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Epidemiological situation and molecular identification of cercarial stage in freshwater snails in Chao-Phraya Basin, Central Thailand

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ABSTRACT

Objective: To investigate the prevalence of cercarial trematode infection in snails and to examine the reconstruction of the phylogenetic relationship to explain the molecular system of cercarial stage trematodes to estimate the infection rate of in the definite host from the Chao-Phraya Basin.

Methods: The snails were collected from 10 provinces of the Chao-Phraya Basin, Thailand by stratified sampling method. The snails were examined for cercarial infection by the crushing method. All DNA specimens were amplified with internal transcribed spacer 3 (ITS3) and ITS4 primer based on PCR technique. The sequence data were aligned and used to reconstruct the phylogenetic tree by unweighted pair-group method with arithmetic means with 10000 bootstraps.

Results: The overall rate of cercarial infection was found to be 5.90% (122/2067). Snails in the family Thiaridae were found to be in the highest prevalence followed by Lymnaeidae, Bithyniidae, Planorbidae, Viviparidae, and Ampullariidae, respectively, while the Buccinidae family (*Clea helena*) did not reveal any infections. The frequently found species of cercariae were parapleurolophocercous cercariae, cercariae and megarulous cercariae. The monophyletic tree separated the snails into five groups comprised of Heterophyidae, Strigeidae, Lecithodendriidae, Philophthalmidae and Echinostomatidae using the sequence of *Angiostrongylus cantonensis* as an out-group.

Conclusions: This study was the first to report on cercarial infection in the Chao-Phraya Basin, Thailand. This revealed that a high variety of freshwater snails were infected by cercariae stage trematodes with a high prevalence. The sequence data of ITS2 can be used to investigate the phylogenetic relationships of trematodes at the family level and in each clade of different families separated by the definitive hosts.

1. Introduction

Digenetic trematodes are widely distributed [1–5] and continue to be an important public health problem in the Greater Mekong Subregion including Vietnam, Myanmar, Cambodia, Laos and Thailand [6]. The life cycle of the trematodes is very complex as they require an intermediate host such as snails or fish for maturation to the infective stage, while the definitive host is often infected by eating raw or half-cooked like fermented fish dishes (pla-ra and pla-som) [6,7]. All digenetic trematodes have been implicated as a cause of various parasitic diseases such as heterophyiasis which often result in significantly high rates of eosinophilic, diarrhea, abdominal pain for the patients who are infected by heterophyid trematodes [8], which are wildly distributed throughout Northern and Northeastern Thailand. Furthermore, the infective larvae stage of blood flukes in the family Schistosomatidae has been known to cause schistosomiasis in humans by penetrating the skin, after which, mild dermatitis

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and Katayama fever would appear [9,10]. Moreover, the human liver fluke, *Opisthorchis viverrini* (*O. viverrini*) that is known to cause opisthorchiasis, is currently reported to have infected about 6 million people in Thailand who have been diagnosed with hepatobiliary diseases and cholangiocarcinoma by chronic infection [11]. The agricultural area located in the central plain of Thailand is one of areas that produce extensive amounts of rice for export [12]. As a result, the activities of agriculturists in this area may produce and discharge waste into the water resources including rivers, irrigation canals, and reservoirs over a long period of time. This is a reason for the widespread occurrence of many trematodes and the high prevalence of cercarial infection in Thailand [4,8,13–16], especially in the Chao-Phraya Basin area. This location has a diverse ecological system comprised of paddy fields, forests and a variety of water resources. This ecological system is suitable for many freshwater snails that play an important role as the intermediate hosts of various trematodes [4,13,14]. Therefore, the current data on the prevalence of cercarial infection in snails have been usefully applied to predict the epidemiological situation of trematode infections in definitive hosts like mammals, Aves, reptiles and humans for the purposes of developing preventative applications in the future.

The classic method used to identify cercaria considered only the morphological characteristics. However, this method consumes more time and requires a high level of experienced-based skills. Nevertheless, the morphology of the larval stage might not be accurately distinguishable other than by a specially trained diagnostic researcher. Moreover, difficulties arise because cercaria are small and soft and also possess only a few stable morphological characteristics and are subject to host-induced phenotypic variations [17]. Therefore, molecular biological methods are the most efficient and accurate tools for the identification of numerous organisms including trematodes [13,18–20]. The internal transcribed spacer 2 (ITS2) of the 18S rDNA gene was selected and used for the identification of various stages and for studying the life cycles of heterophyid trematode (cercaria, metacercaria and adult stages) infections in freshwater fish [21,22]. The sequences of the ITS2 region have been used as potential marker for species or population level [23]. Phan *et al.* [7] separated larval and adult stages of *Haplorchis taichui* (*H. taichui*) and *Haplorchis pumilio* (*H. pumilio*) using the same target gene. Conventional PCR methods have been widely used because the nuclear DNA method is highly accurate, sensitive and can be rapidly applied. Therefore, this sequence data have proven to be beneficial for the purposes of studying species identification, geographical distribution, phylogenetic relationships particularly for *Schistosoma haematobium* and *Schistosoma bovis* [24], *O. viverrini*, *Clonorchis sinensis*, *H. pumilio* and *H. taichui* [25]. In addition, previous reports have used the ITS2 region to characterize *Paragonimus westermani*, *Fasciolopsis buski* and *Fasciola gigantica* collected from Northeast India [19].

Regarding phylogenetic reconstruction, rDNA in the genomes of animals are involved with the evolutionary process and can produce mutations in populations or species. For example, the ITS2 region is a conservative region that appears between 5.8S, and 28S rDNA and becomes homogenized within a given organism and helps the researcher to differentiate between species [26]. Furthermore, this region was used to construct the phylogenetic tree of several organisms. For the example, Prasad *et al.* [19] revealed the relationship of many trematodes in India. A recent experiment conducted by Tang *et al.* [27] involved the construction of the phylogeny tree of the parasitic protozoa *Trypanosoma*. Therefore, this region was deemed to be suitable for use in phylogenetic relationship analysis. Consequently, to identify the species level of cercarial infection in snails, this investigation applied the molecular technique while considering the morphological characteristics for higher levels of accuracy in the results.

The purpose of this study was to investigate the prevalence of each type of cercarial infection present in snails and to reconstruct the phylogenetic tree showing the overall relationship using specific analytical methods based on the PCR technique and focused on the ITS2 region of cercariae found in freshwater snails to estimate the infection rate among the definitive hosts in the Chao-Phraya Basin and to identify beneficial prevention techniques for future investigation.

2. Materials and methods

2.1. Collected samples and cercarial infection

The snail specimens were collected by stratified sampling method [28] during the period of February 2014 to October 2014 from 10 provinces located in the Chao-Phraya Basin, which were Nakhon Sawan, Chai Nat, Sing Buri, Ang Thong, Suphan Buri, Ayutthaya, Nakhon Nayok, Pathum Thani, Nonthaburi and Bangkok (Figure 1). The coordinates for all the collection sites were recorded using the global positioning system. The specimens were classified using a taxonomic key and then they were separated by species level [29].

The cercarial infection was examined in freshwater snails and identified manually under a high magnification stereomicroscope. The living cercariae were vitally stained with 0.5% neutral red dye and identified according to morphological classification as previously described [30]. In addition, the cercarial specimens were stained with Delafield's hematoxylin or acetone orcine, dehydrated in an ethyl alcohol series, cleared with xyline, and mounted in permount. Using a camera lucida,

Figure 1. The location points in the Chao-Phraya Basin where snails were collected for this investigation.