Differential diagnosis of Egyptian *Fasciola* species by deoxyribonucleic acid (DNA) sequences of nuclear ribosomal internal transcribed spacer 1 (ITS1) based on a PCR–RFLP method

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Abstract: Fascioliasis is one of the familiar zoonotic health problems of worldwide distribution including Asian, African countries and Egypt. Morphological differentiation between the liver fluke species *Fasciola hepatica* and *Fasciola gigantica* is difficult. Molecular characterization is important for discriminating *Fasciola* specimens having the deoxyribonucleic acid (DNA) sequences of *Fasciola hepatica*, *Fasciola gigantica*. We have developed a restriction fragment length polymorphism of amplified DNA (PCR–RFLP) of the nuclear ribosomal internal transcribed spacer 1 (ITS1) region in *Fasciola* species. The band patterns of the fragments digested with a restriction enzyme, Rsa I, were accurately distinguished between both forms of *Fasciola*. Amplicons with the sequences of *F. hepatica* and *F. gigantica* were divided into fragments of about 360, 100, and 60 bp, and 360, 170, and 60 bp, respectively, and amplicons with the sequences of both *Fasciola* species yielded fragments of 360, 170, 100, and 60 bp. The results of PCR–RFLP completely coincided with those of sequence analysis, and thus PCR–RFLP is a useful technique for determining the ITS1 type in *Fasciola* species.

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1. Introduction

Fascioliasis is of well-known zoonotic importance and an increasing human health problem. The causative agents *Fasciola hepatica* and *Fasciola gigantica*, which overlap in many regions of Africa and Asia, and in which the differentiation of both species is usually difficult because of the many variations in their morphological characteristics (El-Gozami and Shoukry, 2010). Moreover, in humans, liver fluke classification cannot be achieved by clinical, pathological, coprological or immunological methods. The differential diagnosis between *F. hepatica* and *F. gigantica* infection is very important because of their different transmission and epidemiological characteristics. The two *Fasciola* species have been classified by morphological characters such as body length and width, and they have been shown to be spermic diploid and meiotically functional in cytogenetical characteristics (Sanderson, 1953; Reddy and Subramanyam, 1973). The two species can also be discriminated by deoxyribonucleic acid (DNA) sequences of nuclear ribosomal internal transcribed spacer 1 (ITS1), ITS2, and 28S ribosomal ribonucleic acid genes (Adlard *et al*., 1993; Itagaki and Tsutsumi, 1998; Marcilla *et al*., 2002 and Itagaki *et al*., 2005a). *F. hepatica* mainly occurs in Europe, Americas and Oceania, while *F. gigantica* exist in Africa and Asia (Torgerson and Claxton, 1999). On the other hand, *Fasciola* specimens intermediate between *F. hepatica* and *F. gigantica* in morphology have been found in most countries, including Japan (Watanabe and Iwata, 1954; Itagaki and Akane, 1959 and Oshima *et al*., 1968), India (Varma, 1953), Korea (Chu and Kim, 1967), the Philippines (Kimura *et al*., 1984), Iran (Ashrafi *et al*., 2006) and Egypt (Amor *et al*., 2008 and Allam *et al*., 2011). These intermediate forms are difficult to identify accurately. Moreover, both *Fasciola* forms include aspermic diploid and triploid specimens that are meiotically dysfunctional and gynogenic (Moriyama *et al*., 1979; Sakaguchi, 1980 and Terasaki *et al*., 1998). Aspermic specimens from Japan, Korea, Vietnam and China exhibited heterogeneity between *F. hepatica* and *F. gigantica* in nuclear ribosomal and mitochondrial DNA, suggesting that their origin may be hybridization between the two species (Itagaki and Tsutsumi, 1998; Agatsuma *et al*., 2000; Itagaki *et al*., 2005a, b, 2009 and Peng *et al*., 2009). These findings indicate that *Fasciola* specimens that are diversified in biological nature should be characterized by spermatogenesis and genetic differentiation as well as morphology. Although DNA types based on sequences of the ITS1 region are of value to discriminate species having

characteristics of *F. hepatica, F. gigantica*, and heterogeneity between the two species (Itagaki *et al*., 2005a, b, 2009 and Peng *et al*., 2009), DNA sequencing requires much time and is costly. In this study, we tried to develop a cost-effective PCR–RFLP method to discriminate the ITS1 types of *Fasciola* spp. more quickly.

2. Material and Methods

We used fourteen *Fasciola* specimens that were identified on the basis of their ability for spermatogenesis and nucleotide sequences (600 bp) of the ITS1 region. Total DNA was extracted from individual flukes using an E.Z.N.A. mollusc DNA kit (Omega Bio-Tek, Doraville, USA) according to the manufacturer's instructions. DNA samples were stored at −20°C until use. The DNA fragments (about 680 bp) of the ITS1 region including complete ITS1, partial 18S and 5.8S ribosomal deoxyribonucleic acid (rDNA) were amplified by polymerase chain reaction (PCR) according to Itagaki *et al*. (2005a, b). Although the nucleotide sequence (600 bp) of the DNA fragments has been determined (Peng *et al*. 2009), the remaining sequences (about 80 bp) were analyzed in this study to select an appropriate restriction enzyme for discrimination between fragments with the sequences of *F. hepatica* and *F. gigantica*. The PCR amplicons were directly sequenced using ABI Prism Big Dye terminator v.3.0 ready reaction cycle sequencing kits (Applied Biosystems) in both directions with the use of the same forward and reverse primers as those used in PCR. The sequencing reactions were run on a PE Applied Biosystems 3100 automated sequencer. As a result, four and five restriction sites (GATC) for Rsa I were found in the nucleotide sequence (680 bp) of *F. hepatica* and *F. gigantica*, respectively. The PCR amplicons were precipitated with ethanol/sodium acetate and subjected to digestion overnight at 37°C with the enzyme in a total volume of 50 µl, containing 10 U of Rsa I and a buffer enzyme L (Roche, Mannheim, Germany). The restriction fragments were electrophoresed in 1.8% agarose gels in Tris-acetate-EDTA (TAE) and visualized with ethidium bromide.

3. Results and Discussion

The DNA fragments digested with Rsa I were predicted to be separated into fragments of 367, 104, 68, 59, 54 and 28 bp in the amplicons with the sequence of *F. hepatica* and into fragments of 367, 172, 59, 54, and 28 bp in the amplicons of *F. gigantica* (Fig. 1). However, the resultant bands of the fragments were about 360, 100, and 60 bp in *F. hepatica* amplicons and 360, 170, and 60 bp in *F. gigantica* amplicons (Fig. 2). The band of 60 bp was thought to include the three fragments of 68 bp (only *F. hepatica*), 59 and 54 bp, and the small fragment of 28 bp was not detected on agarose gels. These findings showed that the band patterns between the amplicons with the sequences of *F. hepatica* and *F. gigantica* were evidently different. Allam *et al*. and Marcilla *et al*. (2002) reported that a PCR–RFLP method based on 28S rDNA precisely distinguished the restriction fragments between *F. hepatica* and *F. gigantica* when the PCR amplicons (618 bp) of 28S rDNA were digested by the restriction enzyme Ava II or Dra II. Additionally, El-Gozami *et al*. (2010) and McGarry *et al*. (2007) showed that a PCR method based on RAPD-derived sequences from *F. hepatica* and *F. gigantica* could distinguish the two *Fasciola* species. However, it unfortunately remains unclear whether those PCR techniques are able to detect the heterogeneity between *F. hepatica* and *F. gigantica*, which suggests hybrids or their offspring between the two *Fasciola* species (Itagaki *et al*., 2005a, b, 2009 and Peng *et al*., 2009). The present study showed that amplicons having heterogeneous nucleotides at six variable sites yielded both band patterns of *F. hepatica* and *F. gigantica*, which consisted of bands of about 360, 170, 100, and 60 bp (Fig. 2). The ability to detect the heterogeneity of *Fasciola* species that are widely distributed in most countries will be important for the genetically characterization of *Fasciola* species. The results of PCR–RFLP analysis were consistent with those of sequence analysis in the *Fasciola* specimens used in the present study. These findings indicate that the PCR–RFLP method is a useful method for discriminating DNA types of the ITS1 region in *Fasciola* species.

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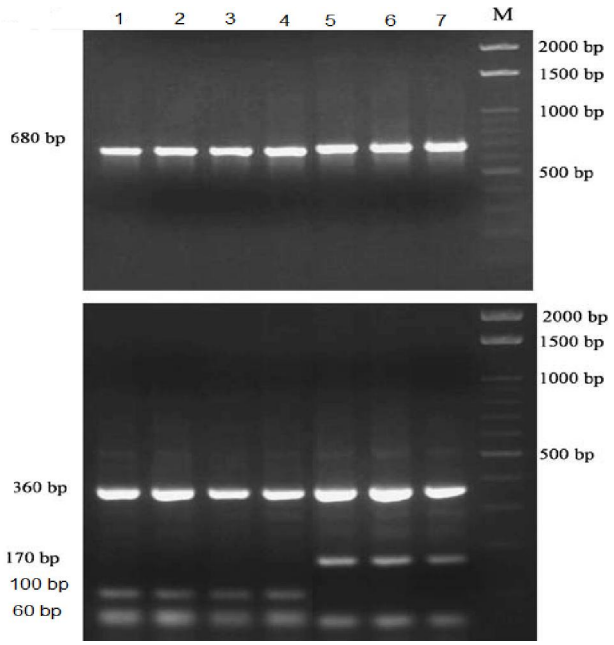

Fig. (1): PCR amplicons (upper) of spermic *F. hepatica* and *F. gigantica* and their restriction fragments (lower) produced by the PCR–RFLP method. The numbers 1 – 7 indicate *Fasciola* specimens that have *F. hepatica* type and *F. gigantica* type, respectively, M 100-bp DNA ladder

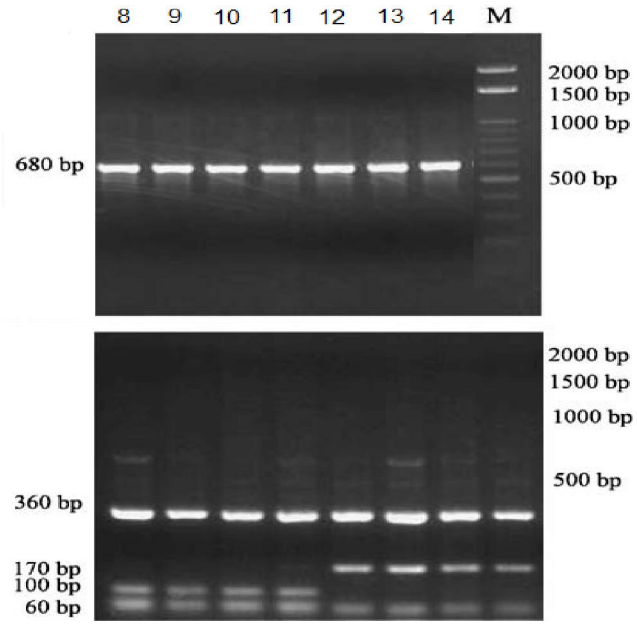

Fig. (2): PCR amplicons (upper) of aspermic *Fasciola* sp. And their restriction fragments (lower) produced by the PCR–RFLP method. The numbers 8 – 14 indicate *Fasciola* specimens that have *F. hepatica* type, *F. gigantica* type, and Fh/Fg type, respectively, M 100-bp DNA ladder.

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