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Phenotypic variability of sheep breeds to fasciolosis in northern Tunisia

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I. Introduction

Fasciolosis is a hepatobiliary parasitic zoonotic disease affecting particularly ruminants, where sheep constitute the most sensitive species. It constitutes one of the main problems affecting the productivity of livestock in several regions of the world, and contributes to global economic losses of more than $3 billion per year (Mas-Coma et al., 2005; Piedrafita et al., 2010) mainly due to the condemnation of livers in slaughterhouses, reduced milk yield, wool and meat production, reduced growth rate, fertility disorders and increased mortality (Sangster, 2001).
The World Health Organization (WHO) has classified fasciolosis as a serious zoonosis with a range of global infestations between 2 and 17 million (Mas-Coma, 2005). It is widespread throughout the world, in more than 90% of countries (Nyindo & Lukambagire, 2015), particularly in temperate zones and cooler high-altitude areas in the tropics and subtropics (Abdulhakim & Addis, 2012) (Figure 1).
In Tunisia, sheep breeding plays a major role in the economy through local production of animal origin food, indeed, meat production by the Tunisian sheep population has been estimated at about 51,200 tons per year (Office de l'Elevage et des Pâturages, 2017). However, the Tunisian sheep population suffers from several health problems, mainly parasitic infections such as fasciolosis, especially in the north and south-west of the country, where animal infection is more widespread in breeding with infestation prevalence rates of 65% and 35%, respectively (Akkari et al., 2020; Hammami et al., 2005). On the other side, *Fasciola hepatica* is resistant to the anthelmintic, triclabendazole, it is becoming very common, and as a result breeders have been left without any means of infection control (Brennan et al., 2007).

The phenotypic and genetic resistance of local sheep breeds to fasciolosis in Tunisia has never been investigated. Therefore, the aim of the present work is to study the phenotypic variability of local sheep breeds to fasciolosis to identify and characterize resistant animals. Indeed, phenotypic measurements have been collected in the following area.

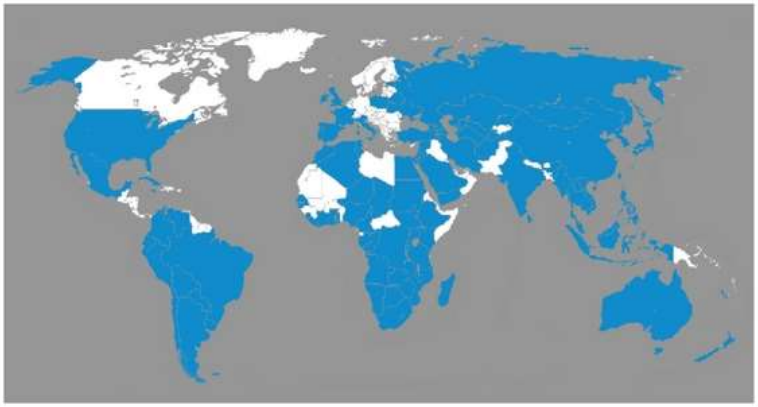

Figure 1 : Global distribution of fasciolosis (Nyindo & Lukambagire, 2015)

II. Materials and methods

Study area

This study was carried out in the region of Sejnane, district of Bizerte, northern Tunisia (37° 06' N; 09° 10' E). Sejnane belongs to the humid bioclimatic status and is characterized by a Mediterranean climate with an annual average rainfall of 1,000 mm, and a hydro-morphic and clay soil, promoting the development of *Galba. truncatula* the intermediate host of *F. hepatica* (Akkari et al., 2020).

The choice of the study area was based on previous studies that have shown that fasciolosis is highly endemic in Sejnane region causing significant impacts on animal health (Ben Said et al., 1979; Jemli et al., 1991; Akkari et al., 2020).

The present study was realized between July 2020 and April 2021, in two seasons: hot (S1) and cold (S2) and on two animal populations: (i) on slaughtered animals in the local slaughterhouse of Sejnane and (ii) on live animals in sheep farms, in total 16 farms were studied (Table1, Figure 2).

Table 1: Characteristics of the studied area

Sampled area	GPS coordinates
Sejnane slaughterhouse	37°3N 9°14E
Farm 1	37°0944N 8°68E
Farm 2	37°0950N 8°68E
Farm 3	37°0953N 8°68E
Farm 4	37°0978N 8°68E
Farm 5	37°0943N 8°68E
Farm 6	37°0812N 9°2189E
Farm e 7	37°0839N 9°216E
Farm 8	37°0849N 9°1634E
Farm e 9	37°0608N 9°2406E
Farm 10	37°0612N 9°24E
Farm 11	37°0641N 9°2252E
Farm 12	37°0609N 9°2406E
Farm 13	37°0564N 9°2381E
Farm 14	37°0564N 9°2326E
Farm 15	37°0571N 9°23269E
Farm 16	37°0571N 9°23269E

Figure 2: Location of sampling area in Sejnane region

Sample collection

Sejnane slaughterhouse

A total number of 603 sheep were randomly sampled. Information about the breed, the sex was collected before animal slaughtering as well as an estimation of the animal age by dental examination (Table 2). The origin of the animal and the use of anthelmintic drugs in the previous months remains poorly known. Faeces, blood (EDTA, and dry tubes) and livers were taken from each animal sampled.

All the samples were placed in identified sterile bags and tubes, then transported to the Laboratory of Parasitology at the National Veterinary School of Sidi Thabet, Tunisia in isothermal coolers and stored at -20°C until analyses.

***Table 2*:** Characteristics of animals sampled in Sejnane slaughterhouse. **Breed**: C: Cross; Q: Queue Fine de l'Ouest; N: Noir de Thibar; B: Barbarine; **Sex**: M: Male; F: Female; **Season**: S1: Season 1; S2: Season 2.

Parameter		**Number of animals (%)**
***Age** (month)*	< 1	498 (82.6)
	[1; 3]	83 (13.8)
	< 3	22 (3.6)
Breed	C	478 (79.2)
	Q	52 (8.6)
	N	69 (11.4)
	B	4 (0.7)
Sex	M	31 (5.1)
	F	572 (94.9)
Season	S_1	293 (48.6)
	S_2	310 (51.4)

Sejnane Farms

The second population studied was sheep farms, A total number of 609 sheep were randomly sampled, Information on breed, sex, conjunctival color, body score is recorded as well as age was estimated by dental examination (Table 3). Also, information on the animal's grazing

pattern and the use of anthelmintic drugs in the previous months is noted. Blood and fecal samples were taken from each sheep, placed in identified sterile bags and tubes, then transported to the Laboratory of Parasitology at the National Veterinary School of Sidi Thabet, Tunisia in isothermal coolers and stored at -20°C until analyses.

***Table 3*:** Characteristics of animals sampled in Sejnane farms. **Breed**: C: Cross; Q: Queue Fine de l'Ouest; N: Noir de Thibar; B: Barbarine; **Sex:** M: Male; F: Female; **Season**: S1: season 1; S2: season 2; **Conjunctival color:** B: White; C: Red; J: Yellow; Lb: Slightly White; Lc: Slightly Red; Rc: Pink.

Parameter		**Number of animals (%)**
Age (year)	< 1	63 (10.3)
	[1,3]	281 (46.1)
	[4,6]	219 (36.0)
	[7,9]	36 (5.9)
	< = 10	10 (1.6)
Breed	C	589 (96.7)
	N	20 (3.3)
Sex	M	54 (8.9)
	F	555 (91.1)
Season	S_1	304 (49.9)
	S_2	305 (50.1)
Conjunctival color	B	32 (5.3)
	C	145 (23.8)
	J	11 (1.8)
	Lb	14 (2.3)
	Lc	31 (5.1)
	Rc	376 (61.7)
Body score	1	120 (19.7)
	2	380 (62.4)
	3	106 (17.4)
	4	2 (0.3)

Laboratory analysis

Haematological analysis

Blood collected in EDTA tubes was used for haematological parameters estimation, mainly red blood cell count (RBC) (×106/ml), haemoglobin (Hb) (g/dl) and haematocrit (Ht) (%). Haematological parameters were measured using an Auto Haematology analyser BC-2800Vet® (Shenzen Mindray Bio-Medical Electronics Co., Ltd).

Livers examination and fluke's collection

At the beginning, an incision along the bile ducts was performed for each liver using a sterile blade. Then the hepatic parenchyma, bile ducts and gallbladder were examined for the presence of immature and adult *Fasciola hepatica*. Liver flukes were collected, recording their number per liver, placed in identified vials and stored in alcohol 70 %.

Bile examination

The working conditions only allowed the examination of 304 bile samples from 603 animals. For each gallbladder, the bile was poured into a stem glass and allowed to sediment for 15 minutes. Then the sediment was aspirated using a pipette, placed in a petri dish and stained with 1 ml of a methylene blue to 1 p. 1000. The solution was later examined under a stereomicroscope x100 to estimate the number of *F. hepatica* eggs (Animale et al., 2021).

Faecal egg counts

For all faeces samples, faecal egg counts of *Eimeria* spp, *Monezia* spp, *Nematodirus* spp and gastro-intestinal helminths were estimated using the flotation technique followed by the McMaster technique for the positive samples. They have been identified according to their specific morphology (Raynaud, 1970).
While, trematode eggs including *Fasciola hepatica* cannot be detected by the flotation technique with usual solutions due to their high weight compared to that of nematodes. *Fasciola hepatica* eggs were estimated by the sedimentation technique as described by Hanson and Perry (1994). Briefly, 5 g of stool collected from each sheep were mixed with 75 ml of

distilled water filtered through a tea strainer and centrifuged at 1500 rpm for 15 minutes. After centrifugation, only 2 ml of sediment was retained and stained by adding 1 ml of methylene blue to 1 p. 1000. The solution was transferred to a Petri dish to estimate the number of *F. hepatica* eggs under a stereomicroscope x100. The number of eggs per gram of feces was calculated by dividing the *Fasciola hepatica* egg count in a sample of 5 g by five.

Fluke's measurement

Morphometric measurements were performed on the collected flukes. Fieldwork conditions made it possible to carry out the measurements on only 335 flukes out of a total number of 458. Total fluke length (**L1**), ventral sucker to tail length (**L2**), oral to ventral sucker length (**L3**), abdomen diameter (**D1**) and tail diameter (**D2**) were the main morphometric parameters measured.

The mean and standard deviation of each parameter were calculated and compared with the values of *Fasciola gigantica* (Animale et al., 2021).

DNA extraction

DNA was extracted from 20 mg liver sheep samples using Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA), and stored at -20°C until used.

Parasitological indicators and statistical analyses

All the data was compiled in an Excel® data file.

The prevalence of infestation (Pr), the mean intensity of infestation (I) and the abundance of infestation (A) were estimated according to the following formula:

Pr = 100 x Number of sheep infested/Number of examined sheep

I = Number of flukes collected in livers / number of infested sheep

A = Number of flukes collected in livers / number of examined sheep

I = Total number of eggs per gram of feces / Number of infested sheep

The prevalences of infestation depending on age, sex and breed were compared using the chi square test at a threshold value of 0.05 (Schwartz, 1993).

Total fluke length (L1), ventral sucker to tail length (L2), oral to ventral sucker length (L3), abdomen diameter (D1) and tail diameter (D2) were compared using the T-student test at a threshold value of 0.05 (Schwartz, 1993).

III. Results

Parasitological indicators

Animals slaughtred

Liver dissection revealed an overall prevalence of *Fasciola hepatica* infestation in sheep was estimated to 11.28%. An overall mean intensity of 25.21 flukes/sheep (and a range from 1 to 195), and the abundance was 2.84 flukes/sheep. While coprological analysis revealed an overall infestation prevalence of 8.39%. The overall mean infestation intensity was 9.96 eggs per gram of feces. Therefore, the Bile examination showed an overall infestation prevalence of 16.78% (Table 4).

In the current study, a higher number of cases of fasciola were detected during bile examination compared to the liver's and coprological examination.

Table 4: Parasitological indicators based on liver dissection, coprological examination and bile examination in Sejnane slaughterhouse.

Parasitological indicators	**Liver dissection**	**Coprological examination**	**Bile examination**
Prevalence	11.28% (68/603)	8.39% (50/596)	16.78% (51/304)
Intensity	25.21 flukes/sheep (Range from 1 to 195)	9.96 eggs/gram of feces	1968,20 eggs/ml of bile
Abundance	2.84 flukes /sheep	_	_

The highest *F. hepatica* infestation prevalence was observed in sheep under one year of age (8.13%), it is also observed in cross sheep breed (10.61%). Female are more infested than male (10.45%). The infestation rate is higher in the hot season (season 1) than in cold one (season 2) (7.13%). There was a significant difference in the over-all infestation prevalence of *F.hepatica* for sheep breed and for the season (p= 0,02111 and p= 0.01548; respectively). While There was no significant difference in the over-all infestation prevalence of *F.hepatica* for neither sheep age nor sex (Table 5).

***Table 5*:** Prevalence of *Fasciola hepatica* infestation in Sejnane slaughterhouse according to risk factors.

Parameter		**Positive / examined (%)**	***P***
Age (month)	< 1	49/498 (8.13)	0,10559
	[1; 3]	15/83 (2.49)	
	< 3	4/22 (0.66)	
Breed	C	64/478 (10.61)	0,02111
	Q	0	
	N	4/69 (0.66)	
	B	0	
Sex	M	5/31 (0.83)	0,44285
	F	63/572 (10.45)	
Season	S_1	43/293 (7.13)	0,01548
	S_2	24/310 (3.98)	

Animals farms

For life animals, coprological analyses revealed an overall prevalence infestation of *F. hepatica* in Sejnane sheep farms was 6.97% with an overall mean intensity was 12.36 eggs/gram of faeces (Table 6).

***Table 6*:** Parasitological indicators based on coprological examination in Sejnane farms.

Parasitological indicators	Coprologie
Prevalence	6,97 % (35/502)
Intensity	12,36 eggs/gram of feces

The highest *F. hepatica* infestation prevalence was observed in sheep age between 1 and 3 years (3.39%); it is also observed in cross sheep breed (6.77%). Females are more infested than males (6.57%). Sheep with conjunctival color pink had have the highest infestation rate (3.59%). In hot season (S1) the infestation rate is higher than in cold season (S2) (4.58%).

There was a significant difference in the over-all infestation prevalence of *F. hepatica* for sheep age (p= 2.20 E-124).

While There was no significant difference in the over-all infestation prevalence of *F. hepatica* for sheep breed; sex; season; conjunctival color and body score (Table 7).

***Table 7*:** Prevalence of *Fasciola hepatica* infestation in Sejnane farms according to risk factors.

Parameter		Positive/ examined (%)	*P*
Age (year)	< 1	3/63 (0.6)	2,20E-124
	[1,3]	17/281 (3.39)	
	[4,6]	13/219 (2.39)	
	[7,9]	2/36 (0.4)	
	< = 10	1/10 (0.2)	
Breed	C	34/589 (6.77)	0,89001
	N	1/20 (0.2)	
Sex	M	2/54 (0.4)	0,519767
	F	33/555 (6.57)	
Season	S1	23/304 (4.58)	0,069099
	S2	12/305 (2.39)	
Conjunctival color	B	2/32 (0.4)	0,404777
	C	8/145 (1.59)	
	J	1/11 (0.2)	
	Lb	2/14 (0.4)	
	Lc	4/31 (0.8)	
	Rc	18/376 (3.59)	
Body score	1	6/120 (1.19)	0,199309
	2	22/380 (4.38)	
	3	6/106 (1.19)	
	4	1/2 (0.19)	

Fluke's measurement

In order to compare the morphology of the collected flukes with that of *Fasciola gigantica*, the morphology measurements showed that the total fluke length (**L1**), the ventral sucker to tail length (**L2**), the oral to ventral sucker length (**L3**), the abdomen diameter (**D1**) and the tail diameter were very significantly different with each other (Table 8).

Therefore, our results showed that the collected flukes are of *F. hepatica*.

Table 8*:* Mean and standard deviation (cm) of morphometric measurements for *F. hepatica* and *F. gigantica*

	F. hepatica	*F. gicantica*	**T student**
L1	2.14 ± 0.25	5.16 ± 0.39	3.3E-157
L2	1.87 ± 0.26	4.76 ± 0.38	3.3E-157
L3	1.87 ± 0.12	0.4 ± 0.06	1.5E-16
D1	0.53 ± 0.14	0.945 ± 0.09	2.3E-33
D2	0.31 ± 0.11	0.7 ± 0.10	2.2E-41

IV. Resistance of small ruminants breeds in Tunisia to fasciolosis

To study the genetic resistance of sheep breeds to fasciolosis in Tunisia, two groups of animals were differentiated based on criteria selection:

- Susceptible animals to fasciolosis: anaemic infected animals with red blood cell (RBC) and haemoglobin (Hb) values below the normal threshold (9 g/dl and 9. 1012/ml, respectively). This group contains 46 animals.
- Resistant animals to fasciolosis: they are non-anaemic infected animals with normal red blood cell (RBC) and haemoglobin (Hb) values (value ≥ to 9 g/dl and to 9 1012/ml, respectively). This group contains 84 animals.

A total of 130 sheep infected by *Fasciola hepatica* were selected for the study of genetic resistance of sheep breeds in the Northwest of Tunisia.

V. Objectives for the following months

- Carry out a biochemical analysis of collected plasma to determine liver enzyme levels and estimate albumin levels of affected animals.
- Study the genetic resistance of sheep breeds to *Fasciola hepatica* and identify the most resistant sheep breeds to fasciolosis in northern Tunisia.
- Study the polymorphism of *Fasciola hepatica* in Tunisia.
- Develop a fasciolosis specific ELISA
- Carry out a perception study on the knowledge of fasciolosis among sheep breeders in the Sejnane region

VI. Conclusions & Recommandations

To conclude, our results showed that *Fasciola hepatica* infestation is frequent in sheep from Sejnane, and that control mesures are ither ineffective or not performed in the region of Sejnane. Therefore, it is necessary to develop a control program to reduce economic losses in sheep flocks.

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