**Effects of physicochemical factors on development and survival of *Opisthorchis viverrini* uterine eggs**

Chanisala Sereewong¹, Monticha Chaiyasaeng¹, Naiyana Senasri², Jukkrid Chaiyos¹, Smarn Tesana¹

¹Food-borne Parasite Research Group, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

²Department of Fisheries, Faculty of Natural Resources, Rajamangala University of Technology Isan Sakonnakhon Campus, Sakonnakhon 47160, Thailand

**ARTICLE INFO**

**Objective:** To investigate the maturity development of miracidia in uterine eggs from four portions of the *Opisthorchis viverrini* uterus and environmental factors possibly affected in maturation and infectivity of distal part uterine eggs. **Methods:** Uteri of adult worms were divided into 4 equal parts. Development of eggs was determined under light microscope. Only embryonated eggs were used to evaluate the effects of physicochemical factors: temperature, salinity, acidity, ultraviolet A, B, C. Infection success was evaluated by feeding treated eggs to intermediate host snails and determining by using a PCR approach. **Results:** Eggs obtained from the uterus closest to the ovary (regions 1 and 2) failed to develop in vitro. Eggs from region 4 of the uterus (close to the genital pore) were used to study effects of physicochemical factors. The highest survival and infection success was in groups of eggs kept at 30 °C (95.20%). The calculated period of loss infection success (LI 50 and LI 95) on miracidia in distal uterine eggs by exposure to UV-A, UV-B and UV-C were 73 and 1 523 d; 8 and 20 d; 1 and 2 d, respectively. Lethal concentrations (LC 50 and LC 95) of salinity, HCl and NaOH on miracidia in distal uterine eggs were 45.43 and 120.09 ppt, 0.01 and 0.25 M; 0.01 and 0.11 M, respectively, after 24 h exposure. **Conclusions:** *Opisthorchis viverrini* eggs display a high tolerance to environmental conditions, especially after snail host eating for infection.

**ABSTRACT**

As many as 7 million Thai people are estimated to be infected with *Opisthorchis viverrini* (*O. viverrini*), with the highest prevalence in northeastern Thailand[1,2]. People become infected by eating fermented, undercooked or raw cyprinid fish dishes harboring infective encysted metacercaria[3]. After ingestion, the excysted larvae migrate into the biliary tree to develop to sexual maturity and produce uterine eggs within 2 weeks[4]. In northeastern Thailand, *Bithynia siamensis goniomphalos* snails serve as the first intermediate host[5,6]. They become infected by ingestion of fully mature eggs containing miracidia.

Distal uterine eggs of mature *O. viverrini* contain developing miracidia[4]. Eggs were laid in feces of definitive hosts need two weeks to become mature before they can infect snails[7]. A previous study on *Clonorchis sinensis* found that immature eggs from the proximal uterus (close to the ovary) could not develop outside the uterine tube[8], a situation that might also occur in *O. viverrini*. In the natural environment, eggs passed in feces and contaminating natural water. This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-Non Commercial-Share Alike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

**How to cite this article:** Sereewong C, Chaiyasaeng M, Senasri N, Chaiyos J, Tesana S. Effects of physicochemical factors on development and survival of *Opisthorchis viverrini* uterine eggs, Asian Pac J Trop Biomed 2018; 8(9): 431-435.
water bodies encounter environmental factors such as temperature changes, salinity, ultraviolet radiation, and changes in water pH. All of these may affect development of eggs to maturity and their survival. There are many environmental factors that can impinge on maturation of trematode eggs. Factors such as temperature and salinity influenced egg development and miracidial emergence of the digenean, Himasthla militaris. The eggs developed normally in the range 2.1 to 3 ppt salinity but in a salinity of 34 ppt, miracidial emergence was markedly inhibited[9]. Embryonation ceased at 12 °C; egg development as well as miracidial emergence occurred at 20 °C and 30 °C, but both processes progressed at a slower rate at 20 °C than at 30 °C. The eggs of Fasciola hepatica developed well at 27 °C when exposed to light[10]. Miracidia of Echinostoma caproni were not tolerant of salinities over 1 ppt but could survive in a pH range 3 to 11 and lived longer at 5 °C[11]. Ultraviolet affected eggs and rhabditoid infective-stage larvae of canine hookworm. Irradiation for one hour at a distance of 10 cm reduced the hatching eggs by half. Those larvae hatched did not develop to the infective stage[12].

In this study, we investigated the maturity development of miracidia in uterine eggs from 4 portions of the O. viverrini uterus. In addition, effects of environmental factors possibly involved in maturation and infectivity of uterine eggs from the distal part of the uterus were investigated. These factors include temperature, salinity, ultraviolet radiation, pH of water.

2. Materials and methods

2.1. Adult O. viverrini preparation

Eight Syrian golden hamsters were infected orally with 50 O. viverrini metacercariae harvested from naturally infected cyprinid fish. Infected animals were provided with water and food ad libitum and maintained for 2 months, were sacrificed to obtain parasites from livers. The worms were washed several times in sterile physiological saline. Animal use was approved by the Animal Ethics Committee of Khon Kaen University under National Council Research of Thailand guidelines (Ethics clearance number 0514.1.75/42).

2.2. O. viverrini egg preparation

Uteri of the adult worms were divided equally into 4 regions between the ovary and the genital pore (Figure 1) and eggs were dissected from each region in Petri-dishes containing sterile normal saline solution. Eggs were maintained at room temperature (25±3 °C). The eggs were sampling to observe on 0 (control), 1, 3, 5, 7, 9, 11, 13 and 15 days post dissection for miracidial development and their viability were determined by staining with trypan blue. Eggs were laid by adult worms maintained in normal saline for 24 h at room temperature were used as positive control.

2.3. Snail sample preparation

Snails (Bithynia siamensis goniomphalos) were collected from natural water reservoirs in Khon Kaen Province, northeast Thailand. After collection, those snails were examined individually for larval trematode infection by the cercarial shedding method every alternate week for two months. Only non-infected snails were used for the studies.

2.4. Observation of survival of O. viverrini miracidia

Survival and development of eggs from different regions of the uterus were investigated. The criteria for egg developmental stages were determined following a previous study[4].

The survival of eggs was determined by using 0.4% trypan blue staining. Dead eggs were permeable and could uptake the dye, therefore appearing blue. Dead miracidia or zygotes appeared as bubbles or vacuoles within degenerating eggs.

2.5. Incubation of O. viverrini eggs at various temperatures

From results of maturity development, all distal uterine eggs (region 4) could develop. They were maintained in plastic cups (250 eggs/cup; 5 replicates for each temperature) with 10 mL distilled water at -20 °C, -15 °C, 0 °C, 15 °C, 30 °C, 45 °C, 60 °C, 75 °C and their viability determined at days 0 (control), 1, 3, 5 and 7.

2.6. Incubation of O. viverrini eggs at different salinities

Distal uterine eggs were maintained in plastic cups (50 eggs/cup; 5 replicates for each concentration) with 10 mL of saline of the following concentrations: 0 (control), 25, 50, 75, 100, 125, 150 ppt. Viability was determined after 24 h exposure.

2.7. Incubation of O. viverrini eggs under ultraviolet radiation

Distal uterine eggs were maintained in a glass box with 2 mL distilled water and exposed to UV-A (17,758 lux, FL 15T8/BL, Thai Toshiba Lighting Co., Ltd., Pathumthani, Thailand), UV-B (9377.59 lux, TL20W/01 RS SLV/25, Philips Lighting Holding B.V., Pila, Poland) or UV-C (4,371.2 lux, UV F17T8, Koninklijke Philips N.V., Eindhoven, Holland). Survival of eggs was observed at day 0 (control), 1, 2, 5, 7, 9, 14, 20 and 30. Five replicates were used, 50 eggs each, for each

![Figure 1. Photograph of O. viverrini adult showing uterus was equally divided to 4 regions.](image)
2.8. Incubation of *O. viverrini* eggs in acid and base solutions

Distal uterine eggs (region 4) were maintained in a glass box with 2 mL of 0 M (control), 1 M, 0.1 M, 0.01 M and 0.001 M HCl. Five replicates were used, 50 eggs each, at each concentration of HCl and viability determined after 24 h exposure.

Distal uterine eggs were maintained in a glass box with 2 mL of 0 M (control), 1 M, 0.1 M, 0.01 M and 0.001 M NaOH. Five replicates were used, 50 eggs each, at each concentration of NaOH and viability determined after 24 h exposure.

2.9. Determining infection success of *O. viverrini* eggs in snails

Fifty *O. viverrini* eggs from each relevant experimental treatment were placed with individual snail (10 snails/group) in a plastic container containing 5 mL distilled water. The snails were stimulated by exposure to electric light at (28±3) °C in water baths for 24 h following a previous report[13]. This was done to encourage them to actively move to seek food and thus encounter the parasite eggs. After that, each snail was washed and reared in a new plastic container for 24 h. The infection success was determined by PCR using species-specific primers for *O. viverrini*. DNA was extracted from the whole soft body of the snail. The negative controls were snails that had not been exposed to *O. viverrini* eggs.

Total genomic DNA was extracted from soft tissue of snail individually using cetyltrimethyl ammonium bromide solution (CTAB) buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v β-mercaptoethanol, 20 mM EDTA, 100 mM Tris HCl, pH 8.0, 0.2 mg/mL proteinase K) and phenol-chloroform phase extraction. The soft tissue was blended by grinder with 600 µL of CTAB buffer, incubated at 55 °C for 3-4 h or overnight. The lysate was phase-extracted with phenol-chloroform (1:1) then centrifuged for 10 min at 12 000 g. Then the supernatant was transferred to a clean tube and extraction repeated with phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 12 000 g for 10 min twice. The DNA in the supernatant was precipitated in isopropanol for 20 min at 4 °C and centrifuged for 10 min at 12 000 g at 4 °C. After that DNA was washed in 75% ethanol and absolute ethanol, centrifuged for 5 min at 12 000 g at 4 °C. Finally, the gel was visualized under UV illumination, and a picture captured using a Bio Doc-It™ Imaging System (Jena Analytik AG, Jena, Germany). One-hundred bp DNA ladder (Vivantis Technologies Sdn. Bhd. Revongen Co, Malaysia) was used as a standard marker for size determination. This ladder consists of 10 fragments between 100 and 1 000 bp in multiples of 100 bp.

2.10. Statistical analysis

Lethal concentrations or lethal effects of 50% and 95% were calculated using probit analysis. The differences between groups were tested by one-way ANOVA and pair-wise comparisons by Duncan’s multiple range test between 2 groups.

3. Results

3.1. Survival and development of *O. viverrini* eggs from 4 regions of uterus

Samples of eggs from all regions of the uterus were assessed for viability using trypan blue at initiation of cultivation (day 0, Figure 2). Viability was in the range 74.4%-99.2%. Viability decreased sharply in regions 1 and 2 on day 3 compared to the control group (laid eggs from adult worms maintained in normal saline) (P<0.05). All eggs from regions 1 and 2 were dead by day 5. But the viability of embryonated eggs in regions 3 and 4 decreased only gradually, not differing significantly from the control group (P>0.05). The viability of eggs in region 3 after 3 days of incubation was lower than in region 4 and in controls. The eggs in region 3, region 4 and the control group slightly decreased in viability till 15 days, as assessed by trypan blue staining (Figure 2).
3.2. Development of eggs from 4 uterine regions.

Immature eggs (type A), found only in region 1 (17.4%), contained small spherical granules, the vitellocytes and the zygote appeared as a grey-brown mass. The diameter of type B was less than that of type A. Eggs of type B were found only in regions 1 (53.8%) and 2 (11.2%). Eggs of types C and D exhibited an operculum and an abopercular knob. Type C was found in regions 1 (25.2%), 2 (53%) and 3 (3.2%). In type D the embryo was multicellular and surrounded peripherally by vitellocytes. Type D was found in regions 1 (3.6%), 2 (29.6%) and 3 (9.6%). Eggs of type E had an eggshell with muskmelon-like appearance and a clearly evident operculum, shoulder, and knob, and was found in regions 2 (8.8%), 3 (58.4%) and 4 (4.6%). Eggs of type F were mature. The miracidium in these was clearly visible. Eggs of this type were more common in region 4 (95.4%) and some of them in regions 3 (28.8%) and 2 (1.8%) of the uterus.

3.3. Survival of miracidia in eggs at various temperatures

The highest percentage survival was in groups of eggs kept at 30 °C (95.2%). All eggs died at -20 °C and at 75 °C, according to trypan blue staining (Figure 3). No snails became infected from eggs kept at -20 °C, -15 °C or 75 °C, according to PCR analyses.

3.4. Survival and development of miracidia in eggs in various salinities

All eggs incubated in salinity of 150 ppt died. Among the remaining groups, those incubated at 125 ppt had the lowest survival rate (1.8%). The highest survival was at 25 ppt (76.8%). No groups kept at salinities of 75 ppt or higher could infect snails. The calculated lethal concentrations (LC\textsubscript{50} and LC\textsubscript{95} of salinity on miracidia were 45.43 and 120.09 ppt, respectively (Figure 4).

3.5. Survival and development of miracidia in eggs exposed to ultraviolet radiation

The eggs could survive in UV-A up to day 30. In UV-B all eggs died by day 14 and in UV-C the eggs had died by day 2 (Figure 5). Snails could not be infected beyond these same numbers of days.

The calculated period of loss infection success (LI\textsubscript{50} and LI\textsubscript{95} values for miracidia in distal uterine eggs due to exposure to UV-A, UV-B and UV-C were 73 and 1,523 d; 8 and 20 d; 1 and 2 d, respectively.

3.6. Survival and development of eggs exposed to acid and base solutions

In acid, the eggs died in 1 M HCl, as determined by trypan blue staining and no infection was detected in snails by PCR analysis even in 0.1 M. In the basic solution, all eggs died at 1 M and 0.1 M NaOH. The concentrations are calculated by using Probit analysis. The loss infection success of 50% and 95% to snails which determined by PCR for HCl and NaOH were of 0.01 and 0.25 M; 0.01 and 0.11 M, respectively.

4. Discussion

In this study the development and survival of O. viverrini eggs from each part of uterus have been described. Physicochemical eggs including ranges of temperature, salinity, alkalinity, acidity and ultraviolet radiation have been tested.

Eggs gradually develop as they move along the uterus from the region of the ovary towards the genital pore. Eggs in the uterus proximal to the ovary were immature and could not complete
maturation outside the uterus. A similar situation has been found in Clonorchis sinensis: eggs maintained in vitro could not mature and become infective[8].

Eggs incubated at temperatures between 0 °C and 60 °C could infect snails, with a peak at 30 °C. The highest and lowest temperatures recorded in Thailand are 44.5 °C and -1.4 °C, respectively (Meteorological Department of Thailand), well within the range of temperatures suitable for infection.

O. viverrini is prevalent in northeast Thailand, an area of surface salt with high salinity of water bodies. We found that eggs could remain viable and infect snails at salinities as high as 125 ppt and 75 ppt, respectively. The host snail in northeastern Thailand, Bithynia siamensis goniomphalos, exclusively occurs in areas with a salinity range of 0.05-22.11 ppt. So eggs of O. viverrini can survive in the full range of salinities in which their hosts occur.

The ability of eggs to survive in rather high concentrations of HCl and NaOH shows that they have a high tolerance for some kinds of chemicals.

The eggs could remain viable in UV-A but died in UV-B by day 14 and in UV–C by day 2. UV-A has a relatively long wave-length at 315–400 nm and comprises 95% of the UV radiation reaching the Earth’s surface. But UV-A is less intense than UV-B and UV-C. UV-B (wavelength 280–315 nm) is very biologically active but cannot penetrate because it is easily absorbed. UV-C has a light spectrum in the range 100–280 nm. This is the most damaging type of UV radiation. However, it is completely filtered by the atmosphere and does not reach the earth’s surface. But in recent type of UV radiation. However, it is completely filtered by the atmosphere and does not reach the earth’s surface. But in recent

Global warming is causing changes to the Earth’s climate: glaciers are melting, sea levels are rising, forests are dying, and the ozone layer is decreasing. While it may be assumed that survival of eggs of O. viverrini which will be affected by these changes, are shown to display a high tolerance to environmental conditions, because they may need to remain viable for long periods until encountered by a snail host.

Conflict of interest statement

The authors declare that there is no conflict of interest.

References

[1] IARC. Opisthorchis viverrini and Clonorchis sinensis. IARC Monogr Eval Carcinog Risks Hum 2011; 100b: 347-376.

[2] Sripa J, Kiatsopit N, Piratae S. Prevalence of trematode larvae in intermediate hosts: Snails and fish in Ko Ae Sub-District of Khueang Nai, Ubon Ratchathani Province, Thailand. Southeast Asian J Trop Med Public Health 2016; 47(3): 399-409.

[3] Donthaisong C, Arunsan P, Suwannatrai K, Prasopdee S, Kulsantiwong J, Wongmaneprateep S, et al. Experimental infection of Opisthorchis viverrini cercariae to the cypridinid fish, Barbonymus gonionotus. Acta Trop 2015; 141: 253-257.

[4] Khampaos P, Jones MK, Lovas EM, Srisawangwong T, Laha T, Piratae S, et al. Light and electron microscopy observations of embryogenesis and egg development in the human liver fluke, Opisthorchis viverrini (Platyhelminthes, Digenaea). Parasitol Res 2012; 110(2): 799-808.

[5] Kulsantiwong J, Prasopdee S, Piratae S, Kampaos P, Thammasiri C, Suwannatrai A, et al. Trematode infection of freshwater snail, family Bithyniidae in Thailand. Southeast Asian J Trop Med Public Health 2015; 46(3): 396-405.

[6] Kiatsopit N, Sithithaworn P, Kopolrat K, Namsanor J, Andrews RH, Petney TN. Trematode diversity in the freshwater snail Bithynia siamensis goniomphalos sensu lato from Thailand and Lao PDR. J Helminthol 2016; 90(3): 312-320.

[7] Chanawong A, Waikagul J. Laboratory studies on host-parasite relationship of Bithynia snails and the liver fluke, Opisthorchis viverrini. Southeast Asian J Trop Med Public Health 1991; 22(2): 235-239.

[8] Uddin MH, Bae YM, Choi MH, Hong ST. Production and deformation of Clonorchis sinensis eggs during in vitro maintenance. PLoS One 2012; 7(12): e52676.

[9] Vanoverschelde R. Studies on the life-cycle of Himasthla militaris (Trematoda: Echinostomatidae): Influence of salinity and temperature on egg development and miracidial emergence. Parasitology 1981; 82(03): 459-465.

[10] Chryssafidis AL, Fu Y, De Waal T, Mulcahy G. Standardisation of egg-viability assays for Fasciola hepatica and Calicophoron daubneyi: A tool for evaluating new technologies of parasite control. Vet Parasitol 2015; 210(1-2): 25-31.

[11] Ford DM, Nollen PM, Romano MA. The effects of salinity, pH and temperature on the half-life and longevity of Echinostoma caproni miracidia. J Helminthol 1998; 72(4): 325-330.

[12] Roh YH. The influence of ultraviolet irradiation upon the development and infectivity of hookworm larvae. Kuoengchunghap Chuphi 1968; 6(1): 23-34.

[13] Prasopdee S, Kulsantiwong J, Piratae S, Kampaos P, Thammasiri C, Suwannatrai A, et al. Temperature dependence of Opisthorchis viverrini infection in first intermediate host snail, Bithynia siamensis goniomphalos. Acta Trop 2015; 141(A): 112-117.

[14] Wongranatanchewin S, Pumidonning W, Sermswan RW. Maleewong W. Development of a PCR-based method for the detection of Opisthorchis viverrini in experimentally infected hamsters. Parasitology 2001; 122(Pt 2): 175-180.

[15] Suwannatrai A, Suwannatrai K, Haruy S, Piratae S, Thammasiri C, Kampaos P, et al. Effect of soil surface salt on the density and distribution of the snail Bithynia siamensis goniomphalos in northeast Thailand. Geopat Health 2011; 5(2): 183-190.

[16] Munakata N, Kazadzis S, Bolsee D, Schuch N, Koskela T, Karpetcho A, et al. Variations and trends of biologically effective doses of solar ultraviolet radiation in Asia, Europe and South America from 1999 to 2007. Photochem Photobiol Sci 2009; 8(8): 1117-1124.