Genetic Variations of the Liver Fluke (*Opisthorchis viverrini*) in Northeast Thailand and Laos PDR by Random Amplified Polymorphic DNA

ความผันแปรทางพันธุกรรมของพยาธิใบไม้ตับในภาคตะวันออกเฉียงเหนือของประเทศไทย และลาว โดยวิธี Random amplified polymorphic DNA

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ABSTRACT

This study aims to explore the genetic variations among population of the liver fluke from different endemic areas using the random amplified polymorphic DNA technique. Liver fluke samples collected from 9 locations in the Northeast namely Khon Kaen, Kalasin, Chaiyaphum, Maha Sarakham, Nakhon Phanom and a location from Laos PDR were analyzed using single strand 10 bases primers. Four out of 15 primers were selected for the analyses. Based on the pattern of DNA fingerprintings, the sampled parasites can be divided into 4 groups. The Laotian isolate showed the most consistent difference in banding pattern to those from Northeast Thailand. The results of this study has demonstrated that there are genetic variations among the liver fluke population in Thailand and Laos PDR. This finding forms basis for further studies on the relationship between the observed genetic variations and other factors such as geographical distribution, variation in prevalence and intensity of infection, pathogenesis as well as its roles in generation of cholangiocarcinoma.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์ที่จะวิเคราะห์หาความแตกต่างด้านพันธุกรรมของพยาธิใบไม้ตับ (Opisthorchis viverrini) ในปลาจากแหล่งระบาดต่าง ๆ ในภาคตะวันออกเฉียงเหนือของประเทศไทยและประเทศลาว โดยใช้เทคนิค Random amplified polymorphic DNA (RAPD) ผลการวิเคราะห์ตัวอย่างพยาธิใบไม้ตับจากแหล่งระบาด 9 แห่งในภาคตะวันออกเฉียงเหนือของไทย ได้แก่ จังหวัดขอนแก่น กาฬสินธุ์ ชัยภูมิ มหาสารคาม นครพนม และจากประเทศลาว 1 แห่ง โดยใช้ไพรเมอร์สายเดี่ยว ชนิด 10 เบสที่คัดเลือกมา 4 สายจากจำนวนที่ทดสอบทั้งหมด 15 สาย มาสร้างลายพิมพ์ DNA ในการวิเคราะห์เปรียบเทียบลายพิมพ์ DNA สามารถแยกตัวอย่างพยาธิใบไม้ตับทั้งหมดออกได้ 4 กลุ่ม ขึ้นอยู่กับชนิดของไพรเมอร์ และพบว่าพยาธิจากประเทศลาวมีลายพิมพ์ DNA แตกต่างจากพยาธิของประเทศไทยอย่างชัดเจน ผลการศึกษานี้ได้แสดงเป็นครั้งแรกว่าประชากรพยาธิใบไม้ตับในประเทศไทยและลาวมีองค์ประกอบทางพันธุกรรมที่แตกต่างกันและเป็นพื้นฐานสำคัญในการศึกษาหาความสัมพันธ์ระหว่างพยาธิใบไม้ตับกลุ่มต่าง ๆ กับปัจจัยอื่นที่เกี่ยวข้อง ด้านแหล่งระบาด ความแปรปรวนของการติดเชื้อ การก่อโรค ความรุนแรงของโรครวมทั้งความสัมพันธ์กับอุบัติการของการเกิดมะเร็งต่อไป

Keywords: *Opisthorchis viverrini*, Genetic variations, Random amplified polymorphic DNA
คำสำคัญ: พยาธิใบไม้ตับ ความผันแปรทางพันธุกรรม

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Introduction

Opisthorchiasis caused by the liver fluke (*Opisthorchis viverrini*; OV) is the most common food-borne trematode infection in Asia and the infection poses serious public health problems in Thailand and neighbouring countries (World Health Organization [WHO], 1995).It has been estimated that at least 7-9 million people in Thailand are infected by this parasite. Human acquires this parasite through consumption of raw or improperly cooked cyprinoid fish containing infective metacercaria. The flukes are luminal parasite as they do not undergo systemic migration, but reside in the small bile ducts and gall bladder. Eggs exit the biliary tract and are excreted in faeces. Chronic and heavy infection in highly endemic areas induces significant morbidities i.e. hepatobiliary disease (cholangitis, cholangiohepatitis, cholelithiasis, cholecystitis, liver abscess, bile duct desquamation, proliferation) and predisposition to the development of cholangiocarcinoma (CHCA)(Elkins *et al.*, 1990; Vatanasapt *et al.*, 1990; Sithithaworn *et al.*, 1994). Most of the hepatobiliary diseases are asymptomatic and may be diagnosed by ultrasonography (Elkin *et al.*, 1990; Mairiang et al, 1992). The mechanisms underlying the association between liver fluke infection and CHCA are not clear, but the flukes are known to produce biliary tract inflammation, hyperplasia, fibrosis and metaplasia. In large autopsy series, it has been shown that a significantly higher percentage of patients who died of CHCA had coexistent opisthorchiasis, compared with those who died of other causes. Furthermore, CHCA is leading malignancy in Northeast Thailand (Vatanasapt et al., 1990) where opisthorchiasis is endenic, in contrast to its rare occurrence in other parts of the country where liver flukes are not present. Base on epidemiological evidence and animal model, OV is now regarded as class I carcinogen.

There are several studies trying to examine the cause of carcinogenesis in *Opisthorchis*- associated CHCA and host factors related to immune response and levels of cancer metabolizing enzymes may play roles in the pathogenesis. Currently, little is known about the genetic variations of the parasite population which may contribute to pathogenesis of CHCA. Korbsrisate *et al.* (1991, 1992) clarified the structure of the ribosomal DNA and nucleotide sequences of 28S and 18S regions but little is known of the existence of genetic variations. Recently there was a report showing some different of the mitochondrial DNA of OV in relation to geographical location (Ando *et al.*, 2002). In this study we examine the genetic variations of OV from Northeast Thailand and Laos PDR.

Materials and Methods

Parasite samples

OV samples were obtained from naturally infected cyprinoid fish in known endemic areas in Northeast Thailand and Laos PDR. The sampling areas in Thailand included Khon Kaen, Kalasin, Chaiyaphum, Maha Sarakham and Nakhon Phanom and Vientiane Laos PDR. The fish samples were collected between October 1999 - February 2000. The fish samples were extracted for metacercariae by pepsin digestion method and the resulting OV metacercariae were given to hamster to produce adult worms. Two months after infection, the worms recovered from infected hamsters were kept at -20 °C until required for DNA analysis.

DNA extraction from adult worms

Frozen adult OVwere disrupted individually in a glass tissue grinder on ice with 100 μl of extraction buffer. Sodium dodecyl sulphate and proteinase K were subsequently added to the final concentration of 1% and 0.1 mg/ml respectively. The homogenate was incubated at 65 °C for 3 hrs. DNA was prepared after the removal of all protein component by extraction with phenol and chloroform. The total DNA was precipitated with 2 volumes of absolute ethanol and then chilled at -20 °C for 3-4 hrs or overnight. After centrifugation, the pellet was washed with 80% ethanol and air dried. The DNA was resuspended in 20 μl of TE buffer and employed for RAPD-PCR.

PCR amplification

The RAPD was performed in a total volume of 30 μl. The reactions contain 2 mM of each dATP, dCTP, dGTP and dTTP, buffer (1.5 mM $MgCl_2$, 30 mM KCl and 10 mM Tris, pH 8.3 in a 30 μl reaction volume), 2 (mol of random primer, 11.25 ng of template DNA and 0.6 unit of Taq DNA polymerase (Pharmacia Biotech, Sweden). RAPD was performed in a DNA thermocycler (Hybaid, Bio-Active Co., Ltd.). The reaction mixture was initially denatured at 95 °C for 5 min 1 cycle followed by running in RAPD cycles. Each cycle consisted of denaturation at 95 °C for 1 min, annealing at 36 (C for 1 min and extension at 72 °C for 2 min. The cycle was repeated for 45 cycles.

Analysis

Amplified products were analysed by electrophoresis in 2% agarose gel using 1(TBE buffer, pH 8.0. After ethidium bromide staining the gel was visualized as fluorescent bands when irradiated with UV transilluminator. The photographs were taken with Image Master® VDS (Pharmacia Biotech, SanFrancisco, USA). Fragments were selected, and base-pair size ranges were determined for each selected fragment by comparing calculated sizes for the same band on several different gels. The presence or absence of fragments within these size ranges for each individual was compared visually and statistically. Discriminant analysis was used to assess the potential for statistic al differentiation between geographic populations using frequency data for various groups of amplified fragments. All data were score as presence (1) or absence(0) of a fragment, For data analysis, bands from each isolates of a parasite-primer combination were counted and the similarity coefficients (F) between two isolates were calculated using the formula F=2nxy/(nx+ny) where nx and ny are the numbers of DNA segments amplified in the isolates x and y respectively, and nxy is the number of segments shared by x and y. The phylogenetic trees generated from the best primer B17 was constructed by using the NTSYS software.

Results

In the initial study, PCR amplifications were performed using fifteen random 10 -mer oligonucleotide primers with a G+C content of 50-80%. Four primers were chosen for further study as they proved to be suitable distinguishing OV isolates by showing clear banding and high reproducibility. The sequence of the selected primers are follow : B5 (5′ TGCGCCCTTC 3′), B17 (5′ AGGGAACGAG 3′), A2 (5′ TGCCGAGCTG 3′), and A17 (5′ GAAACGGGTG 3′). Depending upon the individual worm of *O. viverrini* isolates-primer combination, between 3 to 20 reproducible DNA fingerprints of 0.1 to 2.4 kb were amplified, suggesting both minor and major differences in the RAPD profiles. Each primer amplified several DNA fragments that were polymorphic between the isolates from different geographical areas. Individuals OV from different geographical areas can be classified into 3-6 groups depending on primers.

Figure 1 shows an example of the random amplified polymorphic DNA fingerprints for OV individuals from different areas obtained with primer B17. Each individual worms from each reservoir gives a different pattern and fragments can be scored as being common to 2 or more isolates and some reservoirs give identical pattern. The number of reproducibly amplified fragments produce by this primer varied from 7-16 bands. Amplified products ranged from 227 to 1957 bp. The common bands were 459, 504, 580, 768, 843, 926, 1065, 1284 and 1623 bp.

The random primer B17 could differentiate 9 isolates of OV into 4 distinct banding patterns. Group I consists of isolates from Nam Pong, Ban Phai, Ban Lerngpleuy, Maha Sarakham, Chaiyaphum and Nakhon Phanom. There were 16 fragments (ranging from 399-1623) of discriminating amplified DNA fragments. Group II consists of isolates from Ban Sa-ard and Kalasin. This group included 7 fragments (438-1549 bp). Group III is Ubonratana Dam which has 9 fragments (227-970 bp). Group IV is Lao which has 11 fragments and the DNA banding pattern clearly different from other reservoirs from Northeast Thailand. Table 1 showed the grouping of OV when

using primer B17. Table 2 showed the classification of OV isolates from various endemic areas based on the similarity coefficients calculated from the DNA fingerprinting generated by RAPD-PCR using B17 primer. The number of DNA fragments analysed were 7-16 fragments with the molecular sizes between 227-1957 bp. The grouping of OV was further analysed by a construction of phylogenetic tree. As shown in Figure 2, the Lao isolates by far differs from the isolates from Northeast Thailand. which consists of 3 groups. Group I are Nam Pong, Ban Phai, Ban Lerngpleuy, Maha Sarakham, Chaiyaphum, and Nakhon Phanom, group 2 are Ban Sa-ard, and Kalasin, and group III are Ubonratana Dam.

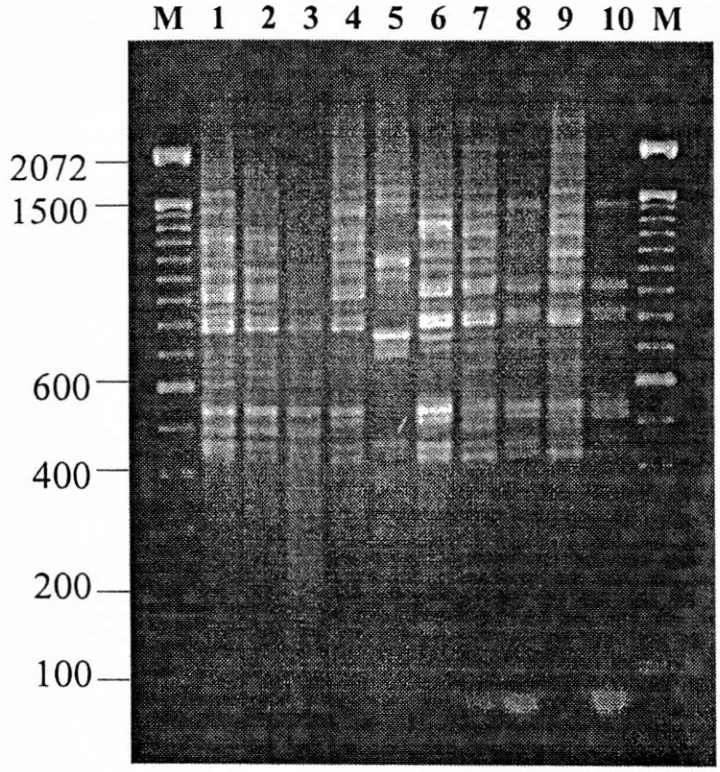

Figure 1 Ethidium bromide stained patterns of the amplified products from 10 geographical isolates using the random primer B17. The amplified products were separated on 2% agarose gel electrophoresis. Lane M = DNA molecular size marker (1-kb ladder)

1 = OV from Nam Pong (Khon Kaen)
2 = OV from Maha Sarakham
3 = OV from Ubonratana Dam
4 = OV from Nakhon Phanom
5 = OV from Laos PDR
6 = OV from Chaiyaphum
7 = OV from Ban Lerngpleuy (Khon Kaen)
8 = OV from Ban Sa-ard (Khon Kaen)
9 = OV from Ban Phai (Khon Kaen)
10 = OV from Kalasin

Table 1 The similarity coefficients between the OV isolates from10 environmental sources using the arbitrary primer B17. Data are expressed as percentages.

	Nam Pong	Maha Sarakham	Ubon ratana Dam	Nakhon Phanom	Laos PDR	Chaiya-phum	Ban Lerng pleuy	Ban Sa-ard	Ban Phai
Nam Pong									
Maha Sarakham	97.1								
Ubonratana Dam	53.3	55.1							
Nakhon Phanom	86.4	83.3	45.1						
Laos PDR	21.4	22.2	18.1	27.5					
Chaiyaphum	84.2	81.0	43.7	92.3	33.3				
Ban Lerngpleuy	80.0	82.3	55.1	83.3	29.6	86.4			
Ban Sa-ard	57.1	59.2	63.6	68.9	30.0	66.6	74.0		
Ban Phai	81.2	83.8	61.5	78.8	25.0	76.4	90.3	75.0	
Kalasin	53.8	56.0	60	59.2	33.3	57.1	64.0	88.8	72.7

Table 2 Classification of OV isolates from various endemic areas based on the similarity coefficients calculated from the DNA fingerprinting generated by RAPD PCR using B17 primer. The number of DNA fragments analysed were 7-16 fragments with the molecular sizes between 227-1957 bp.

OV group	Geographical areas
1	Nam Pong (Khon Kaen) Ban Phai (Khon Kaen) Ban Lerngpleuy (Khon Kaen) Maha Sarakham Chaiyaphum Nakhon Phanom
2	Ban Sa-ard (Khon Kaen) Kalasin
3	Ubonratanana Dam (Khon Kaen)
4	Laos PDR

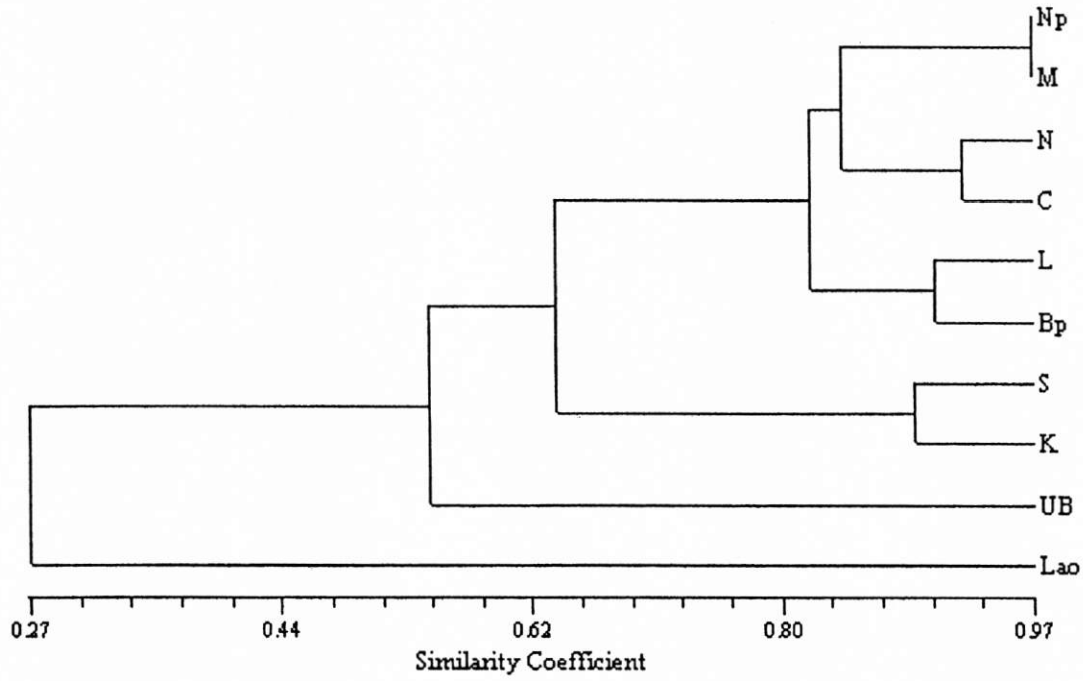

Figure 2 The dendrogram of OV isolates from various geographical sites in Northeast Thailand and Laos PDR constructed by NTSYS software. The isolates locations are Nam Pong (Np), Maha Sarakham (M), Nakhon Phanom (N), Chaiyahum (C), Ban Lerngpleuy (L), Ban Phai (Bp), Ban Sa-ard (S), Kalasin (K), Ubonratana Dam (UB) and Laos PDR (Lao).

Summary and Discussion

This study explores the existence of genetic variations of OV population in Northeast Thailand and Laos PDR. Amongst the 10 base primers tested, 4 primer (B5, B17, A2, and A17), produced patterns that included bands that were polymorphic between isolates of OV from 9 geographic populations, the random primer B17 (figure 1) could differntiate 9 isolates of OV to 4 distinct banding patterns. Isolates of OV from Nakhon Phanom, Chaiyaphum, Maha Sarakham, Ban Phai, Nam Pong and Ban Leungpleuy gave similar banding patterns with the similarity coefficient between the isolates 76.4-97.1%. The DNA pattern OV from Lao was unique and consistently differred from isolates in Northeast Thailand when using all the 4 primers. The basis for the differences between isolates of OV from Thailand and Laos PDR is unknown but may concerned with intermediate hosts (different cyprinoid fish, susceptibility, dispersal ability) host factors (i.e. behavior, immunity, susceptibility and genetic background), and environment (culture, traditional food, life style, geographical condition). The fact that all the Northeastern Thai isolates are originated from the Chi river and its associated branches or reservoirs while the Lao isolate is originated from Nam Ngum reservoir in Laos PDR which has no connection with the Chi river. Thus the spatial distribution may contribute to these differences. Similar study has been reported by Barral *et al.* (1993) discriminating among species, strains and individuals within the genus schistosomes. An important polymorphism was observed among 5 species, allowing a phylogenetic tree to be outlined. These difference can be used for rapid and accurate identification. Polymorphism was revealed among 3 strains of a single species (S. mansoni). A RAPD marker allows sexual discrimination between individuals from the terminal spined-egg species group.

It remains to be investigated whether there is any particular OV groups observed in this study have relation with their virulence, especially in the community with high rate of CHCA. Also, the

approach employed in this study may be applied to other fish-borne trematodes for their evolutionary relation with OV. This would help in understanding the biology of OV and fish-borne trematodes in the region.

In conclusion, RAPD markers are a highly resolving and helpful tool for investigation of variability within the OV. By using this technique, OV can be grouped according to geographical areas and this forms a foundation to explore further the cause of the genetic variations.

Suggestion

1. Confirmation of the findings in this study is clearly needed to verify the genetic variations by other techniques, for example, restriction fragment length polymorphism, and multilocus isoenzyme polymorphism by electrophoretic technique.

2. Studies may be extended to analysis of parasite in human and in cases with overt clinical manifestation i.e. cholangiocarcinoma to find the role of genetic variations on theirs virulence.

3. This method allowed us to discriminate easily between strains, it should be possible to use it as a diagnostic tool in endemic areas where identification of closely related species has always proved to be difficult by means of morphological or biochemical characters. In this way, we would have to increase the number of isolates under assay in order to have an idea of intraspecific polymorphism. Further such studies using a larger number of isolates will be required to confirm the genetic relationship of the grouping found here.

4. The present study identified new and potentially useful primers for *O.* viverrini. Detection of polymorphism is primarily limited by the number of primers tested and, therefore, it is often possible to identify more polymorphism than are needed for a particular study. Accordingly, the best strategy for using RAPD may be to screen by many primers and to select only those that give highly reproducible bands for scoring, rather than trying to optimize every primer-template combination detected

5. Confirmation of the results in this study by separating species of cyprinoid fish from a given geographical areas and compare DNA banding pattern shoud be done.

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