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Genetic Variation of *Fasciola hepatica* from Sheep, Cattle and Buffalo

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Abstract: Random amplified polymorphic DNA (RAPD-PCR) was used to study the genetic variation of *Fasciola hepatica* in sheep, cattle and buffalo. DNA was extracted from adult worms collected from livers of each infected animal in a slaughter house at Gilan province Caspian Sea region of Iran and amplified by the polymerase chain reaction, using four oligonucleotide decamers with arbitrary DNA sequences as primers. RAPD patterns showed the specific but different pattern DNA fragments for each primer. The intraspecific similarity coefficient within three isolates of *Fasciola hepatica* was ranged between 78 to 100%. Present findings showed that the interspecific genetic distance was higher than intraspecific genetic distances (22-50% compare to 0-22%). Which similarity coefficient between sheep and buffalo strains were the lowest values (50%). The inferred phylogenetic tree on the fingerprinting of these isolates clearly demonstrated the existence of population genetic diversity sub structuring within *Fasciola hepatica* of sheep, cattle and buffalo of Iran, raising interesting questions on the host specificity, epidemiology (e.g., zoonotic transmission) and ecology of this fluke.

Key words: Genetic variation, *Fasciola hepatica*, sheep, cattle, buffalo, RAPD-PCR

INTRODUCTION

Fasciolosis is an important socio-economics disease caused by *F. hepatica* and *F. gigantica*. Besides its well known veterinary importance they are recognized also as a serious public health problem. Human infection is estimated up to 17 million people (Mas-Coma *et al.*, 2005). Importance of this zoonotic food-borne disease with a great impact on human development have been emphasized by WHO and other human health institutes, so more recently Fasciolosis is added to the list of important helminthiasis (WHO, 1995; Anonymous, 2004). Whereas in Europe, America and Oceania only *F. hepatica* is present. In Iran and many other areas of Asia and Africa both species co-exist (Lotfy *et al.*, 2002; Rokni *et al.*, 2002; Ashrafi *et al.*, 2004). This geographical overlapping gives rise to many problems in the diagnosis, which finally remains classified as *Fasciola* sp. (Marcilla *et al.*, 2002). The differential diagnosis between *F. hepatica* and *F. gigantica* and study of genetic variation of these flukes is very important, particular where overlapping observe, because of their different epidemiological diagnosis and characteristics the helminthic parasites (Mas-Coma *et al.*, 2005). Molecular techniques based on genomics are very useful for the epidemiological and diagnostic tools as well as for research on genetic variation of the parasitic organisms (Mas-Coma *et al.*, 2005). In the present study RAPD method was used to detect genetic variation within *F. hepatica*.

MATERIALS AND METHODS

Sample Parasites

Mature *F. hepatica* was obtained from the bile duct of sheep (n = 6) cattle (n = 6) and buffalo (n = 6) sacrificed at Gilan slaughter houses (Caspian Sea region, Iran) between April and June 2005. The

worms were identified macro and microscopically through their morphological characteristics such as body length, maximum body width, cone length, cone width using the key presented by Ashrafi *et al.* (2006), the worms of each animal were washed three times with phosphate-buffered saline (PBS, PH 7.3, 37°C) to remove contamination, fixed in 70% ethanol for further study.

DNA Extraction

Genomic DNA was isolated from the apical end of adult flukes. After of cutting this part and place into labeled DNA free microcentrifuge tubes. DNA was extracted and purified using DNA extraction kit (MBST, Iran) according to manufacture protocol. The DNA concentration was estimated spectrophotometrically by reading absorbance at 260 nm and the purity of samples was examined OD 260 nm/OD 280 nm. Additionally the DNA were analysed by electrophoresis on a 1.5% agarose gel in TBE buffer (0.095 M Tris-Borate, 0.001 M EDTA). The gels were stained with ethidium bromide and the DNA was visualized using an UV transilluminator. The DNA solutions were stored -20°C until use.

Random Amplified Polymorphic DNA Analysis

The RAPD analysis was performed in 100 µL total reaction volumes containing 50 ng of genomic DNA, 10 µL PCR buffer 10x (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 µM of one of the following primers (Table 1), (Cinnagen Company, Iran):

5'-GTGACGTAGG-3' (AP₁), 5'-TGCCGAGCTG-3' (AP₂), 5'-GTGGTGGTGG-3' (AP₃) and 5'-GCGAGCGTCC-3' (AP₄).

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 base pair DNA ladder marker (Fermentase Company) was used. The major criteria for taking a fragment into account were reproducibility and distinctness of the fragments.

Data Analysis

RAPD profiles were used to measure genetic similarity among the worms. Each DNA band with different electrophoretic 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 method) and software analysis (Free-Tree-Freeware program). Manually using the formula $F = 2 N_{xy} / (N_x + N_y)$, where, N_x and N_y are the numbers of segments amplified in isolate x and y respectively and N_{xy} is the number of segments shared by them (Nei and Li, 1979). Additionally software analysis was performed using Free-Tree-Freeware program NTSYS-PC (www.natur.cuni.cz/flegit/programs, Pavlicek *et al.*, 1999).

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

Four arbitrary oligonucleotide primers (10 m) of various sequences (Table 1) were used in RAPD-PCR for their ability to generate polymorphic DNA fingerprints of three isolates of *F. hepatica*. Genomic DNA was analyzed from 18 *F. hepatica* of sheep, cattle and buffalo isolated from Caspian Sea region of Iran (Gilan province). The DNA fingerprints of each *F. hepatica* strains were identified and are shown in Fig. 1.

The reproducibility of pattern was confirmed by repeats. Only visible bands shared all PCRs, were used for calculation.

Of the 4 primers produced amplification products, a total of 341 bands were scored of 18 samples, with average of 19 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 11 (AP4). RAPD profiles for all isolates primers were shown to be reproducible on different days and in different laboratories.

Table 1: Nucleotide sequence (nt) of primers

Primers (10 m)	Sequence (5'-3')	GC content (%)
AP1	GTGACGTAGG	60
AP2	TGCCGAGCTG	70
AP3	GTGGTGGTGG	70
AP4	GCGAGCGTCC	80

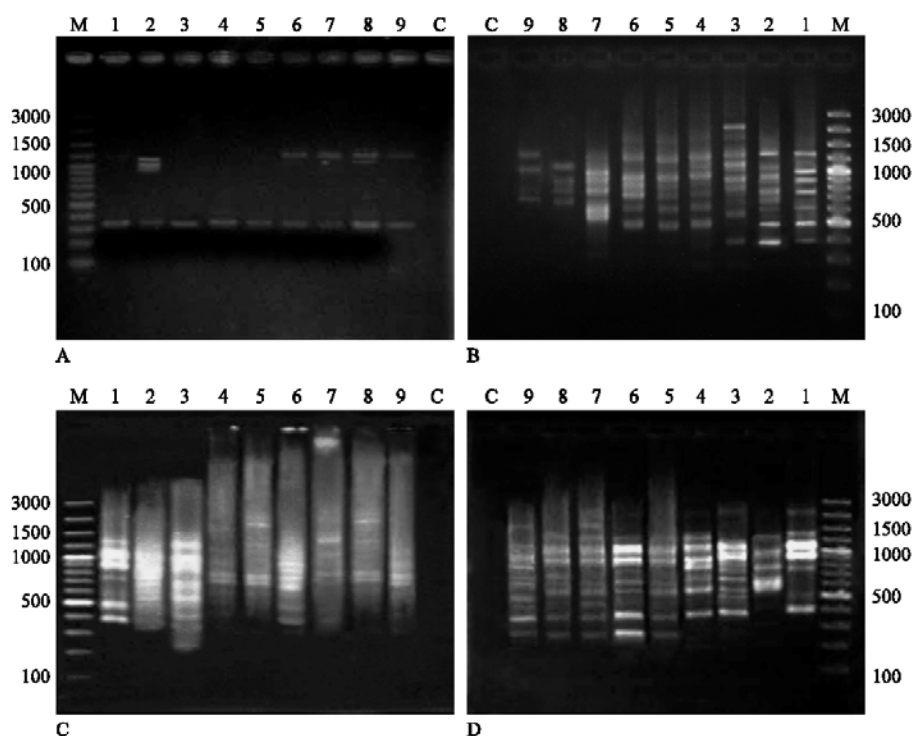


Fig. 1: Portions of four typical agarose gels showing RAPD profiles generated with primers AP1 (panel A), AP2 (panel B), AP3 (panel C) and AP4 (panel D). Lanes 1-3 = *F. hepatica* of sheep, Lanes 4-6 *F. hepatica* of cattle, Lanes 7-9 = *F. hepatica* of buffalo, Lane C = Negative control, Lane M = DNA marker (100 bp DNA ladder marker plus)

Table 2: Similarity coefficient between three strains of *F. hepatica*

Host	Sheep	Cattle	Buffalo
Sheep	0.78-1.00		
Cattle	0.51-0.63	0.92-1.00	
Buffalo	0.50-0.67	0.59-0.78	0.82-1

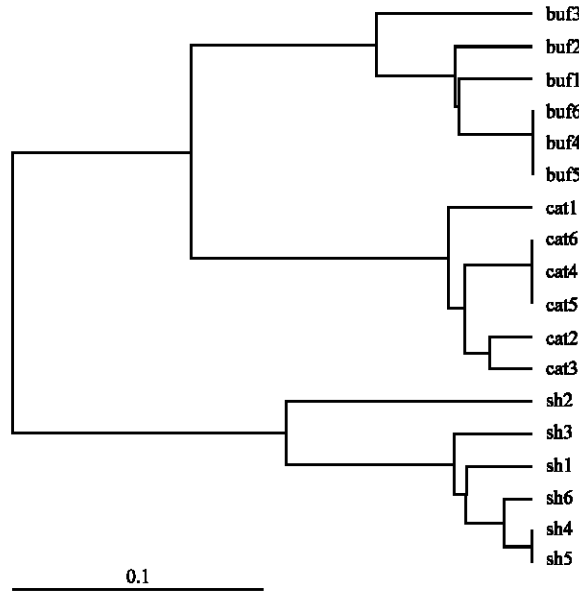


Fig. 2: UPGMA dendrogram based on cluster analysis of RAPD data for 18 individual of *F. hepatica* isolated from sheep (sh), cattle (cat), buffalo (buf). Numbers refers to host identifier. The scale represents the genetic distances between the individuals

Primers AP₂ and AP₄ amplified 3-11 DNA segments from all isolates ranging in 250-2100 bp (base pair). Primer AP₁ amplified a common 350 bp band in all 3 strains of *F. hepatica* and 1100 bp band was shown in buffalo strain and one of cattle strain. Primer AP₃ amplified a 2-8 number of DNA segments from all isolates. Pairwise genetic similarities generated using Nei and Li's coefficient of similarity ranged from as low as 0.5 between sheep and buffalo strains of *F. hepatica* to as high as 1 detected within sheep, cattle and buffalo strains of this trematode. The similarity coefficient between 3 isolates of *F. hepatica* was between 0.5 to 0.78 and within these three isolates of *F. hepatica* was between 0.78 to 1. The summary of inter and intra similarity coefficient of sheep, cattle and buffalo strains of *F. hepatica* are shown in Table 2.

Cluster analysis of the genetic similarity values was based on Nei and Li's 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 (Fig. 2). The constructed phylogenetic tree revealed that considerable genetic diversity exists in population of *F. hepatica*. Study of this phylogenetic tree, demonstrated that three main comprised of each of the three *F. hepatica* strains were formed.

Branches of *F. hepatica* strains on dendrogram cluster divided to 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 annealing temperature beyond 40°C would be resulted substantially decreased efficiency and decreasing this stage temperature under 39°C, caused to decreased specificity and reproducibility.

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 Tris-base, 0.001 M EDTA, pH = 8). These results showed that agarose gel 1% electrophoresis at 100 V for 1.5 h in TBE buffer 0.5x (w/v) electrolyte confirmed 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 only *F. hepatica* is concerned (Marcilla *et al.*, 2002). Whereas the distribution of both species overlap in many areas of Asia and Africa (Mas-Coma *et al.*, 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 *et al.*, 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 and McManus, 1989). Analysis of parasites mitochondrial cytochrome oxidase I (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. gigantica* were found (Itagaki *et al.*, 1998). Differences among individual mitochondrial genomes of *F. hepatica* have shown (Zurita *et al.*, 1988). No intraspecific variations in the restriction endonuclease maps of *F. hepatica* or *F. gigantica* were detected, but length heterogeneity was noted in the intergenic spacer even within individual worms (Blair and McManus, 1989). Difference were detected in the 28 s rRNA gene of *F. hepatica* in sheep and *F. gigantica* in cattle, but were not intraspecific variation performed. Another study demonstrate that individual cows infected by numerous genetically different liver flukes (Semyenova *et al.*, 2003). Difference among ITS₂ of the rDNA of *F. hepatica* and *F. gigantica* were shown, besides proved that ITS₂ sequence is 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 and McClelland, 1990; Williams *et al.*, 1990). The RAPD banding patterns can be affected by a number of factors for example the quality and quantity of template DNA, concentration of reagents and use of different thermocyclers (MacPherson *et al.*, 1993; Meunier and Grimont, 1993; Micheli *et al.*, 1994). However the effect of these factors on the resultant banding patterns is largely due to low annealing temperatures (25-35°C) used in the RAPD-PCR. Thus increased annealing temperatures as in the case in the present study (40°C) increase the stringency of the PCR and insure the reproducibility of RAPD results (de Grujter *et al.*, 2004). The main objective of this study was to investigate genetic variation of *F. hepatica* by RAPD-PCR technique. All individual worms used in this study were isolated from infected host liver from Caspian Sea region of Iran (to delete effect of geographical conditions on genotypes). Using primers AP1, AP2, AP3 and AP4 we detected 341 polymorphic bands from three strains of 18 *F. hepatica* sample. Band profiles ranging 250-2100 bp in sizes. Amplification with primer AP4 products the most bands (145) compared to AP1 (26), AP2 (119) and AP3 (51). These bands pattern could be caused of nucleotide sequences and GC content of each of them, as high GC content of primer AP4 (80%) caused the most bands in compare to low GC content of AP1 and AP2 (60%), produces lower DNA fragments. Smear

appearance of AP3 amplicons, perhaps due to large annealing sites corresponding to the sequence of this primer. Subsequent RAPD fingerprints analysis demonstrated high degree of polymorphism among all individuals of *F. hepatica* (n = 18) examined (associated with a total of 341 polymorphic bands). Similarity coefficient between *F. hepatica* strains was wide range as low as 50% to as high as 100%. The distance indicus observed interspecific (22-50%) was higher than intraspecific (0-22%).

Cluster analysis of the RAPD profiles data (considering all polymorphic bands) showed that *F. hepatica* represented three main clusters, namely distinct isolates of sheep, cattle and buffalo. The results indicated genetic diversity among *F. hepatica* population. According to phylogenetic analysis demonstrated that the higher genetic distance was between three isolates. This dendrogram demonstrated a genetic diversity between each individuals 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* (Semyenova *et al.*, 2003; Ramadan and Saber, 2004). The traditional method used for identification of *F. hepatica* is still based on morphologic distinctions. This traditional method is unreliable (Marcilla *et al.*, 2002; Ashrafi *et al.*, 2006). Detection of genetic variations among microorganisms can be done by DNA fingerprinting using arbitrary primers and PCR (Welsh and McClelland, 1990; Williams *et al.*, 1990). In the present study RAPD analysis proved to be reproducible and rapid method to distinguish *F. hepatica* strains. Each of the three *F. hepatica* strains was shown to be genetically distinct but to share similar migrated DNA bands to some extent. The RAPD patterns produced differences that may be due to the close relation between some strains of same host species (e.g., sheep and cattle strains of *F. hepatica* or different host species (e.g., sheep-buffalo) or to genetic change caused by gene flow between populations (Intapan *et al.*, 2004). 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 a infected host liver consisted of different genotypes. These different genotypes are the most important due to its wide range of intermediate hosts distribution and the fact that it is the main responsible cause of zoonotic disease. The significant variability that was detected suggests a preferential outcrossing mode of reproduction for this hermaphroditic parasite a process which may be influenced by present day livestock transport. These 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 here in Iran and neighboring countries.

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