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Genetic Characterization of *Fasciola* spp. from Tonekabon City (Northern Iran) Based on the Ribosomal Internal Transcribed Spacer Regions

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Abstract.- Fasciolosis caused by *Fasciola* spp. (Platyhelminthes: Trematoda: Digenea) is considered the most important helminth infection of ruminants in tropical countries, causing considerable socioeconomic problems. In the endemic regions of the North of Iran, *Fasciola hepatica* and *F. gigantica* have been previously characterized on the basis of morphometric differences, but the use of molecular markers is necessary to distinguish exactly between species and intermediate forms. Samples identified morphologically as *Fasciola* sp. in buffaloes and goats from Tonekabon city (northern Iran) were genetically characterised by sequences of the first (ITS-1), the 5.8S, and second (ITS-2) Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA). Comparison of the ITS of the North Iranian samples with sequences of *Fasciola* spp. from GenBank showed that the specimens examined had sequences identical to those of *F. hepatica* (n=22, 45.83%) and *F. gigantica* (n=17, 35.42%), which differed from each other in different variable nucleotide positions of ITS region sequences, and their intermediate forms (n=9, 18.75%), which had nucleotides overlapped between the two *Fasciola* species in all the positions. The nucleotide sequencing of ITS rDNA of *F. hepatica* and *F. gigantica* from Tonekabon city showed no nucleotide variation in the ITS-1 and ITS-2 rDNA sequences, versus two ITS-2 haplotypes in standard *F. hepatica* reported in GenBank. The intergenic transcribed spacers ITS-1 and ITS-2 showed to allow a reliable approach for the genetic differentiation of *Fasciola* spp., providing foundation for further studies on *F. hepatica, F. gigantica* and their intermediate forms in the endemic areas.

Key words: *Fasciola* spp., ITS regions, genetic characterization.

INTRODUCTION

Digenean trematodes of the genus *Fasciola* (Platyhelminthes: Trematoda: Digenea) are the common liver flukes of a range of animals with a global geographical distribution (Mas-Coma and Bargues, 1997). Previous studies have shown that *F. hepatica* occurs in temperate areas and *F. gigantica* mainly in tropical zones, and both species may overlap in subtropical areas (Mas-Coma *et al.*, 2005). Fasciolosis caused by *Fasciola* spp. is considered the most important helminth infection of ruminants in tropical countries, and it is involved in considerable socioeconomic problems (Mas-Coma *et al.*, 2005). The infection with *Fasciola* spp. represents a major health problem in diverse parts of Asia such as Iraq (Mahdi and Al-Baldawi, 1987), Pakistan (Qureshi *et al.*, 2005), Saudi Arabia (Over *et al.*, 1992), Vietnam (Tran *et al.*, 2001), Turkey (Turhan *et al.*, 2006), and Iran (Moghaddam *et al.*, 2004; Mas-Coma *et al.*, 2005; Ashrafi *et al.*, 2006).

In the endemic regions of the North of Iran, both *F. hepatica* and *F. gigantica* have been previously characterized on the basis of the morphometric differences using traditional microscopic measurements (Ashrafi *et al.*, 2006, 2007), but the use of molecular methods and markers is necessary to distinguish exactly between species and intermediate forms (Marcilla *et al.*,

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2002). Several studies have previously characterized genetically *F. hepatica*, *F. gigantica* and their intermediate forms from different countries using molecular techniques (Huang *et al.*, 2004; Le *et al.*, 2007; Alasaad *et al.*, 2007; Ali *et al.*, 2008; Li *et al.*, 2009; Farjallah *et al.*, 2009), and there are several studies dealing with the genetic characterization of *Fasciola* spp. from Iran, although they are limited to the Fars and Gilan provinces (Periago *et al.*, 2004; Ashrafi *et al.*, 2007; Karimi, 2008; Rokni *et al.*, 2010). The aim of the present work is to characterize *Fasciola* sp. samples from Tonekabon city (Mazandaran Province, northern Iran) by sequences of the first and second internal transcribed spacers (ITS-1,the 5.8S, and ITS-2) of ribosomal DNA (rDNA).

MATERIALS AND METHODS

Adult trematodes (n=48) were collected at necropsy during slaughter inspection from 47 livers of buffalos and from 43 of goats from Tonekabon city in the Mazandaran province (northern Iran), between February and October 2009. Flukes were identified morphologically as *Fasciola* spp. according to existing keys and descriptions given by Periago *et al.* (2006), and fixed in 70% ethanol until DNA extraction.

Total DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. DNA was eluted in 100 µl of elution buffer (10 mM Tris, 1 mM EDTA) and kept at −20°C until use. The polymerase chain reaction (PCR) carried out in 25 µl of total volume, contained 1 µl of DNA (20–40 ng), 2.5 U AmpliTaq Gold (Applera), 10 mM Tris–HCl (pH=8.3), 50 mM KCl (Applied Biosystems), 3 mM MgCl2 (Promega), 1 mM of dNTPs (Promega) and 0.25 µM of each primer. The DNA region comprising ITS-1, 5.8S rDNA and ITS- 2 (ITS) was amplified by PCR using primers BD1 (forward: 5′-GTCGTAACAAGGTTTCCGTA -3′) and BD2 (reverse: 5′- TATGCTTAAATTCAGCGGGT -3′) (Luton *et al.*, 1992). The PCR was performed in an Amplitron® PCR System II (Thermolyne), and the conditions were as follows: 3 min at 94°C, then 45 cycles of 40s at 94°C, 45 s at 55°C and 1 min at 72°C followed by a final elongation of 5 min at 72°C. A negative control (no DNA) was included in all PCR amplifications. Five millilitres of the amplification products were visualized on 1% ethidium-bromide-stained agarose gels to check the quality of amplification.

The PCR products of rDNA were purified using the commercial kit NucleoSpin Extract (Macherey- Nagel) according to the manufacturer's instructions. Sequencing was performed using the automatic sequencer ABI Prism 310 (Applied Biosystems, Foster City, Calif.). Sequences obtained were aligned using ClustalW (Thompson *et al.*, 1994), and adjusted manually, with previously published *Fasciola* spp. ITS (Table I). The electropherograms were analysed using Chromas 2.13.

RESULTS

The prevalence of fasciolosis in buffaloes and goats from Tonekabon city (northern Iran) by necroscopic examination was 12.76% (n=6) and 6.97% (n=3), respectively.

The ITS fragment amplified from each sample (n=48) using primers BD1 and BD2 was expected to be approximately 1,000 bp in length. The ITS PCR products were subjected to direct sequencing giving products 918bp long. The sequence was composed of the complete ITS-1 sequence of 435 bp, complete 5.8S sequence of 137 bp and complete ITS-2 sequence of 346 bp. The examined specimens showed sequences identical to those of *F. hepatica* (n=22, 45.83%) and *F. gigantica* (n=17, 35.42%), respectively, which differed from each other in different variable nucleotide positions of ITS region sequences, and their intermediate forms (n=9, 18.75%), which had nucleotides overlapped between the two *Fasciola* species in all the positions (Table I).

When comparing ITS-1 sequences with those previously published in GenBank, the only haplotype of *F. hepatica* (FhITS1) differed from the only haplotype of *F. gigantica* (FgITS1) in five polymorphic sites in positions 9, 99, 193, 271 and 291, including three transitions and two tranversions (Table I). While there was no nucleotide variation in the ITS-1, 5.8S and ITS-2 rDNA sequences among the 22 *F. hepatica* samples from Tonekabon city,

Table I.- Comparison of the ITS sequences of *Fasciola* spp. from Tonekabon in the North of Iran with those from different hosts and geographical locations.

Species	Locality	Variable sites of ITS region														Accession number
		ITS-1					ITS-2									
		9	99	193	271	291	210	234	273	279	287	330	337	344	345	
F. gigantica	Niger	T	T	T	A	T	C	C	T	T	C	–	A	A	T	AM900371
	Burkina Faso	T	T	T	A	T	T	C	T	T	C	–	A	T	A	AJ853848
	Kenya	T	T	T	A	T	T	C	T	T	C	–	A	T	A	EF612472-EF612484
	Iran	T	T	T	A	T	–	–	–	–	–	–	–	–	–	FJ756398
		T	T	T	A	T	–	–	–	–	–	–	–	–	–	FJ756397
		T	T	T	A	T	–	–	–	–	–	–	–	–	–	FJ756396
	Niger	C	A	C	T	C	T	T	C	C	C	T	G	T	A	AM900370
	Spain	C	A	C	T	C	T	T	C	C	C	T	G	T	A	AM709649
		C	A	C	T	C	T	T	C	C	T	T	G	T	A	AM709621
	Turkey	–	–	–	–	–	T	T	C	C	C	T	G	T	A	FJ593632
	Egypt	T	T	C	C	C	T	T	C	C	C	T	G	T	A	EF612468-EF612480
	Ireland	C	A	C	T	C	T	T	C	C	C	T	G	T	A	AB207141-AB207148
	Iran	–	–	–	–	–	T	T	C	C	C	T	G	T	A	EF612481
		C	A	C	T	C	–	–	–	–	–	–	–	–	–	FJ756394
		C	A	C	T	C	–	–	–	–	–	–	–	–	–	FJ756392
		C	A	C	T	C	–	–	–	–	–	–	–	–	–	FJ756393
		–	–	–	–	–	T	T	C	C	C	T	G	T	A	EU391412
		–	–	–	–	–	T	T	C	C	C	T	G	T	A	EU391413
		–	–	–	–	–	T	T	C	C	C	T	G	T	A	EU391418
	Tunisia	C	A	C	T	C	T	T	C	C	C/T	T	G	T	A	GQ231546
	Algeria	C	A	C	T	C	T	T	C	C	C/T	T	G	T	A	GQ231547
F. hepatica (n=22)	Tonekabon (Mazandaran province)	C	A	C	T	C	T	T	C	C	C	T	G	T	A	Present study
F. gigantica (n=17)		T	T	T	A	T	T	C	T	T	C	–	A	T	A	
F. hepatica /F. gigantica (n=9)		C/T	A/T	C/T	T/A	C/T	T	T/C	C/T	C/T	C	T/–	G/A	T	A	

published ITS-2 sequences have two haplotypes differing in only one mutation at position 287: haplotype 1 has 'C' (FhITS2-H1), whereas a 'T' appears in haplotype 2 (FhITS2-H2) (Table I). This position is 859 in the alignment of the complete 918-bp long intergenic region including ITS-1, 5.8S and ITS-2. According to sequences deposited in GenBank (Table I), the haplotype distribution showed geographical overlap in several countries and areas: FhITS2-H1 in Niger, Spain, Turkey, Egypt, Ireland, Zanjan (North Iran) and in Tonekabon city in the present study; FhITS2-H2 in Spain, Tunisia and Algeria (Table I).

When comparing ITS-2 sequences, the two haplotypes of *F. hepatica* (FhITS2-H1 and FhITS2-H2) with the only one of *F. gigantica* (FgITS2), five polymorphic sites allow the two species to be distinguished: four transversion in positions 234, 273, 279 and 337, and one indel in position 330 (Table I). Thus, the 10 positions differing between the two fasciolid species represent 1 % of interspecific variation.

DISCUSSION

Several studies have shown that it is difficult, and in certain cases even impossible, to differentiate morphologically between *F. hepatica* and *F. gigantica*, so that several specimens have been considered as intermediate forms (Moghaddam *et al.*, 2004). Therefore, various DNA markers have been considered to identify *Fasciola* species. PCR-based restriction fragment length polymorphism (PCR-RFLP) analyses of the 28S rRNA gene have been used for differentiation of *Fasciola* spp. from South America, Europe and Africa (Hashimoto *et al.*, 1997; Marcilla *et al.*, 2002; Itagaki *et al.*, 2005; Lin *et al.*, 2007). These sequences revealed a few nucleotide differences between the two species, but no intraspecific variations within species (Marcilla *et al.*, 2002). Different studies have also demonstrated that the ITS region (ITS1, the 5.8S and the ITS2) of nuclear rDNA (Itagaki and Tsutsumi, 1998; Marcilla *et al.*, 2002; Periago *et al.*, 2004; Itagaki *et al.*, 2005; Farjallah *et al.*, 2009; Rokni *et al.*, 2010), mitochondrial NDI and COI genes (Hashimoto *et al.*, 1997; Itagaki *et al.*, 2005) provide useful genetic markers for the accurate identification of *Fasciola* species. Previous studies carried out in Asia have shown that *F. hepatica* occurs in various regions, i.e. Iraq (Wajdi and Nassir, 1983), Pakistan (Sharma *et al.*, 1989), Turkey (Turhan *et al.*, 2006), Saudi Arabia (Over *et al.*, 1992), and that *F. hepatica*, *F. gigantica* and their intermediate forms are present in Iran (Fars, Mazandaran and Gilan provinces) (Periago *et al.*, 2004; Karimi, 2008; Ashrafi *et al.*, 2006, 2007), China (Huang *et al.*, 2004; Lin *et al.*, 2007), Japan (Itagaki and Tsutsumi, 1998; Itagaki *et al.*, 2005), Korea (Agatsuma *et al.*, 2000) and Vietnam (Tran *et al.*, 2001).

In the present study, adult specimens of *Fasciola* spp. infecting buffaloes and goats from Tonekabon city (North of Iran) were characterised by sequences of the ITS rDNA. The sequences obtained revealed that the sequences of *Fasciola* spp. from buffaloes and goats were identical to those of previously published for *F. hepatica*, *F. gigantica* and their intermediate forms (Agatsuma *et al.*, 2000; Periago *et al.*, 2004; Itagaki *et al.*, 2005; Ali *et al.*, 2008; Farjallah *et al.*, 2009; Rokni *et al.*, 2010). Five nucleotide mutations appeared between the ITS-1 of *F. hepatica* and that of *F. gigantica* analysed in the present work, as previously reported from different areas, such as Egypt (Periago *et al.*, 2004), Japan (Itagaki *et al.*, 2005), Vietnam (Itagaki *et al.*, 2009; Ichikawa and Itagaki, 2010), Tehran, West Azerbaijan and Khuzestan from Iran (Periago *et al.*, 2004; Rokni *et al.*, 2010).

ITS2 sequences of *F. hepatica* from Tonekabon city showed no nucleotide variations and were identical among them, but comparisons with ITS2 sequences of *F. hepatica* from other geographical regions showed nucleotide differences at least in one position. In fact, the most frequent ITS-2 haplotype (FhITS2-1) showed a widespread distribution, indicating that this is the main haplotype involved in the spread of *F. hepatica* from Spain (Alasaad *et al.*, 2007), Iran (Bargues *et al.*, 2002), Japan (Itagaki *et al.*, 2005), Korea (Agatsuma *et al.*, 2000), Vietnam (Le *et al.*, 2008), Egypt (Periago, 2004), Tunisia, Algeria (Farjallah *et al.*, 2009) and Niger (Ali *et al.*, 2008). The second most frequent *F. hepatica* ITS-2 haplotype (FhITS2-2) differed by a transition in position 287 of the alignment of the two species, but appeared to be less

common, being reported from Spain (Alasaad *et al.*, 2007), Australia (Adlard *et al.*, 1993), Uruguay (Itagaki and Tsutsumi, 1998), Tunisia and Algeria (Farjallah *et al.*, 2009). These findings suggest that the above mentioned variants of *F. hepatica*, occurring in isolated countries, may have a common origin and that they have spread recently throughout these countries because of movement of infected animals.

In the present study the presence of intermediate genotypes of *Fasciola* has been shown using sequences of the ITS rDNA. Karimi (2008) first reported the molecular evidence of an intermediate genotype of *Fasciola* in the Fars province using 18S rDNA–RFLP and sequencing; but recently, Rokni *et al.* (2010) identified ITS1–RFLP patterns as either *F. hepatica* or *F. gigantica* from Iran, reporting no mixed patterns.

In the present study, 12.76% of buffaloes and 6.97% of goats were infected. Epidemiological data obtained by coprological analysis in the same area (Moghaddam *et al*., 2004) have shown total prevalences of 7.3% in sheep, 25.4% in cattle, and no horse was found infected. The same authors, by the analysis of gross pathological liver lesions in slaughterhouses, calculated prevalences of 5.7% in sheep, 4.6% in cattle, 1.6% in goats, and 15.0% in buffaloes. Therefore, Moghaddam *et al.* (2004) suggested that sheep and cattle may be the main reservoir species in this area, that the low number of individuals of buffaloes in Mazandaran suggests that they only play a local role in the transmission, and that goats and horses probably only participate sporadically. The present results confirm those reported by Moghaddam *et al.* (2004), although indicate a most important role of goats, with a prevalence close to that reported by Moghaddam *et al.* (2004) for sheep after coprological analysis, and higher than that reported by the same authors for this species after analysis of gross pathological liver lesions.

In conclusion, the present study showed that the liver flukes in buffaloes and goats from Tonekabon city (North of Iran) are represented by *F. hepatica* and *F. gigantica* species and their intermediate forms. The genetic characterization of *Fasciola* spp. present in this area is a useful tool to achieve the basic information necessary for the field control of this parasite and will have implications for the diagnosis and control of the disease they cause. Although, other investigations, using more variable genetic markers, are needed for further molecular analysis of a wider range of isolates from different host species and geographical locations, in order to better understand the genetic variability and population genetic structure within *Fasciola* spp. and their transmission dynamics in these and in the neighbouring Asian countries.

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