The rapid detection method by polymerase chain reaction for minute intestinal trematodes: *Haplorchis taichui* in intermediate snail hosts based on 18s ribosomal DNA

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Abstract The minute intestinal trematode, *Haplorchis taichui*, is an important parasite species that can infect humans and other mammals. This study investigated the outbreak of *H. taichui* in thiarid snails in the lower part of the Chao Phraya Basin, Thailand by employing morphological and molecular-based methods. In development of a specific primer of *H. taichui*, the PCR reaction was conducted with no cross-reaction to their hosts and other related trematode species. The highest level of sensitivity that could be amplified was 0.50 ng/μl and this was detected with only one egg in the sample. In terms of the epidemic results, the parapleurolophocercous cercaria infected only two species of thiarid snails (*Melanoides tuberculata* and *Tarebia granifera*) with an overall prevalence of 3.80% (23/605). The process of molecular identification revealed positive results indicating that eleven from twenty-three of parapleurolophocercous cercariae specimens in the lower part of the Chao Phraya Basin were *H. taichui*. In conclusion, this study has developed a rapid detection method, which can discriminate *H. taichui* from other parapleurolophocercous cercaria in intermediate snail hosts with a high level of sensitivity. Moreover, the high proportion of *H. taichui* in parapleurolophocercous cercaria (47.83%) indicated that *H. taichui* was the dominant species of this cercarial type and could infect cyprinoid fish in

the lower part of the Chao Phraya Basin leading to public health problems in this area. Thus, a specific primer could be useful in the detection and surveillance of *H. taichui* outbreaks in their hosts. Recognition of this has resulted in the creation of important prevention programs in these infected areas in the further study.

Keywords *Haplorchis taichui* · 18s rDNA · Parapleurolophocercous cercaria · Snail-borne disease · High sensitivity

Introduction

The minute intestinal trematode belonging to the family Heterophyidae has great significance for the public health. It can infect various kinds of birds and mammals, including humans (Pinto et al. 2015; Anucherngchai et al. 2016). Heterophyid trematodes are a widely distributed species and can cause heterophyiasis in humans and other vertebrates via the ingestion of raw or undercooked fish that has been contaminated at the metacercarial stage (Pinto et al. 2015; Onsurathum et al. 2016). This is particularly true for *Haplorchis taichui*, as this trematode species has been continuously reported to be a public health problem in several countries in Southeast Asia (SEA) including Thailand, Lao-PDR, Vietnam, Myanmar and Cambodia (Kumchoo et al. 2005; Van Van et al. 2009; Chai et al. 2013; Rim et al. 2013; Anucherngchai et al. 2016; Onsurathum et al. 2016; Le et al. 2017). *H. taichui* inhabits the intestinal mucosa of patients and causes mild inflammation with acute abdominal pain or appendicitis (Tantachamrun and Kliks 1978; Saenphet et al. 2008). Moreover, it could be a possible etiologic agent for IBS-

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like symptoms in patients (Watthanakulpanich et al. 2010; Mohammadi et al. 2015).

To study the epidemic of *H. taichui*, identification based on morphological appearances was used to identify this trematode species in this study area. However, this method may be difficult to use directly on the feces of patients who have been infected by this trematode. Therefore, most researches focus on larval stages (cercaria and metacercaria) in the intermediate snail hosts when studying an outbreak of trematodes, including *H. taichui* (Boerlage et al. 2013a, b, 2014; Chontanarith et al. 2017; Dzikowski et al. 2004). The investigation of *H. taichui* in snails focused on parapleurolophocercous cercaria, which is known to be a cercarial type of *Haplorchis* species (Chontanarith and Wongsawad 2010; Boerlage et al. 2013a, 2014; Krailas et al. 2014; Chontanarith et al. 2017). This cercarial variety can be distinguished from other pleurophocercous cercaria by the lateral finfolds (Krailas et al. 2014). However, the cercariae are very small and soft-bodied with fewer unique characteristics than specimens at the adult stage. In this context, it is difficult to specifically identify them at the species level. Therefore, misidentification may occur affecting the epidemic results if identification is only based on morphological appearances. Consequently, this study has helped the researchers to develop a rapid detection method based on the PCR method in order to detect *H. taichui* at various stages, including at the egg stage. The primers designed in the present study was used to discriminate *H. taichui* from other parapleurolophocercous cercaria in intermediate snail hosts with a high degree of accuracy and sensitivity. Additionally, this study revealed the accuracy of the epidemic data of *H. taichui* in the lower part of the Chao Phraya Basin. This area is the part of the Chao Phraya River that receives water from northern areas of Thailand. Several studies have reported on the high prevalence of *H. taichui* infection (Wongsawad et al. 2012; Krailas et al. 2016; Onsurathum et al. 2016). This rapid detection method and the epidemic data obtained from this study are useful in the study of *H. taichui* and could lead to the construction of a surveillance plan to prevent the risk of infection.

Materials and methods

Animal ethics

All experimental procedures involving animals were conducted in accordance with the National Research Council of Thailand and were approved by the Biological Experiment Animals Committee, Department of Biology, in the Faculty of Science at Srinakharinwirot University.

Snail specimen collection and prevalence studies

The snail specimens were collected from 99 sampling sites from all districts of five provinces in the lower part of the Chao Phraya Basin (Chainat, Sing Buri, Ang Thong, Suphanburi and Phra Nakhon Si Ayutthaya) by the stratified random sampling method (Freedman et al. 2007). These specimens were classified according to their species and based on morphological appearances using the taxonomic key (Brandt 1974). Each of the snail specimens were then examined using the crushing method (Hung et al. 2015) under light microscopes. The prevalence of parapleurolophocercous cercariae infection in thiarid snail populations was calculated and analyzed in order to construct an epidemic map. Finally, the cercaria specimens were kept in -20°C to prepare for species identification.

Specimen preparation

The related trematode species comprised of *Haplorchis pumilio*, *Stellantchasmus falcatus*, *Echinostoma revolutum* and *Haplorchoides mehrai* were prepared for PCR amplification. These species are commonly found in similar species of intermediate snail hosts of *H. taichui*. Therefore, they were used in the alignment process when designing the specific primers for identification (Fig. 1). Regarding the method of analysis for the adult stage of each trematode species, *H. taichui* and *H. pumilio* were collected at the metacercarial stage from cyprinoid fish using the pepsin digestion technique (Pinlaor et al. 2013). After that, experimental mice (ICR) were infected with the metacercariae samples using the force-feeding method (Ondračková et al. 2015). With regard to the other related species, the adult stage of *Stellantchasmus falcatus* was developed from metacercaria in halfbeak (*Dermogenus pusillus*), while *Haplorchoides mehrai* were directly collected from yellow mystus (*Hemibagrus filamentus*) and *Echinostoma revolutum* was collected from experimental hamsters after 14 days of infection. All of the trematode specimens were confirmed for species identification based on both morphological appearances and DNA sequence data.

Molecular, biological studies and primer design

Genomic DNA of each trematode specimen was extracted using the commercial DNA extraction kit (DNeasy® blood and tissue kit; Qiagen®) according to the manufacturer's protocol. Each DNA sample was amplified using the trematode universal 18S primers (Uni 18S F: 5'-GCT TGT CTC AGA GAT TAA GCC-3' and Uni 18S R-5' ACG GAA ACC TTG TTA CGA C-3') acquired from the

Fig. 1 The multiple alignment of 18s ribosomal DNA sequences shown site of species specific primer base on 18s ribosomal DNA region. The sequence of *H. taichui* *Haplorchoides mehrai*, *H. pumilio*, *S. falcatus* and *E. revolutum* were amplified in this study and another

sequence of *H. taichui* (AY245705.1) was acquired from GenBank Database. The specific primer of *H. taichui* was appeared in the black square box

previous study (Dzikowski et al. 2004). The sequence data of each the PCR products was aligned using the local alignment method and by employing the BLAST program in the NCBI database to confirm that the species were completely identified by taxonomic key (Gibson et al. 2005). The species-specific primers (HT-F: 5'-CCT GAG ATA CGG CTA CCA CTT CCA AGG-3' and HT-R: 5'-AGG CAC AGA GGA CAG GCG TAC TA-3') were manually designed from specific fragments in the sequence data of *H. taichui* based on the 18s ribosomal DNA region. The expected fragment size of *H. taichui* was 1075 base pairs (Fig. 1).

Specificity and sensitivity of primers

The optimal conditions of PCR were optimized with gradient PCR from 55 to 60 °C. The primer set was tested for specificity against three groups of specimens; (1) other intestinal trematodes that were commonly found at the larval stage in similar species of intermediate hosts consisting of *H. pumilio*, *S. falcatus*, *E. revolutum* and *Haplorchoides mehrai*, (2) various cercarial types that were found in intermediate hosts consisting of parapleurophocercous cercaria, furcocercous cercaria, megarulous cercaria, pleurophocercous cercaria, cercariae, xiphidiodercaria, echinostome cercaria and monostome cercaria, along with (3) intermediate snail hosts and cyprinoid fish,

which are also known as second intermediate hosts. The sensitivity test used in this study was divided into two parts. These two groups were comprised of the adult DNA of *H. taichui* and the eggs of *H. taichui*. In the adult stage, the reactions were done with twofold dilution series and DNA was initiated at 2.5–0.08 ng/μl, respectively. For the egg sensitivity test, the initial DNA was extracted from one hundred eggs and the reactions were done in a series of tenfold dilutions.

Epidemic mapping of *Haplorchis taichui*

The parapleurolophocercous cercaria of *H. taichui* was distinguished from other parapleurolophocercous cercaria specimens by the species-specific amplification. The accuracy of the data on *H. taichui* infection was employed to construct an epidemic map of the lower part of Chao Phraya Basin employing the geographic information system program (GIS) to reveal the potential risk areas for infection.

Results

Snail specimen collection and epidemic studies

A total number of 605 thiarid snails from five provinces in the lower part of the Chao Phraya Basin were classified into three species; *Melanoides tuberculata* (318 specimens), *Tarebia granifera* (180 specimens) and *Thiara scabra* (107 specimens). The overall prevalence of parapleurolophocercous cercaria infection in all snail specimens was 3.80% (23/605). Only two snail species; *M. tuberculata* and *T. granifera*, had high prevalence values 4.72% (15/318) and 4.44% (8/180), respectively, while the *Thiara scabra* snail did not reveal parapleurolophocercous cercaria infection.

Specificity and sensitivity of primers

The optimal conditions of the PCR reaction was performed using 58 °C as the melting temperature. The HT primer set was used to amplify the genomic DNA of *H. taichui* with no cross-reactions against other intestinal trematodes (Fig. 2a), other cercarial type (Fig. 2b) and their intermediate hosts (Fig. 2c). With regard to the level of sensitivity, 1075 base pairs of specific fragments of *H. taichui* were generated using the twofold dilution method. The intensity of the DNA band decreased due to low concentrations of DNA templates. The lowest DNA concentration that could be amplified was 0.31 ng/μl (Fig. 3). Moreover, this work was done with the eggs of *H. taichui* specimens to confirm the high sensitivity of this primer set. The initial DNA was

1.50 ng/μl and the reactions were done with a tenfold dilution series. Interestingly, this primer was amplified and had only 0.5 ng/μl of egg DNA (Fig. 4).

Epidemic mapping of *H. taichui*

The genomic DNA of each parapleurolophocercous cercaria specimen was amplified with the HT primer set to discriminate *H. taichui* from other parapleurolophocercous cercaria. The overall prevalence of parapleurolophocercous cercaria in thiarid snails was 3.80%, while the overall prevalence of *H. taichui* confirmed by the HT primer set was 1.82% (11/605). Specimens collected from 23 out of 99 sampling sites were infected with parapleurolophocercous cercaria that is the similar cercarial type of *Haplorchis* spp. However, only 11 from 23 sampling sites comprising Manorom, Sapphaya and Sankhaburi Districts (Chainat Province), Buri and Bang Rachan Districts (Sing Buri Province), Doem Bang Nang Buat Districts (Suphanburi Province), Pa Mok and Wiset Chai Chan Districts (Ang Thong Province), and Phak Hai, Bang Sai and Bang Ban Districts (Phra Nakhon Si Ayutthaya) (Table 1) were identified as *H. taichui* by 1075 bp DNA fragment (Fig. 5). The constructed epidemic mapping revealed the infected area of *H. taichui* as is presented in Fig. 6.

Discussion

This study confirms that two species of thiarid snails; *M. tuberculata* and *T. granifera*, acted as the intermediate hosts of *H. taichui* in the agricultural areas in the lower part of the Chao Phraya Basin. From our results, 11 of 23 parapleurolophocercous cercaria (47.83%) was identified as *H. taichui*. Therefore, this trematode species was the dominant species of this cercarial type. The findings of the study were consistent with those of previous reports, which found the infection of parapleurolophocercous cercaria in thiarid snails (Dechruksa et al. 2007; Ukong et al. 2007; Chontanarith and Wongsawad 2010; Krailas et al. 2011; Anucherngchai et al. 2016; Chontanarith et al. 2017). Moreover, this snail family is the susceptible intermediate snail host of *H. taichui* (Krailas et al. 2012, 2014; Chontanarith and Wongsawad 2013; Anucherngchai et al. 2016). From these results, this situation can be increasing of *H. taichui* infection rate in the cyprinoid fish (the secondary intermediate host) in the lower part of the Chao Phraya Basin and can be led to public health problems in this area. In this study, the overall prevalence of *H. taichui* confirmed by the HT primer set was 1.82% (11/605). This rate of prevalence was higher than that of previous studies conducted in Southern Thailand (0.59%) (Krailas et al. 2011), but remained lower than studies conducted in

Fig. 2 The specificity test of HT primer set with the other related species, cercarial types and hosts. The DEPC treated water was used as negative control (Neg). **a** Related intestinal trematodes species; *HT* *H. taichui*, *HP* *H. pumilio*, *SF* *Stellantchasmus falcatus*, *ER* *Echinostoma revolutum*, *HM* *Haplorchoides mehrai*. **b** Other type of cercariae; *HT* *H. taichui*, *PAR* Other species of Parapleurolophocercous cercaria, *FUR* Furcocercous cercaria, *MEG* Megarulous

cercaria, *PLE* Pleurophocercous cercaria, *CER* Cercariae, *XIP* Xiphidiocercaria, *ECH* Echinostome cercaria, *MON* Monostome cercaria. **c** Host of *H. taichui*; *HT* *H. taichui*, *MT* *M. tuberculata*, *TG* *T. granifera*, *TS* *T. scabra*, *LA* *Lymnaea auricularia*, *BS* *Bithynia siamensis*, *FP* *Filopaludina polygramma*, *FM* *F. martensi*, *RT* *Rasbora tornieri*, *HS* *Henicorhynchus siamensis*, *PP* *Puntioplites proctozysron*

Fig. 3 Twofold dilution was used for sensitivity test: the initial concentration DNA of adult *H. taichui* was 2.5 ng/μl and DEPC treated water was used as negative control (Neg)

Northern Thailand (21.7%) (Chontanarith and Wong-sawad 2010). It seems that *H. taichui* may be discharged from the northern region to the central region of Thailand and then passes into the southern part of Thailand via the rivers and other water sources. In addition, the various and complex freshwater ecosystems found in the study area that are often connected to paddy fields were determined to be a

suitable environment for the cercarial stage of *H. taichui* and their intermediate hosts. The previous reports also supported this finding and it has been suggested that rivers and other freshwater sources with a high level of biodiversity can lead to an increase in the prevalence of trematode infections (Tum et al. 2004).

Fig. 4 Tenfold dilution method was used for sensitivity test of the eggs: Lane number 1 is DNA of 100 *H. taichui* eggs, lane number 2–9 is the tenfold dilution DNA and DEPC treated water was used as negative control (Neg)

Table 1 Twenty-three collection sites, which had parapleurolophocercous cercaria infection in the thiarid snails

Provinces	District	Location	Trematodes infection	Species of snails
Chainat	Manorom	47+621754 1695812	HT PAR ^a	<i>T. granifera</i>
	Sapphaya	47+628456 1673356	HT PAR ^a	<i>T. granifera</i>
	Sankhaburi	47+621707 1659976	HT PAR ^a	<i>T. granifera</i>
	Wat Sing	47+611695 1688758	Other species PAR ^b	<i>M. tuberculata</i>
	Nong Mamong	47+598461 1688866	Other species PAR ^b	<i>M. tuberculata</i>
Sing Buri	In Buri	47+643237 1661938	HT PAR ^a	<i>M. tuberculata</i>
	Bang Rachan	47+641952 1646501	HT PAR ^a	<i>M. tuberculata</i>
	Bang Rachan	47+639952 1645109	Other species PAR ^b	<i>M. tuberculata</i>
	Mueang Sing Buri	47+650026 1647458	Other species PAR ^b	<i>M. tuberculata</i>
Suphanburi	Doem Bang Nang Buat	47+617416 1647008	HT PAR ^a	<i>M. tuberculata</i>
	Nong Ya Sai	47+600230 1633815	Other species PAR ^b	<i>M. tuberculata</i>
	Don Chedi	47+610079 1618066	Other species PAR ^b	<i>M. tuberculata</i>
	Si Prachan	47+613902 1613873	Other species PAR ^b	<i>M. tuberculata</i>
	Bang Pla Ma	47+626074 1591698	Other species PAR ^b	<i>T. granifera</i>
Ang Thong	Wiset Chai Chan	47+646725 1612495	HT PAR ^a	<i>M. tuberculata</i>
	Pa Mok	47+650393 1613230	HT PAR ^a	<i>M. tuberculata</i>
	Samko	47+638391 1617490	Other species PAR ^b	<i>T. granifera</i>
Phra Nakhon Si Ayutthaya	Phak Hai	47+636923 1599604	HT PAR ^a	<i>M. tuberculata</i>
	Bang Sai	47+665056 1563035	HT PAR ^a	<i>M. tuberculata</i>
	Bang Ban	47+660341 1588386	HT PAR ^a	<i>M. tuberculata</i>
	Wang Noi	47+676631 1573740	Other species PAR ^b	<i>T. granifera</i>
	Ban Phraek	47+669935 1619808	Other species PAR ^b	<i>T. granifera</i>
	Bang Pahan	47+666817 1605093	Other species PAR ^b	<i>M. tuberculata</i>

^a*H. taichui* parapleurolophocercous cercaria

^bOther trematode species but they are parapleurolophocercous cercaria

Fig. 5 The total of parapleurolophocercous cercaria specimens were amplified with the HT primer set to discriminate *H. taichui* from other species. Lane 1–23 = parapleurolophocercous cercaria specimens and lane 24 was DEPC treated water (negative control)

The rapid detection approach was constructed for this study. We applied the molecular approach for the rapid identification of *H. taichui* with a high degree of accuracy at the earliest possible stage of infection. This reaction was used to discriminate between the cercarial stage of *H. taichui* and other parapleurolophocercous cercaria, which are related species in heterophyid trematodes. Additionally, the specificity of the primer set was confirmed with no cross-reaction of other trematodes that commonly infect the similar species of snail intermediate hosts. Thus, this primer set can be applied to detect the *H. taichui* infection in various species of fish or snails, such as *Labiobarbus siamensis* (Boonchot and Wongsawad 2005), *Hampala macrolepidota* (Rim et al. 2013). Interestingly, the highest sensitivity of the rapid detection was 0.31 ng/μl, while the concentration obtained from a single egg of *H. taichui* in this study was 1.50 ng/μl. This concentration is lower than the concentration reported by Wongsawad, which revealed the concentration of *H. taichui* eggs as 3.92 ng/μl (Wongsawad et al. 2006). However, the level of sensitivity was found to be sufficient for detection of this parasitic infection, although there was only one egg of *H. taichui* found in the specimen. This is the high performance method used to detect the eggs in patients and is actually

more comfortable for patients than the fecal examination method that was usually used to determine infection in humans (Sohn et al. 2014). Thus, a specific primer could be useful in the next studies for detection and surveillance of *H. taichui* outbreaks in their hosts in the infected areas.

With regard to the epidemic mapping process, after distinguishing the *H. taichui* cercariae species from other parapleurolophocercous cercaria, we found three separate areas that had been infected with *H. taichui*. First was the northern area of Chainat Province and the boundary area of Chainat, Sing Buri and Suphanburi Provinces. These areas are connected to Nakhon Sawan Province, which was confirmed by the existence of *H. taichui* in the previous reports (Anucherngchai et al. 2016). The last area is the connecting area of Ang Thong and Phra Nakhon Si Ayutthaya Provinces. These areas are the in the floodplain, which is comprised of a vast agricultural area. This area provides suitable conditions for the larval stage of trematodes (Tum et al. 2004). It is likely that the adult stage of *H. taichui* in certain definitive hosts, such as birds, mammals and humans, produced eggs and passed them onto the water resource. The contaminated water may be discharged from the agricultural area through the irrigation canals, which would then contribute to the spread of the infection in

Fig. 6 The infection area of *H. taichui* cercariae in the lower area of Chao Phraya Basin, Thailand using molecular identification. The epidemic mapping constructed by the Geographic Information Systems (GIS)

snails. This conclusion is supported by the findings of previous reports that have suggested that the risk of trematode infection was increased when the sampling site was situated near a river or other freshwater resource (Tum et al. 2004; Chontanarith et al. 2017). Therefore, the results of this study can be helpful in estimating the risk of infection in neighboring provinces and has led to creating the surveillance program for Heterophyiasis in infected areas.

Conclusions

The findings of this study were used to establish the high performance rapid detection approach for identifying the important minute intestinal trematode, *H. taichui*, with no cross-reaction with other trematodes and hosts. Furthermore, the limitation of the detection process was found at 0.5 ng/μl. It was sufficient in identifying the parasitic eggs contaminated in the sample using a minute amount of DNA. Additionally, this study also confirmed the existence of *H. taichui* in the lower part of the Chao Phraya Basin based on the molecular data. The epidemic data and rapid detection tools obtained from this study have proven to be beneficial in the epidemiological studies of *H. taichui* in

their intermediate and definitive hosts. Moreover, the findings of this study can be effectively used for the purposes of monitoring and creating the prevention program to determine the prevalence of Heterophyiasis within an area of infection.

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Author contributions CT: Conception and design of study, CT: Acquisition of data, AS and TT: Analysis and/or interpretation of data, AS and TT: Drafting the manuscript, CT: Revising the manuscript critically for important intellectual content. AS, TT, CT: Approval of the version of the manuscript to be published (the names of all authors must be listed).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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