RESEARCH ARTICLE

Simplified Techniques for Killing the Carcinogenic, *Opisthorchis Viverrini* Metacercariae in Cyprinid Fish

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Abstract

Consumption of fluke-free fish is the most important factor in controlling *Opisthorchis viverrini* (OV) infection in endemic areas such as northeast Thailand and thereby reducing the risk of cholangiocarcinoma. Cooking fish is the best way to avoid infection; however, the cultural practice of eating raw or fermented fish is difficult to change. We investigated the food preparation process, using freezing, heating and fermentation to kill OV metacercariae in fish. Uncooked cyprinid fish infected with OV were divided into three groups: refrigerated at 4 °C for 24, 48 or 72 h (control group); frozen at -20 °C for 24, 48 or 72 h; or heated by microwaving (at 400 or 800 W) or boiling at 90 °C for 1, 5 or 10 min. Moreover, pickled (fermented) fish were divided into two groups: refrigerated at 4 °C (control) or frozen at -20 °C for 24 or 48 h. The infectivity of recovered metacercariae was confirmed by infecting hamsters with OV and then evaluating the recovery of adult worms after 1 month. We found that a heating process, by boiling or microwaving at 400 or 800 W for at least 5 min, could kill OV metacercariae, and freezing pickled fish at -20 °C for 48 h could kill OV metacercariae in all sizes of fish. The present study found that heating and freezing processes, as well as the fermentation process under optimal conditions, could kill OV metacercariae in a timely manner. This knowledge is valuable for implementation in endemic areas to control OV infection and cholangiocarcinoma.

Keywords: Liver fluke- Cyprinoids fish- *Opisthorchis viverrini*- fluke free- freezing- microwave- fermentation

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Introduction

Liver fluke infection (opisthorchiasis) in humans has been known for more than 100 years, but the prevalence of this disease remains high in Laos, Cambodia and the northeastern part of Thailand (Leiper, 1915; Chai et al., 2005; Sripa et al., 2010). This is due to the cultural tradition of eating uncooked cyprinoid fish dishes such as raw spicy fish salad (*koi pla*) and fermented, or pickled fish (*pla som*). The fish used to prepare these foods have a high risk of contamination by the infective stage, or metacercariae, of *Opisthorchis viverrini* (OV), which can cause opisthorchiasis in people who consume them (Sukontason et al., 1998; Keiser and Utzinger, 2009; Grundy-Warr et al., 2012; Prasongwatana et al., 2013). This is corroborated by our previous reports showing that metacercariae recovered from pickled fish could cause infection in a hamster model (Onsurathum et al., 2016a; Onsurathum et al., 2016b). In the case of the fermentation process of pickled fish, less than one week (Prasongwatana et al., 2013). Also, our previous report found the presence of OV metacercariae in pickled fish and fresh fish sold in markets in some provinces in northeast Thailand (Onsurathum et al., 2016a). More recently, we confirmed that OV metacercariae were able to remain viable in pickled fish stored under refrigeration at 4 °C, and also found that these metacercariae could infect hamsters (Onsurathum et al., 2016b).

Changing the eating culture of adults who have a preference for raw cyprinoid fish may be too difficult, but

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it is possible to do so in children, even at the pre-school level, if they are given the proper food safety education (Ziegler et al., 2011). Numerous papers have been published describing in great detail the mechanism of fluke-induced cholangiocarcinoma; yet, surprisingly, there is still a general lack of basic knowledge about how to ensure consumption of fluke-free fish, a procedure that is very simple to do but also very important to communicate to people living in endemic areas. Therefore, this study focuses on evaluating various methods (freezing, heating and fermentation) of achieving OV-free fish, and then determining the infectivity of any recovered OV metacercariae by testing on laboratory animals. This knowledge will be very useful for controlling OV in the community and reducing the risk of cholangiocarcinoma in the future.

Materials and Methods

Fish collection and preparation

Cyprinid fish were bought from a market in Mukdahan province. Fish were classified into three sizes, small, medium and large, based on their width and length, as shown in Figure 1 and Table 1. Photos of an OV-infected cyprinoid fish are shown in Figure 2. The experimental design (Figure 3) was as follows: heating in a microwave at 400 or 800 W for 0, 1, 5 or 10 min (Figure 4); boiling at 85–90 °C for 0, 1, 5 or 10 min (Figure 5); freezing at -20 °C or refrigerating at 4 °C (control group) for 24, 48 or 72 h; or fermenting for 24 h and then freezing at -20 °C or refrigerating at 4 °C for 24 or 48 h. Fish in the control group and all experimental groups were minced into small pieces, digested with pepsin-HCL at 37 °C for 1 h and then processed for metacercariae collection, as in previous studies (Sriraj et al., 2013; Aukkanimart et al., 2015). All recovered metacercariae were identified, and only OV metacercariae were counted, photographed and used for animal infection.

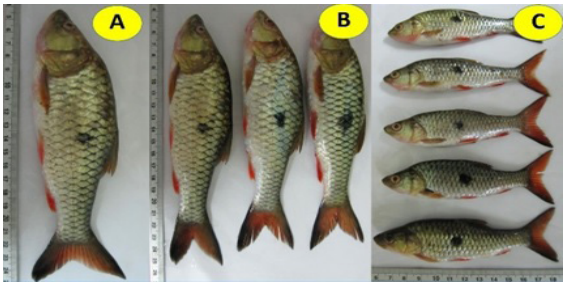

Figure 1. Sizes of Cyprinid Fish. Large (A), Medium (B) And Small (C)

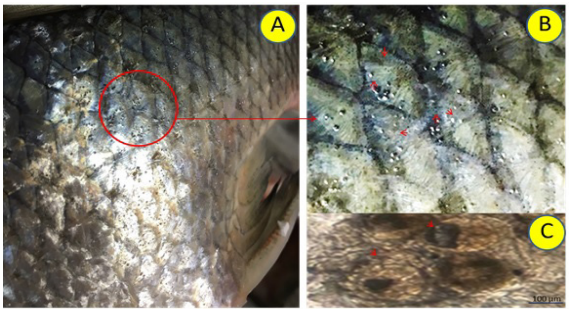

Figure 2. Infected Cyprinid Fish (A), fish scales (B) and metacercariae in scales (C). Circle and arrow indicates metacercariae

Animal infection

Recovered metacercariae from each assigned group were counted, photographed and then used to infect hamsters. All protocols were approved by the Animal Ethics Committee of Khon Kaen University based on the Ethics of experimentation of National Research Council of Thailand (ACUC-KKU-20/2559).

Worm recovery

After 30 days post-infection, all infected hamsters in each assigned group were sacrificed and the livers collected for worm recovery. Livers were squeezed and all worms were collected and counted under a microscope, as in previous studies (Sriraj et al., 2013; Aukkanimart et al., 2015).

Results

Recovery of metacercariae and adult worms after refrigerating at 4 °C or freezing at -20 °C

Recovered metacercariae were observed in all groups, but the highest number was found in the control group refrigerated at 4 °C for 24 h (1,256 metacercariae);

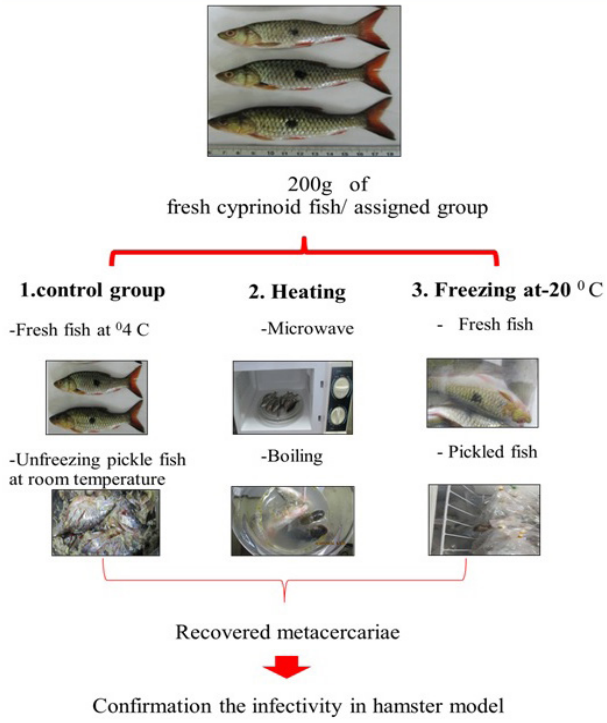

Figure 3. The Experimental Design

Table 1. Sizes of Cyprinid Fish

Size	Width (cm)	Length (cm)	Number of fish/kg	Thickness (cm)
Small (S)	2.5–3.5	<10	36	<1
Medium (M)	3.6–4.5	10–15	19	1.0–1.5
Large (L)	4.6–5.0	>15	7	1.6–2.0

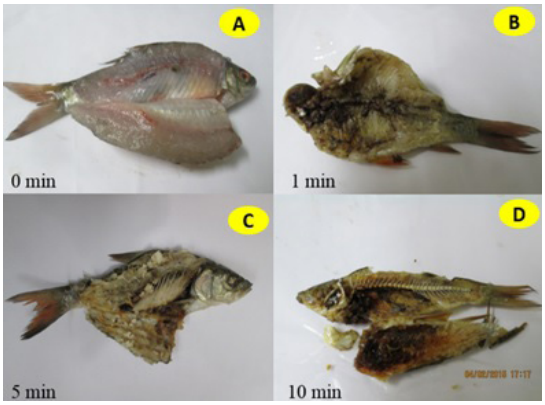

Figure 4. Fish Heated by Microwaving at 800 W for Various Periods of Time. 0 min (A), 1 min(B), 5 min (C) and 10 min (D)

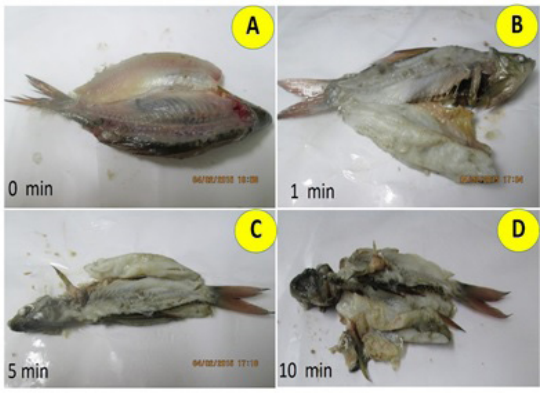

Figure 5. Fish Boiled at 85–90 ºC for Various Periods of Time: 0 min (A), 1 min (B), 5 min (C) and 10 min (D)

the number decreased at 48 and 72 h (604 and 590 metacercariae, respectively). The number of metacercariae in the group frozen at -20 ºC for 24 h (850 metacercariae) was lower than the control at 24 h, and further declined at 48 h (305 metacercariae) and 72 h (190 metacercariae), as shown in Figure 6.

The morphology of metacercariae in the control group refrigerated at 4 ºC for 24 and 48 h was normal and they were able to move, in contrast with the group frozen at -20 ºC for 24 and 48 h which showed abnormal morphology, as evidenced by degradation of the metacercarial bladder (Figure 7). Recovered adults were observed all groups, but the highest number of worms was recovered from the

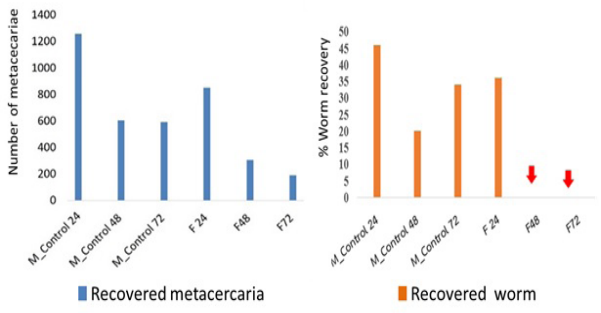

Figure 6. The Number of Metacercariae and Percentage of Worm Recovery from Fish Refrigerated at 4 ºC (Control Group) or Frozen at - 20ºC for 24, 48 or 72 h. F; Freezing, M; Median Size). Red Arrow Indicates 0 Worm

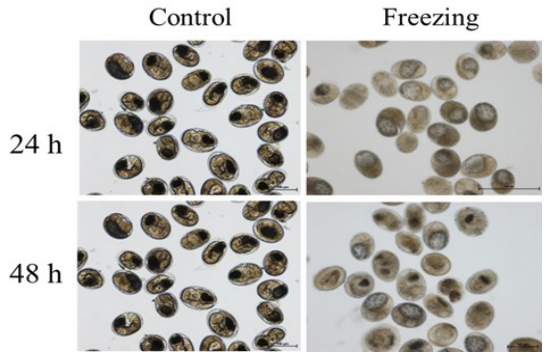

Figure 7. The Morphology of Metacercariae in the Control and Freezing Groups (4ºC and −20 ºC)

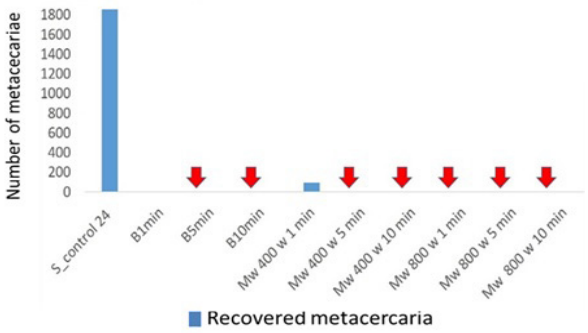

Figure 8. The Number of Metacercariae Recovered After Heating by Boiling (B) or Microwaving (Mw). Red arrow indicates 0 metacercariae

control group refrigerated at 4 ºC for 24 h (23 ± 7; 46%); fewer worms were recovered at 48 h (10 ± 8; 20%) and 72 h (17 ± 9; 34%). The number of worms recovered from the group frozen at -20 ºC for 24 h (18 ± 7; 36%) was lower compared with the control at 24 h; no worms were found in fish frozen for 48 h (0; 0%) and 72 h (0; 0%) (Figure 6).

Recovery of metacercariae after heating by boiling or microwaving

The highest number of metacercariae were recovered from the (unheated) control group (Figure 8). No metacercariae were observed in groups heated by boiling for 5 or 10 min, or by microwaving at 400 or 800 W for 5 or 10 min. However, in fish microwaved at 400 W for 1 min (and to a lesser extent in fish boiled for 1 min), a small number of metacercariae were observed with abnormal morphological changes evidenced by degradation of the metacercarial bladder.. as shown in Figure 9.

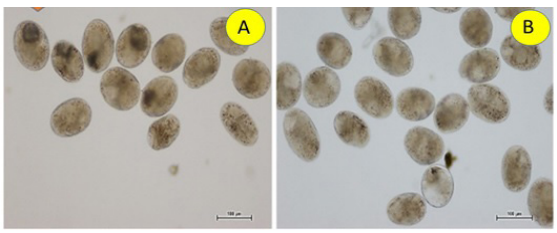

Figure 9. Abnormal Metacercariae Morphology after Boiling for 1 Min (A) or Microwaving at 400 W for 1 Min (B)

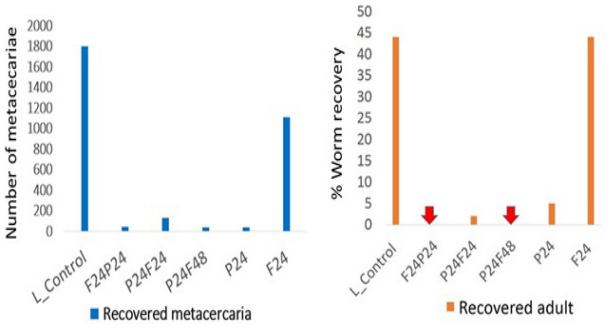

Figure 10. The Number of Metacercariae and Percentage of Adult Worms Recovered from Frozen Pickled fish. L, large size; F, freezing; P, pickled fish; F24P24, fish was frozen for 24 h and then these fish was used for pickle fish which fermented for 24 h; P24F24, fish was used for pickle fish which fermented for 24 h and then frozen for 24 h; P24F48, fish was used for pickle fish which fermented for 24 h and then frozen for 48 h; P24, fish was used for pickle fish which fermented for 24 h; F24, fish was frozen for 24 h; Red arrow indicates 0 worm.

Recovery of metacercariae and adult worms from refrigerated and frozen pickled fish

Recovered metacercariae were observed in all groups, but the highest number of metacerariae was found in the control group refrigerated at 4 °C for 24 h (1,800 metacercariae), followed by the group frozen for 24 h (1,108 metacercariae), pickled fish at room temperature (P24) (40 metacercariae), pickled fish fermented for 24 h and frozen for 24 h (P24F24:131 metacercariae), pickled fish fermented for 24 h and frozen for 48 h (P24F48:40 metacercariae), and finally frozen fish for 24 h and fermented for 24 h (F24P24:42 metacercariae) (Figure 10).

Metacercariae in pickled fish refrigerated at 4 °C for 24 h had normal morphology and were able to move, in contrast with the group frozen at -20 °C for 24 h which showed abnormal metacercarial morphology, as evidenced by degradation of the bladder (Figure 11). In the control group at 4 °C and the frozen group, more than 40% of adults were recovered. The percentage of recovered adults in the P24F24 and P24 (pickled but not frozen) groups was considerably lower, only 2–5%. Interestingly, no worms were recovered from the F24P24 and P24F48 groups.

Discussion

The present study is the first report to show that fish cooking processes, i.e. heating, freezing and fermenting, could reduce the number of OV metacercariae, and under some conditions could kill the metacercariae as well. It was found that a heating process, by boiling or microwaving at 400 or 800 W for at least 5 min, could kill OV metacercariae (Figure 8), as evidenced by abnormal morphology (Figure 9) and confirmed by no recovery of adult worms from hamsters. However, there have been reports on heating methods using gamma rays to kill trematode metacercariae in the Heterophyidae family. A previous study found that chilling of tilapia (fish) muscles

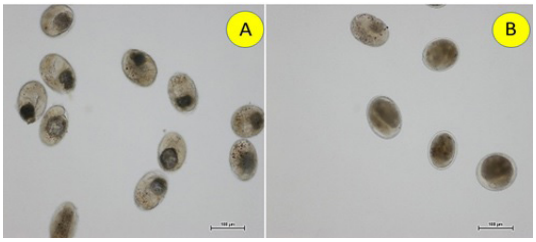

Figure 11. Abnormal Metacercariae Morphology in Pickled Fish Refrigerated at 4 °C for 24 h (A) or Frozen at −20 °C for 24 h (B)

at 5 °C was not cold enough to kill encysted metacercariae, but simple freezing at -10 °C for a period of not less than 3 days or deep freezing at -20 °C for at least 2 days was sufficient to kill all the metacercariae (Elnawawi et al., 2000). For killing O. felineus, freezing fish at -40 °C for 7 h, -28 °C for 14 h, or -13 °C for 32 h were all found to be effective (Iarotski and Be'er, 1993), and for Clonorchis sinensis, freezing at -10 °C for 5 days (WHO, 1989). The results of these previous studies on the freezing process for fresh fish were in accordance with the present findings, i.e. inactive OV metacercariae with abnormal morphology were found in fish frozen for 48 h (Figure 7). However, the optimal duration of the freezing time is based on the size or thickness of the fish.

For pickled fish, our team previously found that at less than 3 days post-fermentation, OV metacercariae were still active and had the ability to infect hamsters (Prasongwatana et al., 2013). This was in agreement with the findings of Onsurathum et al. (2016b), which showed that OV metacercariae in pickled fish kept at 4 °C for less than 3 days were still able to infect hamsters. In the present study, a freezing method was applied to kill OV metacercariae in pickled fish; various time periods were tested. It was found that freezing pickled fish at -20 °C for 48 h could kill OV metacercariae in all sizes of fish. This information should prove to be very useful for people living in endemic areas who like to eat raw pickled fish, in order to help keep them safe from OV infection.

For heating fish, is well known to kill the parasites including metacercarial trematodes in fish. So far, the duration of heating remains problem to answer the people in community. Up to date, heating food using microwave is popular uses in any area of Thailand including urban because it is very cheap now and easy to cook. Thus we heating using microwave and boiling we clarify this question and found that heating by microwave (medium to high electricity, about 400 and 800 W) for a very short time (5–10 min) could kill the OV metacercariae in any part of fish.

In conclusions, the present study found that heating and freezing processes, as well as the fermentation process under optimal conditions, could kill OV metacercariae in a timely manner. And this study suggests that preparing fluke-free fish is possible. Heating fish was found to be the best way to kill OV metacercariae; the second choice was freezing pickled fish at -20 °C for at least 48 h. This knowledge is valuable for implementation in endemic areas to control OV infection.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TB, RA, PSJ, and PSP contributed to the conception and design of the study. RA, PSJ, JS and PSP performed sample collection, performed the experiments. TB and PSP analyzed the results and prepared the manuscript, and critically revised the manuscript. All authors read, revised and approved the final version that was submitted for publication.

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References

Aukkanimart R, Boonmars T, Sriraj P, et al (2015). Anthelmintic, anti-inflammatory and antioxidant effects of Garcinia mangostana extract in *hamster opisthorchiasis*. *Exp Parasitol*, **154**, 5-13.

Chai JY, Darwin Murrell K, Lymbery AJ (2005). Fish-borne parasitic zoonoses: status and issues. *Int J Parasitol*, **35**, 1233-54.

Elnawawi FA, Tawfik MAA, Shaapan RM (2000). Some methods of inactivation or killing of encysted metacercariae in tilapia muscles. *Egypt J Vet Sci*, **34**, 31-8.

Grundy-Warr C, Andrews RH, Sithithaworn P, et al (2012). Raw attitudes, wetland cultures, life-cycles: socio-cultural dynamics relating to *Opisthorchis viverrini* in the Mekong *Basin. Parasitol Int*, **61**, 65-70.

Iarotski LS, Be'er SA (1993). Epidemiology and control of opisthorchiasis in the former USSR. Geneva, World Health Organization.

Keiser J, Utzinger J (2009). Food-borne trematodiases. *Clin Microbiol Rev*, **22**, 466-83.

Onsurathum S, Pinlaor P, Charoensuk L (2016a). Contamination of *Opisthorchis viverrini* and *Haplorchis taichui* metacercariae in fermented fish products in northeastern Thailand markets. *Food Control*, **59**, 493-8.

Onsurathum S, Pinlaor P, Haonon O, et al (2016b). Effects of fermentation time and low temperature during the production process of Thai pickled fish (*pla-som*) on the viability and infectivity of Opisthorchis viverrini metacercariae. *Int J Food Microbiol*, **218**, 1-5.

Prasongwatana J, Laummaunwai P, Boonmars T, Pinlaor S (2013). Viable metacercariae of *Opisthorchis viverrini* in northeastern Thai cyprinid fish dishes-as part of a rational program for control of *O. viverrini*-associated cholangiocarcinoma. *Parasitol Res*, **112**, 1323-7.

Leiper RT (1915). Notes of the occurrence of parasites presumably rare in man in man. *R Army Med Corps*, **24**, 569–75.

Sripa B, Kaewkes S, Intapan PM, et al (2010). Food-borne trematodiases in Southeast Asia epidemiology, pathology, clinical manifestation and control. *Adv Parasitol*, **72**, 305-50.

Sriraj P, Aukkanimart R, Boonmars T (2013). Does a combination of opisthorchiasis and ethyl alcohol consumption enhance early cholangiofibrosis, the risk of cholangiocarcinoma?. *Parasitol Res*, **112**, 2971-81.

Sukontason K, Methanitikorn R, Sukontason K, et al (1998). Viability of metacercariae in northern Thai traditional foods. *Southeast Asian J Trop Med Public Health*, **29**, 714-6.

WHO (1989). Report of the WHO consultation on public health aspects of food-borne zoonotic diseases, Hanover, federal republic of Germany. Geneva, World health organization, **25**.

Ziegler AD, Andrews RH, Grundy-Warr C, et al (2011). Fighting liverflukes with food safety education. *Science*, **331**, 282-3.