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First report of *Fasciola hepatica* in *Equus caballus* host species from Tunisia 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. Samples identified morphologically as *Fasciola* sp. in *Equus caballus* host species from the city of Tunis (north of Tunisia) were genetically characterized, for the first time, by sequences of the 1st (ITS-1), the 5.8S and 2nd (ITS-2) Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA). Comparison of the ITS of the Tunisian samples with sequences of *Fasciola* spp. from GenBank showed that the specimens examined had sequences identical to those of *F. hepatica* sequences selected as reference. The nucleotide sequencing of ITS rDNA showed no nucleotide variation in the ITS-1, 5.8S, or ITS-2 rDNA sequences among all *F. hepatica* samples parasitizing horses from Tunis, versus 2 ITS-2 haplotypes in standard *F. hepatica*, showing a substitution C/T in position 859, reported previously from Tunisia, Algeria, and Spain. The present study is the first demonstration of the existence of the most frequent haplotype (FhITS-H1) of *F. hepatica* species in horses from Tunis (north of Tunisia) by the genetic approach using ITS rDNA as genetic marker, providing a foundation for further studies on *Fasciola* sp. in North African countries.

Key words: *Fasciola hepatica*, *Equus caballus*, Tunis, PCR, ITS, genetic characterization

Introduction

Fasciolosis caused by the genus *Fasciola* (Platyhelminthes: Trematoda: Digenea) is considered the most important helminth infection of ruminants in tropical countries, and it is involved in considerable socioeconomic problems (1). Digenean trematodes of *Fasciola* spp. are common liver flukes of a range of animals with a global geographical distribution (2). Previous studies have shown that *F. hepatica* occurs in temperate areas and *F. gigantica* mainly in tropical zones, and that both species may overlap in subtropical areas (1).

The infection with *Fasciola* spp. represents a major human health problem in diverse parts of Africa such as Egypt, Zambia, Kenya, Algeria, Zimbabwe, Tanzania, and Nigeria (3-10), and human infection cases with *F. hepatica* have been documented from southwest Tunisia, with a prevalence of infection of 6.6% (11). Both *F. hepatica* and *F. gigantica* have been previously characterized on the basis

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of the morphometric differences using traditional microscopic measurements (12,13), but the use of molecular methods and markers is necessary to distinguish exactly between species and intermediate forms (14).

Several studies have previously characterized genetically *F. hepatica*, *F. gigantica*, and their intermediate forms from different countries using the 1st (ITS-1), the 5.8S and 2nd (ITS-2) Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA) sequences (10,15-19), but there are few studies dealing with the genetic characterization of *F. hepatica* from North Africa, and they are limited to specimens parasitizing sheep and cattle hosts species (19). Therefore, the aim of the present work was to characterize *Fasciola* spp. samples parasitizing horses from Tunisia by sequences of the 1st and 2nd internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA).

Materials and methods

Adult trematodes (n = 11) were collected at necropsy during slaughter inspection from bile ducts of livers of *E. caballus* species (n = 6) from the city of Tunis in northern Tunisia, between January and March 2010. Flukes were identified morphologically as *F. hepatica* according to existing keys and descriptions given by Periago et al. (20), and fixed in 70% ethanol until extraction of genomic DNA.

Total genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Body portions from individual trematodes were each placed in 600 µL of a mixture containing 0.5 M ethylene diamine tetraacetic acid (EDTA) plus nuclear lysis solution and then crushed employing a sterile pestle. An aliquot of 17.5 µL of proteinase K (20 mg mL^{-1}; Promega) was added to each tube, which was incubated at 55 °C for 3 h. An aliquot of 3 µL of RNase solution (4 mg mL^{-1}) was added, and the tubes were incubated at 37 °C for 30 min.

Subsequently, 200 µL of protein precipitation solution was added, the tubes vortexed and chilled on ice for 5 min, centrifuged at 4 °C for 4 min, and the DNA precipitated with ethanol. Each DNA pellet was air-dried for 20 min, 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) was carried out in 25 µL of total volume, which contained 1 µL of DNA solution (20-40 ng), 2.5 U of AmpliTaq Gold (Applera), 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl (Applied Biosystems), 3 mM of $MgCl_2$ (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 polymerase chain reaction using primers BD1 (forward: 5′-GTCGTAACAAGGTTTCCGTA-3′) and BD2 (reverse: 5′- TATGCTTAAATTCAGCGGGT -3′) (21). 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 40 s 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 milliliters of the amplification products were visualized on 1% ethidium-bromide-stained agarose gels to check the quality of amplification (19).

The PCR products of ribosomal DNA were purified using the commercial kit NucleoSpin Extract (Macherey-Nagel) according to the manufacturer's instructions. Sequencing was performed using an external sequencing core service (Macrogen Inc., World Meridian Center 908, 60-24 Gasan-dong, Gumchun-gu Seoul, Korea). Sequences obtained were aligned using ClustalW (22), and adjusted manually, with previously published *Fasciola* spp. ITS (Table). The electropherograms were analyzed using Chromas 2.13.

Results

The ITS fragment amplified from each sample (n = 11) using primers BD1 and BD2 was expected to be approximately 1000 bp in length (Figure). The ITS PCR products were subjected to direct sequencing giving products 918 bp 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. Comparison of sequences of the *F. hepatica* samples examined in the present study with those of *F. hepatica* and *F. gigantica* and the "intermediate *Fasciola*" from GenBank confirmed

Table. Comparison of the ITS sequences of *Fasciola* spp. from Tunis parasitizing horses with those from different hosts and geographical locations.

			Variable sites of ITS region													
			ITS-1					ITS-2								
Locality	**Species**	**Hosts**	**9**	**99**	**193**	**271**	**291**	**806**	**845**	**851**	**859**	**902**	**909**	**916**	**917**	**Accession number**
Burkina Faso	FgITS	Cattle	T	T	T	A	T	C	T	T	C	–	A	T	A	AJ853848
Kenya		N/A	T	T	T	A	T	C	T	T	C	–	A	T	A	EF612472-EF612484
Niger	FhITS-H1	*Bos taurus*	C	A	C	T	C	T	C	C	C	T	G	T	A	AM900370
		Bos taurus	C	A	C	T	C	T	C	C	C	T	G	T	A	AM850107
Spain	FhITS-H1	*Rupicapra pyrenaica*	C	A	C	T	C	T	C	C	C	T	G	T	A	AM709649
		Equus caballus	C	A	C	T	C	T	C	C	C	T	G	T	A	AM709646
		Bos Taurus	C	A	C	T	C	T	C	C	C	T	G	T	A	AM709498
		Bos Taurus	C	A	C	T	C	T	C	C	C	T	G	T	A	AM709609
	FhITS-H2	*Cervus elaphus*	C	A	C	T	C	T	C	C	T	T	G	T	A	AM707030
		Dama dama	C	A	C	T	C	T	C	C	T	T	G	T	A	AM709500
Turkey	FhITS-H1	Sheep	–	–	–	–	–	T	C	C	C	T	G	T	A	FJ593632
		Sheep	–	–	–	–	–	T	C	C	C	T	G	T	A	FJ467927
		Sheep	–	–	–	–	–	T	C	C	C	T	G	T	A	FJ459806
Egypt	FhITS-H1	N/A	T	T	C	C	C	T	C	C	C	T	G	T	A	EF612468-EF612480
Ireland	FhITS-H1	*Bos taurus*	C	A	C	T	C	T	C	C	C	T	G	T	A	AB207141-AB207148
Iran	FhITS-H1	Cattle	–	–	–	–	–	T	C	C	C	T	G	T	A	EF612481
		Buffalo	C	A	C	T	C	–	–	–	–	–	–	–	–	FJ756394
		Buffalo	C	A	C	T	C	–	–	–	–	–	–	–	–	FJ756392
		Buffalo	C	A	C	T	C	–	–	–	–	–	–	–	–	FJ756393
		Cattle	–	–	–	–	–	T	C	C	C	T	G	T	A	EU391412
		Cattle	–	–	–	–	–	T	C	C	C	T	G	T	A	EU391413
		Cattle	–	–	–	–	–	T	C	C	C	T	G	T	A	EU391418
Tunisia	FhITS-H1	*Ovis aries*	C	A	C	T	C	T	C	C	C	T	G	T	A	GQ231546
	FhITS-H2	*Bos taurus*	C	A	C	T	C	T	C	C	T	T	G	T	A	Farjallah et al. 2009
Algeria	FhITS-H1	*Ovis aries*	C	A	C	T	C	T	C	C	C	T	G	T	A	GQ231547
	FhITS-H2	*Ovis aries*	C	A	C	T	C	T	C	C	T	T	G	T	A	Farjallah et al. 2009
Tunis (Tunisia)	FhITS-H1	*Equus caballus*	C	A	C	T	C	T	C	C	C	T	G	T	A	Present study JF423939

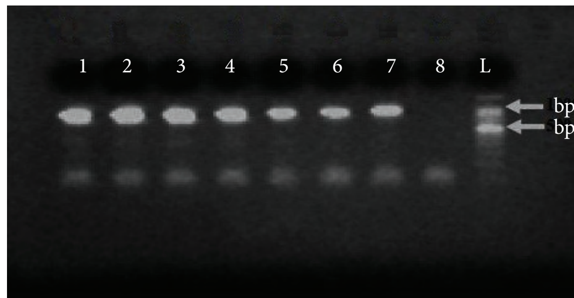

Figure. Agarose gel electrophoresis of ITS PCR products of *Fasciola* spp. samples from Tunis parasitizing horses. Lanes 1-7, sample from Tunis, Lane 8, negative control, M, Molecular weight marker.

that all the samples examined represented a single *Fasciola* species, namely *F. hepatica* (FhITS-H1) (Table). While there was no nucleotide variation in the ITS-1, 5.8S, and ITS-2 rDNA among the examined *F. hepatica* samples from *E. caballus* host species revealing 100% homology with *F. hepatica* sequences selected as reference (FhITS-H1), the published ITS-2 sequence from Tunisia, Algeria, and Spain has 2 haplotypes differing in only one mutation at position 287: haplotype 1 has 'C' (FhITS-H1), whereas a 'T' appears in haplotype 2 (FhITS-H2). This position is 895 in the alignment of the complete 918 bp long intergenic region including ITS-1, 5.8S, and ITS-2 (Table).

When comparing ITS-1 and ITS-2 sequences with those previously published in GenBank, the only haplotype of *F. hepatica* (FhITS-H1), reported in the present study, differed from the only haplotype of *F. gigantica* (FgITS) in 5 polymorphic sites in positions 9, 99, 193, 271, and 291, including 3 transitions and 2 tranversions, and, in 5 polymorphic sites, including 4 transversion in positions 234, 273, 279, and 337, and one indel in position 330, respectively (Table). Thus, the 10 positions differing between the 2 fasciolid species represent 1% of interspecific variation (Table).

Discussion

Previous studies in Africa have shown that *F. gigantica* mainly occurs in Burkina Faso, Senegal, Kenya, Zambia, and Mali (7,9,20,23), while *F. hepatica* has been reported from Morocco (24), and both species have been observed from Egypt and Niger (25,26). From Tunisia and Algeria, the presence of *F. hepatica* was detected in domestic ruminants using serology: Tunisia, 14.3% of cattle, 35%-55% of sheep, and 68% of goats (11,27); and Algeria, 6.3%-27.3% of cattle (5).

Recently, different studies have demonstrated that the ITS region (ITS1, the 5.8S, and the ITS2) of nuclear rDNA (14,19,28-31), mitochondrial NDI, and COI genes (30,32,33) provide useful genetic markers for the accurate identification of *Fasciola* species.

Therefore, in the present study, adult specimens of *Fasciola* spp. infecting *E. caballus* host species from Tunis (north of Tunisia) were characterized by sequencing of the ITS rDNA. This analysis revealed that the sequences of *Fasciola* spp. from horses were identical to those previously published for *F. hepatica* (10,17,19,31). ITS-1, 5.8S, and ITS-2 rDNA sequences of *F. hepatica* parasitizing *E. caballus* host species from Tunis showed no nucleotide variations and were identical to *F. hepatica* sequences selected as reference, but comparisons with some 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 (FhITS-H1), reported also in the present study, showed a widespread distribution, indicating that this is the main haplotype involved in the spread of *F. hepatica* from Spain (17), Iran (34), Japan (30), Korea (35), Vietnam (16), Egypt (29), Tunisia, Algeria (19), and Niger (10). The second most frequent *F. hepatica* ITS-2 haplotype (FhITS-H2) differed by a transition in position 287 of the alignment of this species, but appeared to be less common, being reported from Spain (17), Australia (36), and Uruguay (28).

In the present study, the sequences of the ITS rDNA reported correspond totally to the most frequent haplotype (FhITS-H1) of *F. hepatica*. Recently, Farjallah et al. (19) demonstrated that the liver fluke samples from sheep and cattle host species in Tunisia and Algeria represented the single species *F. hepatica*, but both haplotypes (FhITS-H1 and FhITS-H2) were defined showing the same substitution C/T in position 859 in the ITS-2 sequences.

In conclusion, the present study is the first demonstration of the existence of the most frequent haplotype (FhITS-H1) of *F. hepatica* species in horses

from Tunis by the genetic approach using ITS rDNA as genetic marker. In fact, genetic characterization of *Fasciola* spp. present in this area is a useful tool to obtain 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. Nevertheless, in order to better understand the genetic variability and population genetic structure within *Fasciola* spp. and their transmission dynamics in these and in the neighboring African countries other investigations using this method are needed for further molecular analysis of a wider range of isolates from different horse populations and geographical locations.

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