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Trematodes

Prevalence of *Clonorchis sinensis* infection in freshwater fishes in northeastern China

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ABSTRACT

The prevalence of *Clonorchis sinensis* infection in freshwater fishes was surveyed in Heilongjiang Province, northeastern China, between August 2011 and September 2013. Thirteen species of freshwater fish ($n = 3221$) and one species of shrimp ($n = 93$) were collected from Songhua river, Nenjiang river and other lakes or ponds in 37 sites of 15 representative cities in Heilongjiang Province. They were individually examined by digestion technique, and the *C. sinensis* metacercariae were identified morphologically followed by confirmation using sequences of the second internal transcribed spacer of ribosomal DNA. Ten of the 13 examined species of freshwater fishes were infected with *C. sinensis* metacercariae, while all shrimps were negative. The overall prevalence of *C. sinensis* infection in 3221 examined freshwater fishes was 19.96%, with 42.57% (272/639) in *Pseudorasbora parva*, 22.55% (83/368) in *Hemiculter leuciclus*, 20.44% (121/592) in *Carassius auratus*, 17.71% (68/384) in *Saurogobio dabryi*, 10.85% (23/212) in *Rhodeus ocellatus*, 10.54% (48/455) in *Phoxinus lagowskii*, 8.20% (21/256) in *Perccottus glehnii*, 6.25% (5/80) in *Misgurnus anguillicaudatus*, 4.55% (1/22) in *Xenocypris davidi*, and 1.49% (1/67) in *Cyprinus carpio*. The average infection intensity in *P. parva* was 103.3 encysted metacercariae per gram of fish meat in Zhaoyuan city. The average prevalence of *C. sinensis* infection in Songhua river, Nenjiang river and lakes or ponds were 31.96% (503/1574), 11.30% (102/903) and 7.93% (59/744), respectively. The prevalence of *C. sinensis* infection in Zhaoyuan city (43.68%) was the highest among all sampling locations. These results revealed a high-prevalence of *C. sinensis* infection in freshwater fishes in Heilongjiang Province, northeastern China, posing significant public health concern.

1. Introduction

Fishborne zoonotic trematodes (FZT) are well-known causes of fluke diseases in humans (Chai et al., 2005; Phan et al., 2010). These trematodes include the liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis*, and intestinal flukes of the families Heterophyidae, Leicithodendriidae and Echinostomatidae (Yu and Mott, 1994). Among these

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trematodes, *C. sinensis* is one of the most harmful to humans, which can cause cholangiocarcinoma. Clonorchiasis is a fishborne trematode infection which is considered one of the major parasitic zoonoses in some parts of Asia, including China, Korea, Japan and Vietnam, with approximately 35 million people being infected globally, of whom approximately 15 million are in China (Lun et al., 2005, Lin et al., 2011). Clonorchiasis can cause a series of hepatic diseases such as periductal inflammation, fibrosis, pyogenic cholangitis, cholecystitis, cholelithiasis and liver cirrhosis, and *C. sinensis* is currently classified as carcinogenic to humans for cholangiocarcinoma (Chai et al., 2005; Shin et al., 2010; Sun et al., 2011). *C. sinensis* has been known to be actively transmitted along the rivers, especially among the residents who have the habit of eating raw fish (Kim et al., 2008).

Humans, cats, dogs, pigs and some wild animals are definitive hosts for *C. sinensis*. Some aquatic snails, freshwater fishes and shrimps act as the first and the second intermediate hosts, respectively (Keiser and Utzinger, 2005). The prevalence of *C. sinensis* metacercariae in freshwater fishes has been reported in many countries, such as South Korea, Vietnam (Cho et al., 2006; Kim et al., 2008; Phan et al., 2010; Van et al., 2012). In China, approximately 140 species of freshwater fishes and four species of shrimp have been recognized as complementary intermediate hosts for *C. sinensis* (Zhou et al., 2008). However, only limited data is available regarding the prevalence of *C. sinensis* metacercariae in freshwater fishes in China (Chen et al., 2010; Sohn et al., 2009), and no such survey has been performed in Heilongjiang Province where the residents have the habit of eating raw fishes, resulting in a high *C. sinensis* prevalence of 4.72%, which ranked the third high in China (Fang et al., 2008).

The objective of the present study was to investigate the prevalence of *C. sinensis* infection in freshwater fishes in different river basins in Heilongjiang Province, northeastern China, which would provide "base-line" data for assess the risk for human infection and for execute control programs.

2. Materials and methods

2.1. The study sites for fish collection

Heilongjiang Province (longitude, 121°11′ to 135°05′, latitude, 43°25′ to 53°33′) is located in the most northeastern end of China, which shares borders with Russia in the North and East, with the Inner Mongolian Autonomous Region in the West, and with Jilin Province in the South. It covers an area of 454,000 km^2, with 13 administrative regions, where have numerous rivers and lakes, including the Songhua river, Nenjiang river and a number of lakes and ponds.

From August 2011 to September 2013, a total of 3221 freshwater fishes representing 13 fish species were collected from 37 sites of 15 representative cities. In addition, 93 shrimps representing *Caridina nilotica gracilipes* were collected from Zhaoyuan (28), Zhaodong (40), Qiqihaer (20) and Mulan city (5). Cluster random sampling was performed according to representative rivers and administrative areas. Aquacutured fishes were collected or purchased primarily from fishing grounds, fish farms, fish ponds, and markets, whereas wild fishes were captured by fishermen from Songhua river, Nenjiang river, some lakes and ponds. Songhua river runs through Zhaoyuan, Zhaodong, Harbin, Mulan and Fujin city. Nenjiang river passes through Nehe, Fuyu, Gannan, Qiqihaer city. Daqing, Dumeng, Lindian, Anda, Mudanjiang, and Suihua city have some lakes or ponds. The shrimps examined were sampled from Songhua river and Nenjiang river in four cities mentioned above. All samples were preserved on ice and transported within 24 h to our parasitology laboratory in Daqing (College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University) for examination.

2.2. Examination of encysted metacercariae

Collected fish were kept in a refrigerator at 4 °C for no longer than 3 days before being processed. After the length and weight of each fish was recorded, encysted metacercariae of *C. sinensis* were individually examined by digestion technique (Sohn et al., 2009). In brief, each fish meat (head, scales, bones, viscera excluded) was ground finely with pestle in a mortar, then mixed with artificial gastric juice (8 g of pepsin 1:3000 (Shanghai, China) and 10 ml of concentrated HCl in 1 l of normal saline), and the mixture was incubated at 37 °C for 2–3 h. The fluid containing digested fish meat was filtered with a 40-well/inch copper sieve to remove the large fragments. The filtered fluid was then placed into a 500 ml glass beaker, washed with 0.9% saline until the supernatant became clear. The sediment was carefully examined under a stereomicroscope to count the encysted metacercariae. The metacercariae were identified based on morphological characteristics, such as the size of cysts, folding body displaying vigorous movement within the cyst, and prominent and clearly visible oral and ventral suckers (Scholz et al., 1991; Kaewkes, 2003; Sohn et al., 2009). Finally, the infection intensity of metacrcariae in each fish was recorded. The identity of collected metacercariae was confirmed by molecular identification.

2.3. Molecular identification

The partial sequence of the second internal transcribed spacer (ITS-2) of *C. sinensis* metacercariae was amplified using BD1 and BD2 primers (Luton et al., 1992). One microlitre of DNA template was used in a PCR reaction of 25 μl containing 5 μl of 5× colorless go *Taq* flexi buffer (pH 8.5), 2 μl of $MgCl_2$ (25 mM), 2 μl of dNTP Mixture (2.5 mM), 0.5 μl of each primer (10 pmol/μl) and 0.13 μl of go *Taq* DNA polymerase (5 U/μl) in a thermocycler under the following conditions: 95 °C for 2 min (initial denaturation), followed by 35 cycles of 95 °C for 1 min (denaturation), 50 °C for 1 min (annealing); 72 °C for 1.2 min (extension) for 35 cycles, and then followed by a final extension at 72 °C for 5 min. Each amplicon (25 μl) was examined by agarose gel (1%) electrophoresis and ethidium bromide staining. The positive products were sent to Life Technology Company