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Effects of Physicochemical Factors on Loss of Infectivity of *Opisthorchis viverrini* Cercariae

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Abstract

This study aims to develop an alternative field-based approach to interrupt the life cycle of *O. viverrini*. The effects of temperature, salinity, acidity, ultraviolet A, B, C radiation, and combinations of these physicochemical factors on the loss of infectivity of *Opisthorchis viverrini* cercariae were analyzed to determine values required for 50 and 95 % lethal concentrations (LC_{50} and LC_{95}) and period of loss infection success (LI_{50} and LI_{95}). Newly shed cercariae of *O. viverrini* were used. LC_{50} and LC_{95} values for temperature, salinity, and acidity on cercariae were 33.19 and 37.70 ºC, 7.29 and 8.40 ppt, 4.62 and 4.80 M H_2CO_3, respectively. The values of LI_{50} and LI_{95} on cercariae by exposure to UVA, UVB, and UVC were 19.54 and 20.11 h, 5.03 and 5.12 h, 3.37 and 6.02 min, respectively. Combinations of these factors in the presence of UVC proved damaging to cercariae most rapidly. The shortest loss infection time of LI_{50} and LI_{95} were 1.09 and 2.83 min. Cercariae were deemed to have lost the ability to infect cyprinid fish when they had shed their tails, even though they were still capable of some movement. In nature, temperature, salinity, acidity, ultraviolet radiation, and combinations of these factors, affect the ability of *O. viverrini* cercariae to infect cyprinid fish.

Keywords: Cercariae, Combinations, Physicochemical factor, *Opisthorchis viverrini*

Introduction

Opisthorchis viverrini infection in humans remains a major public health issue in the Greater Mekong Sub-region including Vietnam, Lao PDR, Cambodia, Myanmar, and Thailand [1-4]. In Thailand, 7 million people are infected with this liver fluke, most of them in the northeast of the country. In addition, it has been found that liver fluke is associated with cholangiocarcinoma [2]. Humans acquire the infection by eating raw, fermented, or undercooked cyprinid fish containing the infective stage, mature metacercariae [5]. The parasite life cycle requires the snail *Bithynia siamensis goniomphalos* as 1st intermediate host in the northeast region of Thailand and many species of cyprinid fishes as 2nd intermediate hosts [6].

In the snail host, germinal cells in sporocysts and rediae multiply by asexual reproduction, ultimately producing many free-swimming cercariae. Numerous cercariae of *O. viverrini* are shed daily from naturally infected snails, with an average of 1,728 cercariae/snail, at the daily peak shedding time of 8:00 - 10:00 a.m. [7]. After finding a cyprinid fish host, cercariae penetrate and encyst to become

metacercariae, mainly in the head portion and muscle [8]. Shed cercariae from snail hosts face changes in temperature [9,10], salinity, acidity [11,12], and UV radiation [13]. These changes could cause the death of cercariae or disable swimming. The number of cercariae that are successful at establishing in fish has important ramifications for infection levels (prevalence and infection intensities) in 2nd intermediate hosts and might influence the survival of these hosts through intensity-dependent mortality [14]. To date, most studies on the survival of cercarial transmission stages have focused on a single environmental factor which allows the investigation of a broader range of factor levels.

This study investigated *in vitro* of losing infectivity on the effects of physicochemical factors of temperature, salinity, acidity, ultraviolet A, B, C radiation, and combinations of these, on *O. viverrini* cercariae. If these environmental factors can cause loss of infectivity of cercariae, their uses can be incorporated into control programs to assist the eradication of opisthorchiasis in humans.

Materials and methods

Collection of *O. viverrini* cercariae

Newly shed cercariae of *O. viverrini* were collected from 9:00 a.m. - 12:00 p.m. from naturally infected *B. siamensis goniomphalos*. Snails were placed individually in plastic cups (3 cm in diameter and 2.5 cm in height) that contained dechlorinated tap-water. Cercarial shedding was stimulated by exposure to electric light (40 W) for 2 - 3 h, after which the water in the cup was examined for the presence of cercariae. Cercariae were morphologically identified as those of *O. viverrini* [6] and confirmed by polymerase chain reaction (PCR) using species-specific primers [15]. Animal use was reviewed and approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of National Council Research of Thailand (record No. IACUC-KKU-14/61).

Effects of physicochemical factors on *O. viverrini* cercariae

Preliminary studies were done for calculation of 50 and 95 % lethal concentrations (LC_{50} and LC_{95}) of water temperature, salinity, and acidity for further study to calculate the period (time) of loss infection success (LI_{50} and LI_{95}). Loss of infection success of cercariae was determined by degeneration of body portions or death or loss of tails. Cercariae which lost their tails could not infect to fish hosts [16,17].

Effects of temperature

Preliminary study: Cercariae were maintained in a glass box with 3 mL distilled water (10 cercariae/glass box; 3 replicates for each water temperature) and exposed to water temperature for 30, 31, 32, 33, 34, 35, 36, and 37 ºC, and room temperature for the control group, for 24 h. After that, they were examined periodically for a further 24 h, and numbers of cercariae, which had lost their tails, or which had died, were noted. From the data collected, LC_{50} and LC_{95} of water temperatures after 24 h were calculated.

From preliminary studies, 33.19 ºC was determined as the temperature by which 50 % of cercariae had lost infectivity after 24 h. The corresponding value for 95 % loss of infectivity was 37.70 ºC. Each group of 30 cercariae (10 cercariae/replicate) was incubated at one of these temperatures (control; room temperature, 33.19 and 37.70 ºC) for 0, 1, 3, 6, 12, 24, and 48 h. Period of loss infection success of cercariae was noted at each time interval; then, the period of loss infection success 50 and 95 % (LI_{50} and LI_{95}) values was calculated.

Effects of salinity

Preliminary study: Cercariae were maintained in a glass box (10 cercariae/glass box; 3 replicates for each concentration) with 3 mL of NaCl solutions of the following concentrations: 0 (control), 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 parts per thousand (ppt) for 24 h. After that, they were examined periodically for a further 24 h, and numbers of cercariae, which had lost their tails, or which had died, were noted. From the data collected, concentrations of salinity at which 50 and 95 % of cercariae had a loss of infectivity (LC_{50} and LC_{95}) after 24 h were calculated.

From preliminary studies, 7.29 ppt was determined as the salinity at which 50 % of cercariae had lost infectivity after 24 h. The corresponding value for 95 % loss of infectivity was 8.40 ppt. Each group of 30 cercariae (10 cercariae/replicate) was incubated at one of these salinities (control; distilled water, 7.29 and 8.40 ppt) for 0, 1, 3, 6, 12, 24, and 48 h. Period of loss infection success of cercariae was noted at each time interval; then, LI_{50} and LI_{95} values were calculated.

Effects of acidity

Preliminary study: Cercariae were maintained in a glass box (10 cercariae/glass box; 3 replicates for each concentration) with 3 mL of 0 (control; distilled water), 4.40, 4.60, 4.70, 4.80, 4.90, and 5.00 M H_2CO_3 for 24 h. After that, they were examined periodically for a further 24 h, and numbers of cercariae, which had lost their tails, or which had died, were noted. From the data collected, concentrations of H_2CO_3 at which 50 and 95 % of cercariae had a loss of infectivity (LC_{50} and LC_{95}) after 24 h were calculated.

From preliminary studies, 4.62 M H_2CO_3 was determined as the concentration at which 50 % of cercariae had lost infectivity after 24 h. The corresponding value for 95 % loss of infectivity was 4.80 M H_2CO_3. Each group of 30 cercariae (10 cercariae/replicate) was incubated at one of these acidities (control; distilled water, 4.62 and 4.80 M H_2CO_3) for 0, 1, 3, 6, 12, 24, and 48 h. Period of loss infection success of cercariae was noted at each time interval; then, LI_{50} and LI_{95} values were calculated.

Effects of ultraviolet A (UVA), B (UVB) and, C (UVC) radiation, 10 cm from light source

Effects of ultraviolet A radiation

Cercariae were maintained in a glass box (10 cercariae/glass box; 3 replicates for each time interval) with 3 mL distilled water and constantly exposed to UVA (17,758 lux, FL 15T8/BL, Thai Toshiba Lighting Co., Ltd., Pathumthani, Thailand) for 0 min (control), 19 h 55 min (1,195 min), 19 h 57 min (1,197 min), 19 h 59 min (1,199 min), 20 h 01 min (1,201 min), 20 h 03 min (1,203 min) and 20 h 05 min (1,205 min). After that, they were examined periodically and numbers of cercariae, which had lost their tails, or which had died, were noted. From the data collected, the time of exposure to ultraviolet A radiation by which 50 and 95 % of cercariae had a loss of infectivity (LI_{50} and LI_{95}) was calculated.

Effects of ultraviolet B radiation

Cercariae were maintained in a glass box (10 cercariae/glass box; 3 replicates for each time interval) with 3 mL distilled water and constantly exposed to UVB (9,377.59 lux, TL20W/01 RS SLV/25, Philips Lighting Holding B.V, Pila, Poland) for 0 min (control), 5 h (300 min), 5 h 03 min (303 min), 5 h 05 min (305 min), 5 h 07 min (307 min), 5 h 09 min (309 min), 5 h 11 min (311 min), 5 h 13 min (313 min) and 5 h 15 min (315 min). After that, they were examined periodically and numbers of cercariae, which had lost their tails, or which had died, were noted. From the data collected, the time of exposure to ultraviolet B radiation at which 50 and 95 % of cercariae had a loss of infectivity (LI_{50} and LI_{95}) was calculated.

Effects of ultraviolet C radiation

Cercariae were maintained in glass boxes (10 cercariae/glass box; 3 replicates for each time interval) with 3 mL distilled water and constantly exposed to UVC (3,688.2 lux, UV F17T8, Koninklijke Philips N.V., Eindhoven, Netherlands) for 0 (control), 2, 3, 4, 5, 6 and 7 min. After that, they were examined periodically and numbers of cercariae, which had lost their tails, or which had died, were noted. From the data collected, the time of exposure to ultraviolet C radiation at which 50 and 95 % of cercariae had a loss of infectivity (LI_{50} and LI_{95}) was calculated.

Combinations of physicochemical factors

Thirty cercariae (10 cercariae/replicate) were incubated in each combination of conditions for 24 h. Cercariae were exposed to combinations of 2, 3, 4, 5, and 6 factors at the LC_{50} of water temperature, salinity, and acidity and LI_{50} of UVA, UVB, and UVC, values for each of those conditions. LI_{50} values for UV exposure were less than 24 h in each case. Therefore, in relevant treatments, batches of cercariae were exposed to UV radiation only for the appropriate time, not for the full 24 h. After that, cercariae

were examined. Conditions under which 50 and 95 % of cercariae had lost infectivity (LI_{50} and LI_{95}) were calculated for each combination of factors.

Statistical analysis

The effects of water temperature, salinity, acidity, ultraviolet A, B, C radiation, and combinations of these, on the survival of cercariae, were evaluated by examination of cercariae under a stereomicroscope. The conditions under which 50 and 95 % of cercariae had lost infectivity or infection time were determined using the probit analysis for each parameter.

Results and discussion

Effects of physicochemical factors on the loss of infectivity of *O. viverrini* cercariae

Cercariae exhibited abnormal behaviors, abnormal movement, and loss of tails after exposure to various temperatures, salinities, acidities, UVA, UVB, and UVC radiation. Fifty percent and ninety-five percent lethal concentrations (LC_{50} and LC_{95}) were calculated for temperatures, salinities, and acidities of 33.19 and 37.70 ºC, 7.29 and 8.40 ppt, 4.62 and 4.80 M H_2CO_3, respectively. The values of the period of loss infection success 50 and 95 % (LI_{50} and LI_{95}) for ultraviolet A, B, and C were 19.54 and 20.11 h, 5.03 and 5.12 h, 3.37 and 6.02 min, respectively (**Table 1**). At LC_{50} and LC_{95} conditions of temperature (33.19 and 37.70 ºC, respectively), LI_{50} and LI_{95} of cercariae at 6 and 3 h, respectively, were the same as in the control group. After which they sharply decreased to zero at 48 and 24 h, respectively (**Figure 1A**). Similarly, at LC_{50} and LC_{95} values of salinity (7.29 and 8.40 ppt) both groups were the same as controls at 1 and 3 h, after which they sharply decreased to zero at 48 and 24 h (LC_{50} and LC_{95}, respectively) (**Figure 1B**). When exposed to LC_{50} and LC_{95} levels of acidity (4.62 and 4.80 M H_2CO_3), cercariae in both groups were the same as controls at 1 and 3 h, after which they sharply decreased to zero at 48 and 24 h (LC_{50} and LC_{95}), respectively (**Figure 1C**).

Effects of combinations of physicochemical factors on infectivity of *O. viverrini* cercariae

Cercariae of *O. viverrini* were exposed to combination factors, under the LI_{50} and LI_{95} conditions for each. The most effective combination of any 2 factors was acidity and UVC. In order of efficacy from high to low values, were temperature and UVC, salinity and UVC, UVB and UVC, UVA and UVC, acidity and UVB, salinity and UVB, temperature and UVB, temperature and acidity, UVA and UVB, temperature and salinity, salinity and acidity, salinity and UVA, temperature and UVA, and acidity and UVA (**Table 2**). The most effective combination of 3 factors was salinity, acidity, and UVC. In order of efficacy from high to low values, combinations were temperature, salinity and UVC; temperature, acidity and UVC; UVA, UVB and UVC; salinity, acidity and UVB; temperature, acidity and UVB; temperature, salinity and UVB; temperature, salinity and acidity; temperature, acidity and UVA; salinity, acidity and UVA; and temperature, salinity and UVA (**Table 2**). The most effective combination of 4 factors was temperature, salinity, acidity and UVC. In order of efficacy from high to low values, combinations were temperature, salinity, acidity and UVB; and temperature, salinity, acidity and UVA (**Table 2**). The most effective combination of 5 factors was temperature, salinity, acidity, UVA and UVC. In order of efficacy from high to low values, combinations were temperature, salinity, acidity, UVB and UVC, and temperature, salinity, acidity, UVA and UVB (**Table 2**). The most effective factorial physicochemical factors include temperature, salinity, acidity, UVA, UVB, and UVC (**Table 2**).

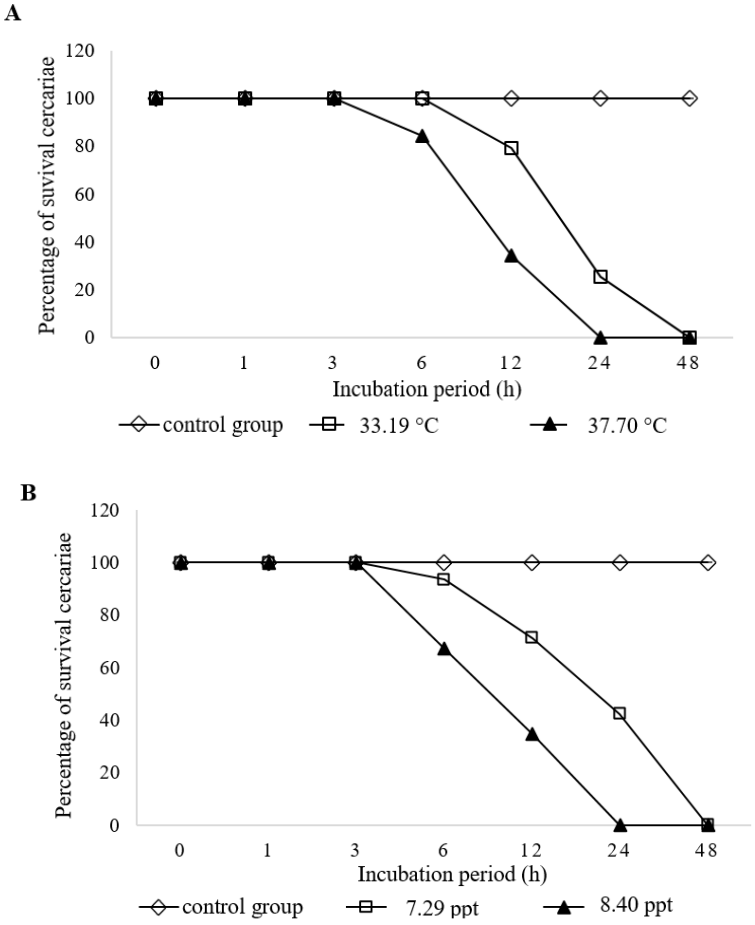
Table 1 The loss of infectivity of *O. viverrini* cercariae exposed to various physicochemical factors.

Physicochemical factors	Effects on *O. viverrini* cercariae	
	50 % loss of infectivity	95 % loss of infectivity
Temperature (ºC)	33.19	37.70
Salinity (ppt)	7.29	8.40
Acidity (M H_2CO_3)	4.62	4.80
UVA (h)	19.54	20.11
UVB (h)	5.03	5.12
UVC (min)	3.37	6.02

Table 2 Times after which 50 and 95 % of *O. viverrini* cercariae had lost their tails (LI_{50} and LI_{95}) after exposure to combinations of physicochemical factors. Values for each factor were those shown in **Table 1**.

Combinations of factors	Time to loss of tail (min)	
	50 % loss	95 % loss
2 factors		
Acidity × UVC	1.49	4.46
Temperature × UVC	1.86	6.52
Salinity × UVC	2.01	4.72
UVB × UVC	2.93	5.64
UVA × UVC	3.47	6.78
Acidity × UVB	60.36	240.92
Salinity × UVB	120.40	240.38
Temperature × UVB	120.87	300.26
Temperature × Acidity	180.48	540.45
UVA × UVB	360.49	540.92
Temperature × Salinity	480.84	840.90
Salinity × Acidity	480.92	780.22
Salinity × UVA	720.24	840.56
Temperature × UVA	720.50	900.48
Acidity × UVA	720.88	840.99
3 factors		
Salinity × Acidity × UVC	1.35	3.82
Temperature × Salinity × UVC	1.54	5.89
Temperature × Acidity × UVC	1.68	4.45
UVA × UVB × UVC	2.92	5.59
Salinity × Acidity × UVB	120.25	240.56
Temperature × Acidity × UVB	120.35	240.14
Temperature × Salinity × UVB	120.65	240.92
Temperature × Salinity × Acidity	420.21	720.14
Temperature × Acidity × UVA	540.69	720.63
Salinity × Acidity × UVA	540.82	780.20
Temperature × Salinity × UVA	780.56	960.00
4 factors		
Temperature × Salinity × Acidity × UVC	1.34	3.84
Temperature × Salinity × Acidity × UVB	120.72	240.43

Combinations of factors	Time to loss of tail (min)	
	50 % loss	95 % loss
Temperature × Salinity × Acidity × UVA	660.79	840.95
5 factors		
Temperature × Salinity × Acidity × UVA× UVC	1.44	4.67
Temperature × Salinity × Acidity × UVB× UVC	1.45	4.52
Temperature × Salinity × Acidity × UVA× UVB	300.62	420.93
6 factors		
Temperature × Salinity × Acidity × UVA × UVB × UVC	1.09	2.83



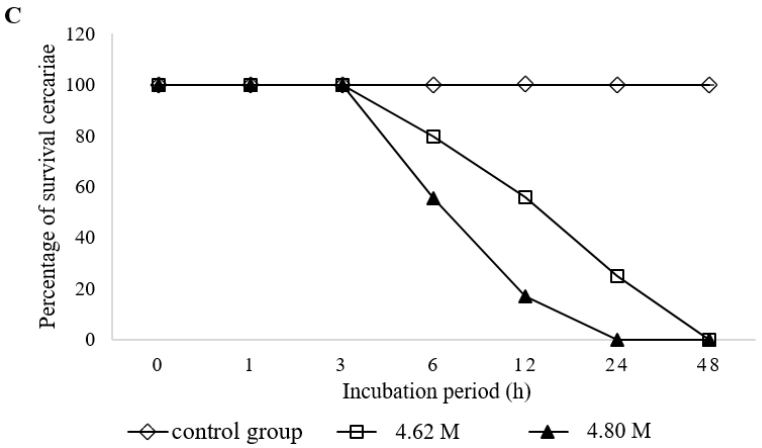


Figure 1 The number of *O. viverrini* cercarial changes at incubation period of temperature (A), salinity (B) and acidity (C).

Discussion

Climate change is expected to have effects on the distributions of parasitic diseases transmitted via aquatic organisms via alterations in natural temperatures, water levels, salinity, and pH [18]. They are environmental factors that have been shown to interactively affect the biota of ecosystems. All 4 environmental factors, separately or in combination, were investigated in this experiment and were found to strongly affect the survival of *O. viverrini* cercariae. UVC was the most effective single factor at causing loss of infectivity in fish intermediate hosts. It is not surprising, therefore, that UVC radiation, in combination with all of the other factors, produced the most rapid killing of cercariae (LI_{50} of 1.09 min and LI_{95} of 2.83 min). Combinations of physicochemical factors accelerate the loss of infectivity of *O. viverrini* cercariae. Similar conclusions were reached previously, in a study of effects of temperature, salinity and UV radiation on the survival of cercariae of an intertidal trematode. Combinations of temperature and UV radiation showed the most deleterious effects on cercariae [19].

Temperature variation is of considerable importance in ecology, as proved to be the case for *O. viverrini* cercariae in our study. Similar conclusions were reported in studies on cercariae of *Echinostoma liei* (Digenea: Echinostomatidae) [20,21]. In this species, survival steadily decreased with increasing temperature, the maximum survival time falling to approximately 8 h at 40 ºC. The increased mortality at the higher temperature is likely directly linked to increased activity levels, which accelerates the depletion of the finite energy reserves of cercariae [22].

Salinity is a major factor influencing the distribution of aquatic organisms [23], including freshwater snail species, since it affects many of their physiological functions [24,25]. High salinities lead to increased metabolism and physiological malfunction and, at very high levels, mortality [26]. Cercariae of *O. viverrini* lost their infectivity more quickly at the higher salinity levels 7.29 and 8.40 ppt (LC_{50} and LC_{95}, respectively), consistent with findings in a previous study [27]. In that study, snails were exclusively found in water with salinity levels ranging between 0.05 and 22.11 ppt and the highest snail population densities were in rice fields, ponds, roadside ditches, and canals within a water salinity range of 2.5 - 5.0 ppt. The presence of *B. siamensis goniomphalos* was negatively correlated with water salinity. However, it seems that snails can tolerate higher salinities than cercariae of *O. viverrini*.

The mineral acidity of water is a measure of the total acid present [28]. Acidity had detrimental effects on *O. viverrini* cercariae with LC_{50} and LC_{95} values of 4.62 and 4.80 M H_2CO_3, respectively. The survival of cercariae decreased with increasing acidity. The pH of the water was generally high but showed greater variations in the pond habitats, which could have an impact on snail populations and distributions.

Ultraviolet radiation is an important environmental factor fluctuating at various spatial and temporal scales and can affect aquatic organisms [29]. We found that to be true in this study. Our observations may thus be more consistent with what has been described for cercariae of *Maritrema novaezealandensis* [30]. The data presented in ref. [19] consistently confirmed that UV has the potential to impair cercariae and cause disruption of cellular processes and that cercariae of *M. novaezealandensis* possess little, if any, the capacity to prevent and cope with such effects. Ultraviolet light may have several effects on water bodies. In addition to the effect of radiation itself, UV light causes an increase in temperature, which in turn may influence salinity and pH. Cercariae of *O. viverrini* must be influenced by the synergistic effects of all these changes.

Conclusions

Our study showed temperature, salinity, acidity, ultraviolet A, B, C radiation, and a combination of these factors to affect the ability of *O. viverrini* cercariae to infect cyprinid fish. The development of an alternative field-based approach to interrupt the life cycle of *O. viverrini* by physicochemical of these factors of cercariae could be of value for fish farm management. Fish farms have to the parasite-free, which relieve peoples of anxiousness about opisthorchiasis.

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