We incubated eggs at different temperature gradients, humidity and pH in phosphate buffer saline (PBS), tap water (TW) and normal saline (NS) using serum and/or liver extract at different concentrations. We obtained maximum hatching of eggs (39.73%) at 26°C by day 5. Moulting started from day 4 and maximum number of L_2 (23.11%) developed at day 5 at 26°C and the highest number of L_3 (26.81%) were detected at day 7 at 26°C. No developments of eggs were observed, and hatching did not occur at pH 2. Better hatching of eggs (43.84%) were recorded in the relative humidity (RH) of ≥ 80-90%. Light had no significant effects on the development and hatching of eggs of *H. contortus*. Maximum eggs hatched in PBS containing 15% serum and 10% liver extract. Besides, in this medium survival rate of larvae was the highest (39.51%). The present study suggests that PBS containing 15% serum and 10% liver-extract may be used as a suitable medium for the incubation of eggs of *H. contortus.*

Key Words: Helminth, *Haemonchus contortus,* Egg, Larvae, Culture media

INTRODUCTION

Agro-ecological and geo-climatic conditions of Bangladesh are highly conducive to the growth and multiplication of a large number of parasitic species. Gastrointestinal parasitism is one of the most important disease complexes of sheep and goats impacting markedly on the livestock health and production. *Haemonchus contortus* Rudolphi 1803, a blood-sucking worm of the abomasum, poses possibly the greatest threat. The percentage of infection with different species of *Haemonchus* ranged from 50-85% (Qadir, 1967). *H. contortus* is the most pathogenic of all nematodes recorded in Bangladesh. The preliminary factors that affect the development and survival of eggs and larvae are temperature and moisture although different parasites vary in their ability to survive in extremes of temperature and humidity. Laboratory informations about the effects of temperature, pH, humidity, light and nutrients on the hatching of eggs and survival of *H. contortus* are more

essential to study the biology and ecology of a parasite. By considering these points the present research work has been designed to investigate the effects of various environmental and nutritional factors on the development and hatching of eggs, and survival of larvae of *H. contortus*.

Materials and Methods

Collection of Abomasii

We collected abomasii from slaughter houses in Mymensingh district. During collection, each and every abomasum was ligated at both cardiac and pyloric ends and carefully separated from mesenteric and other visceral attachment and brought to the laboratory of Parasitology, Bangladesh Agricultural University, Mymensingh taking in plastic bags.

Collection of parasites

Parasites were collected from abomasii following the standard procedure as described by Rahman (1969). Parasites were identified and sexes were differentiated by examining the morphological characteristics following the keys and description given by Soulsby (1965).

Recovery of eggs

Eggs were recovered directly from the gravid *H. contortus*. For this, female parasites were crushed using sterile mortar and pastle containing necessary amounts of PBS. Debris was picked up with sterile needles, and the contents were sieved. The suspension was gently stirred to make uniform solution and eggs were counted by modified McMaster technique (Thienpont *et al.*, 1979).

Study of the effects of different media

To find out the suitable base of the culture media, we incubated pre-counted eggs in phosphate buffer saline (PBS), normal saline (NS) and tap water (TW) at 26^{o}C and pH 6.5 in 80-96% Relative Humidity.

Study of the effects of temperature

To study the effects of temperature, eggs were incubated at various temperature gradients (4-37^{o}C). Identification of different stages of *H. contortus* larvae was made on the basis of morphological features described by Anon (1977), Soulsby (1982) and Rahman *et al.* (1996). Different stages of larvae were detected and percentage was counted.

Study of the effects of pH

To study the effects of pH, we incubated eggs over a pH range of 2.0-11.5 using the following buffers, HCl/KCl for pH 2, sodium citrate for pH 3-7, Tris-HCl for pH 7.5-9 and sodium carbonate-bicarbonate for pH 10-11.5. Observations were made with a dissecting microscope at every 24 hours for 7 days.

Study of the effects of humidity

To determine the effects of relative humidity on the hatching of eggs, and survival of infective larvae, eggs were suspended in PBS and incubated at 70% - ≤80% and ≥80% - 90% RH at constant 26°C temperature. Developmental stages were observed by dissecting microscope at every 24 for 7 days.

Study of the effects of light

To study the effects of light, eggs containing culture media was covered with black cloth to make dark condition having facilities for air exchange and incubated at room temperature. As a control, eggs were incubated in the same media in room temperature exposing to normal light, and observed in similar manner up to 8 days.

Study of the effects of different nutrients

Serum (5, 10 and 15%) and liver extract (5, 10 and 15%) were used to study the effects of nutrients. Combination of serum and liver extract were also tested in similar condition.

Statistical analysis

Student t-test was used to determine the statistical significance with the relevant control.

RESULTS AND DISCUSSION

Maximum eggs hatched in PBS

Of the three culture media used, maximum eggs hatched in PBS (35.1%) followed by TW (24.7%) and NS (13.1%) at day 5. We found that 22.6% infective larvae survived in PBS whereas in TW and NS, 7.9% and 3.5% larvae survived, respectively at day 8. L_3 developed within 8-10 days (Tables 1 and Fig. 1). Mizelle and Berberian (1953) reported that L_3 of *H. contortus* developed within 6.5 days in the laboratory in faeces detection. Veglia (1916) observed that L_1 hatched from eggs within 14-17 hours. The variation in development and hatching of eggs may be due to the media and other condition applied.

Table 1. Effects of media on the hatching of eggs and the survival of larvae of *H. contortus*

Media	Observations (%)										
	D_1	D_2	D_3	D_4	D_5	D_6		D_7		D_8	
						*D	*L	D	L	D	L
NS	-	-	-	10.1	13.1[a]	8.1	5	8.5	4.6	9.6	3.4[a]
PBS	-	-	30.0	32.5	35.1[b]	3.2	31.9	7.8	27.3	2.7	22.6[b]
TW	-	-	20.5	22.4	24.7[ab]	4.3	20.4	9.8	14.9	16.8	7.9[a]

Values in the same column having different superscript are statistically significant ($P<0.05$). *D = dead, *L = live

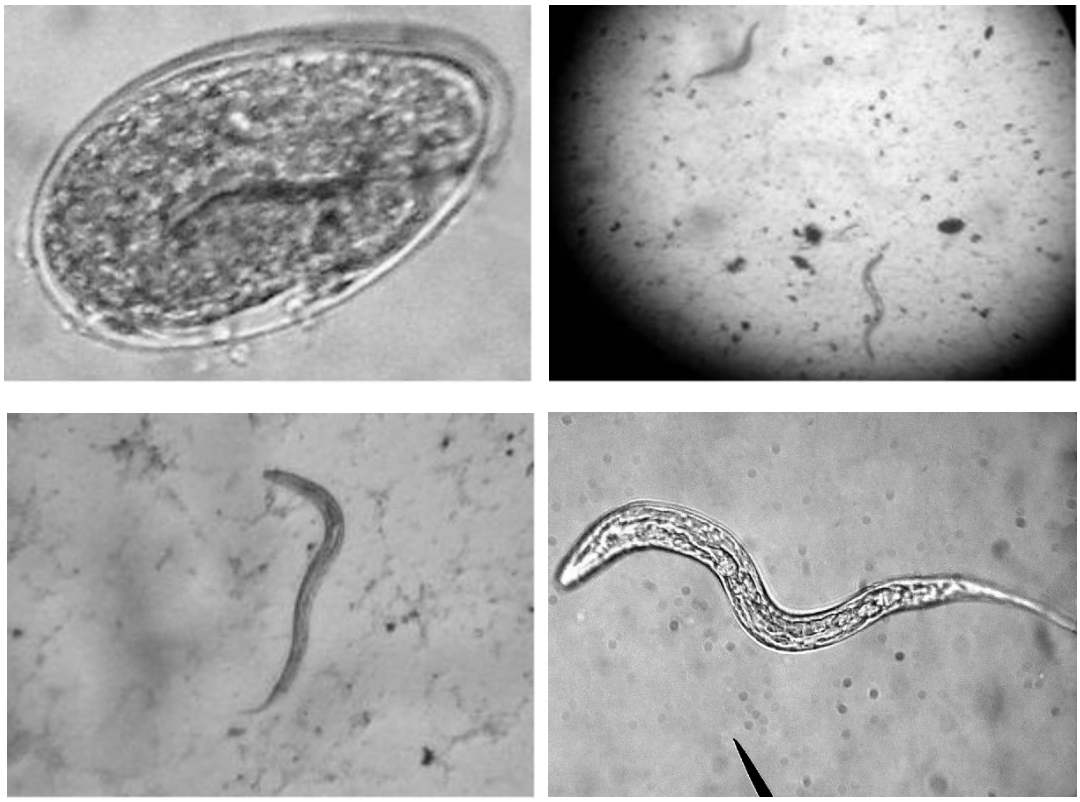

Fig. 1. Eggs and different larval stages of *H. contortus.* We incubated eggs in phosphate buffer saline (PBS) using 15% serum and 10% liver extract at pH 6.5 and 26ºC in 80-96% relative humidity. (a) a developing egg of *H. contortus*, (b) L_1, (c) L_2 and (d) L_3 of the parasite

Temperature 26 ºC is optimum for the hatching of eggs of H. contortus

We observed that developmental changes in eggs of *H. contortus* were arrested at 4 ºC, and failed to develop even when eggs were returned at room temperature (15-20ºC). Detail in the Table 2. Tripathi (1980) and Miro *et al.* (1991) also failed to collect larvae from the culture incubated at 4ºC, and reported that the larvae did not develop when the eggs were returned to warmer conditions. The exact mechanism of inactivation of eggs at lower temperature is not known but may be due to cold injury which squeezed the germinal mass, and the eggs were devitalized. At 9ºC hatching of eggs delayed, which conformed to the findings of Soulsby (1982) who recorded that low temperature retarded the development of eggs, and below 9ºC very few eggs developed. At day 8, only 6.6% larvae survived. Shorb (1944) observed that survival rate of larvae decreases in cold. At 17ºC hatching of the eggs started at day 3, and 17% eggs developed to infective stage. Conway (1964) and Narain and Chowdhury (1979) found that eggs of *H. contortus* start to hatch at day 4 at 15ºC - 17ºC, and also attained infective L_3 stage. At 26ºC temperature 30% eggs hatched at day 3 and maximum 39.7% egg hatched at day 5, and some larvae (14.0%) attained infective stage at day 5 (Table 2). Laha *et al.* (2000) observed that 17.24%, 37.55% and 24.80% eggs hatched by 24 hours, 48 hours and 72 hours, respectively at 25-30ºC.

Table 2. Effects of temperature on the hatching of eggs, development and survival of larvae of *H. contortus*

Temp. °C	Hatching of eggs (%)			Development of different larval stages (%)								Survival of larval stages (%)		
	D_3	D_4	D_5	D_4		D_5		D_6		D_7		D_6	D_7	D_8
				L_2	L_3	L_2	L_3	L_2	L_3	L_2	L_3			
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	7.0	10.0[a]	-	-	3.2	-	6.1	3.4	1.9	6.1[a]	9.6	8.0	6.6[a]
17	18.3	26.5	34.2[b]	13.5	-	21.9	6.7	10.6	13.1	2.5	18.5[b]	23.6	21	17.0[b]
26	30.0	37.4	39.7[b]	21	-	23.1	14.0	13.3	21.0	6.1	26.8[ab]	34.3	32.9	27.5
37	12.5	19.3	20.4[ab]	10	-	13.3	2.6	9.8	10.1	3.7	14.1	19.9	17.9	14.6[b]

Values in the same column having different superscript are statistically significant ($P<0.05$)

Tripathi (1977) found that hatching of eggs of *H. contortus* required 4 days at 20°C -30°C. L_1 to L_2 development required 4-12 days. L_2 moulted into L_3 within 13.5 days. Misra and Ruprah (1973a) observed that 22-35°C was suitable for development and survival of pre-parasitic stages of *H. contortus.* Interestingly, we found that at 37°C hatching of eggs and the percentage of infective larvae were markedly decreased. Only 20.5% eggs hatched at 37°C and 14.6% L_3 survived up to day 8. Narain and Chowdhury (1979) and Tripathi (1977) found that very few larvae attained to the infective stage. Hernandez *et al.* (1992) and Tembely (1998) also found that the eggs failed to develop and larval development was low at 37°C. Possibly, the higher temperature prevents the process of embryonic development and also kills the larvae after hatching.

pH 6.5 is optimum for the development and hatching of H. contortus eggs

Eggs did not develop and hatch at pH 2 and very negligible number of eggs (4.4%) hatched at pH 3. Best results (39.4%) were obtained at pH 6.5 by day 6 (Table 3). Sommerville and Murphy (1983) also observed that eggs failed to hatch at low pH, which is possibly due to the inactivation of related enzymes. Stringfellow (1986) and Misra and Ruprah (1973a) found that maximum hatching of eggs and development of *H. contortus* larvae occurred at pH 6.5-8.5 in laboratory condition. On the other hand, hatching of the eggs were markedly reduced at pH 11.5 (Table 3), which is possibly due to the denaturation of the egg mass in high alkaline pH.

Maximum eggs hatched at >80 - 90% relative humidity (RH)

Maximum (43.8%) eggs hatched at ≥ 80 - 90% RH whereas at 70 - ≤80% RH 34.5% eggs hatched (Table 4). Rahman *et al.* (1996) described that 80% RH was favourable for the hatching of eggs of *H. contortus*, and the larvae reached in infective stage within 3-4 days. Levine and Todd (1975) found more than 50% eggs developed into infective stage in 90% RH in laboratory condition. Rossanigo and Gruner (1994) recorded 54% hatchability of eggs when the faecal moisture content is sufficient. Krecek *et al.* (1992) considered RH as an important microclimatic variable for *H. contortus* since number of larvae increased in

pasture with the increase of RH. In field condition, Fernandez *et al.* (1994) showed the significance of seasons on the development of eggs into infective larvae, and he made the highest recovery of L_1 and L_2 in monsoon. In fact, rainy season is considered as a suitable season for the development and hatching of eggs of *H. contortus* (Ndamukong and Ngone 1996 and Swarnkar *et al.*, 1997). Moisture is considered as the most important climatic condition affecting the development and survival of infective larvae.

Table 3. Effects of pH on the hatching of eggs of *H. contortus*

Observations	Effects of different pH							
	2	3	4	5.5	6.5	7.5	9.5	11.5
Day 1	-	-	-	-	-	-	-	-
Day 2	-	-	-	-	5%	-	-	-
Day 3	-	-	8.8% ^{b}	21.1% ^{a}	24.7% ^{a}	23.0% ^{a}	18.6% ^{b}	2.1% ^{ab}
Day 4	-	4.4% ^{a}	12.2% ^{b}	27.5% ^{a}	30.1% ^{a}	29.4% ^{a}	20.9% ^{b}	6.9% ^{ab}
Day 5	-	7.1% ^{a}	14.5% ^{b}	32.3% ^{a}	34.9% ^{a}	31.0% ^{a}	23.0% ^{ab}	10.2% ^{ab}
Day 6	-	9.3% ^{a}	16.0% ^{ab}	35.7% ^{a}	39.4% ^{a}	37.0% ^{a}	26.2% ^{b}	-
Day 7	-	9.6% ^{a}	-	-	-	-	27.6% ^{b}	-

Values in the same row having different superscript are statistically significant ($P<0.05$)

Table 4. Effects of humidity, dark and light on the hatching of eggs of *H. contortus*

Observations	Effects of different humidity (%)		Effects of light (%)	
	70-≤80	≥80-90	Dark	Light
Day 1	-	-	-	-
Day 2	-	-	-	-
Day 3	27.9 ^{a}	35.6 ^{b}	29.8	31.1
Day 4	31.3 ^{a}	43.0 ^{b}	35.5	36.4
Day 5	34.5 ^{a}	43.8 ^{b}	38.3	39.9
Day 6	36.1	-	-	-

Light has no effect on the hatching of eggs

We could not detect any significant effect of light on the development and hatching of eggs of *H. contortus*. Hatching of eggs started at day 3 on both the dark and light condition. Maximum 38.3% and 39.9% eggs hatched at dark and light condition, respectively (Table 4). Islam and Ahmed (1987) also found that there was no significant effect of light on the hatching of eggs and survival of larvae.

Nutrient is essential for the survival of larvae

Best results were obtained in PBS containing 15% serum and 10% liver extract. In this media 35.3% of eggs hatched at day 2 and gradually increased up to 45.3% at day 5. Mortality of the larvae decreased and survival increased, and 39.5% larvae survived at day

8. Percentage of egg hatching, survival and longevity of the larvae increased in the media that contained serum and liver extract (Table 5). This combined media ensured a balanced environment and provide protein and several vitamins. This finding conformed to that of Paul (1965) who recorded the increased survival of larvae in a uniform manner in a medium containing plentiful food sources. He also obtained fourth stage *H. contortus* within nine days after inoculation into vitamin supplemented media. Furthermore, he observed the maximum larval survival in a medium containing 0.5% liver extract together with some extract of backer's yeast. Taken together, our results suggest that PBS containing 15% serum and 10% liver-extract may be used as suitable media for the incubation of eggs of *H. contortus.* Furthermore, better results may be obtained if eggs are incubated at 26^{o}C temperature, pH 6.5 and ≥80-90% RH.

Table 5. Effects of nutrient on the hatching of eggs and the survival of larve of *H. contortus*

Media	Observations (%)										
	D_1	D_2	D_3	D_4	D_5	D_6		D_7		D_8	
						D	L	D	L	D	L
PBS + 5% serum	-	22.0	31.2	37.9	37.9[a]	2.7	35.2	5.0	32.9	8.0	29.9
PBS + 10% serum	-	27.6	35.1	38.4	39.0 [a]	1.0	37.9	3.0	36.0	6.1	29.9 [a]
PBS + 15% serum	-	33.4	37.3	40.7	41.8 [b]	0.5	41.3	2.5	38.8	8.6	33.2 [b]
PBS + 5% LE	-	21.6	27.0	38.2	38.5 [a]	1.9	36.6	4.0	34.5	8.7	29.8 [a]
PBS + 10% LE	-	31.8	36.0	39.1	39.8 [a]	0.6	39.2	3.5	35.7	5.0	34.8 [b]
PBS + 15% LE	-	28.2	32.4	37.5	38.7 [a]	2.8	35.9	3.0	35.6	6.6	32.0 [b]
PBS + 15% serum + 10% LE	-	35.3	38.2	42.0	45.3 [b]	1.5	43.8	3.9	41.4	5.8	39.5[ab]

Values in the same column having different superscript are statistically significant ($P<0.05$), LE = Liver extract, D = Dead; L = Live

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