SURVIVAL LONGEVITY OF *Clonorchis sinensis* METACERCARIAE COLLECTED IN RAY - FINNED FISH (*Toxobramis houdemeri*) *IN VITRO* CONDITION

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ABSTRACT

Clonorchis sinensis is a biological carcinogen inducing human cholangiocarcinoma (CCA). Clonorchiasis is one of the important endemic infectious diseases in Vietnam. The present study investigated the survival longevity of *C. sinensis* metacercariae in various *in vitro* conditions to find the best way of inactivating metacercariae in fish muscle. The fish (n=910; 6.74 - 9.85 cm in length, and body weight 4.52 - 7.42 g/fish) were randomly sampled from Thac Ba reservoir, Yen Bai province. A total of 2358 *C. sinensis* metacercariae were detached from soaked fish individuals with different solutions of Lemon juice, Vinegar, Alcohol 55%, and NaCl 10% at different immersion times (1, 6, 12, 24, 48, 72 hours) to evaluate the inactivated rate of metacercariae. The results showed that the metacercariae did not die after 1 and 6 hours in 4 different solutions. However, a proportion of *C. sinensis* metacercariae (40%) immersed in Alcohol 55% became inactivated after 12 hours. The mortality rate of *C. sinensis* metacercariae in the Alcohol was higher than in Vinegar, Lemon juice, and NaCl during all the time of the experiment. These data suggest that Alcohol 55% are effective for inactivating metacercariae of *C. sinensis* after 60 hours.

Keywords: *Clonorchis sinensis*, *metacercariae*, *survival*, *fish, Thac Ba reservoir, Yen Bai, Vietnam.*

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1. INTRODUCTION

Small liver flukes (clonorchiasis, opisthorchiasis) are of important significance for public health in Asia [1]. It occurs primarily in East Asia, and it is currently endemic in South Korea, China, Taiwan, and Vietnam [1, 2, 3, 4]. The number of people infected in this region is estimated at 7 - 15 million [5, 6] and prevalence varies widely, from less than 1.0% in Guang Xi, China, more than 40% in North Vietnam, and more than 70% in Guangdong Pr., China, and 1.5 - 2.0 million people show symptoms or complications [7, 8, 9, 10]. Importantly, infected people had heavy infections up to 1.1 million with more than 1.000 eggs/g feces [5]. Currently, it is estimated that more than 200 million people are at risk of *C. sinensis* infection, and over 15 million are infected worldwide [11]. In Vietnam, at least 1 million people are infected with liver flukes with *C. sinensis* in the North and *Opisthorchis viverrini* in the Central and South regions [12, 13]. *C. sinensis* is endemic in 21 northern provinces, whereas *Opisthorchis* spp. in 11 central and southern provinces [14]. In general, all the liver fluke (*C. sinensis*, *O. viverrini*, and *O. felineus*) infections induce chronic inflammatory diseases of the hepatobiliary system, and in chronic high worm burden infections this may lead to bile duct cancer termed cholangiocarcinoma (CCA) [15, 16, 17]. Most of these manifestations are mild and

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asymptomatic. However, once advanced CCA develops, clinical manifestations such as jaundice occur in approximately half of the cases, while the other half may have no specific symptoms [1]. People infected with less than 100 worms have no symptomatic [16, 17, 18, 19]. In which, people infected with one-hundred to thousands of worms may cause jaundice, indigestion, epigastric discomfort, anorexia, general malaise, diarrhea, and mild fever [5]. Over time, infected people without treatment may have serious symptom such as liver enlargement, allergic lesions, congestion of the spleen, bile stone development, cholecystitis, and liver cirrhosis. The most serious possible outcome is the development of cholangiocarcinoma. Benign hepatobiliary diseases are characterized by cholangitis, obstructive jaundice hepatomegaly, periductal fibrosis, cholecystitis, and cholelithiasis [1, 15]. Clonorchiasis is a major health problem in endemic areas, it is estimated global loss of 275,370 disability–adjusted life years (DALYs) on health status in endemic areas [17]. In Western Siberia where in highly endemic foci of *O. felineus*, CCA was detected in 77% of patients infected with *Opisthorchis* and in 34.2% of patients without *Opisthorchis* [16]. In Vietnam, a new endemic area of *C. sinensis* has been identified in residents living around Thac Ba Lake, Yen Bai province with the prevalence of 35% [20]. Recently, wild - caught fish from this lake were found infected with high prevalence and intensity of *C. sinensis* metacercariae [21]. In these mountainous regions, the risk for human infection is closely related to social and cultural traits that determine food behaviors [22]. Eating habit of raw or undercooked fish is common in localities near lakes, reservoirs, streams and ponds where fresh fish are readily available which can lead to infection of *C. sinensis* in the fish muscle.

However, there is little information and big gaps of knowledge on the viability of metacercariae of *C. sinensis* in different treatment methods. The previous study conducted by Fan (1998) revealved that metacercariae of Clonorchis were not effective to inactivate in frozen freshwater fish [23]. The viability of *C. sinensis* metacercariae obtained from cyprinid fish-*Pseudorasbora parva* in different temperatures and saline solutions showed that, metacercariae had lived for 10 - 20 days at -12°C, but died after 3 - 7 days at -20°C. With saline solution (3 g salt /10 g fish) metacercariae can survived for 5 - 15 days at 26°C [23]. At room temperature, metacercariae of *C. sinensis* lived for 5 - 12 days (under water conditions) and for 3 - 7 hours (without water). At 65°C, metacercariae of *C. sinensis* died after 3 hours but at 40^0C metacercariae live within 2.5 hours. Metacercariae isolated from dead fishes are alive for 3 - 7 days at room temperature and for 30 days at 4°C. There were over 50% of metacercariae alive up to 60 days after being isolated. Under freezing conditions, metacercariae died after 35 hours at -12°C and after 30 seconds at -196°C [1]. This study investigates the survival longevity of *Clonorchis sinensis* metacercariae *detached from soaked fish* in different sollutions.

2. METHODOLOGY

Fish collection: A total of 910 the ray-finned fish (*Toxobramis houdemeri*) with average length from 6.74 to 9.85 cm, bodyweight from 4.52 - 7.42 g/fish were collected from Thac Ba reservoir, Yen Bai province.

Experimental design

We used Lemon juice, Vinegar, Alcohol 55%, and NaCl 10% to inactivate metacercariae in fish because they are the common ingredients used in food processing and the making of raw fish food "Gỏi cá". In the habit of eating raw fish food is sliced thin and then soaked with these solutions.

Each fish will be measured the total length by a ruler, and weighted by analytical scales. After that, each of 10 fish was grouped together in one glass (a total of 30 fish for one experiment). Fish samples were kept in 4 different solutions (NaCl 10%, Alcohol 55%, Vinegar and Lemon juice) in different times (1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 60 hours, 72 hours) in room temperature 37^0C. Fish samples were stored in NaCl 0.85% in 4° as a control group. And then, fish samples from each experiment were crushed and

incubated for 3 hours at 37°C and then digested and examined the mortality of isolated metacercariae by observing the ability of movement of small live fluke under Olympus-CX31 (Japan).

Metacercariae from the soaked fish in 4 different solutions were detached by using the pepsin tissue digestion method and morphologically identified according to the key published by Sohn (2009) [24].

Statistic

Data were inserted into an Excel and converted to the R software for statistical analysis. The effect of different solutions for inactivating metacercariae infected in fish was compared by using prop.test in R software. All statistical tests with p value < 0.05 were considered statically significant.

3. RESULTS

Table 1 shows the average length and body weight of the ray-finned fish (*Toxobramis houdemeri*). The length and body weight of *T. houdemeri* in Alcohol solution were statically different compared with NaCl, Lemon juice, and Vinegar ($p < 0.01$).

Table 1. Fish length and body weight

Solutions treatment	No. Fish	Mean length (±SE)	95% CI	Mean body weight (±SE)	95% CI
NaCl	230	$9.73^{b} \pm 0,61$	9.48 – 10.00	$7.42^{b} \pm 1.64$	6.71 - 8.13
Lemon juice	240	$9.57^{b} \pm 0.75$	9.25 - 9.89	$7.08^{b} \pm 1.86$	6.29 - 7.87
Alcohol	230	$6.74^{a} \pm 2.09$	5.82 - 7.64	$4.52^{a} \pm 1.64$	3.81 - 5.24
Vinegar	210	$9.85^{b} \pm 0.85$	9.46 - 10.23	$6.83^{b} \pm 1.67$	6.07 - 7.59

Superscript letters in the column indicate significant ($p \leq 0.05$) differences in length and body weight intensity; the same letters indicate no significant difference, SE standard error, CI confident interval

Table 2. The mortality rates of *C. sinensis* metacercariae of infected fish after being immersed with different solutions

Time post immersion (hours)	NaCl 10%	Alcohol 55%	Vinegar 100%	Lemon juice 100%
1	0% (0/44)	0% (0/243)	0% (0/48)	0% (0/48)
6	0% (0/67)	0% (0/106)	0% (0/47)	0% (0/50)
12	6.85% (5/73)	40% (10/25)	0% (0/217)	0% (0/210)
24	16.28% (7/43)	54.05% (20/37)	10.29% (7/68)	10.38% (11/106)
48	37.5% (12/32)	60.61% (20/33)	10% (5/50)	11.59% (8/69)
60	98.48% (65/66)	100% (28/28)	54.55% (36/66)	25.21% (61/242)
72	97.83% (45/46)	100% (47/47)	35.68% (66/185)	58.06% (36/62)

A total of 2358 *C. sinensis* metacercariae from 910 soaked fish individuals in 4 different solutions experiments were detached including 371 metacercariae from fish fixed in NaCl 10%, 787 metacercariae in Lemon juice, 519 metacercariae in Alcohol 55% and 681 metacercariae in Vinegar. The mortality rate and live rate of metacercariae were calculated after 1, 6, 12, 24, 48, 60, and 72 hours (Table 2). After 1 and 6 hours, no metacercariae died. However, *C. sinensis* metacercariae in fish fixed in alcohol became inactivated after 12 hours 40% (10/25). The mortality rate of *C. sinensis* metacercariae which detached from soaked fish in the Alcohol was higher than in Vinegar, Lemon juice and NaCl in all most of the time at 12, 48, 60, and 72 hours with the following values 40, 17.16, 5.84; 5.25, 5.20, 3.32; 6.06, 5.23 1.61; 2.80, 3.97, 1.01; 1.83, 1.72, 1.02 times respectively. The mortality of *C. sinensis* metacercariae in 4 different solutions after 12, 24, 48, 60, and 72 hours were statistically significant ($p < 2.2e-16$).

There has been little doubt about the mortality of *C. sinensis* metacercariae in the infected fish in different solutions. The present study addressed a small part of the problem by using common ingredients used in food processing and the making of raw fish food every day. We found that (1) all 4 different solutions were associated with the mortality of *C. sinensis* metacercariae, (2) NaCl 10% and alcohol 55% can be inactivated *C. sinensis* metacercariae after 12 hours and (3) the Alcohol 55% was the best solution to inactivated *C. sinensis* metacercariae. Our finding is consistent with previous studies. In previous study, the metacercariae isolated from fish were exposed to Lemon juice 100% and Ethanol 30%, 40%, and 50% directly. The survival rates were 100% within 1 hour, 95.3% within 3 hours, and about 70% after 6 and 12 hours in Lemon juice. In Alcohol, the survival rates of the metacercariae varied on different concentrations in the range of 83.3 - 98.5% after the first 30 minutes and 85.4 - 96.6% after 60 minutes and 62.1 - 83.3% after 90 minutes by Phan *et al.* (2016) [25]. However, in the present study, the survival rate is 100% in Lemon juice and Vinegar, 60% in Alcohol, and 93.15% in NaCl 10% after 12 hours, suggesting that *C. sinensis* metacercariae in infected fish can live longer than those isolated when are soaked in solutions.

The other studies detected metacercariae remained viable and ineffective after storing at a heavy salt concentration (30%) for 7 days by Fan (1998) [23] and these findings gave results similar to a study by Wongsawad *et al.* (2005) [26], which showed that Lemon juice (pH = 3) had no killing effect on metacercariae of *Stellantchasmus falcatus* and also agreed with that by Prasongwatana *et al.* (2013) [27], which showed that metacercariae of *O. viverrini* could survive in fermented fish, pla-ra and plasom. However, there is little difference with time for inactive metacercariae in salt, our findings indicate that no metacercariae were viable after 72 hours but Fan (1998) [23] observed metacercariae still alive after 7 days. Among 4 different solutions, our results also show that using NaCl 10% and Alcohol can inactivate metacercariae better than using Lemon juice and Vinegar for treatment. The potential mechanisms for causing the higher death of metacercariae remain to be elucidated, but there are several reasonable hypotheses: Alkalosis had an effect on the metacercariae wall. The mortalily rate of *C. sinensis* in Alcohol solution treatment is highest after 12 hours experiment. The reason may be due to the mean body weight of fish in Alcohol treatment is smaller than NaCl, Lemon juice, and Vinegar that make alkalosis, dehyration process is faster than those. The mortality rate of *C. sinensis* in Lemon juice and Alcohol and NaCl solutions treatment increased with time, while in Vinegar changed at 72 hours. The reason may be due to fish being infected with a number of *C. sinensis* metacercariae are larger than in NaCl, Lemon juice, and Alcohol solutions which cause the different.

These results have taken together suggest that keeping the fish in NaCl 10%, Lemon juice, Alcohol 55% and, Vinegar in a short time are ineffective for inactivating metacercariae. The present findings must be interpreted in the context of a number of potential limitations such as

increased sampling to increase the fish samples for analysis in the long time with another treatment, and collect the same fish size for all solutions treatment. In conclusion, these data suggest that we should not eat raw fish or undercooked fish.

4. CONCLUSION

The mortality rate of *C. sinensis* metacercariae detached from fish immersed in the Alcohol 55% was higher than those in Vinegar, Lemon juice and NaCl. Alcohol 55% are effective for inactivating *Clonochis sinensis* metacercariae recollected from fish after 60 hours. The result of this study provides important information on the Survival longevity of *Clonorchis sinensis* metacercariae detached from soaked fish in NaCl, Alcohol, Lemon juice, and Vinegar, which can be used to warn people like eating uncooked fish, and raising awareness for everyone to prevent infection of *C. sinensis* from fish.

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