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Prevalence of *Fasciola hepatica* in *Galba truncatula* detected by Multiplex PCR in the province of El Tarf (Algeria)

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ABSTRACT. The prevalence of *Fasciola hepatica* infection in the snail *Galba truncatula* was studied for the first time in Algeria using Multiplex PCR. A total of 722 individuals were collected from 11 typical habitats (temporary ponds, road ditches and puddles) distributed in five localities of the El Tarf province, known for endemicity of fascioliasis. Snails were divided in 75 groups and DNA extraction was performed using Chelex® (Biorad, Nazareth Eke). Two primers were used, the first is specific for 124 bp DNA fragment of *Fasciola* sp and the second one represents the ITS-2 lymnaeids sequence (500–600 bp). The prevalence of the infection was estimated to be 46.66% for pools (35/75 were positive). The bursting out of the pools show that 75 snails were carriers of *F. hepatica* which gives an overall infection rate of about 10.74%. The results of snails infection according to their sizes showed significant differences in the studied size classes, thus snails of big sizes were the most infected. Based on these data, epidemiological implication of Multiplex PCR as a fully reliable technique to highlight high risks periods of fasciolosis will be of a great interest.

INTRODUCTION

Fascioliasis caused by *Fasciola hepatica*, is one of the most widespread parasitoses in the world. It affects mainly the domestic ruminants and leads to important economic losses (liver condemnation) as well as zootechnical performance reduction (Mage, 1988, 1989). In Algeria, *Galba truncatula* is the intermediate host of this parasitosis and it is very frequent, particularly in the north of the country where conditions are more favourable to its development and extensive farming is most practiced (Mekroud, 2004a). A relatively recent study on the definitive host and snails intermediate host was undertaken during nine years in north Algeria. The study of the disease prevalence in livestock using a serology technique have shown that it was approximately of 26.7% in cattle and 23.5% in sheep in the area of Jijel (littoral zone), whereas it did not exceed 6.8% in cattle and 6.3% in sheep in the semi-arid areas (Constantine). (Mekroud, 2004a).

Many diagnostic tools are used to detect the disease in the definitive host. However, the detection methods of the natural infection in snails are based essentially on crushing, snail dissection and/or cercarial shedding. The prevalence of the natural infection of *G. truncatula* in three areas (Jijel, Constantine and El Tarf) was detected by observation under binocular microscope. It ranges from 0.3% to 10.4% (Mekroud, 2004a). PCR technique, a more specific and highly sensitive method, was developed using a specific DNA sequence of the *Fasciola* sp genome (Kaplan *et al*., 1995; Caron *et al*., 2007). This technique was optimized using an innovative, rapid and inexpensive protocol for DNA extraction and the establishment of an internal control (multiplex PCR) to

eliminate false negative results (Caron *et al.*, 2011). The present study aims to set the prevalence of *F. hepatica* in *G. truncatula* based on a molecular basis using multiplex PCR for the first time in Algeria.

MATERIALS AND METHODS

Snails (*Galba truncatula*) collection

The snails samples used in this study were taken from five locations in the province of El Tarf (Boutheldja, Lac des oiseaux, El Matrouha, El Guergour and Oued El Hout). This province (Figure 1) is located in north-eastern Algeria and comprised two clearly different regions. The northern part is characterized by alluvial plains, clay-sandy soil structure and sub-humid to hot humid climate, while the south zone had a red clayey soil with moderate permeability. The climate is mainly humid.

The province of El Tarf has moreover a wetland complex made of several lakes, streams and dams. The samples were taken in typical biotopes (temporary ponds, rivers, streams, and puddles). The quadrant method (1 m^2) was used for snail collection. The quadrant method consists in collecting snails present in soil, in water or on vegetation in 1 m^2 perimeter for each biotope (Mekoud, 2004a). Snails were rinsed with water and subsequently conserved in labeled tubes containing 70% ethanol. The density of snails for each habitat was recorded and expressed as the mean of random counting on 3 m^2. 177, 101, 323, 80 and 41 snails were collected respectively in Boutheldja, El Guergour, Lac des oiseaux, El Matrouha and Oued El Hout (Figure 2). The size of all collected snails was measured before being analyzed by multiplex PCR. They have been divided into four size classes ranging from 3 to 4.9 mm, 5 to 6.9 mm, 7 to 8.9 mm and 9 mm and more.

DNA extraction

DNA extraction was performed using Chelex® (Biorad, Nazareth Eke). Each snail was crushed in 100 µl of 5% Chelex®. The mixtures were incubated first at 56°C for one hour and secondly at 95°C for 30 min before being centrifuged at 13,000 g for 7 min. After DNA concentration measurement and quality check using a spectrophotometer (Thermo

Figure 1. Location of the El Tarf province in northeastern Algeria

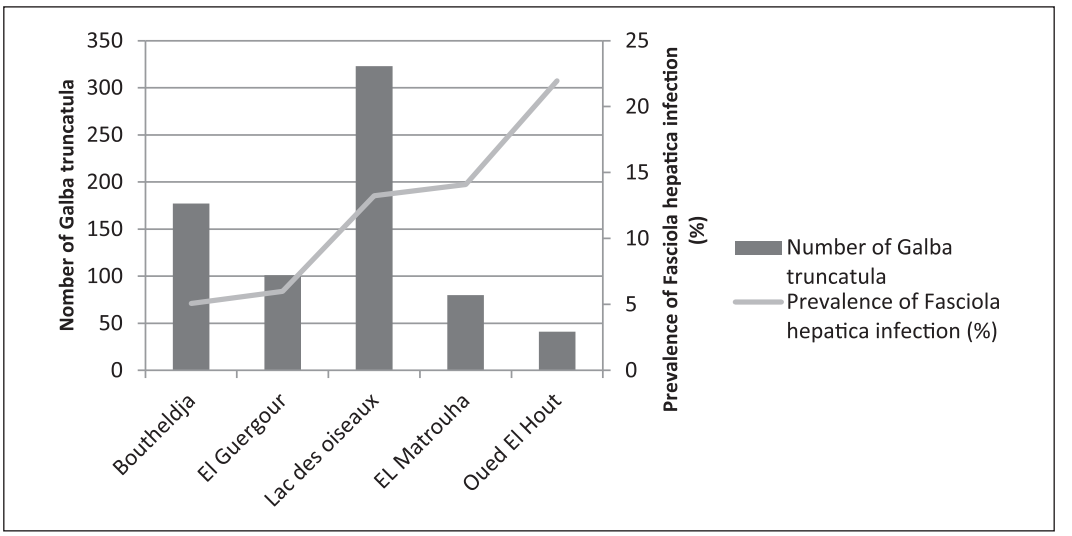

Figure 2. Number of *Galba truncatula* collected from the five localities and prevalence of *Fasciola hepatica* infection

Scientific, NanoDrop 1000), the supernatant was stored at -20°C. Pools of 6 or 10 snails were made by mixing together their DNA at a rate of 1µl of each snail sample in order to minimize the PCR number. Thereafter, the mixtures have been diluted (1/10, 1/100).

Multiplex PCR

Multiplex PCR used in our study has been previously described (Caron *et al.*, 2011). The DNA of 722 snails was amplified in a Peltier Thermal Cycler (MJ Research) using a commercial kit (Taq PCR Master Mix, Qiagen). This PCR amplifies with specific primers : Fsh1 (sense) 5'-GAT-CAA-TTC-ACC-CAT-TTC-CGT-TAG-TCC-TAC-3' and Fsh2 (antisense) 5'-AAA-CTG-GGC-TTA -AAC-GGC-GTC-CTA-CGG-GCA-3' a highly repeated 124 bp sequence specific for *Fasciola* sp (Kaplan *et al.*, 1995; Caron *et al.*, 2007), and with News2 (sense) 5'-TGT-GTC-GAT-GAA-GAA-CGC-AG-3' and Its2Rixo (antisense) 5'-TTC-TAT-GCT-TAA-ATT-CAG-GGG-3' the ITS-2 rDNA sequence specific for lymnaeids (500–600 bp) (Almeyda-Artigas *et al.*, 2000; Bargues *et al.*, 2001). The PCR mixture was prepared according to the manufacturer's recommendations with 1 µl DNA in a final volume of 25 µl. Amplification was performed as follows: initial denaturation at 95°C for 5 min; 40 cycles, each comprising denaturation at 95°C for 60 sec, annealing at 56°C for 60 sec and extension at 72°C for 60 sec; final extension at 72°C for 10 min. In order to detect possible PCR inhibition, several dilutions were tested to find the band of the internal control. The samples were initially tested after being diluted 10x. If the internal control band was not visible, the samples were diluted again 100x then tested undiluted. At the end, if the band of internal control still not visible, 0.05% Bovine Serum Albumin (BSA) is added to the 10x diluted sample. (Cucher *et al.*, 2006). Moreover, the DNA of 6 or 10 snails was pooled then tested by multiplex PCR to limit the PCR number. If the *Fasciola* sp DNA was amplified, all snails belonging to the group were tested individually by PCR.

Electrophoresis

After amplification, electrophoresis was performed on 2% agarose gel prepared in TAE buffer with ethidium bromide.

Data Analysis

Statistical analysis was performed using SAS (Statistical Analysis System, 2000). Chi2 test was used to compare the prevalence of the snails infection by *Fasciola* sp in the studied areas and the infection according to the snails size classes. The internal control band

of 24 snails on 722 snails could not be amplified (PCR inhibitors), so they were excluded from the different prevalences calculation.

RESULTS

Multiplex PCR

The results of the multiplex PCR carried out on 75 pools (722 snails) showed a prevalence of 46.6% (35/75 pools were positive) (Table 1). In the 35 positive pools, 75 snails revealed to be *Fasciola* sp carriers with a total infection rate of 10.7% (Table 2 and 3, Figure 3).

Prevalence by localities

Significant differences were noted for the prevalence in the various localities ($p<0.05$), it ranged from 5.1% to 21.9%. The recorded prevalence values in Boutheldja, El Guergour, Lac des oiseaux, El Matrouha and Oued El Hout are respectively: 5.1%; 6%, 13.2%, 14.1% and 21.9% (Table 3, Figure 2).

Table 1. Dilution factor of DNA for pools (75 pools corresponding to 722 snails)

1	1/10	1/100	1/10 + BSA
	I = 13/75 = 0.17		
	IC<0: 13 / IC>0: **62**		
		LI = 9/13 = 0.69 I = 4/13 = 0.30	
		IC<0: 4 / IC>0: **9**	
LI = 1/4 = 0.25 I = 3/4 = 0.75			
IC<0: 3 / IC>0: **1**			
			I = 0/3= 0
			IC<0: 0 / IC>0: **3**
	P = 35/75 = 46.66% E = 0		
	F<0: 40 / **F>0: 35**		

I : Inhibition; LI : loss of inhibition; IC: Internal control; P : Prevalence; E : Eliminated

Table 2. Dilution factor of DNA for snails (338 snails distributed in 35 positive pools)

1	1/10	1/100	1/10 + BSA
	I = 192/338 = 0.56		
	IC<0 | IC>0		
	192 | **146**		
		LI= 142/192 = 0.73 I = 50/192 = 0.26	
		IC<0 | IC>0	
		50 | **142**	
LI = 5/50 = 0.1 I = 45/50 = 0.9			
IC<0 | IC>0			
45 | **5**			
			LI = 21/45 = 0.46 I = 24/45 = 0.53
			IC<0 | IC>0
			24 | **21**
	P = 75/314 = 23.88% E = 24/314 = 7%		

I : Inhibition; LI : loss of inhibition; IC : Internal control; P : Prevalence; E : Eliminated

Table 3. Prevalence of *Fasciola hepatica* infection in localities studied

Locality	Number of *Galba truncatula* collected	Number of *Galba truncatula* eliminated (inhibition)	Number of *Galba truncatula* infected by *Fasciola hepatica*	Prevalence (%)
Boutheldja	177	0	9	5,1
El Guergour	101	1	6	6
Lac des oiseaux	323	21	40	13,2
EL Matrouha	80	2	11	14,1
Oued El Hout	41	0	9	21,9

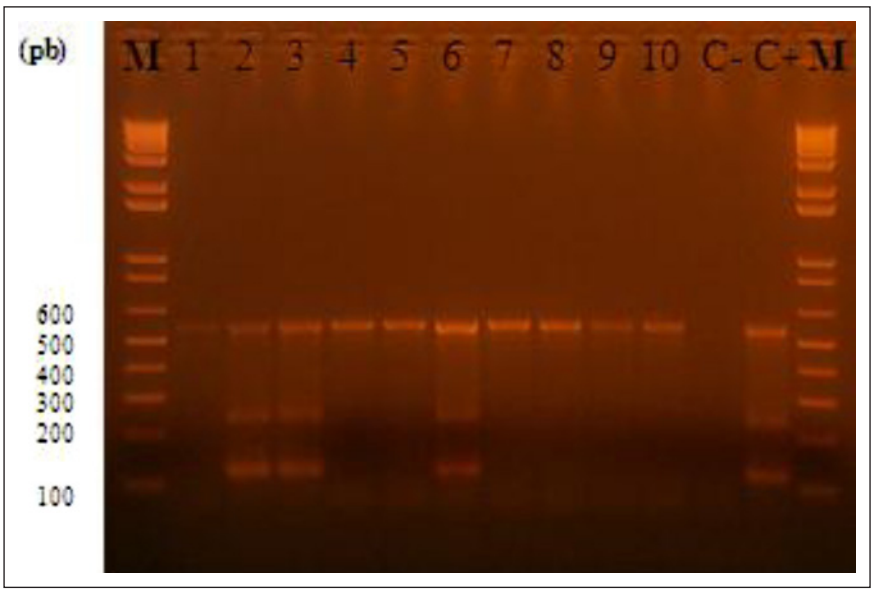

Figure 3. Agarose gel electrophoresis following multiplex PCR. M, molecular size; 1, 4, 5, 7, 8, 9 and 10, *Fasciola hepatica* negative snails; 2, 3 and 6, *Fasciola hepatica* positive snails; C-, negative control; C+, positive control.

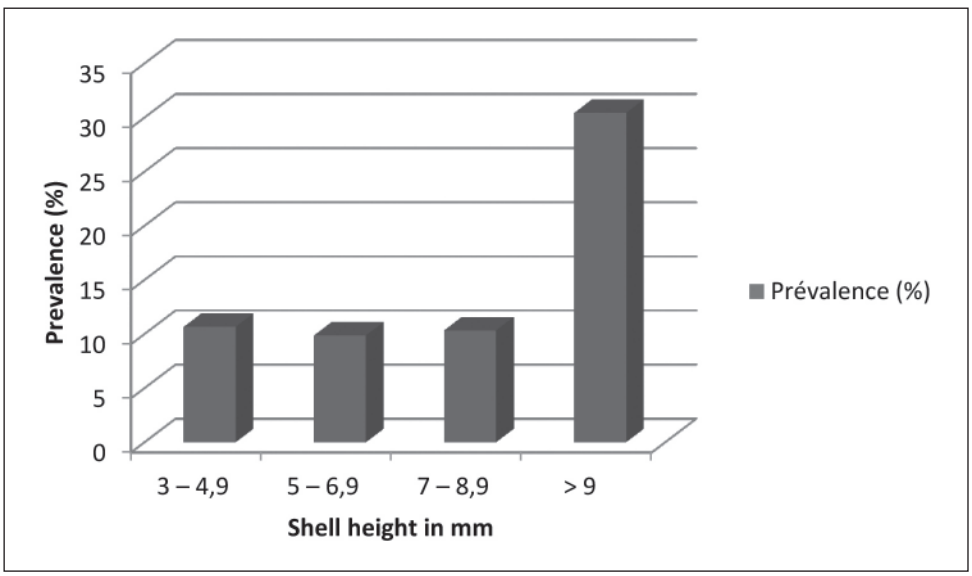

Figure 4. Prevalence of *Fasciola hepatica* infection in relation to the shell height of snails

Prevalence according to the snails size

The results of the snails infection according to their sizes showed significant differences between the studied size classes ($p<0.05$). Infection rates recorded in the size classes ranging from 3 to 4.9 mm, 5 to 6.9 mm, 7 to 8.9 mm are respectively: 10.7%, 9.8% and 10.3%, when the highest infection rate (30.4%) was recorded in the size class of 9 mm and more (Figure 4).

DISCUSSION AND CONCLUSION

The use of Chelex® for snail DNA extraction have several advantages. Indeed, this chelating resin provides amplifiable DNA in a very short time, with lower costs and without use of toxic solvent and multiple tube transfers. (Caron *et al.*, 2008; Garcia Gonzalez *et al.*, 2004). Therefore, this DNA extraction technique is ideal for dealing with large samples. Multiplex PCR technique was adopted to remove possible inhibitions. The use of internal control and the appearance of ITS2 band, snail parasite could be deduced contrary to the other techniques of molecular biology with which false negatives can't be removed.

Our work is the first attempt to study the prevalence of *Fasciola* sp. in *G. truncatula* using multiplex PCR technique in Algeria. Note that many relatively simple PCR protocols (conventional PCR, real-time PCR) have been developed for parasite detection in an intermediate host because of their very high sensitivity compared to basic microscopic techniques (Krämer & Schnieder, 1998; Mostafa *et al.*, 2003; Cucher *et al.*, 2006 ; Caron *et al.*, 2007, 2008), but just few studies have used these techniques of molecular biology to diagnose *Fasciola* sp. in *G. truncatula.* In a study carried out in Poland on a total of 192 *G. truncatula* analyzed by PCR, an infection rate of 26.6% have been recorded (Kozak & Wedrychowicz, 2010). In Switzerland, the prevalence of the infection in 130 populations of *G. truncatula* collected from 70 *F. hepatica*-infected farms was estimated at 7%, obtained by real-time PCR. (Schweizer *et al.*, 2007) These prevalences are similar to those obtained in our results, since El-Tarf is known as fascioliasis endemic area.

Multiplex PCR technique was used for the first time in Brazil to detect *F. hepatica* in experimentally infected snails. In this study, mitochondrial DNA was used to identify different larval stages (Magalhães *et al.*, 2004), but the interpretation of the results was disadvantageous because of the appearance of many bands. Microscopic techniques were also used in some studies conducted in North Africa about the detection of *G. truncatula* infection by *F. hepatica*. In Algeria's extreme east, a study was conducted in two areas where two infection rates were recorded for each: 3.1% and 2.4% in Constantine and 5.9% and 4.6 % in Jijel (Mekroud *et al.*, 2004b). The first explored area is characterized by a semi-arid climate unlike Jijel wich have a humid climate like the explored localities in our study.

In Tunisia, the investigations undertaken in the oases of Gafsa and Tozeur using a microscopic technique have recorded respective infection rates: 19.4% and 26%. These prevalence values can be partly explained by the fact that the studied areas are permanently exposed to humidity (irrigation canals and waterways) which constitute a very favorable microclimate to snails development. (Hammani & Ayadi, 2000; Hammani *et al.*, 2007).

In Morocco, the prevalence obtained always by microscopy remains clearly lower even if the explored areas have the same climate: 0.17% (Khallaayoune *et al.*, 1991); 2.9% (Khallaayoune & El Hari, 1991); 1.01% (Goumghar *et al.*, 1997) and 1.5% (Ghamizi, 1998). However, compared to microscopic usual techniques, molecular biology techniques are clearly more specific and sensitive. The use of pools for the PCR analysis has shown great interest in reducing the number of PCR tests particularly during large epidemiological studies. Indeed, on the 75 analyzed pools, just 35 pools appeared to be *Fasciola* sp carriers and were subjected to individual snail analysis for the determination of positive snails. Noticing that few studies have used snails pooling process for PCR analysis (Rognlie *et al.*, 1996; Fu-Rong Wei *et al.*, 2010).

The study of the infection rates according to the sampling areas showed a highest value in Oued El Hout where the samples were taken from a road ditch with a total prevalence of about 21.9%. In El Matrouha area, the infection rate was similar to that found in the Lac des oiseaux area. The highest value in this area was recorded in a waterway crossing partly the Institute of Veterinary Sciences, where the farm animals (cattle and sheep) can reach it. Moreover, the lowest values of the infection prevalences

were observed in El Guergour and Boutheldja areas with respective overall rates of 6% and 5.1%. These variations noted in the various studied areas can be explained by the sampling and the explored biotopes. Nevertheless, the global infection of the various areas remains below the real infection rate in the province of El-Tarf, not only because the animals of the different prospected regions in this work are raised in extensive breeding, but also this humid province is known as one of the most heavily affected provinces by fascioliasis that causes great losses in the slaughterhouses.

The results concerning the snails infection according to their sizes showed a most significant infection in lymnaeid whose size was 9 mm and more, with a prevalence of 30.4%. The lymnaeid belonging to the other size classes showed a significantly identical prevalence for snails whose size ranging from 3 to 4.9 mm, 5 to 6.9 mm and 7 to 8.9 mm. Our results corroborate those obtained by Kaplan *et al.*, 1997.

However, our sample is biased because just 23 snails of the 722 analyzed snails belong to the size class of 9 mm and more. According to Rondelaud *et al.* (2009), the adult snails are often more infested than the young ones and there is some variability in the results because of the sampling techniques based generally on either time unit (20 minutes for example) or surface unit.

In conclusion, epidemiological studies using multiplex PCR analysis carried out over all the year will be of a great interest to highlight the periods of high risks and then to take adequate control measures.

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