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In vitro study of factors related to the hatching of eggs of *Oesophagostomum columbianum*

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Abstract

To determine the *in vitro* effects of various environmental and nutritional factors on the hatching of eggs, development and survival of larvae of *Oesophagostomum columbianum,* eggs were cultivated at different temperature gradients, humidity and P^H in phosphate buffer saline (PBS), tap water (TW) and normal saline (NS) using serum and liver extract at different concentrations during the period from November 2008 to October 2009. In this study, no development of eggs was seen at 4^0C temperature within 15 days. The eggs also did not hatch while transferred to room temperature within 15 days. Hatching of eggs were maximum (38.7%) at 26^0C on day 5. While hatching of eggs of *O. columbianum* did not occur at p^H 2. Maximum number of hatching of eggs was seen on day 6 (40.00%) at p^H 6.5 and lowest in (3.65%) at p^H 11.5. Maximum hatching of eggs (51.21%) were recorded in the relative humidity of ≥80%-90% on day 5. Light had no significant effect on the development and hatching of eggs of *O. columbianum.* Maximum eggs hatched in PBS containing 15% serum and 10% liver extract (42.16%) but for PBS containing 10% liver extract also produced about to same hatching rate (42.15%). The present study suggests that PBS containing 15% serum and 10% liver extract may be used as suitable media for the incubation of eggs of *O. columbianum* due to continuous increasing of hatching rate of eggs according to the advancement of days. Further more, for *in vitro* culture better results may be obtained if eggs are incubated at 26^0C, p^H 6.5 and relative humidity ≥80%-90%.

Keywords: *O. columbianum,* Factors, Hatching of eggs

Introduction

Livestock industry of Bangladesh faces a number of obstacles hindering its development of which parasitic diseases are of great importance. Ruminants of Bangladesh are affected by various types of helminth parasites (Rahman and Razzak, 1973; Rahman and Mondal, 1983). According to FAO (1990), the estimated loss of productivity of animals in terms of mortality, loss of meat and milk, generation loss, loss of reproductive rate due to animal parasites to the extent upto 50% in Bangladesh. Among the parasitic diseases, the gastro-intestinal nematodes especially *Oesophagostomum columbianum* infection is a major problem all over Bangladesh, especially in small ruminants. The percentage of infection with different species of *Oesophagostomum* ranged from 92% in sheep and goats (Mohanta *et al.*, 2007). The preliminary factors that affect the hatching of eggs are mainly temperature and moisture. Embryonated eggs appeared to be most susceptible to adverse environmental conditions than the unembryonated eggs. The unembryonated eggs of *Oesophagostomum columbianum* are more susceptible to high or low temperatures. Prevalence of *Oesophagostomum* spp. infection was much higher during rainy season. The percentage of infection ranged from 57.0-72.0% (Hernandez *et al*., 1992; Xeupeng *et al*., 1994 and Achi *et al*., 2003). Eggs deposited on the pasture hatch and develop to the infective larval stage but hatching and develop more slowly at lower temperatures. The eggs hatching factors have been studied in natural condition but very little attention has been paid to determine the environmental and nutritional factors related to hatchability of eggs of *O. columbianum* in laboratory condition. Laboratory information is more essential to study the biology and ecology of a parasite.

Therefore, the present work was undertaken to study the effects of different temperature, nutrients (serum and liver extract), p^H, humidity and light on the hatching of eggs, detection of a suitable media for the incubation of eggs of *O. columbianum.*

Materials and Methods

The present study was carried out during the period from November 2008 to October, 2009. Large intestines of goats were collected from slaughter houses at Mymensingh town and Kamal Ranjit market of Bangladesh Agricultural University (BAU), Mymensingh. Parasites collection, identification, recovery of (*O. columbianum)* eggs and their incubation were done by following the techniques.

Female parasites were crushed by using mortar and pestle containing necessary amount of phosphate buffer solution (PBS). Eggs were counted by modified McMaster technique (Thienpont *et al.*, 1979) by using Mcmaster counting chember.
Total number of eggs/ml = (Total eggs×10)/ 0.3.

Study of the effects of temperature:

To study the effects of temperature, eggs were incubated at various temperature gradients that is 37^0C, 26^0C, 17^0C and 9^0C. The pre-counted numbers of eggs were suspended in a petri dish containing culture media. The petri dishes were kept in an aluminium tray covered with a thin layer of moist tissue paper to prevent the evaporation of culture media. Then they were incubated in an incubator separately at above mentioned temperature gradients upto 5 days and examined regularly at 24 hours interval by using dissecting microscope. A drop of culture media was examined for the detection of development of eggs. To study the effect of 4^0C temperature, the culture media were kept in refrigerator. After 15 days of observation the culture media were replaced in room temperature to record the further development described by Soulsby (1982) and Rahman *et al.* (1996).

Study of the effects of p^H

To study the effects of p^H, the different p^H levels (2, 3, 4, 5.5, 6.5, 7.5, 9.5 and 11.5) of the culture media were adjusted by adding glacial acetic acid or by adding sodium hydroxide in PBS drop by drop with the help of dropper and stirred the media. The p^H was detected by p^H meter. Observations were made by dissecting microscope in every 24 hours up to 7 days.

Study of the effects of humidity

To determine the effects of relative humidity on the hatching of eggs, the pre-counted number of eggs were mixed in the PBS and incubated at 70%-≤80% and ≥80%-90% of relative humidity at constant 26^0C temperature. Developmental stages were observed by dissecting microscope at 24 hours at regular interval up to 7 days.

Study of the effects of light

There were two groups of samples and each containing three media to identify the light effect on those groups. Pre-counted number of eggs counting culture media was covered with hard paper to make dark condition having facilities for air exchange keeping in room temperature. Then observation was made in similar manner up to 8 days.

Study of the effects of different media

To find out a suitable media for the cultivation of eggs of *O. columbianum*, pre-counted eggs were cultured in phosphate buffer solution (PBS), normal saline (NS) and tap water (TW) and incubated at room temperature and observed in similar procedure.

Study of the effects of different nutrients on the hatching of eggs

For the preparation of different nutrient media 5%, 10%, 15% serum and 5%, 10% and 15% liver extract were added separately to the PBS. A pre-counted number of eggs were suspended in each and every culture medium mentioned above and all were incubated at room temperature up to 8 days. Then the percentage of hatching eggs was calculated at every 24 hours.

Statistical analysis

Student t-test was used to determine the significance among the different variables (Steel and Torrie, 1980).

Results and Discussion

Effects of temperature

In this present study development of eggs did not occurred at 4^0C temperature during the period of observations up to 15 days. Hatching of eggs were maximum (38.7%) at 26^0C on day 5 of this study. Hatching started from day 4 in all cases except at 9^0C temperature where eggs hatched on day 3 and hatching continued up to day 5 (Table 1). It was observed that all developmental changes in eggs and larvae of *O. columbianum* were arrested at 4^0C and failed to develop even when returned to room temperature (15-20^0C). Tripathi (1980) recorded that absence of larval development in eggs at 4^0C temperature during an observation period of 30 days. Lunsmann (2001) noted that range of temperature tolerated by *Oesophagostomum* eggs in pig was not wider. Xuepeng *et al.* (1995) reported that eggs of *Oesophagostomum* were able to survive in the cold conditions in an undeveloped state. The exact mechanism of inactivation of eggs at 4^0C could not be explained. However, it was observed that germinal mass was somewhat squeezed. Possibly low temperature caused cold injury to the germinal mass of eggs and the eggs were revitalized.

Table 1. Effects of temperature on the hatching of eggs, development and survival of larvae of *O. columbianum*

Temperature (^{0}C)	Hatching of eggs (%)				
	D_1	D_2	D_3	D_4	D_5
4	-	-	-	-	-
9	-	-	1.90d	5.10d	6.90d
17	-	9.43a	17.91b	25.25b	32.80b
26	-	4.10c	28.65a	35.90a	38.7a
37	-	7.96b	11.69c	17.99c	23.10c

Values in the same column having different superscript are statistically significant ($p<0.05$); D= day of experiment

At 9^0C hatching of eggs started at day 3 which was relatively delayed than in other temperature. This finding conformed to the findings of Soulsby (1982) recorded that low temperature retarded the development of eggs. Difference was observed by Islam and Ahmed (1987) who recorded the hatching of eggs in days 11 at 10^0C and also observed that eggs hatched in 2-4.6 days at 15-34^0C which is supported by the result in this experiment at 17^0C (day 5).

At 26ºC temperature 21.10% eggs were hatched on day 2 and maximum 38.7% of eggs hatched on day 5 and larvae attained infective stage on day 5. Whereas, Rahman *et al.* (1996) found that eggs were hatched within 1-2 days at 26ºC. Shahiduzzaman *et al.* (1999) found that hatching of eggs of *O. columbianum* required 4 days at 20ºC -30ºC but present author observed that hatching of eggs started on day 2 and this might be some factors about internal condition of laboratory and procedure differ from that experiment. Besides Islam and Ahmed (1987) recorded the hatching of *Strongyloides* larvae by second day in laboratory condition at 20ºC -30ºC which also supported the study result. Tripathi (1977) made similar observation's of this author where he mentioned that 20ºC-30ºC was the most suitable temperature for hatching of eggs of *O. columbianum*.

At 37^0C, the hatching of eggs was markedly decreased. Tripathi (1980) showed that at 40ºC, some eggs hatched out within 24 hours. Islam *et al.* (2005) found that most of the eggs did not hatch in high temperature (≥40^0C). These above three findings confirmed the results of this study. Lunsmann (2001) concluded that eggs can be quickly inactivated by temperatures above 60ºC, or above 40ºC in combination with dryness. Berbigier *et al.* (1990) observed that fecal temperature was greater than 40-45ºC at midday and dehydration, egg mortality was greater on short than on tall grass and higher in morning than in evening deposits. It is concluded that the high temperature prevents the process of embryonic development and also can kill the larvae once they are embryonated.

Effects of p^H

Hatching of eggs were initiated from day 4 in p^H 3 but for rest of the p^H, eggs started to hatch from day 3. Ceasation of hatching of eggs were started from day 7 and for that cesation of hatching eggs were maximum at p^H 4 (4.6%) and minimum at p^H 6.5 (19.00%). Hatching of eggs were maximum on day 6 in all cases and for that highest hatching rate of eggs was at p^H 6.5 (40.00%) and lowest rate of hatching was at p^H 11.5 (3.65%) (Table 2). Hatching of eggs did not occur at p^H 2 of the incubation periods and it might be due to coagulation of germinal mass of the eggs due to high acidic p^H level of the media. Marcia *et al.* (2004) observed that neutral p^H level may produce highest level of viability of eggs which also support author's findings. In this study eggs hatchability and survival of larvae greater in lower p^H that the higher p^H level which also supported the study of Blotkamp and Pit (2000) where they found that larvae also survived in the acidity of an artificial mixture.

Table 2. Effects of p^H on the hatching of eggs of *O. columbianum*

Observations	Effects of different p^H							
	2	3	4	5.5	6.5	7.5	9.5	11.5
Day 1	-	-	-	-	-	-	-	-
Day 2	-	-	-	-	4	1	-	-
Day 3	-	-	7.74d	20.10b	22.66a	18.60c	12.9c	2.21e
Day 4	-	3.40f	10.10e	22.00c	34.34a	24.40b	16.91d	3.67f
Day 5	-	7.02f	12.52e	28.00c	36.36a	30.90b	17.55d	4.49g
Day 6	-	8.12f	17.60e	30.20b	40.00a	28.19c	20.12d	3.65g
Day 7	-	8.56d	4.60f	5.19e	16.00a	9.93c	11.17b	0.25d

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

Effects of humidity

Hatching of eggs were started on day 3 of the incubation on both the humidity gradients 70%-≤80% and ≥80%-90% of relative humidity at constant temperature (26^0C). Maximum 51.21% of hatching of eggs were recorded at relative humidity of ≥80%-90% on day 5 (Table 3). Maximum 51.21% of eggs were hatched in case of relative humidity ≥80-90% and egg hatching started on day 3. These confirmed the finding of Rahman *et al.* (1996) who described that 80% relative humidity was the favorable for the hatching of eggs of *Oesophagostomum columbianum* and also proved the finding of Rossanigo and Gruner (1994) that was 54% of hatchability of eggs when the fecal moisture content is sufficient. Berbigier *et al.* (1990) found that minimal fecal water content during the first 36 h explained the 74% mortality rate for eggs of *Oesophagostomum columbianum*. This also confirmed the findings of Fossing *et al.* (1995) that optimum egg hatching was in the temperature range from 15ºC to 20ºC and at humidity from 79.5 to 95.5%. Nath (1978) studied that eggs are affected by the changes in the humidity level around them and eggs do not develop below 87% relative humidity. Shahiduzzaan *et al.* (1999) studied that matured eggs were incubated at room temperature humidity varied from 85% to 94% and eggs of *Oesophagostomum radiatum* required 2.5 to 7.5 days with an average of 6.18 days for hatching of eggs.

Effects of light

No significant variation was observed on the development and hatching of eggs of *O. columbianum* incubated in dark and light condition. Finally 36.61% and 39.9% of eggs were hatched in dark and light condition, respectively on day 6 (Table 3). Hatching of eggs were started on day 3 in both the dark and light condition. Maximum 30.21% and 31.26% eggs were hatched out at dark and light condition, respectively which is more or less similar to the findings of Islam and Ahmed (1987) who found that there was no significant variation in the days required for the hatching of eggs and survival of larvae in light and darkness.

Effects of culture media

Hatching of eggs were initiated on day 3 in all cases, as in Phosphate buffer saline (PBS), normal saline (NS) and tap water (TW). On day 5 the rate of hatching of eggs were maximum (42.52%) in PBS and minimum (14.12%) in normal saline (NS) (Table 4).

Table 3. Effects of humidity, dark and light on the hatching of eggs of *O. columbianum*

Observations	Effects of different humidity (%)		Effects of light	
	70-≤80	≥80-90	Dark (%)	Light (%)
Day 1	-	-	-	-
Day 2	-	-	-	-
Day 3	20.12d	31.19c	30.21c	31.26c
Day 4	22.68c	33.35b	32.25b	33.92b
Day 5	28.29b	51.21a	36.61a	39.02
Day 6	31.01a	10.50d	10.90d	12.16d

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

Table 4. Effects of media on the hatching of eggs and the survival of larvae of *O. columbianum*

Media			Observations (%)		
	d_1	d_2	d_3	d_4	d_5
Normal saline (Ns)	-	-	6.12c	12.60c	14.12c
Phosphate Buffer Solution (PBS)	-	-	35.0a	38.02a	42.52a
Tap water (TW)	-	-	28.5b	32.92b	36.50b

Values in the same column having different superscript are statistically significant ($p<0.05$). d= day of experiment

Effects of nutrients on the hatching of eggs of *O. columbianum*

Hatching of the eggs were recorded on day 2 in all nutrient containing media namely 5%, 10%, 15% serum in PBS and 5%, 10% and 15% liver extract in PBS. Initially on the day 2, the highest hatchability of eggs (28.96%) were recorded in case of PBS containing 15% serum and 10% liver extract but only 13.6% eggs were hatched in PBS containing 5% serum. Hatching continued up to 5 days where the highest hatchability was recorded 42.16% and the lowest was 28.1% in PBS containing 15% serum and 10% liver extract and 5% serum, respectively on day 5 (Table 5). On the seven nutrients containing media the best results were obtained in PBS containing the combination of 15% serum and 10% liver extract in which 28.96% of eggs were hatched on day 2 and gradually increased upto 42.16% on day 5. Percentage of egg hatching increased due to the media contained serum and liver extract are the source of protein and several vitamins, respectively. These conformed to the findings of Paul (1965) that the egg hatching took place in a medium reasonably favorable and containing plentiful food.

Table 5. Effects of nutrient on the hatching of eggs and the survival of larvae of *O. columbianum*

Media		Days of	Hatching		
	d_1	d_2	d_3	d_4	d_5
PBS+5% serum	-	13.60g	16.62e	26.19e	28.10d
PBS+ 10% serum	-	15.17f	28.22c	28.60d	34.25c
PBS+15% serum	-	21.21c	32.31b	34.16b	35.95c
PBS+ 5% liver extract	-	18.19d	31.11b	31.01c	34.00c
PBS+10% liver extract	-	24.44b	38.66a	38.98a	42.15a
PBS+15% liver extract	-	16.65e	26.50d	32.25c	38.18b
PBS+15% serum+ 10% liver extract	-	28.96a	32.55b	35.10b	42.16a

Values in the same column having different superscript are statistically significant ($p<0.05$). d= day of experiment

This study suggests that PBS containing 15% serum and 10% liver extract may be used as suitable media for the incubation of eggs of *Oesophagostomum columbianum*. Furthermore, better results may be obtained if eggs are incubated at 26ºC temperatures, p^H 6.5 and relative humidity of ≥80%-90%.

With this study, temperature and humidity were most important environmental factors for *in vitro* cultivation of eggs of *Oesophagostomum columbianum*.

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