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RESEARCH

MOLECULAR IDENTIFICATION OF *FASCIOLA* SPP. – REPRESENTATIVE SAMPLES FROM THAILAND BASED ON PCR-RFLP

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ABSTRACT

Fasciola spp. are large liver flukes in family Fasciolidae (Railliet, 1895), subfamily Fasciolinae (Stiles et Hassall, 1898). *Fasciola hepatica* Linnaeus, 1758 and *F. gigantica* Cobbold, 1855 are known as the cause of human fascioliasis. *Fasciola* intermediate form was also reported in the overlapping endemic area between *F. gigantica* and *F. hepatica*. The infection of *Fasciola* has become increasingly important because of the recent widespread emergence related to climate change and human activity. However, the accuracy for morphological identification of the *Fasciola* has still been problematic. To solve this problem, ribosomal internal transcribed spacer 1 a`nd 2 (ITS1 and ITS2 rDNA) and mitochondrial cytochrome c oxidase (mt *cox*1) gene, have been used as the genetic markers. However, there is no information about the relative ability of those markers for identifying species of *Fasciola*. In this study, fifty-seven representative *Fasciola* spp. were collected from the slaughterhouse, Pathum Thani Province, Thailand and identified by using PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) techniques. The results of molecular identification were used to evaluate the efficacy of the ITS1, ITS2 and *cox*1 markers for the molecular identification. The results indicated the band patterns of the ITS1 and ITS2 markers were congruent, while the pattern of *cox*1 marker was different. Based on ITS1 and ITS2 markers, most of the representative samples were *F. gigantica*, while the *Fasciola* intermediate form was the minority in the group. This study suggested that the ITS1 marker is the most potential genetic marker for molecular identification of *Fasciola* spp.

Keywords: *Fasciola* spp., Morphological identification, Molecular identification, Genetic marker, PCR-RFLP.

INTRODUCTION

Fasciola hepatica Linnaeus, 1758 and *F. gigantica* Cobbold, 1855 are the parasitic trematodes in class Trematoda, family Fasciolidae (Railliet, 1895) and subfamily Fasciolinae (Stiles and Hassall, 1898). The adult worm infects the liver of various species of mammals, particularly human and livestock (Mas-Coma *et al.*, 1999). The infection of *F. hepatica* and *F. gigantica* is the cause of fascioliasis (Mas-Coma *et al.*, 2009a). About 2.4-17 million people in tropical and sub-tropical areas have the experience to infect from *Fasciola* spp. (Toledo *et al.*, 2009). In Thailand, *Fasciola*

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species have the high prevalence, particularly in the northern part, because of consuming fresh vegetables without good cleaning (Wannasan *et al*., 2014). The infections of *Fasciola* have been increasingly important by climate change, which affects to the widespread distribution of such parasites (Mas-Coma *et al*., 2008; 2009b). *F. hepatica* is reported in America and Europe, while *F. gigantica* is found in Asia and Africa. However, the area distribution of those two parasites can overlap, particularly in equator zone. The hybrid form between *F. hepatica* and *F. gigantica* is often reported and so-called *Fasciola* intermediate form (Mas-Coma *et al*., 2009a, Periago *et al*., 2008).

In Thailand, *F. hepatica* and *F. gicantica* firstly discovered in 1970 and in 1989, respectively (Buranasin and Harinasuta, 1970; Tesana *et al*., 1989). Afterward in 2014, *Fasciola* intermediate form was explored in the northern part of Thailand (Wannasan *et al*., 2014). Based on the previous studies, *Fasciola* intermediate form was reported unclear-morphological characters by similar in their size and shape with both *F. gigantica* and *F. hepatica* (Periago *et al*., 2008; Srimuzipo *et al*., 2000). It was reported from livers of cattle in slaughterhouses and identified as the hybrid status by presenting of the heterosequence of nuclear DNA and ribosomal internal transcribed spacer 1 (ITS1) (Chaichanasak *et al*., 2012; Wannasan *et al*., 2014).

Recently, many molecular markers, five mitochondrial genes (cytochrome *c* oxidase subunit 1 and 3 (*cox*1 and *cox*3), nicotinamide adenine dinucleotide dehydrogenase subunit 1, 4 and 5 (*nad1, nad4 and nad5*)) and 3 nuclear ribosomal RNA regions/gene (internal transcribed spacer 1 and 2 regions (ITS 1 and ITS2) and 28S ribosomal RNA gene (28S rRNA), were used to identify *Fasciola* spp. (Blair and McManus, 1989; Itagaki *et al*., 2001; Marcilla *et al*., 2002; Shafiei *et al*., 2013; Yakhchali *et al*., 2015). The *cox*1 sequences and PCR-RFLP of the ITS1 and ITS2 regions have been used as the genetic markers to detect/identify species complexity of *F. hepatica*/ *F. gigantica* or *Fasciola* intermediate forms. (Ichikawa *et al*., 2017; Wannasan *et al*., 2014). However, the results obtained from those 3 genetic markers were sometimes difficult to interpret due to the presence of unclear restriction bands pattern, particularly those from PCR-RFLP of the ITS1 and ITS2 (Mas-Coma *et al*., 2009a).

In this study, therefore, we aimed to identify *Fasciola* species collected from Thailand, where the overlapping distribution of *F. hepatica*, *F. gigantica* and the intermediate form have been reported using the ribosomal ITS1 and ITS2 regions (ITS1 and ITS2) and the mitochondrial *cox*1gene (*cox*1) as genetic markers and to evaluate the most effective markers for *Fasciola* identification.

MATERIALS AND METHODS

Sample collection and morphological identification of *Fasciola* species

Fifty-seven adult worms of *Fasciola* spp. were collected from the livers of cattle in slaughterhouse at Pathum Thani Province. The cattle were transported from Kanchanaburi Province, Thailand. The infected livers were kept on ice and transferred to the laboratory at the Department of Helminthology, Faculty of Tropical Medicine within an hour. *Fasciola* adult worms were gently separated from the livers and then fixed between two glass slides with a little pressure in normal saline for 1 hour. The fixed adult worms were then preserved in absolute alcohol and stored at 4 °C. Afterward, morphological identification was performed under the stereo-microscope according to the basic morphological characters (Periago *et al*., 2008; Valero *et al*., 1996). The identified adult worms were then cut individually at the posterior part of the body and stored in absolute alcohol at -20 °C until use.

Polymerase Chain Reaction

The small pieces of *Fasciola* adult worms were cut from lateral part of the body. Genomic DNAs were extracted individually using the tissue genomic DNA mini kit (Geneaid, Taipei, Taiwan), according to the manufacturer's protocol. PCR primers were designed from mtDNA (*cox1*) of the complete genome sequences of *F. hepatica*, *F. gigantica* and *Fasciola* intermediate form (GenBank accession nos. NC_002546, M93388,

AF216697, NC_024025, KF543343, respectively). The ribosomal ITS and ITS2 regions were amplified using the primers ITS1-F, ITS1-R for the ITS1 (Ichikawa and Itagaki, 2010) and the primers 3S and BD2 for the ITS2 (Huang *at al*., 2004) All primer sequences and the size of PCR amplicons were described in Table 1. PCR amplifications were conducted three times for each sample to amplify the ITS amplicons. Each PCR reaction was 50 µl in a final volume, composing of 1xTop*Taq* master mixed kit (1U Top*Taq* polymerase, 1.5 mM $MgCl_2$, and Top*Taq* polymerase buffer), 20 *p*mol of each primer, and 10 ng/µl genomic DNA template. The PCR cycles consisted of initial denaturation at 95 °C for 3 min following by 29 cycles of denaturation at 95 °C for 30 s (for *cox1*) and 45 s (for the ITS1 and ITS2), annealing at 54 °C (for *cox1*) and 55 °C (for consisted of the initial denaturation ITS1 and ITS2) for 30 s, and extension at 72 °C for 30 s (for the *cox*1) and 60 s (for the ITS1) and 45 s (for the ITS2). Final extension of each PCR reaction was conducted at 72 °C for 8 min.

Table 1 PCR primer used for *cox*1, ITS1 and ITS2 markers.

Primer	Markers	Sequence (5′ → 3′)	PCR amplicons (bp)	Reference
FascoiF	*cox*1	AAATGCTTTGAGTGCTTGGTTG	836	This study
FascoiR	*cox*1	ATGAGCAACCACAAACCACG	836	This study
ITS1-F	ITS1	TTGCGCTGATTACGTCCCTG	680	(Ichikawa and Itagaki, 2010)
ITS1-R	ITS1	TTGGCTGCGCTCTTCATCGAC	680	(Ichikawa and Itagaki, 2010)
3S	ITS2	GGTGGATCACTGGGCTCGTG	550	(Huang *et al*., 2004)
BD2	ITS2	TATGCTTAAATTCAGCGGGT	550	(Huang *et al*., 2004)

Table 2 Restriction sites of PCR amplicons of *cox*1, ITS1 and ITS2 markers for discriminating species of *Fasciola*.

Species	Molecular markers (restriction enzyme)	PCR amplicons (bp)	Restriction site (nucleotide position)	Restriction fragment size (bp)
Fasciola hepatica	*cox*1 (*Hpy*188III)	836	420, 807	420, 378, 29
F. gigantica			420, 664, 807	420, 244, 143, 29
Fasciola intermediate form			103, 420, 807	378, 317, 103, 29
F. hepatica	ITS1 (*Rsa*I)	680	28, 395, 499, 567, 625	367, 103, 68, 59, 55, 28
F. gigantica			28, 395, 567, 625	367, 172, 59, 55, 28
Fasciola intermediate form			28, 395, 499, 567, 625	367, 172, 103, 68, 59, 55, 28
F. hepatica	ITS2 (*Nla*III)	550	73, 186, 421	235, 130, 113, 73
F. gigantica			73, 186, 344, 421	158, 130, 113, 77, 73
Fasciola intermediate form			73, 186, 421, 344	235, 158, 130, 113, 77, 73

PCR-RFLP

Restriction sites of the mitochondrial *cox*1 and the rDNA ITS1 and ITS2 sequence were indicated in Table 2. The DNA sequences of each genetic marker were obtained from GenBank to design the restriction sites using BioEdit version 7.1.9 (Hall, 1999). The PCR amplicons were conducted as described above. After purifying, the PCR products with the ethanol precipitation method, 500 ng of purified PCR products from each sample were digested with restriction enzyme as follows *Hpy188*III for the *cox1*, *Rsa*I for the ITS1 and *Nla*III for the ITS2 (New England BioLab Inc., Massachusetts, USA) at 37 °C for 60 min, 37 °C for 150 min and 37 °C for 60 min, respectively. The enzymatic digestion was inactivated by heating at 65 °C for 20 min, 80 °C for 20 min and 65 °C for 20 min, respectively. The digested PCR amplicons were run *electrophoresis* on a 2% agarose gel at 50 V for 180 min. The band patterns were visualized on a UV transilluminator. The PCR-RFLP figure was photographed with the Gel Documentation (G-Box (HR); Syngene, UK).

RESULTS

Molecular identification based on PCR-RFLP

Fifty-seven samples of *Fasciola* spp. were morphologically identified as the *F. gigantica*-like because they were similar in shape and size. All of them were then examined using the PCR-RFLP method. The species-specific bands of the ITS1, ITS2 and *cox*1 markers used to identify the representative samples of *Fasciola* (Figs. 1, 2 and 3). The results of the PCR-RFLP of the ITS1 and ITS2 markers indicated that samples in lane Nos. 4 and 15 were *Fasciola* intermediate form. Whereas, the band patterns of the *cox*1 showed that they were *F. gigantica*. In lane No. 10, the *cox*1 marker provided the band pattern of *F. hepatica*, while the ITS1 and ITS2 markers presented the patterns of *F. gigantica*.

Based on the *cox*1 marker, 2 (4%) of the 57 samples were identified as *F. hepatica* and 55 (96%) of them were *F. gigantica*. The PCR-RFLP patterns from the ITS1 and ITS2 markers revealed the different results from those of the *cox*1 marker. Based on ITS1 and ITS2 markers, 3 (5%) of the samples were identified as *Fasciola* intermediate form, while 54 (95%) of them were *F. gigantica*.

DISCUSSION

Regarding species identification of *Fasciola* in Thailand, 57 adult worms of *Fasciola* collected

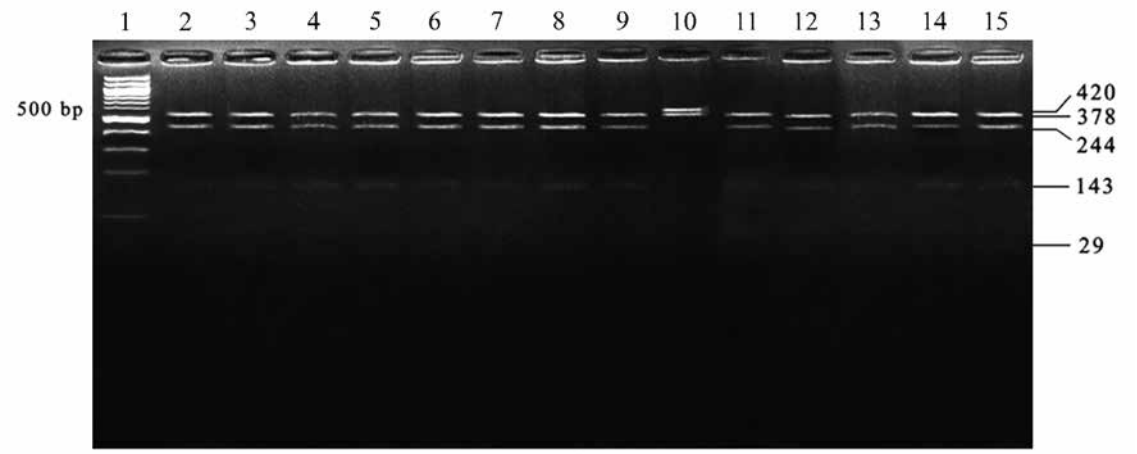

Fig 1 PCR-RFLP patterns of *cox*1 amplicon (832 bp) digested by *Hpy188*III enzyme. Lane 1: 100 bp DNA ladder, Lanes 2 to 9: *F. gigantica*, Lane 10: *F. hepatica*, Lanes 12 to 15: *F. gigantica*. The PCR digested amplicons were run on 2% agarose gel at 50 V for 180 min.

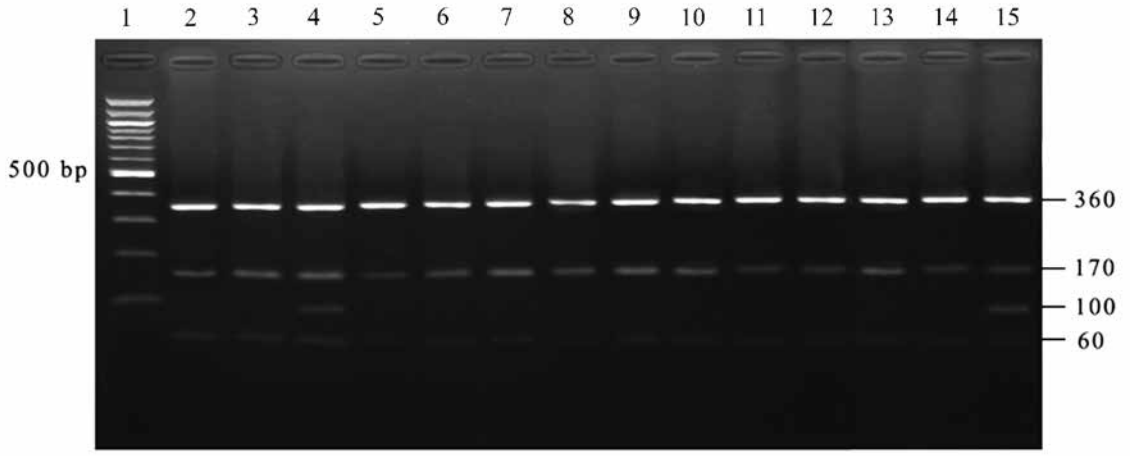

Fig 2 PCR-RFLP patterns of ITS1 amplicon (680 bp) digested by *Rsa*I enzyme. Lane 1: 100 bp DNA ladder, Lanes 2 to 3: *F. gigantica*, Lane 4: *Fasciola* intermediate form, Lanes 5 to 14: *F. gigantica*, Lane 15: *Fasciola* intermediate form. The PCR digested amplicons were run on 2% agarose gel at 50 V for 180 min.

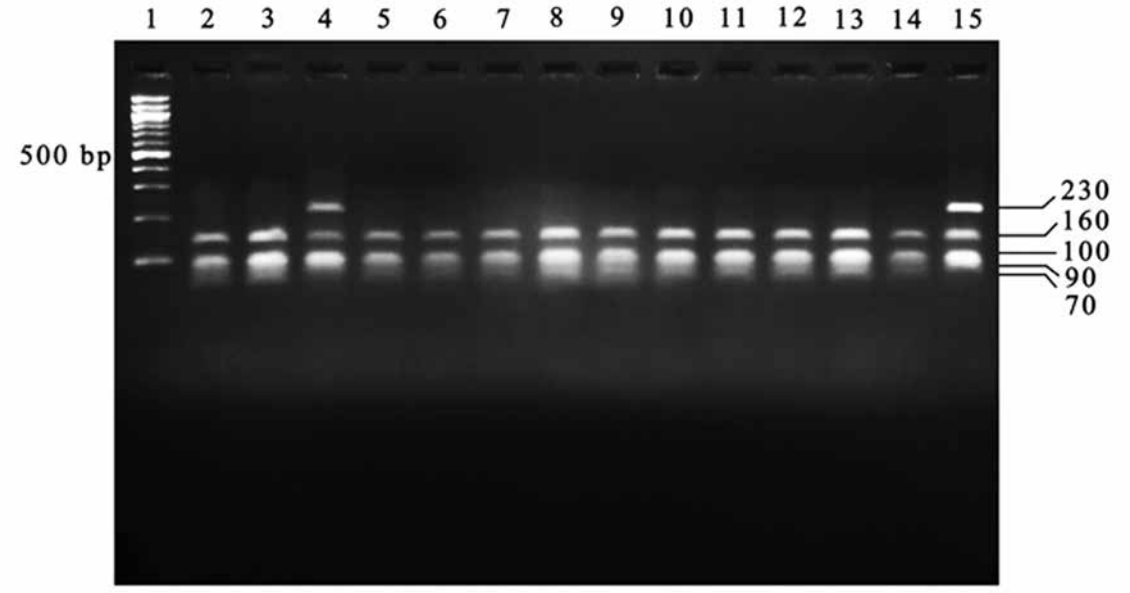

Fig 3 PCR-RFLP patterns of ITS2 amplicon (550 bp) digested by *Nla*III enzyme. Lane 1: 100 bp DNA ladder, Lanes 2 to 3: *F. gigantica*, Lane 4: *Fasciola* intermediate form, Lanes 5 to 14: *F. gigantica*, Lane 15: *Fasciola* intermediate form. The PCR digested amplicons were run on 2% agarose gel at 50 V for 180 min.

from the slaughterhouse at Pathum Thani Province were used as the representative *Fasciola* samples. Based on their morphological characteristics (shape, size, and position of testes), the species of *Fasciola* cannot be distinguished. The size and shape, which normally used to identify are varied because of the age of worm, geographic locality of the host, and worm burden in the liver of host (Phalee *et al.*, 2015; Wannasan *et al.*, 2014). Therefore, in this study, only *F. gigantica*-like species were noted.

To solve the problem of morphological

identification, molecular markers were then applied to the group of samples. The results of molecular identification based on PCR-RFLP between the ITS1 and ITS2 markers indicated congruent results. Most of the representative samples were *F. gigantica*, while *Fasciola* intermediate form was the minority among the group. *F. hepatica* was not detected based on those markers. It was contrary to the results from PCR-RFLP based on the *cox*1 marker, which could not find the *Fasciola* intermediate form from the representative samples but detecting *F. gigantica* as the *F. hepatica*.

Considering the heterozygous band patterns PCR-RFLP of the ITS1 and ITS2 markers between *F. giganica* and *F. hepatica*, our results supported the status of hybrid species of *Fasciola* intermediate form (Ichikawa and Itagaki, 2010). The hybridization evidence can be detected in the nuclear DNA because of the presence of the heterosequence. We suggested that the *cox*1 marker could not be used as the genetic marker for identifying the hybrid form. It is because the *cox*1 is a haploid gene in the mitochondrial genome that no recombination occurs between male and female genomes (Liu *et al*., 2014). Therefore, the heterosequence is not found in the mitochondrial genes (Lin *et al*., 2007). Comparing the results of PCR-RFLP between the ITS1 and ITS2 markers, band patterns of the ITS1 was more easily to distinguish than those of the ITS2 (Figs. 2 and 3). Comparison among the three markers, we suggest that the ITS1 is the most effective genetic marker to be used for *Fasciola* identification.

For molecular identification, the ITS1 as the genetic marker could be used as a good alternative approach to solve the problem of morphological identification. Using only size, shape and position of the testes might not be enough to use for the diagnostic morphological characteristics. To increase the accuracy for species identification of *Fasciola*, we suggest using at least the ITS1 marker to confirm the species.

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