Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

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

Document heading

Multilocus enzyme electrophoresis analysis of *Echinostoma revolutum* and *Echinostoma malayanum* (Trematoda: Echinostomatidae) isolated from Khon Kaen Province, Thailand

Weerachai Saijuntha[1], Sumonta Tapdara[2], Chairat Tantrawatpan[3*]

[1]*Walai Rukhavej Botanical Research Institute (WRBRI), Mahasarakham University, Mahasarakham, Thailand*
[2]*Clinical Pathology Laboratory, Amnatcharoen hospital, Amnatcharoen, Thailand*
[3]*Division of Cell Biology, Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Rangsit Campus, Pathumthani, Thailand*

ARTICLE INFO

Article history:
Received 5 July 2010
Received in revised form 17 July 2010
Accepted 1 August 2010
Available online 20 August 2010

Keywords:
Echinostoma
Echinostomes
Genetic variation
Multilocus enzyme electrophoresis
Allozyme

ABSTRACT

Objective: To explore the genetic variation and differentiation of 2 echinostomes from genus *Echinostoma*, i.e. *Echinostoma revolutum (E. revolutum)* and *Echinostoma malayanum (E. malayanum)* from Khon Kaen Province, Thailand. **Methods:** These parasites were compared at 22 enzymes encoding a presumptive 30 loci by using multilocus enzyme electrophoresis (MEE) technique. **Results:** Twenty-two loci can be used as diagnostic markers to differentiate these 2 species. *E. revolutum* and *E. malayanum* had fixed genetic differences at 70% of loci, whereas both species had fixed genetic differences from the liver fluke, *Opisthorchis viverrini* at 91% of loci. Intraspecific variation within a population of *E. revolutum* was observed at 5 polymorphic loci. **Conclusions:** MEE is a powerful technique to investigate genetic variation and differentiation of *E. revolutum* and *E. malayanum*.

1. Introduction

Human echinostomiasis is caused by at least 19 species of echinostomes (from 8 genera). The most speciose genus, *Echinostoma*[1], has been reported in China, India, Indonesia, Japan, Korea, Malaysia, Philippine, Russia, Taiwan and Thailand[2-4]. In Thailand, 5 species of echinostomes have been reported to infect humans, three of which are *Echinostoma ilocanum (E. ilocanum)*, *Echinostoma revolutum (E. revolutum)* and *Echinostoma malayanum (E. malayanum)*[2]. Morbidity and mortality due to echinostomiasis are difficult to assess because of a prolonged latent phase, a short acute phase and clinical symptoms similar to other intestinal helminthiasis. Moreover, clinical symptoms are related to parasite load[1]. The significant pathology of human echinostomiasis is the intestinal mucosa damage and cause extensive intestinal and duodenal erosions[1]. Echinostomiasis is also a very important disease in domestic animals and wildlife.

Echinostomiasis differs from other disease causing from other food-borne trematodes because of it has a much broader second intermediate host range. A wide variety of aquatic animals, namely snails, crustaceans, fish and amphibians serve as the second intermediate hosts[5]. These parasites propagated in wide range of host but the genetic variation of echinostomes is have no more concern especially in Southeast Asian echinostomes. The predominant differences of gloss morphology among these echinostomes are circumoral disc and testes characteristic[3]. However, distinguishing between these parasites is probably confusing if external and internal organs of these worms are not well developed, especially testes shape. Moreover, co-infection of *E. revolutum* and *E. malayanum* has been founded in rat and human host. Then, molecular markers are needed to confirm the case of unidentified species morphologically. The molecular markers have been developed to identify or differentiate species, and also examine genetic relationships of

*Corresponding author: Chairat Tantrawatpan, Division of Cell Biology, Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Rangsit Campus, Pathumthani, Thailand.
Tel: +66-2926-9768
Fax: +66-2926-9755
E-mail: talent3003@yahoo.com

American, African and Australian echinostomes[6,7]. Multilocus enzyme electrophoresis (MEE) is a powerful technique that has been effectively used to investigate genetic variation and population genetic of many food-borne parasitic trematodes previously[8,9], including *Echinostoma* spp.[10].

The aims of the present study were to explore the genetic variation within and between 2 medically important echinostomes from genus *Echinostoma* (*E. revolutum* and *E. malaynum*) using MEE, and to establish genetic makers for their identification and differentiation.

2. Materials and methods

2.1. Sample collection

Adult worms of *E. revolutum* were collected from intestines of domestic ducks from a slaughterhouse in Khon Kaen Province, Thailand. *E. malayanum* were grown in hamster with metacercarial cysts obtained from Indoplanorbis exustus snail collected from a natural swamp in Khon Kaen Province, Thailand. These snails were digested by 0.3% pepsin A solution, as method of the extraction of *Opisthorchis viverrini* metacercariae from cyprinid fish as described previously[9]. The metacercariae of *E. malayanum* were identified under a stereomicroscope, after that 50 live metacercariae were given orally to each of a male golden Syrian hamster, aged 6-8 weeks, by intragastric intubation. The hamsters were sacrified 30 days after infection. The adult of these trematodes were examined using a light microscope and identified to species-level according to the size of the circumoral disc, testes morphology and the number of collar spines[3]. Several individual worms of each species were randomly selected for carmine staining to confirm their species identity by morphology[3]. Other individuals were washed extensively in normal saline, and then frozen at -80 ℃ for subsequent electrophoretic analyses.

2.2. Sample preparation for electrophoresis

Individual worms were placed in a microcentrifuge tube to which 10-30 μL of lysing solution (100 mL distilled H_2O, 100 μL β-mercaptoethanol, 10 mg NADP) was added. Samples were manually homogenized with a glass rod. The homogenates were then centrifuged at 10 000 rpm for 10 min while maintained at a temperature of 4 ℃. Supernatants were stored in capillary tubes as 5 μL aliquots at -20 ℃ until required.

2.3. MEE

MEE was performed using cellulose acetate gels (Cellogel; Milan) as the support medium, with 0.02 M phosphate buffer (pH 8.0) used as the running buffer. MEE gels were run for 120-150 min at a constant voltage (200 V) and at a constant temperature (4 ℃). The histochemical staining methods were used. Parasite samples were compared using 22 enzymes (abbreviation, enzyme commission No.), namely: adenylate kinase (Ak, 2.7.4.3), aldolase (Ald, 4.1.2.13), enolase (Enol, 4.2.1.11), fructose-1,6-diphosphatase (Fdp, 3.1.3.11), fumarate hydratase (Fum, 4.2.1.2), glyceraldehyde-3-phosphate dehydrogenase (Gapd, 1.2.1.12), glucose-6-phosphate dehydrogenase (G6pd, 1.1.1.49), aspartate amino transferase (Got, 2.6.1.1), glucose-phosphate isomerase (Gpi, 5.3.1.9), hexokinase (Hk, 2.7.1.1), isocitrate dehydrogenase (Idh, 1.1.1.42), malate dehydrogenase (Mdh, 1.1.1.37), malic enzyme (Me, 1.1.1.40), nucleotide diphosphate kinase (Ndpk, 2.7.4.6), peptidase valine-leucine (PepA, 3.4.13.11), peptidase leucine-glycine-glycine (PepB, 3.4.11.4), peptidase phenylalanine-proline (PepD, 3.4.13.9), phosphoglycerate mutase (Pgam, 2.7.5.3), phosphoglucomutase (Pgm, 2.7.5.1), pyruvate kinase (Pk, 2.7.1.40), triose phosphate isomerase (Tpi, 5.3.1.1), and uridine monophosphate kinase (Umpk, 2.7.1.48).

2.4. Data analyses

For each enzyme, the alleles with the least electrophoretic mobility from the cathode were designated as allele a. The multiple banding patterns of individual worm at a particular locus were consistent with the expectations of heterozygous individuals for enzymes with a quaternary structure, e.g. two-banded and triple-banded patterns for heterozygous individuals for monomeric and dimeric enzyme, respectively[9]. The fixed allelic (hence genetic) differences among samples representing the 2 echinostome species were recorded. A fixed genetic difference occurs when 2 group of samples do not have any alleles in common at an enzyme locus[11]. A phenogram was constructed based on an unweighted pair group method with arithmetic mean (UPGMA) to measure the fixed differences between and within species.

3. Results

In this study, genetic variation within and between 2 medically important species of echinostomes, *E. revolutum* and *E. malayanum* were analysed using 22 enzymes which encoded presumptive 30 loci. Of this, up to 17 loci can be used to differentiate *E. revolutum* from *E. malayanum* (Figure 1). Intraspecific variation within *E. revolutum* population was observed at 5 loci, namely *G6pd-1*, *Got-1*, *Me-1*, *PepA-2* and *PepB-2* (Figure 2). Of these, two alleles were detected at for *G6pd-1*, *Got-1* and *PepB-2*, whereas 3 alleles were detected fort *Me-1* and *PepA-2*. Within a population of *E. revolutum* showed individually fixed difference ranged between 0-12%, whereas a fixed difference was not detected

within a population of *E. malayanum*. Fixed genetic difference between *E. revolutum* and *E. malayanum* was detected at 21 loci (70% of loci examined). The echinostomes showed genetically differ from the liver fluke, *O. viverrini* at 91% fixed difference (Figure 3).

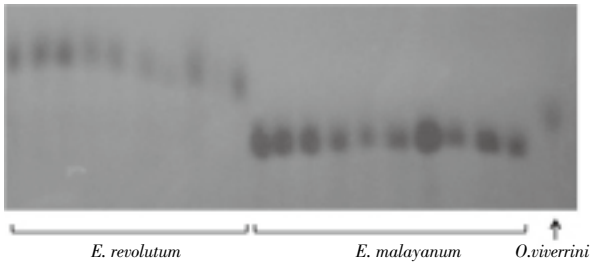

Figure 1. The electrophoretic banding patterns of Ald, a diagnostic locus among *E. revolutum*, *E. malayanum* and *O. viverrini*.

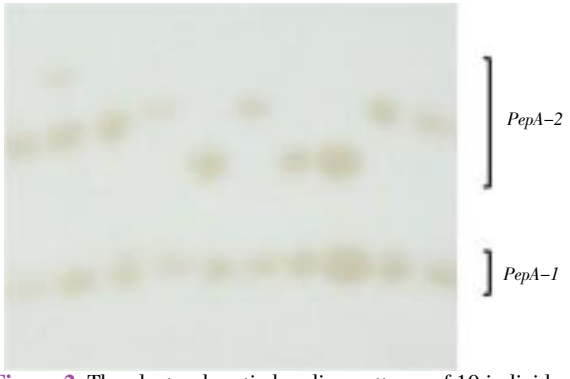

Figure 2. The electrophoretic banding patterns of 10 individuals of *E. revolutum* for PepA.

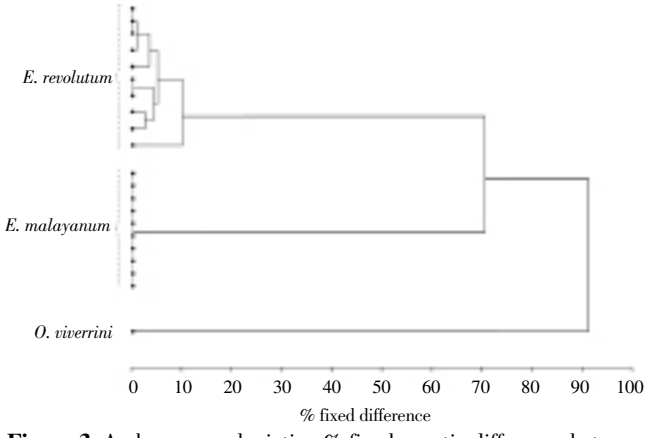

Figure 3. A phenogram depicting % fixed genetic difference between *E. revolutum*, *E. malayanum* and *O. viverrini*. Each dot represents an individual adult sample, except for *O. viverrini* (represents a pool of 10 adultworms).

4. Discussion

The number of enzyme loci examined (30 loci) for echinostomes in this study is a larger than in previous studies[12]. Seventeen diagnostic loci between *E. revolutum* and *E. malayanum* observed herein strongly supported that allozyme markers were potentially used to investigate genetic variation and differentiation of echinostomes in Thailand as in previous study[10]. Moreover, the 5 polymorphic loci, *G6pd-1*, *Got-1*, *Me-1*, *PepA-2* and *PepB-2* may provide the opportunity for further comprehensive analyzes of the population genetics in these parasites as previous studies in other trematodes, e.g. *O. viverrini sensu lato*[13]. As known that various freshwater snails, fish, crustaceans and amphibians act as first and second intermediate host of echinostomes, while several species of mammals and poultry have been reported as definitive hosts[5]. Then, allozyme markers established in this study could be used for a more comprehensive genetic investigation within and/or among species of echinostomes, as well as co-evolutionary relationships of their hosts, as has been done between *O. viverrini* and its first intermediate host, *Bithynia* snail[9]. In addition, diversity of host species should importantly influence the genetic variation of these parasites, in term of subdivision populations, the spatial patterns of selection, and the extent of gene flow between populations[14].

Fixed genetic difference between *E. revolutum* and *E. malayanum* (70%) is greater than the genetic differences reported previously among other three echinostomes from genus *Echinostoma*, i.e. *Echinostoma caproni (E. caproni)*, *Echinostoma paraensei (E. paraensei)* and *Echinostoma trivolvis* (*E. trivolvis*)[12]. The level of percent fixed differences that typically distinguishes different species when allopatric populations are being compared should be greater than 15%. Then, within a population of *E. revolutum* showed individually fixed difference ranged between 0-12%, which suggested that intraspecific variation should exist in this population. A fixed difference was not detected within an *E. malayanum* population, which may cause from high inbreeding in this sympatric population. However, these echinostomes endemic through Southeast Asia, probably the species complex may be existed if more isolates were examined. Additional independent analyses such as morphological comparisons may provide evidence to support this hypothesis. This may potentially be the case as has been shown for two and five geographical isolates of Brazilian *E. paraensei* and South-East Asian *O. viverrini sensu lato*, respectively which have some distinct biological characteristic[15,16]. Genetic investigations across a wider geographical range, temporal and spatial population dynamics of these medically important intestinal parasites could be investigated as has been done in other trematodes[17,18]. In addition, comprehensive population genetics studies of more species and isolates of echinostomes are essential to understand the micro- and macro-evolutionary changes in Thailand and also other countries in South-East Asia.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This research was supported by the Thailand Research Fund and the Commission on Higher Education (grant no. MRG5180102 to Weerachai Saijuntha).

References

[1] Chai JY. Echinostomes in humans. In: Toledo R, Fried B. *The biology of echinostomes: from the molecular to the community*. New York: Springer; 2009, p. 1-37.

[2] Chai JY, Shin EH, Lee SH, Rim HJ. Foodborne intestinal flukes in Southeast Asia. *Korean J Parasitol* 2009; **47** Suppl: S69-102.

[3] Miliotis MD, Bier JW. *International handbook of foodborne pathogens*. New York: CRC Press; 2003.

[4] Fried B, Graczyk TK, Tamang L. Food-borne intestinal trematodiases in humans. *Parasitol Res* 2004; **93**(2): 159-70.

[5] Toledo R, Esteban JG, Fried B. Recent advances in the biology of echinostomes. *Adv Parasitol* 2009; **69**: 147-204.

[6] Kostadinova A, Herniou EA, Barrett J, Littlewood DT. Phylogenetic relationships of *Echinostoma Rudolphi*, 1809 (Digenea: Echinostomatidae) and related genera re-assessed via DNA and morphological analyses. *Syst Parasitol* 2003; **54**(3): 159-76.

[7] Detwiler JT, Bos DH, Minchella DJ. Revealing the secret lives of cryptic species: Examining the phylogenetic relationships of echinostome parasites in North America. *Mol Phylogenet Evol* 2010; **55**(2): 611-20.

[8] Vilas R, Sanmartin ML, Paniagua E. Genetic variability of natural populations of trematodes of the genus *Lecithochirium* parasites of eels. *Parasitology* 2004; **129**(Pt2): 191-201.

[9] Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Pipitgool V, Tesana S, et al. Evidence of a species complex within the food-borne trematode *Opisthorchis viverrini* and possible co-evolution with their first intermediate hosts. *Int J Parasitol* 2007; **37**(6): 695-703.

[10] Saijuntha W, Sithithaworn P, Andrews RH. Genetic differentiation of *Echinostoma revolutum* and *Hypodereaum conoideum* from domestic ducks in Thailand by multilocus enzyme electrophoresis. *J Helminthol* 2010; **84**(2): 143-8.

[11] Andrews RH, Chilton NB. Multilocus enzyme electrophoresis: a valuable technique for providing answers to problems in parasite systematics. *Int J Parasitol* 1999; **29**(2): 213-53.

[12] Sloss B, Meece J, Romano M, Nollen P. The genetic relationships between *Echinostoma caproni*, *E. paraensei*, and *E. trivolvis* as determined by electrophoresis. *J Helminthol* 1995; **69**(3): 243-6.

[13] Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Satrawaha R, Chilton NB, et al. Genetic variation at three enzyme loci within a Thailand population of *Opisthorchis viverrini*. *Parasitol Res* 2008; **103**(6): 1283-7.

[14] Brandt M, Fischer-Blass B, Heinze J, Foitzik S. Population structure and the co-evolution between social parasites and their hosts. *Mol Ecol* 2007; **16**(10): 2063-78.

[15] Maldonado AJr, Zeitone BK, Amado LA, Rosa IF, Machado-Silva JR, Lanfredi RM. Biological variation between two Brazilian geographical isolates of *Echinostoma paraensei*. *J Helminthol* 2005; **79**(4): 345-51.

[16] Laoprom N, Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Ando K, et al. Biological variation within *Opisthorchis viverrini sensu lato* in Thailand and Lao PDR. *J Parasitol* 2009; **95**(6): 1307-13.

[17] Vilas R, Sanmartin ML, Paniagua E. Temporal allozyme divergence in infrapopulations of the hemiurid fluke *Lecithochirium fusiforme*. *J Parasitol* 2004; **90**(1): 198-201.

[18] Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Satrawaha R, Chilton NB, et al. Genetic variation at three enzyme loci within a Thailand population of *Opisthorchis viverrini*. *Parasitol Res* 2008; **103**(6): 1283-7.