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Full Article

Molecular differentiation of sheep and cattle isolates of *Fasciola hepatica* using RAPD-PCR

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ABSTRACT

Understanding genetic structure and status of genetic variation of *Fasciola hepatica* isolates from different hosts, has important implications on epidemiology and effective control of fasciolosis. Random amplified polymorphic DNA (RAPD-PCR) was used to study the genetic variation of *F. hepatica* in sheep and cattle. DNA was extracted from adult helminthes removed from livers of each infected animal in slaughterhouse at East-Azerbaijan province, North-West of Iran. DNA template amplified by the polymerase chain reaction, using three oligonucleotide decamers with arbitrary DNA sequences as primers. RAPD patterns showed the specific but different pattern DNA patterns for each primer. The intraspecific similarity coefficient within two isolates of *F. hepatica* was ranged between 69 to 100%. Present findings showed that the interspecific genetic distance was higher than intraspecific genetic distances (19-47% compares to 0-19%). Pair wise similarity matrices generated from each isolates-primer combination were totaled and the similarity coefficient between strains were calculated both manually (Nei and Li method) and software analysis (Free-Tree-Freeware program). The inferred phylogenetic tree on the fingerprinting of these isolates clearly demonstrated the existence of population genetic diversity sub structuring within *F. hepatica* of sheep and cattle of Iran, raising interesting questions on the host specificity, epidemiology (e.g., zoonotic transmission) and ecology of this fluke. RAPD-PCR is useful for both individual identification and epidemiological investigations in endemic regions.

Keywords: Molecular differentiation, *Fasciola hepatica*, sheep, cattle, RAPD-PCR

INTRODUCTION

Fasciolosis, caused by genus *Fasciola* is one of the most important plant borne helminthes infections of human and livestock in many parts of the world. It is an emerging public health problem in the Middle East including Iran and Egypt (Alcaino 1989, Hussein *et al* 2000). In Iran, annual economic loss caused by the disease to be two billion Rials that mainly due to condemned livers, reduced milk yield, fertility disorders and reduced meat production (Mas-Coma *et al* 2005, Rokni1 *et al* 2010). Understanding genetic structure and status of genetic variation of the parasite populations has important implications on epidemiology and effective control of fasciolosis (Ashrafi *et al* 2006). During the last decade, the causative agents of infectious disease have identified

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using nucleic acid-based techniques. Serologic detection of antibodies to parasites is useful as a screening device, but often there is cross-reaction, and generally is not applicable to discriminate between active and latent infection (Wolstenholmel *et al* 2004). The results with nucleic acid-based assays are independent of immunocompetence or previous clinical history and can distinguish between organisms that are morphologically similar and/or share antigenic epitopes, and the organisms do not need to be viable or culturable. An inherent disadvantage of these assays is that isolates containing variant DNA sequences may be missed even though the sequence is not associated with virulence (Zhao *et al* 1998). The main objective of this study was to investigate genetic variation of *F. hepatica* by RAPD-PCR technique. This work aimed to study the genetic variability of *Fasciola hepatica* collected from cattle and sheep origins by RAPDs-PCR technique. This study is not particularly aimed to phylogenically analyze *Fasciola* spp. However, it helps to better understand the evolution of these helminthes.

MATERIALS AND METHODS

Parasites. Adult *F. hepatica* were removed from the livers of sheep (n = 6) and cattle (n = 6) slaughtered at East-Azerbaijan slaughterhouses (North-West region, Iran) between July-September 2009. Parasites were identified microscopically (body length and width, cervical cone lengthens width) using the key identification by Ashrafi et al. (2006). Each fluke were washed three times by phosphate-buffered saline (PBS, pH 7.3, 37°C) and fixed in 70% ethanol.

DNA extraction. Genomic DNA was extracted using DNA extraction kit (MBST,cinnagen company, Iran) and purified the DNA concentration was estimated at 260 nm absorbance and the purity of samples was examined OD260 nm/OD 280 nm. Additionally, the DNA was analyzed on 1.5% agarose gel in TBE buffer (0.095 M Tris-Borate, 0.001 M EDTA). The gels were stained by ethidium bromide and DNA bands were visualized using an UV transilluminator. The DNA templates were stored at -20 °C.

Random Amplified Polymorphic DNA Analysis. The RAPD analysis was performed in100 µl total reaction volumes containing 50 ng of genomic DNA, 10 µl PCR buffer lO×(Cinnagen Company, Iran), 0.2 mM, dNTPs (10 mM of each), 1.5 mM MgCl2, 2.5 U *Taq* DNA polymerase (Cinnagen Company, Iran) and 0.2 µl of one of the following primers (Table 1), (Cinnagen Company, Iran):
5'-GTGACGTAGG-3' (AP1), 5'-TGCCGAGCTG-3' (AP2), 5'-GCGAGCGTCC-3' (AP3) (GenBank).
According to Iranian cinnagen company protocol, the thermal cycling profile consisted of one cycle of 5 min at 94 °C (Primary denaturation) and 40 cycles of 1 min at 94 °C (denaturation), 45 sec at 40 °C (Annealing), 1 min at 72°C (extension) and one cycle of 5 min at 72°C (Final extension) carried out in the MWG thermocycler (MWG, Germany). Negative control tube without DNA template was included in each PCR reaction. For agarose gel electrophoresis, individual RAPD products (10 µl) were loaded on 1% agarose gel (w/v TBE 0.5x) and subjected to electrophoresis at 100 V for 1.5 h using TBE buffer (0.5x), stained with ethidium bromide and then visualized with UV illumination followed recorded photography. For estimation of band profiles, a 100 bp DNA ladder marker (Fermentase Company) was used. The major criteria for taking a fragment into account were reproducibility and distinctness of the fragments.

Table 1. Nucleotide sequences (nt) of primers (GenBank).

Primers(10µl)	Sequence(5'-3')	Length (no. of nucleotides)	GC content (%)
AP1	GTGACGTAGG	10	60
AP2	TGCCGAGCTG	10	70
AP3	GCGAGCGTCC	10	80

Data Analysis. RAPD profiles were used to measure genetic similarity among the helminthes. Each DNA band with different electrphoretic mobility was assigned a position number scored as either 1 or 0 for

present or absent respectively. Pair wise similarity matrices generated from each isolates-primer combination were totaled and the similarity coefficient between strains were calculated both manually (Nei and Li 1979) and software analysis (Free-Tree-Freeware program). Manually using the formula F = 2 Nxy/Nx+Ny, where, Nx and Ny are the numbers of segments amplified in isolate x and y respectively and Nxy is the number of segments shared by them (Nei & Li 1979). Additionally software analysis was performed using Free-Tree-Freeware program NTSYS-PC. An unrooted dendrogram was constructed from the similarity coefficient data based on the unweighted pair group method with arithmetic average (UPGMA) clustering (Nei 1972, 1978).

RESULTS

Three arbitrary oligonucleotide primers (l0 ml) of various sequences (Table 1) were used in RAPD-PCR for their ability to generate polymorphic DNA fingerprints of two isolates of *F. hepatica.*Genomic DNA was analyzed from F. *hepatica* of sheep and cattle The DNA fingerprints of each *F. hepatica* strains were identified (Figure 1). The reproducibility of pattern was confirmed by repeats. Only visible bands shared all PCRs, were used for Calculation. Of the three primers produced amplification products, a total of 256 bands were scored of 12 samples, with average of 22 bands scored per sample. The number of scorable bands generate by a single primer ranged from as few as 1 (AP1) to as many as 9 (AP3). RAPD profiles for all isolates primers were shown to be reproducible on different days and in different laboratories. Primers AP2 and AP3 amplified 3-9 DNA segments from all isolates ranging in 230-2150 base pair (bP). Primer AP1 amplified a common 350bp band in all two strains of *F. hepatica* and 1100 bp band was shown in one of cattle strain. The similarity coefficient between two isolates of F. *hepatica* was between 0.5 to 0.78 and within these two isolates of *F. hepatica* was between 0.78 to 1. The summary of inter and intra similarity coefficient of sheep and cattle strains of *F.*

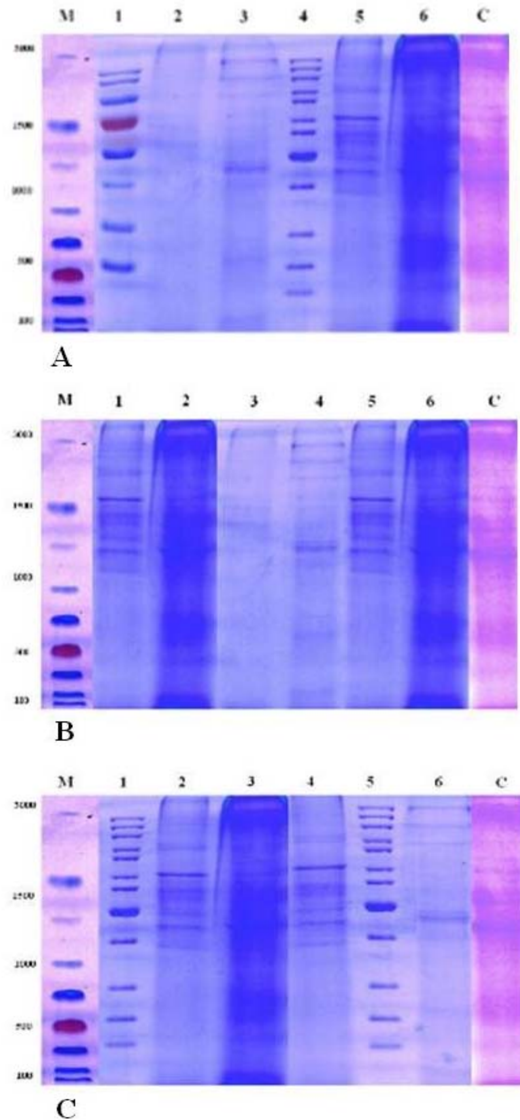

Figure 1. Portions of three typical agarose shoing RAPD profiles generated with primers API (panel A), AP2 (panel B) and AP3 (panel C). Lanse 1-3= F. hepatica of sheep, Lanse 4-6= F. hepatica of cattle, Lanse C= negative control, Lane M= DNA marker (100 bp DNA ladder marker plus).

hepatica are shown in Table 2. Cluster analysis of the genetic similarity values was based on Nei and Li's 1979 similarity coefficient performed to generate a dendrogram illustrating the overall genetic relationships within *F. hepatica* and the accessions and individuals between these isolates. The dendrogram was constructed using UPGMA clustering (Figure 2). The constructed phylogenetic tree revealed that considerable genetic diversity exists in population of

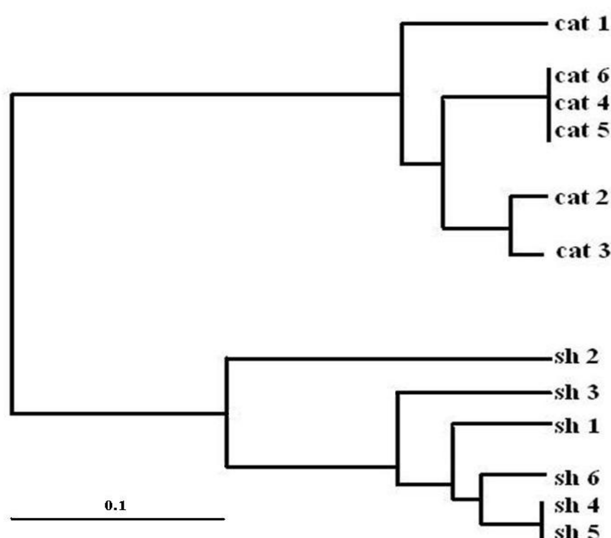

Figure 2. UPGMA dendrogram based on cluster analysis of RAPD data for 12 individual of fasciola hepatica from sheep (sh), cattle (cat). Numbers refers tohost identifier. The scale represents the genetic distances between the individuals.

F. hepatica. Study of this phylogenetic tree demonstrated that two main comprised of each of the two *F. hepatica* strains were formed. Branches of *F. hepatica* strains on dendrogram cluster divided into several sub branches consisted of each strain. Different annealing temperatures (36, 37, 39, 40 and 42 °C) were tested and 40 °C achieved the highest amplification efficiency and reproducibility. Increasing the armealing temperature beyond 40 °C would be resulted substantially decreased efficiency and decreasing this stage temperature under 39 °C, caused to decreased specificity and reproducibility.

Table 2. Similarity coefficient between two strains of *Fasciola hepatica.*

Host	Sheep	Cattle
Sheep	0/78-1/00	—
Cattle	0/51-0/63	0/92-1/00

Additionally, in order to optimize electrophoresis conditions, different agarose gel (Cinnagen, company) concentrations were assayed (0.6, 0.75, 0.9, 1, 1.5 and 1.7%, w/v, TBE 0.5x, 0.95 M TB, 0.001 MEDTA, pH 8). These results indicated that 1% agarose gel at 100 V for 1.5 h in TBE buffer 0.5x (w/v) electrolyte confirmed the best band patterns. Increasing the agarose gel concentration over 1.5% (w/v) caused smear appearance and decreased it under 0.9% resulted low and weak bands pattern that don't differentiate efficiency.

DISCUSSION

In Iran, two well-known digeanian trematode *F. hepatica* and *F. gigantica* were involved in both ruminant and human Fasciolosis. In Europe, America and Australia almost *F. hepatica* is more concerned (Marci1la 2002). Whereas the distribution of both species overlap in many areas of Asia and Africa (Mas-Coma 2005). Differentiation between *F. hepatica* isolated from Australia, *F. gigantica* isolated from Malaysia and *Fasciola* spp. from Japan was performed by analyzing of their mtDNA using PCR-RFLP method (Hashimoto 1997). Using restriction maps of the ribosomal genes, demonstrated that a *Fasciola* isolated from Japan was identified to *F. gigantica* but different from *F. hepatica* (Blair & McManus 1989). Analysis of parasites mitochondrial cytochrome oxidase 1 (*co1*) and nicotinamide adenine dinucleotide dehydrogenase subunit (*nd1*) showed that Japanese *Fasciola* forms were more closely related to *F. gigantica* than to *F. hepatica.* Moreover high intraspecific variations of the *nd1*sequence of *F. giganitca* were found (Itagaki 1998). Differences among individual mitochondrial genomes *of F. hepatica* have shown (Zurita 1988). No intraspecific variations in the restriction endo nuclease maps of *F. hepatica* or *F. gigantica* were detected, but length heterogeneity was noted in the intergenic spacer even within individual helminthes (Blair & McManus 1989). Differences were detected in the 28srRNA gene of *F. hepatica* in sheep and *F. gigantica* in cattle, but were not intraspecific variation performed. Another study demonstrated that individual cattle infection by numerous genetically different liver flukes (Semyenova 2003).

Differences among ITS2 of the rDNA of *F. hepatica* and *F. gigantica* were shown, besides proved that ITS2 sequence was identical for *F. hepatica* that differ in various geographic origins (Mas-Coma *et al* 2005).

Profiles of whole-body proteins and Excretory/ Secretory (ES) products of *Fasciola* differed among hosts such as sheep and calves (Lee *et al* 1992). Random amplified polymorphic DNA has been widely used as a genetic screening method (Gasser 2006) because it is rapid relatively simple to perform and requires only a small amount of genomic DNA (1-20 ng) without genome sequence information prior to analysis (Welsh & McClelland 1990, Williams *et al* 1990). The RAPD banding patterns can be affected by a number of factors such as quality and quantity of template DNA, concentration of reagents and using different types of thermocyclers (MacPherson et al 1993, Meunier & Grimont 1993, Micheli *et al* 1994). However, the effect of these factors on the resultant banding patterns was largely due to low annealing temperatures (25-35 °C) used in the RAPD-PCR. Thus increased annealing temperatures as in case of present study (40 °C) increased the stringency of the PCR and insure the reproducibility of RAPD results (De Gruijter *et al* 2004). Band profiles ranging 230-2150 bp in sizes. Amplification with primer AP3 products the most bands (125) compared to AP1 (33), AP2 (98**)** These bands pattern could be caused of nucleotide sequences and GC content of them, as high GC content of primer AP3 (80%) caused the most bands in compare to low GC content of API and AP2 (60%), produces lower DNA fragments. Smear appearance of AP1 amplicons, perhaps due to large armealing sites corresponding to the sequence of this primer. Subsequent RAPD fingerprints analysis demonstrated high degree of polymorphism among all examined *F. hepatica*. Similarity coefficient between *F. hepatica* strains was wide range as low as 50% to as high as 100%. The distance indices observed interspecific (19-47%) was higher than intraspecific (0-19%).

Cluster analysis of the RAPD profiles data (considering all polymorphic bands) showed that *F. hepatica* represented two main clusters, namely distinct isolates of sheep and cattle. The results indicated genetic diversity among *F. hepatica* population. According to phylogenetic analysis demonstrated that the higher genetic distance was between these two isolates. This dendrogram demonstrated a genetic diversity between each individual of *F. hepatica.* Although shown that genetic distance between strains was less than observed within strains. An important finding in relation to previous studies refers to existence of strains in population of *F. hepatica* (Ramadan & Saber 2004, Semyenova *et al* 2003). The traditional method used for identification of *F. hepatica* is still based on morphologic distinctions. This traditional method is unreliable (Ashrafi *et al* 2006, Marci1la *et al* 2002). Detection of genetic variations among micro-organisms could be done by DNA fingerprinting using arbitrary primers and PCR (Welsh & McClelland 1990, Williams *et al* 1990). In present study, RAPD analysis proved to be reproducible and rapid method to distinguish *F. hepatica* strains. Each of the two *F. hepatica* strains was shown to be genetically distinct but to share similar migrated DNA bands to some extent. The results of the present study showed that the *F. hepatica* strains are able to infect several hosts and on the other hand *F. hepatica* population of an infected host liver including different genotypes. These different genotypes are the most important factor due to its wide range of intermediate host's distribution and the fact that it is the main responsible cause of zoonotic disease. The detected significant variability suggested a preferential out crossing mode of reproduction for this hermaphroditic parasite a process which may be influenced by present day livestock transport. This finding may have important implications for gaining a better understanding of the isolates of *F. hepatica* may fulfill the requirements of control programs in zoonotic disease in the region and neighboring countries.

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