ENZYME MARKERS TO IDENTIFY AND CHARACTERIZE *OPISTHORCHIS VIVERRINI* IN THAILAND AND LAO PDR

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Abstract. We conducted an allozyme electrophoretic study to explore potential enzyme markers to distinguish *Opisthorchis viverrini* in Thailand and Lao PDR. Twenty-eight enzymes encoding presumptive 32 loci were established. The enzymes glucose-6-phosphate dehydrogenase and pyruvate kinase were diagnostic between two geographically separate isolates from Thailand. Twelve enzymes, *ie*, aconitate hydratase, aldolase, creatine kinase, enolase, esterases, fumarate hydratase, aspartate aminotransferase, glucose-phosphate isomerase, alanine aminotransferase, isocitrate dehydrogenase, malic enzyme, and pyruvate kinase, also provided diagnostic markers for these two isolates from Thailand and one isolate from Lao PDR.

INTRODUCTION

The fish-borne liver fluke, *Opisthorchis viverrini*, is endemic in Lao PDR, Vietnam, Cambodia, and Thailand (WHO, 1995). In Lao PDR, a high prevalence of *O. viverrini* infection (average 50%) was reported by Kobayashi *et al* (2000). Endemic areas in Thailand are mainly in the northeast (Preuksaraj, 1984; Jongsuksuntigul, 2002). Previous studies have shown significant differences in the prevalence and intensity of infection of *O. viverrini* between geographical areas (Jongsuksuntigul *et al*, 1992; Jongsuksuntigul and Imsomboon, 1998). For example, prevalence in the northeast has ranged from 14% in Khon Kaen Province to 33.6% in Nakhon Phanom Province (Jongsuksuntigul, 2002). Recently, Sriamporn *et al* (2004) reported that *O. viverrini* infection was still high, ranging from 2.1-70.8% (average 24.5%) in different districts within Khon Kaen Province. Other studies have shown variation in intensity of infection among fish of different endemic areas in northeast Thailand (Sithithaworn *et al*, 1997). As the initiator of cholangiocarcinoma (CCA), *O. viverrini* is classified as a type-1 carcinogen (IARC, 1994). Whether this epidemiological variation reflects the extent of genetic variation within *O. viverrini* remains to be determined.

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Multilocus enzyme electrophoresis (MEE) is a powerful technique for resolving problems in the systematics and population structure of many parasites (Andrews and Chilton, 1999). Many previous studies have used the MEE technique to identify and characterize species of parasites, including protozoa, arthropods and helminths (Andrews and Chilton, 1999), for example, studies of helminth taxa, such as *Clonorchis sinensis* (Park *et al*, 2000), *Schistosoma japonicum* (Chilton *et al*, 1999), *Fasciola* spp. (Agatsuma *et al*, 1994), *Paragonimus* spp. (Agatsuma *et al*, 1986), *Echinococcus granulosus* (Lymbery and Thrompson, 1988). Park *et al* (2002) found that 2 of the 12 enzyme loci examined were diagnostic between an isolate of *C. sinensis* from Korea and from China. Sueblinvong *et al* (1993) applied MEE to *O. viverrini* and found activity in the enzymes glucose-phosphate isomerase, glucose-6-phosphate dehydrogenase, and phosphoglucomutase, in adult worms from human autopsy. Whether these enzymes prove useful diagnostically remains to be determined. Chilton *et al* (1999) also showed that 2 of 23 enzyme loci could distinguish 2 laboratory-derived strains of *S. japonicum*. As Andrews and Chilton (1999) emphasized, it is important to establish as many enzyme loci as possible to be confident of the diagnostic accuracy of specific enzyme loci and at what level they resolve taxonomically, *eg*, individual, population, subspecies, or species.

To our knowledge, only two molecular genetic studies have been conducted to date on *O. viverrini*. Sithithaworn *et al* (unpublished) used RAPD analysis

to examine genetic variation, and Ando *et al* (2001) investigated nucleotide base variation in the cytochrome c oxidase subunit I (CO I) gene of mitochondrial DNA and in the internal transcribe spacer region II (ITS II) encoded in ribosomal DNA. Genetic variations were detected among *O. viverrini* isolates from different geographical areas by RAPD analysis and at the CO I gene, but not ITS II. However, these data were insufficient to clarify the significance of the extent of genetic variation in this liver fluke.

It is apparent that comprehensive analyses are necessary to determine which genetic markers will prove useful in identifying and characterizing isolates of *O. viverrini*. Our preliminary allozyme studies, to determine the presence of enzyme activity, detected 46 enzymes in *O. viverrini* (unpublished). The present study applies the technique of allozyme electrophoresis to *O. viverrini* from Thailand and Lao PDR to establish as many enzyme loci (*ie* genetic markers) as possible for comprehensive studies to examine the extent of genetic variation in *O. viverrini*.

MATERIALS AND METHODS

Parasite isolates

Metacercariae of *O. viverrini* were obtained from various species of cyprinid fish intermediate hosts, from two areas [Ban Lerngpleuy (KLp) and Ban Phai (KBp)] in Khon Kaen Province, Thailand, and one area in Vientiane Province, Lao PDR. Fifty to 100 metacercariae were used to infect hamsters for 4-6 months, when adult worms were recovered from the bile duct. They were washed extensively in physiological saline, placed in pools (20 worms or more) into microcentrifuge tubes, frozen live, and stored at -70 ˚C for future analysis.

Homogenate preparation

Frozen homogenates of *O. viverrini* and uninfected hamster liver (as a host enzyme control) on gels were prepared for electrophoretic examination by adding an equal volume of lysing solution (100 ml distilled H_2O, 100 μl β-mercaptoethanol, 10 mg NADP) to the thawed sample, sonicating (Vibra Cell, Sonics and Materials, USA) and centrifuging at 10,000 rpm for 10 minutes at 4 °C. Supernatants were stored in capillary tubes as 5 μl aliquots at -20 °C until used.

Allozyme electrophoresis

Allozyme electrophoresis was conducted on pools of worms from each geographical area using cellulose acetate (Cellogel, Milan) as the support medium according to Andrews and Chilton (1999) for studies of parasite systematics and population structure. Histochemical staining followed the methods described by Richardson *et al* (1986).

Data analysis

The electrophoretic banding patterns of isolates at each of the 32 enzyme loci were interpreted allozymically, with allele α designating the allozyme with the least electrophoretic mobility from the cathode. For each enzyme, bands detected in different isolates were scored alphabetically as allozymes in order of increasing anodal migration. A fixed genetic difference occurs when a sample or group of samples being examined does not share any alleles in common with another sample or group of samples at a particular locus. A phenogram was constructed using the GWBasic program based on an UPGMA (Unweighted Pair Group Method of Analysis; Sneath and Sokal, 1978) of pair-wise comparisons of the proportion of loci that showed fixed allelic difference between isolates.

RESULTS

Twenty-eight enzymes encoding presumptive 32 enzyme loci, gave sufficient staining intensity and resolution to enable reliable genetic interpretation, *ie*, (abbreviation, enzyme commission no.); aconitate hydratase (ACON, 4.2.1.3), adenylate kinase (AK, 2.7.4.3), aldolase (ALD, 4.1.2.13), creatine kinase (CK, 2.7.3.2), enolase (ENOL, 4.2.1.11), esterases (EST, 3.1.1.1), fructose-1,6-diphosphatase (FDPase, 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 aminotransferase (GOT, 2.6.1.1), glucose-phosphate isomerase (GPI, 5.3.1.9), alanine aminotransferase (GPT, 2.6.1.2), hexokinase (HK, 2.7.1.1), isocitrate dehydrogenase (IDH, 1.1.1.42), lactate dehydrogenase (LDH, 1.1.1.27), 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 (PEP-A, 3.4.13.11), peptidase leucine-glycine-glycine (PEP-B, 3.4.11.4), peptidase phenylalanine-proline (PEP-D, 3.4.13), phosphoglycerate mutase (PGAM, 2.7.5.3), phosphoglucomutase (PGM, 2.7.5.1), 6-phosphogluconate dehydrogenase (6PGD, 1.1.1.44), pyruvate kinase (PK, 2.7.1.40), triose-phosphate isomerase (TPI, 5.3.1.1), and uridine monophosphate kinase (UMPK, 2.7.1.48).

The following 12 enzyme loci distinguished between the 2 isolates of *O. viverrini* from Thailand and the isolate from Lao PDR: *Acon, Ald, Ck, Enol, Est,*

Fum-2, Got-1, Gpi, Gpt-1, Idh, Me-2, and *Pk*. Two loci, *G6pd* and *Pk*, distinguished between the two isolates from different geographical areas in Khon Kaen Province. The isolate from KBp in Thailand can be distinguished from the isolate from Lao PDR at enzyme locus *G6pd*, but this locus does not distinguish the Lao isolate from the Thai KLp isolate. Interestingly, each isolate can be distinguished at enzyme locus *Pk*.

The allelic profiles for the allozyme patterns of the pooled *O. viverrini* isolates from three different geographical localities are shown in Table 1. The phenogram generated from these data (Fig 1) shows that the Lao isolate had fixed genetic differences at 38% of the 32 enzyme loci established from the 2 Thai isolates, which had fixed genetic differences from each other at 6% of the loci examined.

DISCUSSION

The results of this study have provided 32 enzyme loci that can be used to identify and characterize isolates of the liver fluke *O. viverrini* from different geographical areas in Thailand and Lao PDR.

At least 13 independent markers were detected that can distinguish *O. viverrini* from Khon Kaen, Thailand and the Lao isolate, as well as distinguishing between the 2 Khon Kaen isolates, KLp and KBp. For instance, 12 enzyme loci markers distinguished *O. viverrini* from Khon Kaen and Lao PDR, whereas 2 enzyme loci could distinguish 2 isolates from Khon Kaen Province, and one enzyme loci distinguish all 3 isolates.

Sueblinvong *et al* (1993) used MME to investigate genetic variations among *O. viverrini* and found that the enzyme loci *Gpi, Pgm,* and *G6pd* showed electrophorectic banding variation. The adult worm samples were collected from human autopsy, but the geographical origin of the metacerceriae and/or the subject hometown were not given. In our study, these three enzyme loci revealed variation in banding patterns between *O. viverrini* isolates only in *Gpi* and *G6pd*, but not in *Pgm*. *Gpi* is diagnostic between Khon Kaen and Lao isolates; *G6pd* is diagnostic only

Table 1
Allelic profiles of enzyme variation in *O. viverrini,* from different isolates.

Enzyme[a]	KLp	KBp	VT
Acon	a	a	b
Ald	a	a	b
Ck	a	a	b
Enol	b	b	a
Est	b	b	a
Fum-2	b	b	a
G6pd	b	a	b
Got-1	a	a	b
Gpi	a	a	b
Gpt-1	a	a	b
Idh	b	b	a
Me-2	a	a	b
Pk	b	a	c

[a]The following enzyme loci were monomorphic between isolates; *Ak, Fdp, Fum-1, Gapd, Got-2, Gpt-2, Hk, Ldh, Mdh, Me-1, Ndpk, Pep-a, Pep-b, Pep-d, Pgam, 6Pgd, Pgm, Tpi* and *Umpk*.

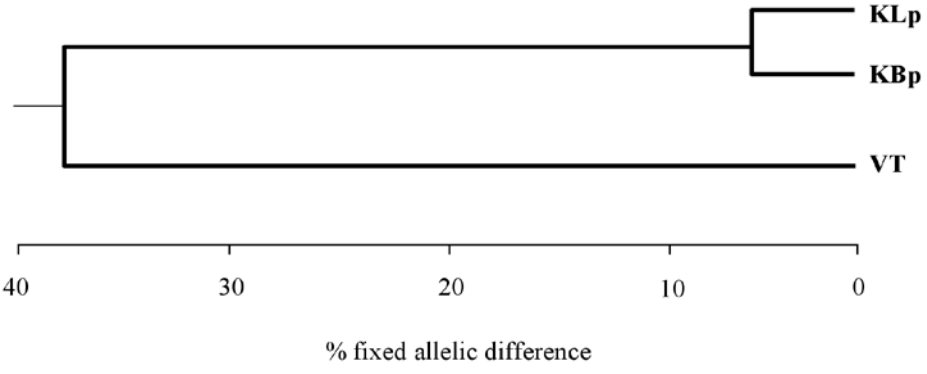

Fig 1- Phenogram depicting the number of percent genetic differences between 3 different isolates, KLp = Ban Lerngpleuy (Khon Kaen Province, Thailand), KBp = Ban Phai (Khon Kaen Province, Thailand), VT = Vientiane (Vientiane Province, Lao PDR).

between KBp and VT; while *Pgm* is monomorphic and has no diagnostic value. Therefore, using only these 3 enzymes as markers, it may be inferred that the KLp isolate is more similar to the VT isolate than the KBp isolate. Analysis at 32 enzyme loci, however, showed that both Khon Kaen isolates were very different genetically from the Lao isolate, with 38% of loci sharing fixed genetic differences. As suggested by Andrews and Chilton (1999) more than 15 enzyme loci should be used to investigate genetic variations in parasites. A preliminary study, using RAPD analyses of *O. viverrini* isolates from different geographical areas in Thailand and Lao PDR, provides supporting evidence for the genetic variation detected by MEE here.

This level of genetic dichotomy (38%) has been found in other allozyme studies of parasites, including helminths; for example, Merenlender *et al* (1987) found that *S. japonicum* from China and the Philippines had fixed genetic differences at 37.5% of the 16 enzyme loci examined. The level of fixed genetic differences may reflect possible population variation (*eg* KBp versus KLp) or the presence of very distinct genetic groups or perhaps subspecies/species of *O. viverrini*.

The utility and rigor of these markers in accurate identification and characterization of *O. viverrini* requires further investigation, so that they can be used appropriately to explore genetic variations and population structures of *O. viverrini* in Southeast Asia.

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