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Original paper

Identification and differentiation of *Fasciola hepatica* and *F. gigantica* using multiplex PCR technique

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ABSTRACT. We aimed to present an alternate method instead of PCR-RFLP and also develop an optimized method for rapid, time-saving and affordable molecular-based approach to discriminate species of liver fluke, *Fasciola hepatica* and *F. gigantica*. Seventy-six samples of *F. hepatica* and 28 *F. gigantica* were collected from the slaughterhouses of endemic regions in Iran. Following a comprehensive analysis of the mitochondrial complete sequences of both *F. hepatica* and *F. gigantica*, the extracted DNAs from all samples were used as templates in multiplex PCR reactions containing two sets of primers specific for cytochrome c oxidase I (*cox I*) gene of both species. In a parallel experiment, PCR-RFLP was performed for each sample using internal transcribed spacer (*ITS1*) sequence. Furthermore, following a PCR amplification for *cox I* gene, the amplicons were purified for sequencing. To assess the validity of the multiplex PCR approach, the obtained data from the multiplex PCR and PCR-RFLP experiments were compared with each other. By sequence analysis of 104 samples, 76 and 28 samples were identified as *F. hepatica* and *F. gigantica*, respectively. Results revealed 100% and 92% of accuracy as for multiplex PCR and PCR-RFLP. The designed multiplex PCR strategy offers a valid alternative approach to the conventional methods with distinctive features including convenience, cost-effectiveness, time-saving (3 hours from sampling to obtain final results) and high efficacy.

Keywords: *Fasciola hepatica*, *Fasciola gigantica*, multiplex PCR, PCR-RFLP

Introduction

The common liver trematodes *Fasciola hepatica* and *F. gigantica* are the etiological agents of fasciolosis, a global disease that affects both ruminants and humans. *Fasciola* species in wet areas cause a lot of economic and health losses because of liver damage, high morbidity rates and reduction in livestock products such as: reducing the production of meat and milk [1].

Iran has had two major outbreaks of fasciolosis in the northern regions of the country, with approximately 10,000 cases in each outbreak [2,3]. Fasciolosis due to *F. hepatica* could be a health issue in numerous nations with moderate climates such as in Europe, the Americas and Australia, though the major endemic regions for *F. gigantica* are huge tropical locales of Africa, and numerous ranges of Asia including Uzbekistan, Turkmenia, Iran, Iraq, India and Pakistan, [4,5].

In Asia and Africa, the distribution of *F. gigantica* and *F. hepatica* overlaps and this issue makes it troublesome to distinguish the specific species included in human diseases, so that it is frequently indicated to as *Fasciola* spp. [6]. In Iran based on molecular studies in Fars, Khuzestan, Kohgiluyeh and Boyer-Ahmad (southern of Iran) [7–9], Tehran (center of Iran) [10], Guilan (north of Iran) [11], West Azerbaijan (northwest of Iran) [8,10,12], Ardabil (northwest of Iran) [13], Kermanshah, Lorestan (west of Iran) [14,15] and Khorasan [8] provinces both of *Fasciola hepatica* and *Fasciola gigantica* were reported. In Zanjan province (northwest of Iran) only *Fasciola hepatica* was reported [16.17].

Accurate diagnosis of these two species is difficult due to high similarity of morphological features. Furthermore, hybridization between different genotypes of the *Fasciola* increases various forms of this trematode [18]. Because of the differences in the epidemiological aspects, intermediate host, control strategies, and variances in the forms of pathological lesions, differentiation between *F. hepatica* and *F. gigantica* infection is important.

Fasciola species are generally identified based on morphological and molecular methods. Since the host type affects the size of the parasite, and also lack of distinctive morphological keys using morphological methods is challenging, especially in areas where both species overlapped. To date, various molecular methods have been used to identify the parasite species including conventional PCR [19–22], PCR-restriction fragments length polymorphism (RFLP) [23–25], loop-mediated isothermal amplification (LAMP) [26], PCR-linked single-strand conformation polymorphism (PCR-SSCP) [27], random amplified polymorphic DNA (RAPD) [28], TaqMan real-time PCR [29], and sequence□related amplified polymorphism (SRAP) [30]. Among the mentioned methods, one of the most commonly used methods in different parts of the world is the PCR-RFLP [23,31–33]. However, due to low precision, high cost, and time consuming, developing a valid, user-friendly, and time-saving method for differentiation of *Fasciola* spp. seems necessary.

This study therefore, aimed to develop a multiplex PCR method to distinguish between *F. gigantica* and *F. hepatica* based on species-specific variations in the mitochondrial encoded cytochrome c oxidase I (MT-*cox I*) and species-specific primers for amplification of *cox I* gene.

Materials and Methods

Fasciola spp. collection

One hundred and four (104) specimens were collected during slaughterhouse inspection from livers of buffalo, cattle and sheep from three provinces of Iran (Lorestan, Sistan and Baluchistan, and Khuzestan) in 2018 (Tab. 1). The flukes were washed with phosphate-buffered saline (PBS) for three times and fixed in 70% ethanol and stored at room temperature until testing.

DNA extraction and design of primers

DNA extraction was performed by the commercial QIAamp DNA extraction kit (Qiagen Germany) according to the manufacturer's protocol. Genomic DNA was stored at –20°C for further processing. The cytochrome oxidase 1 (*cox1*)

Table 1. Total number of examined liver flukes from different definitive hosts

	Origin	Host of collected fluke			Total number
		cattle	sheep	buffalo	
	Lorestan	17	35	–	52
	Sistan and Baluchistan	10	15	–	25
	Khuzestan	8	8	11	27
Total number		35	58	11	104

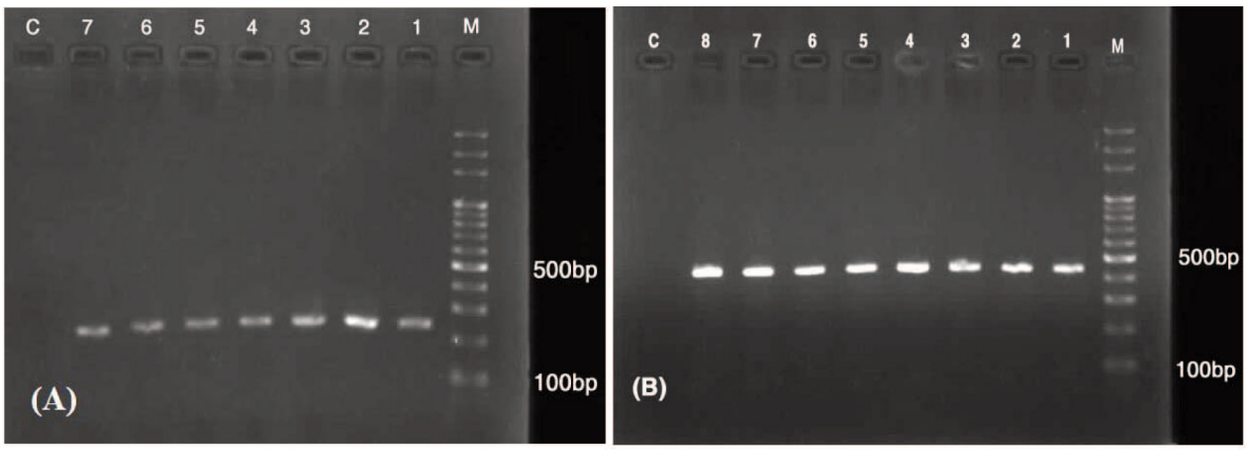

Figure 1. Agarose gel electrophoresis of *cox 1* gene PCR amplification. (A) lanes 1–7: *F. gigantica* (240 bp) from different hosts, lane C: negative control, lane M: 100-bp DNA ladder. (B) lane 1–8: *F. hepatica* (440 bp) from different hosts, lane C: negative control, lane M: 100-bp DNA ladder

sequences of *F. hepatica* and *F. gigantica* were obtained from the Genbank databases. Subsequently, sequence alignment was performed for each of them separately to determine the conserved regions. Afterwards, a pairwise alignment was carried out to distinguish the differences between them. The obtained sequences were utilized for designing specific primers. The primer sets were designed to amplify a region of the *cox1* gene, different sizes of amplicon were expected as follows: forward primer (F.h: AGATT TGGGCTTTGGTTGCTCGG) and reverse primer (F.h:GACAAACAAACACAAGCA GGCAAT) to amplify a 440 bp DNA fragment in the *cox1* region for *F. hepatica* and forward primer (F.g:GCTTTGA GTGCTTGGTTGTTGC) and reverse primer (F.g:TATATGACGACCA GTACCCTCGC) for amplify a 240 bp DNA fragment in the *cox1* region for *F. gigantica*.

Multiplex PCR reaction

The multiplex PCR was performed based on a similar study [21]. The target sequences were amplified using commercial kits (Taq PCR Master Mix, Qiagen) that contained 400 µM of each dNTP, $MgCl_2$ (1.5 mM) and finally adding 10 pmol of each primer. PCR was done in a reaction volume of 25 µl. The PCR mixture reaction was amplified in a thermocycler (Corbett Research, Sydney, Australia) under the following temperature conditions: initial denaturation step at 94°C for 5 min followed by 30 cycle 94°C for 30 s as denaturation, 60°C for 30 s as annealing, 72°C for one minute as extension and final extension at 72°C for 10 min. The resulting PCR product was electrophoresed on 2% agarose gel containing safe stain. DNA marker of 100 bp was used to determine and estimate the size of DNA. The size of amplicons was monitored on the agarose gel corresponding to the expected band sizes of 440 bp and 240 bp for *F. hepatica* and *F. gigantica*, respectively.

PCR-RFLP

The *ITS1* region was amplified using two forward primer (5'-TTGCGCTGATTACGTCCCT G-3') and reverse primer (5'-TTGGCTGCGCTC TTCATCGAC-3'). The primers were synthesized by Macrogen Company (South Korea). PCR examination was done in 15 µl reactions containing of 7.5 µl master mix (Amplicon), 0.5 µl of each forward and reverse primers (5 pmol), 1.5 µl genomic DNA, and 5 µl distilled water. The reactions were performed as follows: pre-denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 45 s, extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. In order to differentiate specifically *F. gigantica* from *F. hepatica*, PCR-RFLP on *ITS1* region was performed using the enzyme *RsaI*. The size of the PCR product was 700 bp.

PCR product were subsequently digested using the *RsaI* restriction enzyme. The enzyme in *F. hepatica* produces fragments 28, 54, 59, 68, 104 and 367 bp, and it produces fragments 28, 54, 59, 172 and 367 bp in the *F. gigantica*.

Sequencing

All of PCR product was purified and sequenced with ABI 3130X sequencer. Resulting sequences were adjusted manually by Chromas (version

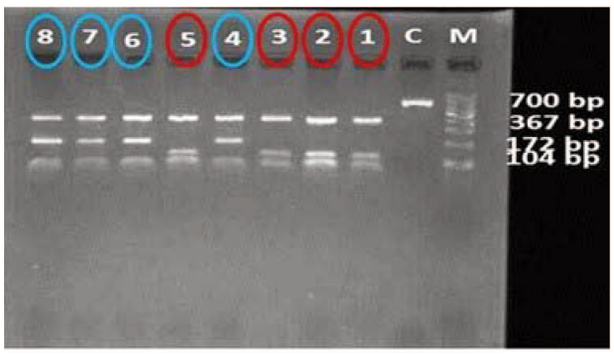

Figure 2. The pattern of PCR products after digestion with *RsaI*: lane M: 100 bp DNA marker, lane C: undigested PCR product to control the *ITS1* primer, lanes 1–3 and 5 are *F. hepatica* from sheep, lanes 4 and 6–8 are *F. gigantica* from buffalo and cattle

1.0.0.1) and then compared with available reference sequences in BLAST GenBank database by software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Ethical statement

The preserved samples of parasites were available in the archive of Helminthology Unit, Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences. Ethical approval of the study was obtained by the Medical Ethics Committee of the Tehran University of Medical Sciences (IR.TUMS.REC.1395.2404).

Results

In multiplex PCR method, an electrophoretic size discrimination of bands on agarose gel the lengths of 440 bp and 240 bp was considered for *F. hepatica* and *F. gigantica*, respectively (Fig. 1). Each DNA sample was used in an individual reaction of PCR containing two sets of primers and following gel electrophoresis only a single band was observed.

The results of the PCR-RFLP patterns of *F. gigantica* and *F. hepatica* flukes after digestion of the PCR products with *RsaI* restriction enzymes, showed the size of 104 bp fragment for *F. hepatica* and a band size of 172 bp for the *F. gigantica* (Fig. 2). Achievement of sequencing clarified, multiplex PCR is one hundred present accuracy, while PCR-RFLP results is not accurate completely (Tab. 2). The different band patterns generated after digestion and used to differentiate between the *F. gigantica* and *F. hepatica*.

Afterwards, to evaluate the validity of the current method, data from the multiplex PCR and PCR-RFLP were compared (Tab. 2).

Discussion

Fasciolosis or liver rot is one of the most important parasitic diseases that transmitted via infected water and food, so caused economic and public health challenge. The disease is one of the Neglected Tropical Diseases (NTD) in humans and animals in most parts of the world [34,35]. In order to prevention and control of fasciolosis, the identification of *F. hepatica* and *F. gigantica* species is essential.

Fasciolosis has a wide speared distribution in the world, so different species of worm have showed variable geographical pattern. Despite, the surveys declare *F. hepatica* is more exist in temperate areas and *F. gigantica* is in tropical areas, both of species have overlap in subtropical zones [36,37]. The result of the present study clarify *F. hepatica* and *F. gigantica* species are co-exist in south of country, similar founded some studies was detected both of worms in different parts of country [9,38,39].

Table 2. Comparison between results of PCR-RFLP and multiplex PCR method

Method	Detected species	Number of specimens	Number (%) of samples confirmed by sequencing
PCR-RFLP	*F. hepatica*	76	72 (95)
	F. gigantica	28	24 (86)
	Total number	104	96 (92)
Multiplex PCR	*F. hepatica*	76	76 (100)
	F. gigantica	28	28 (100)
	Total number	104	104 (100)

Traditional methods including morphological characteristics cannot accurately determine the nature of both species. On the other hand, these two species can mate with each other and create hybrid specimens, in which it cannot be identified by the only morphological methods [40]. The presence of these intermediate shapes of the worms in some countries such as Iran, Egypt, Japan, Taiwan, Philippines, and Korea can cause problems, in addition misdiagnosis based on parasitological, immunological and pathologic methods [41,42]. For this reason, in some reports, it is described as *Fasciola* spp. So, it is necessary to use a precise and reliable method that can identify these species from each other [43], also some researchers are believed that the reason of adaptation of parasite to new definitive host and environment is due to high genetic variability of worm [1,37].

Since genotype characteristics are not influenced by geographical and environmental factors, so these methods can easily differentiate between two species from each other. This makes molecular methods useful for accurately detecting trematodes, especially in areas where both species exist. So far, various molecular techniques based on DNA markers have been used to detect *Fasciola* spp. Immunological and parasitological tests can not differentiate *F. hepatica* and *F. gigantica* from each other [44]. Molecular methods such as PCR for amplification of specific DNA regions, including ribosomal regions (*ITS1* and *ITS2*) and mtDNA fragment (*cox1*, *ND1*) are able to verify morphometric studies [25,33,37,45].

During recent decades, numerous PCR-based methods, such as PCR-RFLP, PCR-SSCP and other certain assays, have been designed for a more accurate identification of *Fasciola* spp. [23–25, 27,46]. Results of the current study showed of the 104 samples by PCR-RFLP, 96 were correctly identified however all of the 104 samples were correctly identified by the multiplex PCR method. The reason for this result is probably due to the quality of the DNA or the lower efficiency of the PCR-RFLP method.

So far, several studies have been done in different parts of the world using PCR-RFLP to identify *Fasciola* spp. [25,31,32,47]. One of the requirements of this approach is to select the suitable restriction enzyme, but sometimes these enzymes are not able to identifying species for instance. A study was conducted on *Fasciola* spp. of cattle and sheep by the PCR-RFLP method with BamH1 and Pag1 enzymes via targeting *ITS2* region. Conclusively, for *F. hepatica* fragments of 230, 340, and 341 bp were observed, but these enzymes did not have a specific fragment for *F. gigantica* [47]. In China, using the *ITS2* region and restriction enzymes Hsp92II and *RcaI* in different hosts, the Hsp92II enzyme was effective than RcaI in comparison between two species [25]. In addition, low DNA quality may affect the restriction enzymes and the enzyme cannot cut it, and thus the identification of the species may become problematic.

In the current study, PCR-RFLP was incapable of detecting 8 (8%) samples in comparison with the multiplex PCR method, which was probably due to the DNA quality of the samples or the quality of the enzyme. It should be noticeable, PCR-RFLP like conventional PCR have false negative results due to inhibitors [48]. PCR-RFLP moreover is time-consuming and may be could not identify near species carefully, also the cost should be spent on the purchase of suitable enzyme. Therefore, it is necessary to use accurate method to overcome the problems.

Multiplex PCR assists can amplify more than one target of interest in a PCR by using multiple primer pairs and produce amplicons in different sizes [49]. The design and selection of the multiple primer pairs can make the reaction specific for the target organism. In this study, the design of the primers was done to specifically attach to their targets without the proliferation of non-specific fragments and of course, without a primer dimer and high functionality.

For the first time, Chamberlain et al. [50] described this method, first of all they investigated deletion within the Duchenne Muscular Dystrophy gene. Subsequently, this method was used in several studies to detect parasite species in faecal specimens, salivary, serum and other environmental samples [48,51–53]. The multiplex PCR method used in the present study focuses on *F. hepatica* and *F. gigantica*. Specific primers were designed to allow amplification of each species of parasites, in which differentiation was done according to specific band size. The accuracy of this new molecular method was confirmed by sequencing of mitochondrial and nuclear gene. This method was able to identify all samples in comparison with the PCR-RFLP method. Several advantages of multiplex PCR are as follows: simple method, suitable in cost and time-saving in accurate species

identification than other conventional PCR and PCR-RFLP methods. This method can be introduced as a substitute for other common methods, and may also be useful for downstream projects like phylogenic studies.

In conclusion, using multiplex PCR, two specific pair primer sets were selected for identification of *F. hepatica* and F. *gigantica*. The mitochondrially encoded MT-*cox1* or *cox1* gene were used to identification the worms. Multiplex-PCR was developed to allow the coincident detection of both species of parasite in a single reaction. Low volumes of reagents and short time was spent to set up and examine in this method compare to conventional PCR and PCR-RFLP, therefore multiplex-PCR is appropriate molecular method to routine diagnostic of *F. hepatica* and *F. gigantica* species. The results were reproducible and established over time.

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