DEVELOPMENT OF PCR-BASED DIAGNOSIS OF MINUTE INTESTINAL FLUKE, *HAPLORCHIS TAICHUI*

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Abstract. *Haplorchis taichui* specific primers were designed using a high annealing temperature random amplified polymorphic DNA (HAT-RAPD) PCR method and 18 arbitrary primers (Operon Technologies) to generate polymorphic DNA profiles for 13 different parasites. The *H. taichui* specific fragment was screened. A 256 bp HAT-RAPD marker generated from OPP-11 primer specific for *H. taichui* was cloned and sequenced. From the sequence data, specific primers were designed that generated a 256 bp amplicon. The minimum DNA template needed for PCR detection was 10 fg. The successful development of the *H. taichui* specific DNA-based detection will be beneficial in management and epidemiological control programs.

INTRODUCTION

Heterophyiasis is an important clinical problem through out the world and especially in Asia. Many heterophyid parasites have been reported as infecting humans in different countries, such as *Metagonimus* spp in Korea (Chai *et al*, 1998), *Stellantchasmus falcatus* (Sripalwit *et al*, 2003) and *Haplorchis taichui* in Thailand. Humans acquire these parasites by eating raw or undercooked freshwater fish containing metacercariae (Kumchoo *et al*, 2003). Fish-eating animals including dogs, cats and rodents can also become infected and serve as reservoirs of infection (Le *et al*, 2006).

Heterophyid flukes, *S. falcatus*, *Centrocestus caninus* and *H. taichui*, have been reported as endemic species in northern Thailand. *H. taichui* has a wide geographical distribution and a high prevalence of infection in Chiang Mai Province (Sripalwit *et al*, 2003) and is sympatric with the liver fluke, *Opisthorchis viverrini*, in northeastern Thailand (Maleewong *et al*, 2003). The most susceptible snail host for *H. taichui* is *Tarebia granifera*, which is the same snail host for *S. falcatus*. The fish host belongs to the mud carp group *Henicorhynchus siamensis* (Kumchoo *et al*, 2003). *O. viverrini* metacercariae and several heterophyid flukes, *S. falcatus*, *C. caninus*, *Haplorchoides* sp. and *H. taichui*, may be found in the same fish host. As their morphology, particularly egg forms, closely resemble each other, diagnosis through microscopic examination is difficult. Consequently, a specific and accurate detection method is needed for parasitic differentiation. Recently PCR methods have been developed for specific detection of different parasites species. For instance, PCR and filter hybridization have been introduced to detect bird schistosome cercariae in lakes (Hertel *et al*, 2002), in snail hosts, in fecal and water samples and in plankton (Hamburger *et al*, 1998; 2001; Pontes *et al*, 2002). In Thailand, specific DNA probes have been developed for the detection of *O.viverrini*,

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(Sermswan *et al*, 1991) and *Paragonimus heterotremus* (Intapun *et al*, 2005). PCR-RFLP has been developed to detect the large liver fluke, *Fasciola hepatica*, cercariae infection in the *Lymnaea columella* snail (Magalhães *et al*, 2004). Due to their rapidity, accuracy and specificity, PCR-based methods have also been introduced to use in copro-diagnosis compared with a standard method in the detection of several parasites, including *Echinococcus multilocularis* in the definitive host (Dinkel *et al*, 1998) and *O. viverrini* (Wongratanacheewin *et al*, 2002).

Random amplified polymorphic DNA PCR (RAPD-PCR) is a useful approach for DNA fingerprinting as no prior DNA sequence information is needed. Anuntalabhochai *et al* (2000) reported that a high annealing temperature-random amplified polymorphic DNA (HAT-RAPD) technique (annealing temperature of 46-48ºC) can produce good polymorphism, reproducibility and high resolution results. The present study was carried out to design a pair of species specific primers based on a fragment generated by the HAT-RAPD technique which could be used as a diagnostic tool for *H. taichui* specific detection and identification.

MATERIALS AND METHODS

Parasite specimens

The 13 parasite species used in this study consisted of 9 trematodes (*H. taichui*, *S. falcatus*, *Haplorchoides* sp, *C. caninus*, *O. viverrini*, *Ganeo tigrinus*, *Fischoederius elongatus*, *Orthocoelium streptocoelium*, *Paramphistomum epiclitum*), 2 nematodes (*Heterakis gallinarum* and *Ascaridia galli*) and 2 acanthocephalan (*Acanthocephalus lucidus* and *Pallisentis* sp).

Genomic DNA extraction

Genomic DNA from all parasites was extracted and purified from adult worms using the Dneasy Tissue Kit (QIAGEN) according to the instructions of the manufacturer. The *H. taichui* specific fragment obtained from HAT-RAPD PCR was purified from agarose gel using a QIAEX Gel Extraction Kit (QIAGEN). Extracted genomic DNA was diluted to a working concentration of 50 ng/μl and stored at -20ºC until used.

HAT-RAPD PCR

Eighteen commercially available arbitrary 10-mer primers (Operon Technology, USA) were used individually in HAT-RAPD PCR. The reaction was carried out in a final volume of 20 μl in a MyCycler™ Thermocycler (Bio RAD) as follows: 1 cycle at 95ºC for 5 minutes; 30 cycles at 95ºC for 45 seconds, 48ºC for 45 seconds, 72ºC for 1 minute; and a final extension at 72ºC for 7 minutes. The HAT-RAPD PCR products were separated by 1.4% agarose gel-electrophoresis stained with 1 μg/ml ethidium bromide and photographed (Kodak digital camera Gel Logic 100).

Cloning and sequencing of the HAT-RAPD fragment

The *H. taichui* specific HAT-PAPD fragment was cloned in a pGEM-T Easy Vector Cloning Kit (Promega, USA) and transferred into *Escherichia coli* (DH5α) by electroporation (Bio RAD) and subjected to sequencing.

Design of *H. taichui* specific PCR primers

H. taichui specific primers (forward/reverse) were designed based on sequence data of a serotype fragment selected from a HAT-RAPD marker using Genetyx-MAC ver.11. The primer pair was tested for specificity with all 13 parasite samples. The optimum PCR condition for *H. taichui* specific detection was determined by varying amount of $MgCl_2$ and adjusting the annealing temperature until only the specific PCR product was clearly generated. The sensitivity of the reaction was investigated by am-

plifying with a 10 fold serially diluted *H. taichui* DNA template ($1x10^{-8}$- $1x10^{-15}$g).

RESULTS

Screening for the *H. taichui* specific fragment

After HAT-RAPD PCR amplification, DNA fingerprint patterns were analyzed for an *H. taichui* specific fragment. A 256 bp fragment generated by OPP-11 primer was found to generate an *H. taichui* specific fragment (Fig 1), which was purified, cloned and sequenced.

Development of *H. taichui* specific PCR detection

A pair of primers was designed using Genetyx-MAC ver.11 software based on the sequence of the 256 bp *H. taichui* specific fragment. The sequences of the primers were Hapt-F , 5'- GGC CAA CGC AAT CGT CAT CC-3'and Hapt - R,5'-GCG TCG GGT TTC AGA CAT GG-3'. The PCR conditions for specific amplification of *H. taichui* were the same as those for HAT - RAPD PCR, except the $MgCl_2$ concentration was 1.0 mM and the annealing temperature was 68ºC. This primer pair always generated the species-specific fragment (256 bp) in only the *H. taichui* DNA sample (Fig 2) and in both the larval and metacercarial stages (Fig 3).

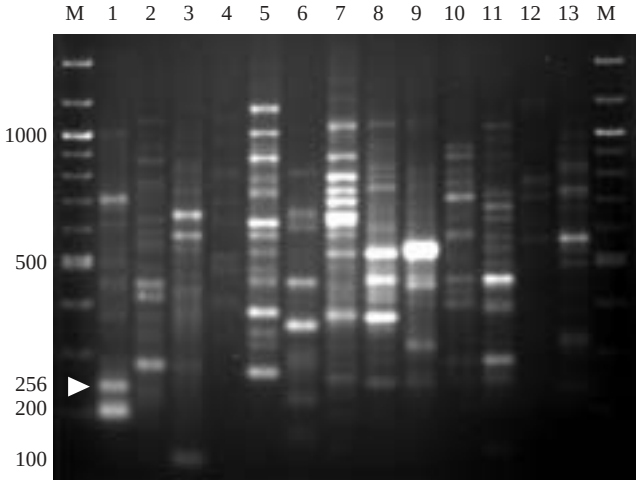

Fig 1–HAT-RAPD markers generated by OPP-11 primer. Lane M, 100 bp molecular marker; lane 1, *H. taichui*; lane 2, *S. falcatus*; lane 3, *Haplorchoides* sp; lane 4, *C. caninus*; lane 5, *O. viverrini*; lane 6, *Ganeo tigrinus*; lane 7, *F. elongatus*; lane 8, *O. streptocoelium*; lane 9, *P. epiclitum*; lane 10, *H. gallinarum*; lane 11, *A. galli*; lane 12, *A. lucidus*; lane 13, *Pallisentis* sp. Arrow indicates *H. taichui* specific 256 bp fragment.

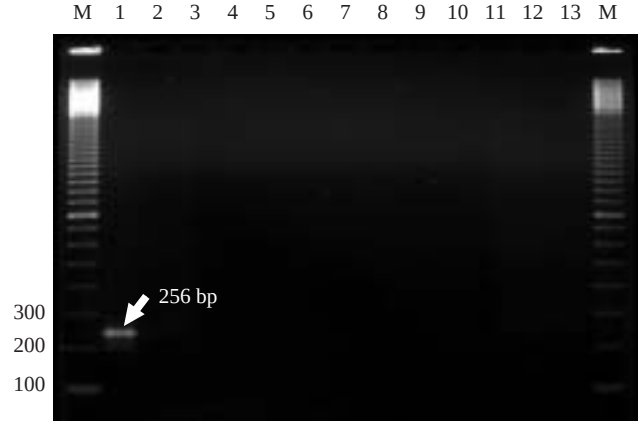

Fig 2–Specificity of *H. taichui* specific primers. Lane M, 100 bp molecular marker; lanes 1-13, as described in legend of Fig 1.

DISCUSSION

In this study, we developed a primer pair designed from an *H. taichui* specific marker obtained from a HAT-RAPD profile. The specificity and sensitivity of the detection were confirmed in independent reactions. Tests of specificity were conducted in both the adult and metacercarial stages. The minimum DNA template needed for detection by PCR was 10 fg, similar to previous reports that indicated a minimum DNA template of 1 fg for detection of larval *S. mansoni* in water samples (Hamburger *et al*, 1998), 10 fg for larval *S. haematobium* in water samples (Hamburger *et al*, 2001) and 2.5 pg for the detection of *O. viverrini* (Wongratanacheewin

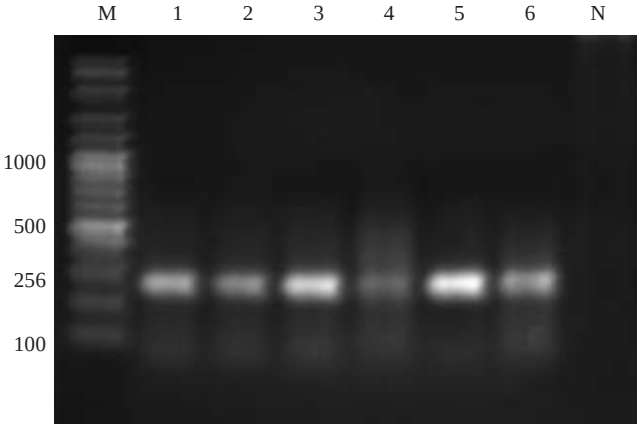

Fig 3–PCR detection of *H. taichui* amplification was performed as described in Materials and Methods. Lane M, 100 bp molecular marker; lanes 1-3, *H. taichui* worm (DNA extracted from an individual adult worm); lanes 4-6, *H. taichui* metacercaria (DNA extracted from an individual sample); N, negative control.

et al, 2002). The sensitivity of detection depends on the length of designed primers. Longer primers give more specificity with DNA template than shorter primers (Magalhães *et al*, 2004) as they can be annealed with template in more stringent conditions (Pontes *et al*, 2002). Most primers are designed based on known sequence data, such as the highly repetitive sequence used to detect *Schistosoma mansoni* (Hamburger *et al*, 1998; Pontes *et al*, 2002), the tandem repeat DNA sequence used to detect *Trichobilhazia ocellata* (Hertel *et al*, 2002), the mitochondrial 12S rRNA gene used to detect *Echinococcus multilocularis* (Dinkel *et al*, 1998), and the mitochondrial sequences used to detect *Fasciola hepatica* (Magalhães *et al*, 2004), *Clonorchis sinensis* and *O. viverrini* (Le *et al*, 2006). The specific primers used in this study were designed base on a fragment of HAT-RAPD marker without a prior DNA sequence data and/or known location, which is the advantage of using this technique to construct specific PCR primers for the detection of parasites.

The successful development of *H. taichui* specific primers will allow application of PCR in the detection of cercariae in snails, metacercariae in intermediate fish hosts and eggs in fecal specimens of definitive hosts, which are required in management and epidemiological control programs.

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