Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Epidemiology and molecular genotyping of echinostome metacercariae in *Filopaludina* snails in Lamphun Province, Thailand

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ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form 15 October 2013
Accepted 15 December 2013
Available online 20 January 2014

Keywords:
Echinostoma sp.
Filopaludina sp. snails
RAPD-PCR
Epidemiology
Metacercariae

ABSTRACT

Objective: To analyze the prevalence of echinostome metacercariae in *Filopaludina dorliaris* (*F. dorliaris*) and *Filopaludina martensi martensi* (*F. martensi martensi*) and genotype variation of echinostome metacercariae by using random amplified polymorphic DNA (RAPD) analysis. **Methods:** *Filopaludina* sp. snails were collected from eight localities of Lamphun Province, Northern Thailand and examined for echinostome metacercariae. RAPD-PCR was used to analyze genotype variation of echinostome metacercariae. **Results:** A total of 3 226 *F. dorliaris* and *F. martensi martensi* snails were collected from eight localities. The overall prevalences of echinostome metacercariae in *F. dorliaris* and *F. martensi martensi* were 40.89% and 36.27%, while the intensity of infection was 20.37 and 12.04, respectively. The dendrogram constructed base on RAPD profiles, 4 well supported domains were generated; (i) group of metacercariae from Ban Hong, Mae Ta, Meaung, Pa Sang, Toong Hua Chang, and Weang Nong that were clustered in the group of *E. revolutum*, (ii) Ban Thi, (iii) Lee, and (iv) 3 adults of an out group. **Conclusions:** This research demonstrated RAPD profiling has been a useful tool to detect DNA polymorphisms to determine genetic relationship between echinostome metacercariae in Lamphun Province, Northern Thailand.

1. Introduction

Echinostomes are important intestinal parasitic flatworms invading a wide range of definitive hosts, such as aquatic birds, mammals and occasionally humans[1,2]. Echinostome cercariae can penetrate into several second intermediate hosts: such as gastropods, bivalve snails, crustaceans, fish and amphibians including tadpoles, where, at this stage, they can further develop into infective metacercariae[3,4]. The ingestion of these second intermediate hosts through improper cooking, that have harbored metacercariae is the main cause of echinostome infection[5]. In Thailand, four echinostomes have been reported for infecting humans; *Echinostoma malayanum (E. malayanum), Echinostoma revolutum (E. revolutum), Echinostoma ilocanum (E. ilocanum)* and *Hypoderaeum conoideum (H. conoideum)*[4]. The identification of echinostome metacercariae is generally conducted using unique morphological characteristics, but due to a large number of morphological similarities, inadequate descriptions, poor specific diagnostic characters and extensive synonymy[6], the identification system has not been considered fully reliable[7]. Unique morphological characteristics of echinostomes are focused on the head collar with collar spines around the oral sucker. The number and arrangement of the collar spines are considered important evidence for taxonomic purposes[8]. Different numbers of arranged collar spines have been reported, varying from a minimum of 31 to a maximum of 51[9]. The number of collar spines presented in each echinostome species can be used as identical evidence in both their larvae and adult forms. However, relying only on the number of collar spines to be used

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for the identification aspect is still considered inadequate due to the inconsistencies in appearance, particularly in different location. In accordance with previous studies, reported that the average collar spine number of most *Echinostoma* spp. is 43 (39–45)[10], and these are arranged in two alternating rows at the dorsal side with a long–large cirrus sac that reaches beyond the ventral sucker. The average number of collar spines of *E. malayanum* is 41[11], whereas, the investigated four human echinostomes, which possessed different numbers of collar spines: *E. revolutum* (37), *E. malayanum* (43), *Echinoparyphium recurvatum (E. recurvatum)* (43–50), and *H. conoideum* (41–45)[12]. As mentioned previously, relying only on the number of collar spines may not enough for the purpose of identification of echinostomes, specifically. Sibling morphology and/or cryptic trematode species can cause problematic cases of identification. Moreover echinostomes populations present complex intraspecific variabilities as showed in a number of research papers. The genetic variability of parasites have been well demonstrated by RAPD profiles[13–17]. Moreover, the procedure of applying high annealing temperature to random amplified polymorphic DNA (HAT–RAPD) has been used to detect *Opisthorchis viverrini (O. viverrini)* and *Haplorchis taichui (H. taichui)* in human feces from Chomtong District, Chiang Mai Province, Thailand[18]. In this study focused on the status of echinostome metacercariae infections in freshwater snail, *F. dorliaris* and *F. martensi martensi* and genetic variation by RAPD–PCR analysis from different location of Lamphun Province, Northern Thailand.

2. Materials and methods

2.1. Sample collection

A total of 3 226 freshwater snails (*F. dorliaris* and *F. martensi martensi*) were collected by hand during January 2011 to December 2012 from eight locations (Ban–Hong, Ban–Thi, Lee, Mae–Ta, Meaung, Pa–Sang, Toong–Hua–Chang, and Weang–Nong–Long) of Lamphun Province, Northern Thailand. Metacercariae were removed from *F. dorliaris* and *F. martensi martensi* by crushing method under light microscope and were counted and used as a source of materials for molecular identification.

2.2. Molecular study

Genomic DNA of all collected echinostomes will be extracted and purified from both adults and metacercariae by using GF–1 Tissue extraction kit (Vivantis), according to the manufacturer's instructions. The specific fragment for echinostome metacercariae, obtained from RAPD PCR, will be screened and purified from agarose gel, using the GF–1 Gel DNA Recovery Kit (Vivantis). All extracted genomic DNA will be diluted to a working concentration of 50 ng / μL and stored at –20 ℃ before usage and each 1 μL will be used in PCR reaction. Ten commercially available arbitrary 10–mer primers (Operon technology, USA) will be used individually for RAPD PCR and the reaction will be carried out in a final volume of 20 μL, with common PCR composition and performed in a MyCyclerTM Thermocycler (Bio–RAD). PCR conditions as follows: 1 cycle of 95 ℃ for 5 mins, 30 cycle of 95 ℃ for 45 s, 57 ℃ for 1 min, 72 ℃ for 1 min and 1 cycle of final extension at 72 ℃ for 7 mins. RAPD PCR products will be separated on 1.4% TBE agarose gel electrophoresis, stained with 0.5 μg/mL ethidium bromide and photographed by using a Kodak Digital Camera Gel Logic 100.

3. Results

A total of 19 048 metacercariae were removed from 3 226 *Filopaludina* spp. snails which were morphologically pre–identified as echinostomes (Figure 1). The overall prevalence and intensity of metacercarial infection was 38.07% (1 228/3 226) and 15.51% (19 048/1 228) respectively. The prevalence of infection peak in rainy season (41.73%), and gradually decreased in cool–dry season (36.38%) and hot–dry season (34.73%). In addition, the total prevalence of metacercarial infection in *F. dorliaris* was higher than *F. martensi martensi* as 40.89% and 36.27%, respectively. The prevalence of metacercarial infection in *F. dorliaris* in three seasons was higher than *F. martensi martensi* and highest in rainy season (46.01%) (Table 1).

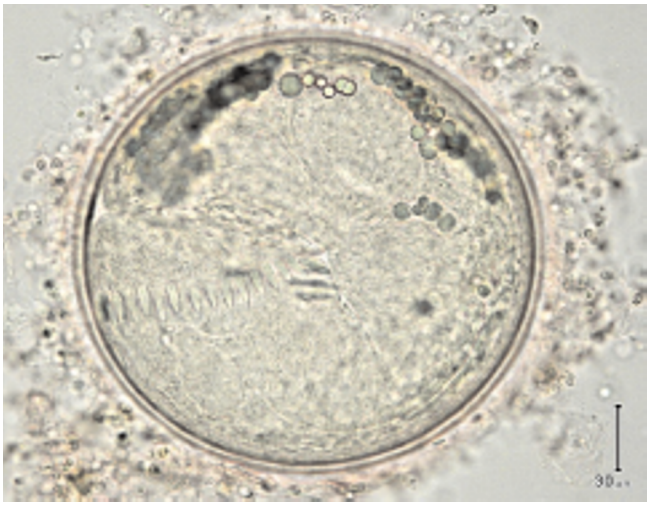

Figure 1. Echinostome metacercariae infected in *Filpaludina* sp. which present collar spines.

Table 1
Prevalence of metacercarial infection in *Filopaludina* sp. discovered in three seasons.

Season	*F. dorliaris*	*F. martensi martensi*	Overall
Hot-dry	37.42 (122/326)	33.22 (192/578)	34.73 (314/904)
Rainy	46.01 (242/526)	38.80 (298/768)	41.73 (540/1 294)
Cool-dry	37.00 (148/400)	35.99 (226/628)	36.38 (374/1 028)
Overall	40.89 (512/1 252)	36.27 (716/1 974)	38.07 (1 228/3 226)

All metacercariae and adult worms used in this study were genetically profiled using RAPD PCR with ten random arbitrary primers: OPA01, OPA03, OPA06, OPA09, OPN04, OPN06, OPN07, OPN08, OPN10, and OPP11. The polymorphic DNA profiles generated were compared with the adult stages of *E. revolutum*, *H. taichui*, *Centrocestus caninus (C. caninus)*, and *Fasciola gigantica (F. gigantica)* (Figure 2). The results showed that the DNA profiles of echinostome metacerariae are shared bands among different locations.

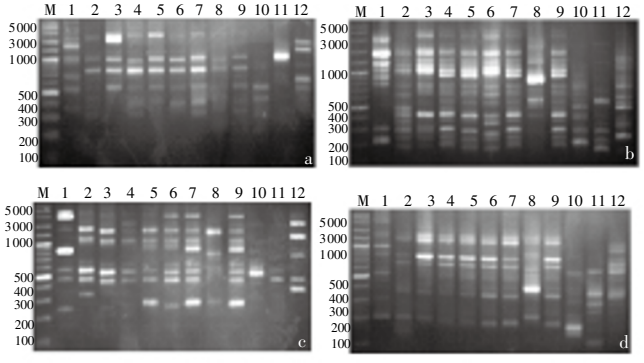

Figure 2. RAPD DNA profiles of metacercariae and adults obtained with the primers.
(a) OPA06, (b) OPN07, (c) OPN08, (d) OPP11 primer Lane M, 100 bp ladder; Lane 1, Ban Thi; Lane 2, Meaung; Lane 3, Pa Sang; Lane 4, Ban Hong; Lane 5, Mae Ta; Lane 6, Weang Nong Long; Lane 7, Toong Hua Chang; Lane 8, Lee; Lane 9, *E. revolutum* (adult); Lane 10, *H. taichui* (adult); Lane 11, *C. caninus* (adult); Lane 12 *F. gigantica* (adult)

The dendrogram demonstrated the relationships of all matacercariae and adult worms based on RAPD profiles that had been constructed. Three adult worms: *H. taichui*, *C. caninus*, and *F. gigantica* were used as an out group. The representative phenogram RAPD-PCR profiles with the ten primers showed the clustering of these populations in four main groups was defined as: (i) *E. revolutum* and six echinostome metacercariae from different localities (Meaung, Pa Sang, Mae-Ta, Ban Hong, Weang Nong Long, Toong Hua Chang), (ii) Ban Thi, (iii) Lee, and (iv) 3 adults of an out group. This result confirmed the genetic differences between echinostome metacercariae from 2 locations (Ban Thi and Lee) and the group of *E. revolutum*, and it has been proposed that they from a different *Echinostoma* species than the group of *E. revolutum* (Figure 3).

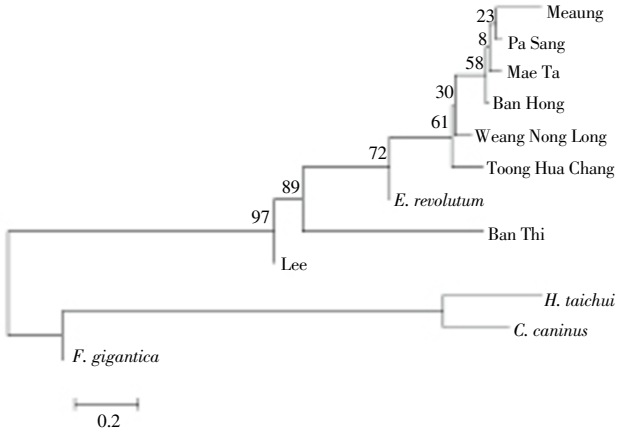

Figure 3. Phenogram tree based on the percentage of shared bands obtained by the average with ten primers by RAPD-PCR.
The percentages correspond to bootstrap values (percentage of times which a clustering was maintained during 1 000 replicates).

4. Discussion

The study of epidemiology of echinostome metacercariae in *Filopaludina* snails in Lamphun Province, Northern Thailand was found to have different prevalence of infection during the three seasons. This may be due to the water level in the rainy season period being more than during other seasons. The water level is very critical to cercariae to finding secondary host snail[18]. The identification of echinstome metacercariae, based on morphology is difficult, due to a large number of morphological similarities and inadequate descriptions. Unidentified metacercariae, particularly the echinostome species, which possess similar morphological features pose significant identification problems. Specific and accurate methods are required to overcome these limitations. The molecular methods have been reported to be used to distinguish among morphologically similar parasites[19]. ITS sequences and mitochondrial DNA are used as genetic markers and for phenogram analyses including the identification of several platyhelminhs[6,20]. In this study, the RAPD PCR method was used for the purpose of comparing DNA profiles with those of the adult stage. The results of dendrogram indicated that Ban Thi and Lee were placed in the different clade of *E. revolutum* adult worms which confirmed a different of genetic, while Meaung, Pa Sang, Mae-Ta, Ban Hong, Weang Nong Long, Toong Hua Chang were clustered in the same clade of *E. revolutum* adult worms. Although six locations show the same clade of *E. revolutum*, they are also divided into two subgroups. Echinostomes reports the epidemiology in several Thailand species; *E. malayanum*, *E. revolutum*, *E. ilocanum*, and *Hypoderaem conoideum*[21]. An increase of the annealing temperature in RAPD PCR conditions to over 46 ℃ provides greater polymorphism and highly reproducible results, which are suitable for determining genetic variations.

Moreover, diagnostic evidence has been provided for the purpose of identification of such organisms. This evidence, which requires only common molecular facilities, is also included as it was described in a former report[17,22].

This research demonstrated RAPD profiling has been a useful tool to detect DNA polymorphisms to determine genetic relationship between echinostome metacercariae in Lamphun Province, Northern Thailand. However, if a further study was to be conducted a thorough comparative morphology of these metacercariae, and their adult stage, should be analyze.

Conflict of interest statemert

We declare that we have no conflict of interest.

Acknowledgements

This work was supported by the Ministry of Science and Technology and the Graduate School, Chiang Mai Univeraity. Special thanks are extended to the Applied Parasitology Research Laboratory, Department of Biology, Faculty of Science, and Chiang Mai University for the use of their lab facilities. We also thank Miss Lauren Cammock for assistance.

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