ELIMINATION OF *HAPLOCHIS TAICHUI* METACERCARIA IN CYPRINOID FISH WITH FREEZING TEMPERATURE AND SOURED FISH (*PLASOM*) WITH SALINITY

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Abstract. Human *Haplorchis taichui* intestinal fluke infection occurs via consumption of raw or undercooked cyprinoid fish and products made of this fish, *eg* soured fish (*plasom*). This study investigated the elimination of the *Haplorchis taichui* metacercaria in cyprinoid fish by freezing temperature (-20°C) and in *plasom* made from cyprinoid fish by salinity. Moving score and movability index were used as criteria for determining viability of the metacercaria. Fish samples were derived from a natural reservoir at village Phu Khambao, Tambon Ubolrattana, Ubonrattana District, Khon Kaen, Thailand (GPS location: 16°43′22″N 102°37′28″E). The results showed that fish samples stored at -20°C in a commercial freezing (ice-cream) cabinet must be kept for at least 72 hours to completely eliminate the metacercaria. In addition, salinity at 41.2 ppt or higher effectively eliminated the metacercaria in *plasom* samples kept at room temperature for two days. Since *plasom* with high salinity is too salty and unhealthy, households or home-based producers should be suggested to produce fluke-free *plasom* by using fish stored in commercial ice-cream cabinet for 72 hours.

Keywords: *Haplochis taichui*, cyprinoid fish, *plasom* (soured fish), freezing temperature, salinity

INTRODUCTION

Cyprinoid fish are the secondary intermediate hosts for metacercaria of the intestinal fluke, *Haplorchis taichui* (Faust and Nishigori, 1926). This parasitic infection is found in humans throughout Southeast Asian countries, with most Thai cases confirmed in the north and northeast regions (Ratanasritong and Kliks, 1972; Radomyos *et al*, 1998; Wongsawad *et al*, 2000; Boonchot and Wongsawad, 2005; Chai *et al*, 2005, Kumchoo *et al*, 2005; Nithikathkul and Wongsawad, 2008; Chai *et al*, 2009a, b). *H. taichui* infection occurs through human ingestion of metacercaria via raw or undercooked fish or fish products, such as soured

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fish (*plasom*). After arriving at duodenum, *H. taichui* metaceraria excysts and attaches to the intestinal mucosa (Sukontason *et al*, 2001). Development and penetration of the adult fluke into the mucosal layer or microvilli of the intestine can cause irritable bowel syndrome-like symptoms with excessive mucous production and superficial mucosal necrosis, leading to colicky pain and mucous diarrhea (Beaver *et al*, 1984; Watthanakulpanich *et al*, 2010). *Plasom*, an indigenous food of northern and northeastern Thailand, is usually made from raw small cyprinoid fish by mixing with salt, chopped garlic and steamed rice, and fermenting in a container or plastic bag at room temperature for two days. Since the people in these two regions have long established traditions of eating raw and fermented fish dishes like *plasom*, *H. taichui* infection is a persistent public health problem (Manning *et al*, 1971; Harinasuta *et al*, 1987; Waikagul, 1991; Radomyos *et al*, 1998; Sukontason *et al*, 1999; Sukontason *et al*, 2005; Chai *et al*, 2005; Chai *et al*, 2009a; Wongsawad *et al*, 2009).

Temperature is a crucial factor for the survival of the metacerariae of many fish-borne trematodes, though the survival rate varies amongst studies. Encysted *H. taichui* metacercaria can survive in *Tilapia zillii* fish stored at 5°C for 11 days, -5°C for 24 hours, and -10°C for 16 hours, respectively (Abdallah *et al*, 2009). Wongsawad *et al* (2009) also found that *H. taichui* metacercaria can survive in fish stored in refrigerator for over two weeks. *Clonochis sinensis* larvae in *Pseudorasbora parva* fish have survived even being stored at -12°C for 10-20 days and -20°C for 3-7 days (Fan, 1998). However, Fattakov (1989) found that *Opisthorchis* spp metacercaria in fish could be eliminated if stored at -20°C for 3-7 days, -28°C for 15-18 hours, -35°C for 8 hours, and -40°C for 2 hours, respectively. If very low temperatures are effective at eliminating metacercaria, then storage at -20°C is probably the most practical and effective temperature to promote as commercial freezing (ice-cream) cabinets with temperatures of -20°C are widely available at prices affordable for small scale producers or households.

Salinity levels could also be a possible factor in eliminating *H. taichui* metacercaria in *plasom*. Usually, *plasom* produced in local endemic areas has salinity of 15-30 ppt which was not effective in eliminating *H. taichui* metacercaria in *plasom* being stored at room temperature for 2-3 days. Wongsawad *et al* (2009) found live *H. taichui* metacercariae in *plasom* containing 15 ppt salt stored at room temperature for 14 days and in refrigerator for 21 days. These findings do not rule out the potential for higher salinity levels to impact on metacercaria survival rates.

Endemic *H. taichui* infection rates are an ongoing public health problem in some northern and northeastern Thai communities. There is suggestive potential for varying salinity levels and low temperature storage of fish to reduce metacercarial survival rates in raw fish. Given this, this study was designed to assess the effectiveness of these two relatively simple practices for eliminating encysted metaceraria in cyprinoid fish and consequent human *H. taichui* infection burden.

MATERIALS AND METHODS

Fish samples

Cyprinoid fish used in this experiment were caught in a northeastern Thailand natural reservoir at Village Phu Khambao, Tambon Ubolrattana, Ubonrattana District, Khon Kaen, Thailand (GPS location: 16°43′22″N 102°37′28″E).

Design

A group comparison design was used. The fish were randomly assigned without predilection into two equal groups: group 1 for treatment with freezing temperatures, and group 2 for fermented fish (*plasom*) preparation using different salinity levels. Experimentation was performed in the Department of Veterinary Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Thailand.

Varying temperature storage condition: Group 1

Group 1 fish were randomly allotted into 7 sets of 0.5 kg. Each set was stored at different temperatures and durations; set 1 at 4°C (in a refrigerator) for 48 hours, and sets 2-7 at -20°C in a commercial freezing cabinet (Sanyo chest freezer, SF-C697, Sanyo, Bangkok, Thailand), for 12, 24, 36, 48, 60 and 72 hours, respectively. Freezer temperature was monitored with a digital thermometer at 08.30AM and 04.30PM daily during the whole experiment. The average temperature of the freezer was -20.6°C in the morning and -20.7°C in the afternoon. Then, the remaining *H. taichui* metacercariae in each fish sample was investigated at the end of each experimental period. This experiment was repeated three times.

Varying salinity condition: Group 2

To prepare *plasom* all fish in group 2 were cut into small pieces, mixed together, and equally allotted into eleven bags of 160 g fish per bag. Then, chopped garlic (16 g), steamed rice (8 g) and differing amounts of cooking salt (0, 0.1, 0.5, 1, 2, 5, 10, 20, 50, 70 and 100 g, respectively) were mixed into each bag. The ingredients were mixed manually and the bags were shaken until all salt was dissolved and no ingredient clumps were left. This was to ensure as thorough mixture of ingredients dissolved salt as possible. The *plasom* bags were then tied tightly with rubber bands and stored at room temperature for two days. *Plasom* salinity in each bag was measured prior to investigating the existence of metacercariae (PL-700PC bench top meter, Gondo Electronic, Taipei, Taiwan) and was found to be 0.62, 8.45, 9.06, 9.76, 10.9, 10.4, 16.0, 34.7, 41.20, 49.40 and ≥ 50 ppt, respectively. The experiment was repeated three times.

Isolation and identification of *H. taichui* metacercaria

Isolation of *H. taichui* metacercaria was performed by mincing the samples and digestion with pepsin solution (0.25% w/v pepsin A, 0.85% w/v NaCl and 1.5% v/v HCl) at a ratio of 1 kg sample per 3 liters of pepsin solution. The number and microscopic morphology of the existing metacercaria was identified via a stereoscope or compound microscope following Pearson and Ow-Yang (1982) and Scholz *et al* (1991). In general, the intact *H. taichui* metacercaria with gelatinous coat has round or oval shape and is 188-220x155-185 μm in size. The C-shaped larva contains a set of clearly detectable oral and ventral suckers. A dark round excretory bladder and 11-18 baseball glove-shaped ventro-genital sacs observable at the posterior part were specific marker structures for the identification of this parasite.

Moving score and movability index as criteria for determining viability of *H. taichui* metacercaria

Moving score (MS) and movability index (MI) of the collected metacercariae were determined following Wongsawad *et al* (2009). Based on degree of internal structures and movement of metacercaria, MS was defined into 4 categories; MS0 (degenerated or dead: could not identify internal structures and movement); MS1 (non-active: could identify internal

structures but no movement within 30 seconds); MS2 (slightly active: could identify movement within 5-30 seconds); and MS3 (active: could identify movement within 5 seconds). Then, MI was calculated from the formula ($\sum \frac{nxNn}{\sum N}$) to determine viability of the parasite, where n=MS, N_n=number of metacercariae of all MSs, and $\sum N$=number of all existing metacercariae. MI was then classified into four categories: 0 (all metacercariae were dead); 0-1 (some were still alive); 1-2 (most were still alive); and ≥ 2 (all were still alive).

Statistical analysis

Descriptive statistic was used to present MS, either as total metacercariae or percentage (number of metacercariae in each MS/total metacercariae). The Kolmogorov-Smirnov normality test showed that the transformed MI was normally distributed. Then, one-way ANOVA was performed to compare mean±SD values of MI and Tukey's Post Hoc Test was used for paired comparisons (SPSS version 19; IBM, Armonk, NY). Statistical significance was considered at a p-value < 0.05.

RESULTS

Existence of *H. taichui* metacercaria in cyprinoid fish after freezing

The total number of *H. taichui* metacercariae found in fish stored at 4ºC for 48 hours was 207±20. These were rated for movability as 17.3±2.5 (8.3%) in MS0 level (degenerated or dead), 64.3±12.1 (30.9%) in MS1 (non-active), 39.4±4.7 (9.1%) in MS2 (slightly active), and 80±6.2 (33.5%) in MS3 (active), respectively (Table 1). The majority of the metacercariae in this condition were still alive. Further, the movability index (MI) for these samples (2.06±0.04) also indicated that active metacercariae were abundant in these fish samples. Thus, risk of infection from ingesting these fish was still high.

The total numbers of *H. taichui* metacercariae in fish stored at -20ºC for 12, 24, 36, 48, 60, and 72 hours were 185.3±42.6, 169.3±43.0, 107.7±15.7, 90.3±11.0, 62.0±3.6, and 59.7±24.4, respectively (Table 1). Metacercaria survival decreased with the increasing duration of -20ºC freezer storage. After storage for 60 hours, only 1.7±0.6 (2.7%) active metacercariae were found (MS3 level), with almost all metacercariae (60.3±3.1, 97.3%) in MS0 (degenerated or dead level). After fish were stored for 72 hours, all metacercariae were degenerated or dead, rated as MS0. Movability indices for fish samples stored at -20°C for 12, 24, 36, 48, 60, and 72 hours were 1.98±0.07, 1.35±0.11, 0.59±0.01, 0.19±0.06, 0.08±0.03 and 0, respectively (Table 1). These data indicated that number and movability of the metacercariae decreased with longer freezer storage durations. The complete elimination of metacercariae was only successful in the samples stored at 20ºC for 72 hours. Statistical differences at p<0.05 among MIs of fish samples stored at -20ºC for 12-60 hours were obtained.

Existence of *H. taichui* metacercaria in *plasom* with different salinities

In general, the total average number of movable *H. taichui* metacercariae in *plasom* decreased significantly (from 27±4, 24±8, 27±5, 23±6, 27±10, 17±3, 9±1, 9±2, 10±4, 7±2 to 6±1) following the increases of salinity levels (from 0.62, 8.45, 9.06, 9.76, 10.9, 11.4, 16.0, 34.7, 41.2, 49.4 to ≥ 50 ppt), respectively (Table 2). Metacercariae were found in all moving scores (MS0 to MS3) if the fish had salinity of 16 ppt or lower. However, they were constrained in the MS0 to MS2 ranges with majority in MS0 if the salinity was 34.7 ppt. If salinity was 41.2 ppt or higher, all metacercariae were

Table 1
Average number and percentage of *H. taichui* metacercariae distributed in different moving scores (MS) and movability indices (MI) in fish samples stored at 4°C for 48 hours and -20°C for 12, 24, 36, 48, 60 and 72 hours.

Temperature/ Duration (°C/Hours)	Average number of metacercariae±SD (%)					MI (Mean±SD)
	MS0	MS1	MS2	MS3	Total	
4/48	17.3±2.5 (8.3)	64.3±12.1 (30.9)	39.3±4.7 (19.1)	86.0±6.2 (33.5)	207±20 (100)	2.06±0.04
-20/12	43.0±15.6 (23.1)	26.0±16.1 (14.7)	16.7±12.5 (9.2)	99.7±38.6 (52.9)	185.3±42.6 (100)	1.98^{a}±0.07
-20/24	73.7±25.5 (43.0)	39.7±9.7 (23.6)	10.3±2.1 (6.5)	45.7±12.1 (27.0)	169.3±43.0 (100)	1.35^{b}±0.11
-20/36	79.3±11.2 (73.7)	11.7±0.6 (10.9)	5.0±2.0 (4.6)	11.7±2.5 (10.8)	107.7±15.7 (100)	0.59^{c}±0.01
-20/48	84.0±11.4 (92.9)	0.3±0.6 (0.4)	0.7±0.6 (0.8)	5.3±2.1 (5.9)	90.3±11.0 (100)	0.19^{d}±0.06
-20/60	60.3±3.1 (97.3)	0 (0)	0 (0)	1.7±0.6 (2.7)	62.0±3.6 (100)	0.08^{a}± 0.03
-20/72	59.7±24.4 (100)	0 (0)	0 (0)	0 (0)	59.7±24.4 (100)	0

Mean MI with different superscripts (a-d) indicates statistically significant (p<0.05), (n=7).

strictly confined only in the MS0. These results were consistent with the calculated movability index (MI). In samples having salinities of 16 and 34.7 ppt, MI was lower than 1, and became zero in samples with salinities of 41.2 ppt or higher. All metacercariae in *plasom* samples with salinities of 41.2 ppt or higher were completely degenerated or dead within two storing days. MIs of the samples with salinities of 0.62 to 41.2 ppt were significantly different (p<0.05) (Table 2).

DISCUSSION

In this experiment, all *H. taichui* metacarcariae in the fish were completely eliminated after storage at -20°C for at least 72 hours. How *H. taichui* metacercariae in fish were eliminated by deep freezing is not clearly understood, although information regarding freezing injury of other cell types is useful to consider. At freezing temperature (-20°C), ice crystals form and can disrupt both physical cell structures and biochemical activities; thus affecting survival of the metacercaria. It was apparent that a fatal environment was successfully achieved for the group stored at -20°C for at least 72 hours when all metacercariae were eliminated. However, a wide distribution of the moving scores (MS) and movability indices (MI) of the metecarariae in fish stored at 4°C for 48 hours and -20°C for shorter than 72 hours indicates variable viability where fish of these groups still contained numerous active metacercariae.

A gelatin coat surrounding each *H. taichui* metacercaria could play an important role as the first line of natural defense against such a potentially fatal environment. The intact coat could provide temperature and osmotic tolerance for metacercaria, especially during

Table 2
Average number and percentage of *H. taichui* metacercaria distributed in different moving scores (MS) and movability index (MI) in *plasom* samples having different salinity levels.

Salinity (ppt)	Average number of metacercariae±SD (%)					MI (Mean±SD)
	MS0	MS1	MS2	MS3	Total	
0.62	2±1 (6.2)	3±2 (9.9)	4±2 (16.0)	18±2 (67.9)	27±4 (100)	2.46^{a} ±0.11
8.45	3±1 (12.7)	2±1 (8.5)	8±4 (32.4)	11±2 (46.5)	24±8 (100)	2.14 ±0.13
9.06	3±1 (11.0)	6±2 (20.7)	10±4 (37.8)	8±1 (30.5)	27±5 (100)	1.89^{a} ±0.10
9.76	5±1 (21.4)	6±2 (24.3)	5±2 (22.9)	7±3 (31.4)	23±6 (100)	$1.61^{a,b}$ ±0.19
10.90	7±2 (24.4)	9±2 (32.9)	7±6 (25.6)	5±1 (17.1)	27±10 (100)	$1.33^{b,c}$ ±0.10
11.40	7±2 (38.5)	5±2 (30.8)	3±1 (19.2)	2±1 (11.5)	17±3 (100)	1.04^{c} ±0.04
16.00	4±2 (48.1)	3±1 (29.6)	1±1 (14.8)	1±1 (7.4)	9±1 (100)	0.82^{c} ±0.11
34.70	5±2 (61.5)	3±1 (30.8)	1±1 (7.7)	0 (0)	9±2 (100)	0.47^{d} ±0.09
41.20	10±4 (100)	0 (0)	0 (0)	0 (0)	10±4 (100)	0.00
49.40	7±2 (100)	0 (0)	0 (0)	0 (0)	7±2 (100)	0.00
≥50	6±1 (100)	0 (0)	0 (0)	0 (0)	6±1 (100)	0.00

Mean MI with different superscripts (a-d) indicates statistically significant ($p<0.05$), ($n=7$)

early storage. Therefore, severe physical assault of sufficient duration could be needed to overcome the gelatinous barrier and allow cellular structures and mechanisms to be injured or disrupted, leading to metacercaria degeneration. Since cryoprotectant was not used in this study, the freezing temperature could have directly damaged the gelatin coat and had direct contact with the metacercaria. Varying thicknesses of metacercaria gelatin coats may explain the varying freezing tolerance levels found and reflected in unevenly distributed MS and MI values. Similar phenomenon has been observed in other parasitic species with different thickness of gelatin coat. For example, metacercaria of *Clonorchis sinensis* with thicker gelatin coats can preserve their infectivity in experimental rats and rabbits even stored at -12°C for 10-20 days and -20°C for 3-7 days (Fan, 1998). Fattakhov (1989) also found that metacercariae of *Opisthorchis* spp in fish were eliminated if stored at -28°C for 15-18 hours, -35°C for 8 hours and -40°C for 2 hours, respectively. Encysted metacercariae of parasites in the family Diplostomatidae, Haplorchidae and Prohemistomatidae can survive in tilapia fish kept at 5°C for 12, 11 and 14 days, but at -10°C for 16, 32 and 40 hours, respectively (Abdallah *et al*, 2009). Variability across these data and ours suggests further study on these differences is crucial.

Injury or degeneration of metacercariae could be the fatal sequel of both intracellular and extracellular ice crystal formation (Han and Bischof, 2004; Takamatsu and Zowlodzka, 2006). During ice crystal formation, the solution changes would segregate the solute to the non-solidified water thereby increasing its concentration. Then, such increased concentration would directly affect cell viability. Surrounding ice crystals could block cellular transportation, and simultaneously press or scratch cells and damage cell membranes. Effluxion of the intracellular fluid drawn by the extracellular ice would lead to cellular dehydration. The resulting osmotic stress would cause the loss of osmotic equilibrium so that the concentration inside the cell could not be retained. These conditions would be the severe physical actions to cause cell injury. If the freezing treatment continues, intracellularly-formed ice crystals could directly block the relocation of various cytoplasmic components and biochemical mechanisms so that all vital cellular activities are stopped either partially or completely (Takamatsu and Zawlodzka, 2006). Metacercariae would die or degenerate if all cellular structures and biochemical mechanisms involving intracytoplasmic movements were disrupted, strongly supported by the MS and MI values in Table 1. For metacercariae found only in MS0, their calculated MI would be zero as well, indicating a complete degeneration or death of the metacercariae in that sample.

Plasom is typically made in village households by mixing chopped garlic, steamed rice and salt with cyprinoid fish, and fermenting for only 2 days. A longer fermenting period is not preferred because *plasom* will become too sour or off-flavor due to high amount of lactic acid being produced by lactic acid bacteria. Our preliminary survey had found that salinity levels in the two-day fermented *plasom* made by the village households ranged from 15 to 30 ppt. Wongsawad *et al* (2009) demonstrated that the metacercariae could survive in uncooked *plasom* with salinity of 15 ppt for 14 days at room temperature or 21 days in refrigerator. Thus, *H. taichui* infection risk is still high in people consuming raw or uncooked plasom with salinity levels of 15-30 ppt after a 2 day storage period. Since this experiment was to study the direct effect of salinity on the metacercarial survival, the even distribution of salinity in the whole fish was crucial. Thus the thorough mixing of fish being chopped into small pieces with ingredients was to allow the melted salt to effectively and rapidly penetrate into the whole sample, and provide an even salinity environment. Our results clearly showed that salinity of 34.7 ppt and lower did not totally eliminate the infective *H. taichui* metacercariae within two days. All metacercariae in the *plasom* were completely eliminated only with salinity levels of 41.2 ppt or higher (Table 2). However, *plasom* having this salinity level is too salty and not healthy for consumption.

Intracellular and extracellular concentrations of sodium chloride determine intracellular and extracellular osmolarity. A 2% or higher concentration of sodium chloride can immensely increase osmotic pressure which is truly critical to the viability of the metacercaria of certain trematodes (Oshima, 1957 cited by Yokogawa, 1965). Addition of salt into fish initially increases salinity or tonicity of the extracellular fluid. Since the cell membrane performs as a selectively permeable membrane, increased tonicity surrounding the cells is the determining factor for differences in osmotic pressure between inside

and outside cellular components of the *H. taichui* metacercaria. It was apparent that metacercaria still existed in any MSs and their MIs were still high at the lower salt concentrations. This was because the cell membrane can naturally tolerate a certain osmotic pressure, especially at salt concentrations lower than 30 ppt for 2 days. Increased tonicity and osmotic pressure established hypertonicity overload with hyperosmolarity (hyperosmotic concentration) at a concentration of 41.2 ppt for 2 days and affected the effluxion of the intracellular fluid. Consequent shrinkage of the cell occurs to prevent plasmolysis. This loss of equilibrium destines the metacercaria to stop moving and finally die.

In conclusion, *H. taichui* metacercariae were eliminated in cyprinoid fish stored at -20°C for at least 72 hours and in *plasom* with salinity levels of 41.2 ppt or higher for 2 days. These two simple treatments have important public health implications for reducing *H. taichui* infection among at-risk communities. Thus, we suggest households or home-based producers to produce fluke-free *plasom* or other fish products by using fish stored at -20°C (in commercial ice-cream cabinet) for 72 hours.

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