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Temperature dependence of *Opisthorchis viverrini* infection in first intermediate host snail, *Bithynia siamensis goniomphalos*

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ABSTRACT

Determining of the success of a parasite's infectiveness in its snail host clearly depends on environmental conditions. Temperature, one of the most influential factors impinging on metabolism of cold-blooded animals, is believed to be an important factor in parasitic infection in snails. In order to elucidate the influence of temperature, sex and size of snails on infectivity of *Opisthorchis viverrini* to its first intermediate host, *Bithynia siamensis goniomphalos*, 960 snails were divided into 2 groups by sex. Each group was subdivided by their size into small and medium sub-groups. Each snail was fed with embryonated uterine-eggs of *O. viverrini* at different temperatures (16–37 °C, 3 °C intervals). Dissections were carried out 1, 7, 14, 28 and 56 days thereafter and detection of *O. viverrini* infection was undertaken by PCR using specific primers. Infection was strongly temperature-dependent, as temperature increases of 1 °C resulted in increased odds of infection 5.4% ($P < 0.01$). A temperature of 34 °C gave the highest rate of infection of 44.14%. We also found that the odds of infection in small sized snails was 39.8% higher relative to medium sized snails ($P < 0.05$). Relative to day 1, the decrease in the odds of infection was detected when the day post infection was longer ($P < 0.01$). Proportion of infection in female was not different to male significantly.

1. Introduction

Food-borne trematode infections including the liver flukes, lung flukes and intestinal flukes, are considered to be a public health and socioeconomic problem worldwide. These diseases are mostly found in developing countries, especially in the Asian continent. Opisthorchiasis is one of the most important of the food-borne trematodiases in Asia. An estimated 10 million people are infected with *Opisthorchis viverrini*, whereas *O. felineus* parasitizes approximately 1.2 million people (Keiser and Utzinger, 2009). *O. viverrini* is predominantly endemic in the Lower Mekong region where an estimated 6 million people harbor the infection in Thailand. Human infections occur by eating raw, fermented or undercooked cyprinid fishes containing infectious metacercariae. The worm inhabits the bile ducts and provokes hepatobiliary damage as a result of chronic inflammation of the bile duct wall. Chronic inflammation caused by opisthorchiasis is generally accepted to be a precursor for bile duct cancer, or cholangiocarcinoma (CCA) (Schwartz, 1980; Rim, 1986; Holzinger et al., 1999; Sirica, 2005; Kawanishi and Hiraku, 2006). CCA has the highest incidence of any cancer in men and the third highest in woman in Thailand (Khuhaprema and Srivatanakul, 2007).

The *O. viverrini* life cycle requires two intermediate hosts – *Bithynia* snails and cyprinid fishes. Three species/subspecies have been reported as the first intermediate host of *O. viverrini* in Thailand – *Bithynia funiculata*, *B. siamensis siamensis* and *B. siamensis goniomphalos* (Brandt, 1974). *B. siamensis goniomphalos* is distributed throughout northeast Thailand, where the prevalence of *O. viverrini* infection and incidence of CCA are the highest of anywhere in the world (Vatanasapt et al., 1990, 2000; Parkin et al., 2002; Jongsuksuntigul and Imsomboon, 2003). To date, the incidence of CCA is the highest in Udornthani Province followed by Khon Kaen Province (Khuhaprema and Srivatanakul, 2007). Surprisingly, the infection rate of *O. viverrini* in *B. siamensis goniomphalos* in Khon

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Kaen Province was extremely low (0.11%), but the infection rate in the same area of both cyprinid fishes (97%) and humans (90%) was very high (Vichasri et al., 1982; Upatham et al., 1984; Brockelman et al., 1986). Furthermore, cats and dogs have been recognized as reservoir hosts for *O. viverrini*, with the prevalence of infections of 35.5–36.4% in cats and 0.4–3.8% in dogs (Enes et al., 2010; Aunpromma et al., 2012). In 2005, Sri-aroon et al. (2005) reported that the infection rates of *O. viverrini* in *B. siamensis goniomphalos* varied from 0.61 to 1.3%. More recently however, average prevalence of 3.04% has been reported, with the highest prevalence in Sakon Nakhon Province of 6.93% (Kiatsopit et al., 2012). Natural infection in snails is influenced by snail size, with only adult snails over 8 mm in shell length being infected (Brockelman et al., 1986). In contrast, laboratory studies indicate that small sized snails had higher infection rates than larger snails (Chanawong and Waikagul, 1991).

Snails are cold-blooded animals, so consequently their activities and metabolism are dependent on environmental temperature. Under natural conditions, seasonal changes play an important role on the infection of the snail intermediate hosts of *O. viverrini* (Upatham and Sukhapanth, 1980; Brockelman et al., 1986). Upatham and Sukhapanth (1980) observed that prevalence of *O. viverrini* infection in *B. siamensis siamensis* was detected almost all year round, but was more frequent during periods of moderate rainfall. Brockelman et al. (1986) described a detailed study of *B. siamensis goniomphalos* natural populations where snail reproduction, density and *O. viverrini* infection were subjected to seasonal variation. When rainfall was adequate 2 generations could be produced each year with peaks occurring during spring rains and after the fall monsoon floods. With the onset of the drought, a population density of 400–500 snails/m^2 was observed to decrease to 5–15 snails/m^2. The density of snails was depended on certain levels of salty water with the highest density (69.2 snails/man power in 5 min) was in salinity of 2.5–5 ppt (Suwannatrai et al., 2011). In the cold season, infection rates were higher than other periods.

Snail intermediate hosts are required for multiplication of *O. viverrini* to facilitate growth, development and asexual reproduction. To establish the infection, *B. siamensis goniomphalos* ingests parasite eggs excreted in the feces of humans and other mammalian hosts such as cats and dogs. Once in the host, miracidia hatch from the eggs and subsequently penetrate snail tissues and develop and reproduce through the stages of sporocysts, rediae and cercariae (Wykoff et al., 1965). A classical method for *O. viverrini* detection in *Bithynia* snails is cercarial shedding, usually performed by exposing the snails to light sources to activate the release of free-swimming cercariae (Adam et al., 1993). However, this method is unsuitable for the pre-patent period of infected snails. Alternatively, crushing of snails has been used to detect sporocysts and rediae, but these intramolluskan stages are difficult to identify to species level due to similar morphology with other trematodes. Moreover, parasite detection and identification in these situations faces additional limitations including low parasite burdens, pre-patent infections, snail fatality after collection, and aborted development of sporocysts (Barbosa, 1992; Hanelt et al., 1997). Accordingly, the actual prevalence remains murky and we believe it is grossly underestimated.

Intramolluskan stages (sporocysts and rediae) of trematode parasites are reliably detected using polymerase chain reaction (PCR)-based procedures (Müller et al., 2007), but are lacking for detection of *O. viverrini* in infected snails. PCR assays have been developed to detect *O. viverrini* DNA in experimentally infected hamsters (Wongratanacheewin et al., 2001), where the development of specific primers was based on the pOV-A6 specific DNA probe sequence (Sermswan et al., 1991). Additionally, the pOV-A6 probe has been used to detect *O. viverrini* eggs in feces (Sermswan et al., 1991; Sirisinha et al., 1991), displaying high sensitivity and specificity.

The present study aimed to investigate the influence of temperature and also sex and size of snails on the infectivity of *O. viverrini* to *B. siamensis goniomphalos*. The PCR assay, a highly sensitive and specific technique for detecting intramolluskan stages of *O. viverrini*, was designed to achieve the objectives. Moreover, this would allow possible applications of the detection technique in other fluke infected snails.

2. Materials and methods

2.1. Preparation of snail samples

B. siamensis goniomphalos adult snails were collected from public ponds in Muang district, Khon Kaen Province, Thailand and maintained in ceramic aquaria containing de-chlorinated tap water in the laboratory and provided them with synthetic snail food (Sumethanurungkul, 1970). The snails were examined for trematode infection by cercarial shedding weekly for 8 weeks. A number of *O. viverrini* infected and non-infected field snails based on cercarial shedding outcomes were observed. Snails that were free from trematode infection were then used for experimental infections.

2.2. Gut emptying time

The gut emptying time of snails was investigated to ensure that the ingested eggs were depleted from the snail's gut before processing of DNA and PCR analyses. One hundred snails (5–10 mm in shell length) were individually fed with 2 different colored artificial foods (edible dyes). Red colored food was initially fed to snails until red colored feces was detected; thereafter green colored food was used. Changes in fecal color (from red to green) were monitored every 10 min. The duration between first detection of red feces and first detection of green feces was determined to be the gut emptying time.

2.3. Preparation of O. viverrini eggs

Twenty golden syrian hamsters (*Mesocricetus auratus*) were experimentally infected with 50 *O. viverrini* metacercariae obtained from naturally infected cyprinid fish. The infected animals were euthanized 6 weeks post-infection (p.i.). *O. viverrini* adults were obtained from biliary tracts and gall bladders of hamsters and then washed with 0.85% sodium chloride solution. Mature eggs were dissected from the distal portion of the uterus of adult flukes under a stereoscope (Khampoosa et al., 2012). The eggs were washed several times with distilled water and kept at room temperature for 2 weeks to undergo full maturation for further experimental infection (Chanawong and Waikagul, 1991).

2.4. Experimental infection

Nine hundred and sixty *B. siamensis goniomphalos* were used for experimental infections divided into 2 equal groups based on sex (male and female) then subdivided into sub-groups of small and medium sizes by shell lengths of 2.0–6.0 mm and 6.1–10.0 mm respectively. These snails were placed individually in transparent plastic containers with 6 ml of de-chlorinated tap water and exposed to 50 embryonated *O. viverrini* eggs (Chanawong and Waikagul, 1991). Eight groups (30 snails/temperature group of each size and gender) were placed into plastic containers covered with porous lids and placed in water baths of temperature ranges of 16–37 °C with 3 °C intervals, and activated by exposure to electric light (8 W globe). Snails ate parasite eggs freely under these conditions for 24 h. Feces of two hundred randomly selected snails (25

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